A ROLE FOR THE TRUNCATED TRKB RECEPTOR IN NEURONS

by

BARBARA MURRAY FENNER

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This dissertation was presented

by

Barbara Murray Fenner

It was defended on

December 16, 2004

and approved by

Dr. Cristian L.Achim, MD, PhD, Department of Pathology

Dr. Donna Beer-Stolz, PhD, Department of Cell Biology and Physiology

Dr. Reza Zarnegar, PhD, Department of Pathology

Dr. Yong-Jian Liu, PhD, Department of Neurobiology

Dr. Robert Bowser, PhD, Department of Pathology
Dissertation Director

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A ROLE FOR THE TRUNCATED TRKB RECEPTOR IN NEURONS

Barbara Murray Fenner, PhD

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Brain derived neurotrophic factor (BDNF) promotes cell survival, proliferation, differentiation, and enhances neurotransmission. In several neurodegenerative diseases, including Parkinson's disease (PD), BDNF mRNA and protein are altered in regions of pathology.

The cellular response to BDNF is mediated by trkB. There are two trkB receptor isoforms abundantly expressed in the brain, full-length (fl) and truncated trkB (tc). TrkB.fl is a tyrosine kinase receptor that activates intracellular signaling cascades. Although the extracellular and transmembrane domains of trkB.tc are 100% homologous to trkB.fl, its intracellular domain is unique and lacks the catalytic amino acids. TrkB.fl functions as a signaling receptor while trkB.tc binds to and internalizes BDNF. The intracellular function of trkB.tc is unclear because it lacks the cytoplasmic signaling domain. The purpose of this dissertation was to identify a novel role for the truncated trkB receptor in mature neurons of the central nervous system. Our study had two goals: 1) to investigate the hypothesis that trkB.tc facilitates the endocytic sorting of BDNF and 2) to investigate the changes in trkB.tc protein distribution in PD. Finally, we correlated the changes in trkB.tc distribution in PD with its potential role in BDNF transport.

Our organelle studies revealed co-localization of trkB.tc and internalized BDNF within endosomes, showing that the two proteins were transported as a complex. This protein complex is maintained within recycling endosomes. Although we did see co-localization of trkB.tc and internalized BDNF within lysosomes, it was not as extensive as the sorting of BDNF to recycling vesicles endosomes.

Immunofluorescence studies of human autopsy striatum and substantia nigra revealed that trkB.tc and trkB.fl are differentially distributed in the control and PD brains. Furthermore, changes in the distribution of both trkB isoforms are seen in PD and correspond to regions of pathology. We conclude that upregulation of striatal trkB.tc in PD is an early response to neurodegeneration and regulates the effects of BDNF.

In summary, trkB.tc facilitates the intracellular sorting of internalized BDNF to recycling endosomes. The altered distribution of trkB.tc in PD suggests enhanced trkB.tc transport, and potentially BDNF transport. This may enhance the neuroprotective effects of BDNF in PD.

1. Introduction

The fate of a cell, from genesis to death, is influenced by a group of molecules known as growth factors. These factors act as fate determining agents during embryogenesis. Growth factors bind to cell surface receptors to induce cell survival, proliferation, or differentiation. They also act as chemotactic agents directing cell migration and in the central nervous system (CNS), targeting of neurites.

Rita Levi-Montalcini first identified NGF in 1952. She observed that sensory or sympathetic nerve cultures exposed to tumor extracts undergo extensive neurite outgrowth. Six years later, this growth factor was isolated by Stanley Cohan and identified as Nerve Growth Factor [refer to (Levi-Montalcini, 1987) for a comprehensive review of NGF]. Over the next four decades, five new members of the nerve growth factor family have been identified. These include brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), neurotrophin-4/5 (NTF-4/5), neurotrophin-6 (NT-6), and neurotrophin-7 (NT-7) (Altar and DiStefano, 1998) (Huang and Reichardt, 2001).

BDNF is abundantly expressed in many regions of the central nervous system. Neurons exposed to BDNF show increased cell survival, proliferation, differentiation, and enhanced neurotransmission (Atwal et al., 2000) (Huang and Reichardt, 2001) (Encinas et al., 2000). These functions are mediated by the binding of BDNF to its high affinity tyrosine receptor kinase B (trkB). Disruption of the neurotrophic effects of BDNF leads to cell death. In neurodegenerative diseases like Parkinson's disease (PD), BDNF and trkB expression and

function are altered (Howells et al., 2000) (Mogi et al., 1999) (Parain et al., 1999). The aberrant distribution and function of BDNF, in PD, contributes to pathology through survival factor deficiency and disruption of synaptic transmission. Understanding the mechanisms that regulate BDNF intracellular activities will identify new therapeutic targets in chronic neurodegenerative diseases.

1. BDNF is a potent neurotrophic factor

BDNF is a potent neurotrophic factor that targets neurons in several regions of the CNS. The major functions of BDNF promote: 1) survival and proliferation, 2) neuronal differentiation, and 3) modulation of neurotransmission. BDNF and its activities in CNS development, maintenance, and disease have become a major focus in neuroscience research.

1.1 BDNF promotes neuronal survival and proliferation

BDNF functions as a typical growth factor by promoting cell survival. *In vivo* and *in vitro* data show that BDNF promotes survival both in developing and post-mitotic neurons (Liebl et al., 1997) (Jungbluth et al., 1997) (Alcantara et al., 1997). Developing neurons require BDNF for cell survival. In the mature CNS, post-mitotic neurons require BDNF to sustain their viability. Neurons compromised by disease or injury prevent cell death by activating BDNF signaling cascades (Hagg, 1998), (Alcantara et al., 1997).

The role of BDNF in neuronal cell survival is well illustrated in the developing nervous system. During neurogenesis, neural crest and neural tube cells differentiate to become

peripheral and central nervous system progenitor cells, respectively. Neuronal progenitor cells follow chemical cues, including BDNF, to develop into mature phenotype-specific neurons. Its temporal and regional regulation in neuronal and non-neuronal tissues is imperative to the growth and integrity of the peripheral and central nervous system.

There are two key timepoints in CNS development where BDNF is most influential: 1) the early embryonic stage of neuronal cell division and 2) the peri-natal stage of naturally occurring apoptosis. Numerous studies show that embryonic sensory and motor neurons require BDNF for their survival (Liebl et al., 1997) (Jungbluth et al., 1997). Sensory neurons require a time-sensitive exposure to multiple neurotrophic factors (Pinon et al., 1996). Mutant mice lacking the proteins for BDNF, NT3, NGF, or their high affinity trk receptors have greatly impaired development of sensory neurons (Liebl et al., 1997). Experiments in the embryonic trkB-/- mouse show a significant increase in pyknotic nuclei and cell death in trigeminal ganglion neurons at embryonic stages 11-12 (E11-12) (Pinon et al., 1996). Antisense RNA studies showed that sensory neurons, including those in the cochleovestibular ganglion, are dependent on BDNF support at E11-12 for their survival (Staecker et al., 1996). Motor neurons, derived from the ventral tube, also rely on BDNF for their survival during prenatal development (Jungbluth et al., 1997). BDNF treatment of the ventral neural tube induces a significant increase (40%) in motor neuron number. In summary, regulating the availability of BDNF to sensory and motor neurons establishes their survival and growth rate.

In the post-natal brain, BDNF regulates apoptotic cell death in both proliferating and post-mitotic neuronal populations. If BDNF or its receptor expression is disrupted, there is a substantial increase in apoptosis. BDNF-/- or trkB-/- mice show increased apoptosis in two proliferating brain regions (the subventricular zone of the olfactory bulb and subgranular layer

cells of the dentate gyrus) (Linnarsson et al., 2000), and in the post-mitotic cells of the hippocampus, cortex, striatum, and thalamus (Alcantara et al., 1997). Conversely, BDNF over-expression, like in the dopaminergic cells of the DBH:BDNF transgenic mice, leads to a 52% increase in midbrain cell survival (Alonso-Vanegas et al., 1999).

There is now strong evidence that cell survival is induced by anterograde transport of BDNF to its target neurons (Fawcett et al., 1998) (Spalding et al., 2002). In DBH:BDNF transgenic mice, noradrenergic neurons were induced to synthesize BDNF. Not only do these neurons show an increase in cell survival, but they also promote cell survival of their target neurons in the neocortex (Fawcett et al., 1998). There is also evidence showing that anterograde transport of BDNF occurs between targets in the visual system (Spalding et al., 2002).

BDNF prevents chemically or physically-induced cell death (Alcantara et al., 1997) (Hagg, 1998) (Takei et al., 1999). Neuronal axotomy, a physically induced injury, is a common model used to study the neurotrophic effects of BDNF. Alcantara et al. (1997) showed that trkB-/- mice show an increase in apoptosis of hippocampal neurons following axotomy. Hippocampal explants from the trkB-/- mice were used to show that these trkB-/- cells did not respond to BDNF stimulus. The preparation of hippocampal explants inherently axotomizes CA3 and subicular neurons. There was a significant increase in cell death in the trkB-/- explants compared to explants from control mice. BDNF treatment of control mice increased cell number, but had no effect of trkB-/- explants (Alcantara et al., 1997). In addition to hippocampal neurons, BDNF also protects dopaminergic, motor, corticospinal, and sensory neurons from axotomy induced cell death (Hagg, 1998) (Alcantara et al., 1997) (Giehl et al., 1998) (Tonra et al., 1998).

BDNF promotes cell survival, and prevents cell death, by signaling through its high affinity receptor, trkB. Specifically, BDNF binds to the full-length trkB (trkB.fl) receptor to activate intracellular adaptor proteins of the PI3K pathway (Atwal et al., 2000). The PI3K pathway is activated by trkB.fl-mediated activation of Ras or N-Shc/Grb-2 (Nakamura et al., 1996). This induces activation of the Akt/protein kinase B adaptor proteins, which induce anti-apoptotic signaling. The apoptotic pathway is directly inhibited by Akt phosphorylation of BAD, which prevents its binding to and subsequent activation of the pro-apoptotic protein Bcl-xL. Finally, PI3K can also regulate the phosphorylation of IκB and its subsequent activation of NFκB. Nuclear localization of NFκB induces transcription of pro-survival proteins (Huang and Reichardt, 2001).

1.2 BDNF promotes neuronal differentiation

Neuronal differentiation is a broad term that encompasses 1) fate determination or phenotype selection, 2) neurite extension and targeting, and 3) the modulation of synaptic transmission. Because neurotrophin modulation of synaptic transmission is a unique function of BDNF, it will be addressed separate from the other two types of BDNF-induced neuronal differentiation.

1.2.1 Neurite complexity and targeting

BDNF is a pluripotent neurotrophic factor that can induce differentiation as well as survival in many neuronal cell populations. In the developing nervous system, BDNF controls

cell number by inducing proliferation and inhibiting apoptosis. Later in the post-natal brain, there is a period of neurite extension and path finding. At this stage, neuronal processes are guided by chemical cues to direct elongation and ultimately formation of synaptic connections with their target neurons. This process results in an extensive and complex neural network, containing feedback circuits. BDNF plays a major role in the outgrowth and path-finding events of several neuronal populations.

BDNF promotes neurite outgrowth by activating trkB.fl to induce prolonged activation of the MAPK/Erk pathway (Huang and Reichardt, 2001). This pathway is initially activated by trkB.fl binding to FRS2 (fibroblast growth factor receptor substrate) or SNT (suc-associated neurotrophic factor-induced tyrosine phosphorylated target) (Easton et al., 1999). FRS2 forms a complex with Grb2 and activates adaptor proteins: Crk, Src, $p13^{suc1}$, and SH-PTP-2. These adaptor proteins then signal to activate the Erk pathway which in turn, induces phosphorylation of microtubule-associated proteins and neurofilament proteins. Thereby FRS2-mediated activation of MAPK/Erk pathway can directly influence the outgrowth of dendrites and axons, respectively (Huang and Reichardt, 2001).

Neurite outgrowth is defined by dendritic and axonal extension and complexity. Depending on the neuronal phenotype, BDNF either enhances or inhibits dendritic growth (McAllister et al., 1997) (Mertz et al., 2000). For example, BDNF has opposing effects on dendritic complexity in different cortical layers of the cortex. BDNF increases dendritic growth in layer IV but inhibits growth in layer VI. When cortical neurons were exposed to BDNF neutralizing antibodies, existing dendrites retracted from their targets (McAllister et al., 1997). BDNF promotes either proliferation or dendritic growth in an individual cell population. It does

not promote proliferation in neurons that undergo dendritic changes in response to BDNF (Mertz et al., 2000).

BDNF can also have opposing roles in neurite outgrowth within the same neuronal population. In retinal ganglion cells (RGCs), BDNF inhibits dendritic arborization but stimulates their axonal arborization (Lom and Cohen-Cory, 1999). The affects of BDNF are highly regulated. BDNF induces innervation of neocortical serotonergic axons but has no effect on cell survival, proliferation, or neurite extension from non-serotonergic neuronal populations (Mamounas et al., 2000). In addition to inducing neurite outgrowth, BDNF also mediates specialized target innervation. Random outgrowth of neurites is not sufficient for proper neuronal connections to develop. It is essential for these neurites to make appropriate connections with their target neurons and to make these connections in the appropriate region of the brain or periphery. This is evident in the gustatory system (Ringstedt et al., 1999), where BDNF is necessary for proper innervation and localization of taste buds and gustatory papillae. Developing gustatory fibers find their appropriate targets by extending to localized BDNF-rich regions of the tongue. When BDNF is overexpressed, gustatory fibers are unable to reach their appropriate target, as BDNF is diffusely distributed throughout the tongue. Appropriate and functional neuritic outgrowth and targeting is reliant on the distinct temporal and regional distribution of BDNF.

1.2.2 Neuronal phenotype determination

Mature neurons express phenotype specific markers that identify their specialized role in the nervous system. This phenotype determination occurs in the developing nervous system. There is abundant evidence that BDNF mediates phenotype marker expression in several

neuronal populations, including calbindin, serotonergic, and cholinergic neurons (Fawcett et al., 2000) (Galter and Unsicker, 2000a) (Ward and Hagg, 2000).

Calbindin-containing neurons are found in the lateral septum. These neurons express trkB receptors and respond to BDNF stimulus. Fawcett et al. (2000) studied a population of BDNF-expressing noradrenergic neurons that project to the lateral septum. They showed that BDNF stimulus induces increased calbindin synthesis and expression in lateral septum neurons, but does not affect cell proliferation or survival (Fawcett et al., 2000).

In embryonic raphe nucleus cultures, Galter et al (2000) identified an autocrine/paracrine feedback loop with BDNF that induces serotonergic phenotype selection (Galter and Unsicker, 2000). In this system, cAMP activation of raphe nucleus neurons induces BDNF synthesis. Synthesized BDNF is released to activate trkB receptors on raphe target neurons. This induces secretion of tryptophan hydroxylase, the precursor for serotonin. Thus, a feedback loop is formed to regulate BDNF-induced phenotype selection of serotonergic neurons.

Finally, BDNF can also induce phenotype selection in forebrain cholinergic neurons. There is a reduction in cholinergic neurons and overall neuronal size in the absence of BDNF. Knockout studies show that developing cholinergic neurons of the forebrain are dependent on BDNF for their phenotype selection (Ward and Hagg, 2000).

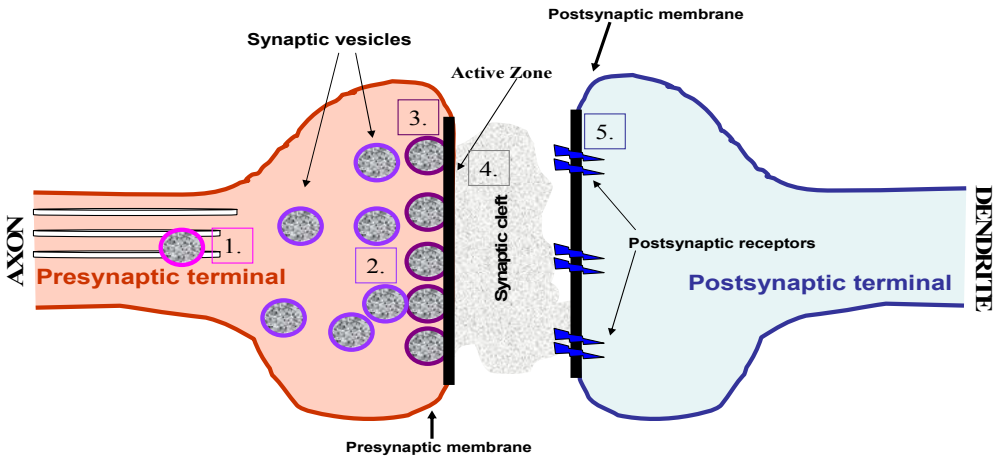
BDNF-mediated phenotype selection is often associated with anterograde signaling. Activity in the presynaptic neuron induces BDNF synthesis. Synthesized BDNF is then transported to the axon terminal where it can be released at the synapse. The post-synaptic neuron responds to the BDNF stimulus by inducing the gene expression of phenotype markers such as calbinding, serotonin, and choline acetyltransferase (Fawcett et al., 2000) (Galter and Unsicker, 2000) (Ward and Hagg, 2000).

1.3 BDNF modulates synaptogenesis and neurotransmission

Synaptic transmission is a highly complex transneuronal signal that occurs between two neuritic terminals, called the synapse. The synapse is comprised of a presynaptic (axonal) terminal and a postsynaptic (dendritic) terminal. Neurotransmitters and neuromodulators are packaged in presynaptic vesicles. These vesicles accumulate at the presynaptic membrane and release proteins into the synaptic cleft upon depolarization. Receptors on the post-synaptic membrane bind to and internalize these proteins (Figure 1). BDNF-mediated regulation of synaptic protein expression, synaptic morphology, and depolarization can alter synaptic efficiency.

Figure 1. The synapse.

Neurotransmitters and neuromodulators are transported to the presynaptic terminal (1). Here, they are packaged into synaptic vesicles. These vesicles accumulate at the active zone (2). Vesicles fusion, or docking (3), occurs and upon depolarization of the presynaptic cell, the contents of the vesicles are released into the synaptic cleft (4). Receptors on the postsynaptic membrane bind to and internalize the released proteins (5).



1.3.1 Reciprocal interactions between BDNF and neuronal activity

The reciprocal interactions of BDNF and neuronal activity modulate neurotransmission. High frequency neuronal stimuli enhance BDNF activity by increasing BDNF translation and by translocating intracellular trkB to the somal plasma membrane (Tao et al., 1998) (Du et al., 2000). A calcium response element, CaRE1, and subsequent transcription factor, CaF (Tao et al., 1998), mediate this increase in BDNF synthesis. BDNF synthesis is also upregulated in response to kainic acid (KA)–induced excitation (Goutan et al., 1998).

The interaction between neuronal activity and BDNF is reciprocal. Neuronal activity induces BDNF synthesis while BDNF synthesis modifies neuronal activity. Specifically, BDNF influences high fast excitatory synapses (Balkowiec et al., 2000) (Itami et al., 2000) (Pozzo-Miller et al., 1999). In glutamatergic neurons, these changes are mediated through AMPA-receptors. The opposing effects of BDNF on synaptic activity vary between cell types. BDNF inhibits AMPA currents in brainstem neurons (Balkowiec et al., 2000) and enhances AMPA current in thalamocortical neurons (Itami et al., 2000). K252a, a tyrosine kinase inhibitor, prevents BDNF-mediated changes in AMPA current (Balkowiec et al., 2000). Therefore, trkB.fl activation is necessary for BDNF to regulate AMPA current. TrkB.fl activation may directly interact with AMPA receptors, may activate intracellular adaptor proteins that modify the AMPA receptors, or may stimulate membrane trafficking of the receptor subunits (Balkowiec et al., 2000). Although the mechanism of BDNF-mediated changes in AMPA currents is unclear, BDNF can modify neurotransmission by altering synaptic proteins and morphology.

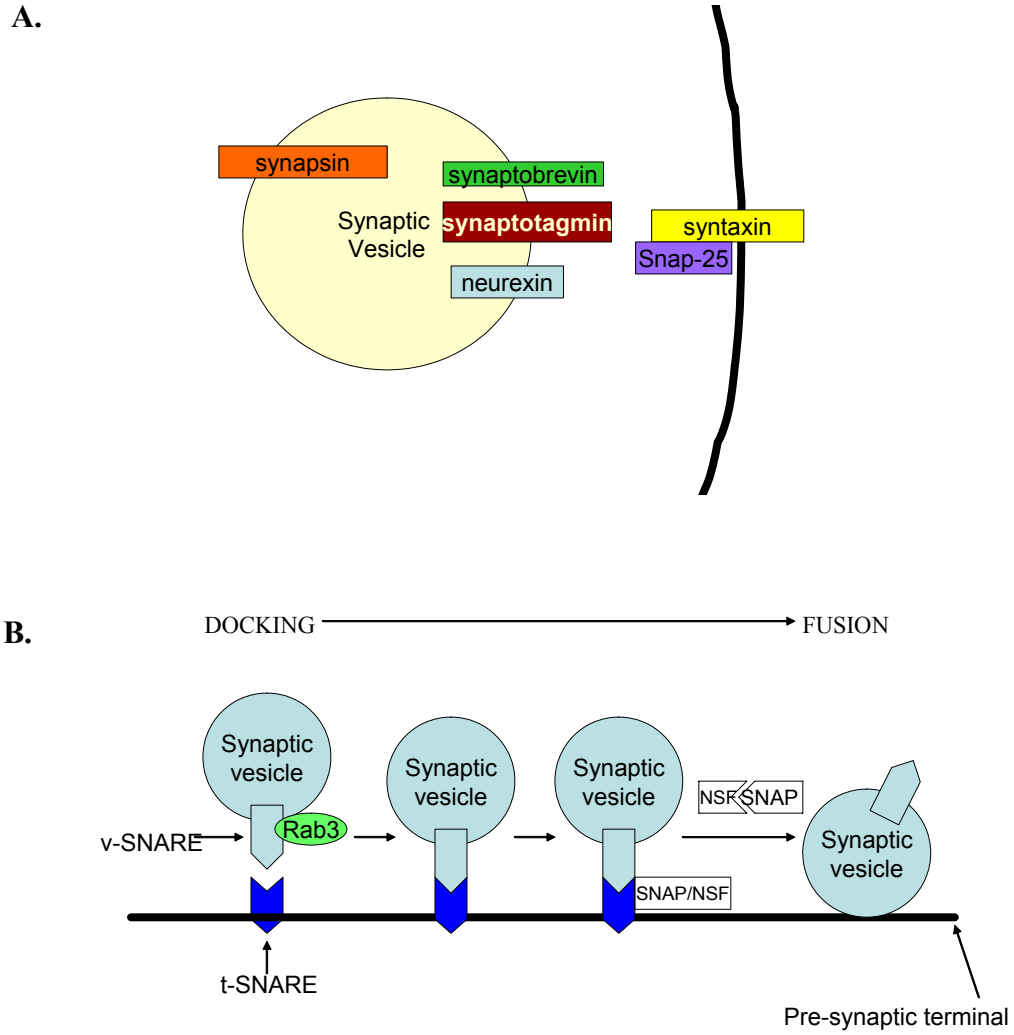
1.3.2 Mechanisms of BDNF-modified neurotransmission

BDNF modifies neurotransmission by altering the expression of synaptic proteins and the physical composition of the presynaptic terminal. BDNF-/- mice show an increase in total synaptic vesicles but a decrease in docked vesicles (Carter et al., 2002). This results in an overall decrease in neurotransmitter release (Pozzo-Miller et al., 1999). These changes are also observed in trkB-/- mice (Martinez et al., 1998). BDNF stimulates synaptic exocytosis via three mechanisms: 1) increasing the number of synaptic vesicles at the active zone, 2) increasing the synaptic density, and 3) increasing the overall number of synapses per neuron (Bradley and Sporns, 1999) (Tyler and Pozzo-Miller, 2001). Synaptic vesicle packaging, translocation to the active zone, fusion to the synaptic membrane, and subsequent neurotransmitter release are controlled by presynaptic protein function (Figure 2).

BDNF is necessary for the appropriate expression and localization of synaptic proteins in vesicle docking, fusion, and exocytosis (Pozzo-Miller et al., 1999) (Martinez et al., 1998) (Jovanovic et al., 2000). In BDNF-/- or trkB-/- mice, synaptic vesicles formation and neurotransmitter packaging occurs normally. Instead, vesicle docking and fusion is disrupted. Synaptophysin, synaptobrevin, and Rab3a are proteins necessary for the docking and fusion of synaptic vesicles. In the absence of BDNF, the expression of these proteins is altered (Pozzo-Miller et al., 1999) (Martinez et al., 1998). This phenomenon alone would be sufficient to inhibit neurotransmitter release. But, BDNF deficiency further disrupts the synapse by regulating the expression of syntaxin, snap-25, synaptotagmin, and synapsin I (Martinez et al., 1998) (Jovanovic et al., 2000). These proteins are necessary for exocytosis to occur. BDNF modulates neurotransmission through its effects on vesicle docking, fusion, and exocytosis.

Figure 2. Synaptic vesicle docking and fusion.

Vesicular synapsin I (A) binds to actin and neurofilaments to mediate synaptic vesicle transport to the presynaptic terminal. Once at the terminal, the synaptic vesicle must fuse with the pre-synaptic membrane (B). v-SNAREs (A, synaptobrevin and synaptotagmin) bind to t-SNAREs (A, syntaxin) via hydrolysis of Rab3 (B). Docking is followed by the recruitment of SNAP-25 to syntaxin (A). The release of SNAP intiates the fusion of the synaptic vesicle with the pre-synaptic membrane (B).



2. Tyrosine receptor kinase B mediates BDNF activity

The cellular response to BDNF is mediated by the high affinity trk receptor, trkB. There are two trkB receptor isoforms abundantly expressed in the brain, full-length (fl) and truncated trkB (tc). TrkB.fl functions as a traditional tyrosine kinase receptor. Conversely, the trkB.tc receptor binds to and internalizes BDNF but it lacks the cytoplasmic signaling domain. TrkB.tc does not function as a tradition tyrosine kinase receptor and its intracellular function is still unclear. But, the expression and function of trkB.fl alone is not sufficient to regulate the complex and abundant effects of BDNF. We propose that trkB.tc has an active role in regulating BDNF function.

2.1 Biochemical properties of trkB receptor isoforms

The trkB gene is located on chromosome 9q22, is over 590kbp, and contains 24 exons (Stoilov et al., 2002) (Valent et al., 1997). This gene encodes over 100 RNA transcripts ranging from 0.7-9kb (Middlemas et al., 1991). There are two abundantly expressed trkB isoforms in the brain, the full-length tyrosine kinase receptor (gp145, trkB.fl), and the truncated receptor (gp95, trkB.tc) (Klein et al., 1989). Recent evidence has shown that there is a third trkB receptor expressed in the brain, trkB.shc (Stoilov et al., 2002). TrkB.shc is a truncated trkB receptor that lacks the putative tyrosine kinase domain but contains the Shc substrate binding site. Regulation of trkB isoform synthesis is driven by alternative gene splicing, use of multiple promoters, and multiple poly-adenylation sites (Barettino et al., 1999) (Stoilov et al., 2002). There are two

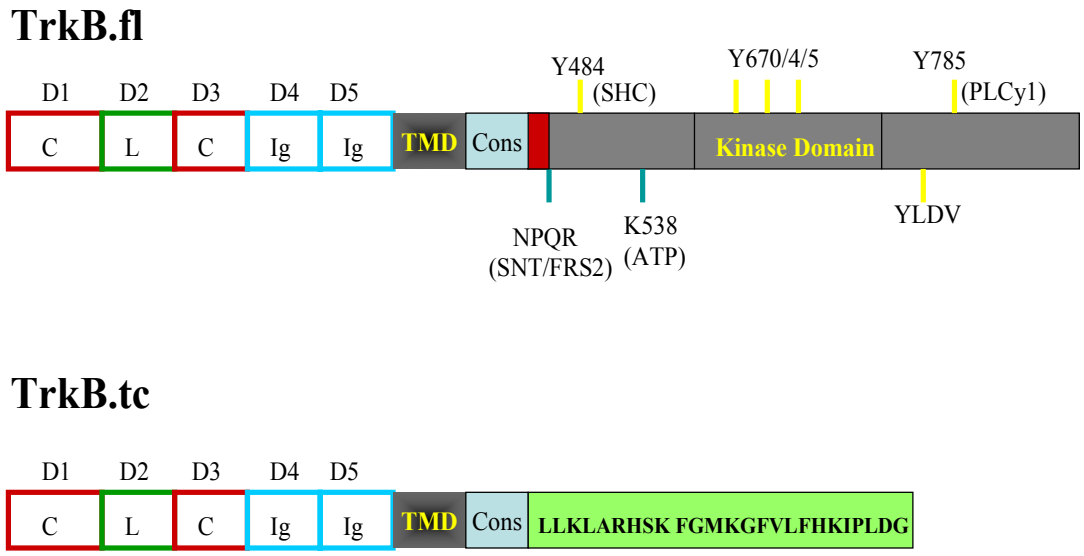
promoters upstream of the trkB translation start site: P1 and P2. P2 is located within the P1 promoter (Barettino et al., 1999). TrkB.fl is generated from alternative splicing at exon 24; trkB.tc from exon 16; and trkB.shc from exon 19 (Stoilov et al., 2002). Although there are many polyadenylation and promoter sites identified within the trkB gene, their regulation of trkB isoform translation is unknown.

The extracellular domain of trkB is highly glycosylated, 398 amino acids long, and has a molecular weight of 60.6kDa (Haniu et al., 1995). There is 100% homology between the extracellular domains of full-length (trkB.fl) and truncated (trkB.tc) trkB. Both receptors contain five binding domains (D1-D5) (Ultsch et al., 1999) (Figure 3). D2, the leucine-rich region, is essential for high affinity binding and D5, the immunoglobulin-like domain, directs ligand-binding specificity (Ultsch et al., 1999) (Haniu et al., 1995) (Ninkina et al., 1997).

Cytoplasmic domains of trkB.tc and trkB.fl are isotype specific after the first 12 intracellular amino acids. TrkB.fl contains a complex intracellular domain with a tyrosine rich catalytic region (McCarty and Feinstein, 1998). The short cytoplasmic tail of trkB.tc has a serine residue and the SNT (src-associated neurotrophic factor induced tyrosine) activation sequence 'KFG' (lysine-phenylalanine-glycine) (Baxter et al., 1997) (Figure 3).

Figure 3. Structure of the trkB.fl and trkB.tc receptor isoforms.

The extracellular domain of the truncated receptor is 100% homologous to that of the full-length receptor. The first 12 intracellular amino acids are conserved between isoforms. The remaining intracellular domains are unique. [Abr.: C (cysteine rich domain), L (leucine rich domain), Ig (Ig-like domain), D# (domain number), TMD (transmembrane domain), cons (intracellular region conserved between trkB.fl and trkB.tc), Y# (tyrosine residue and amino acid number, and K# (lysine residue and amino acid number). The parenthesis indicate substrate-binding site.]



TrkB receptors can form three types of dimers: trkB.fl:trkB.fl, trkB.fl:trkB.tc, and trkB.tc:trkB.tc (Ohira and Hayashi, 2003). The level of trkB expression varies between isoforms. In the early developing brain, the full-length isoform of the trkB receptor is abundant (Fryer et al., 1996) (Ohira et al., 1999). Rat forebrain expression of trkB.fl reaches adults levels by birth, while trkB.tc does not increase to adult levels until post-natal day 10-15 (Fryer et al., 1996). This expression pattern is conserved in the monkey brain. TrkB.tc and trkB.fl distribution vary between brain regions and intracellular structures. In the adult brain, trkB.fl is most abundantly expressed in the cerebral cortices while trkB.tc is most abundantly expressed in

the hippocampus and cerebellum. Overall, trkB.tc is the predominant isoform expressed in the adult brain (Ohira et al., 1999). The specific mechanisms regulating the developmental versus adult differential expression of trkB receptors in the brain are unclear.

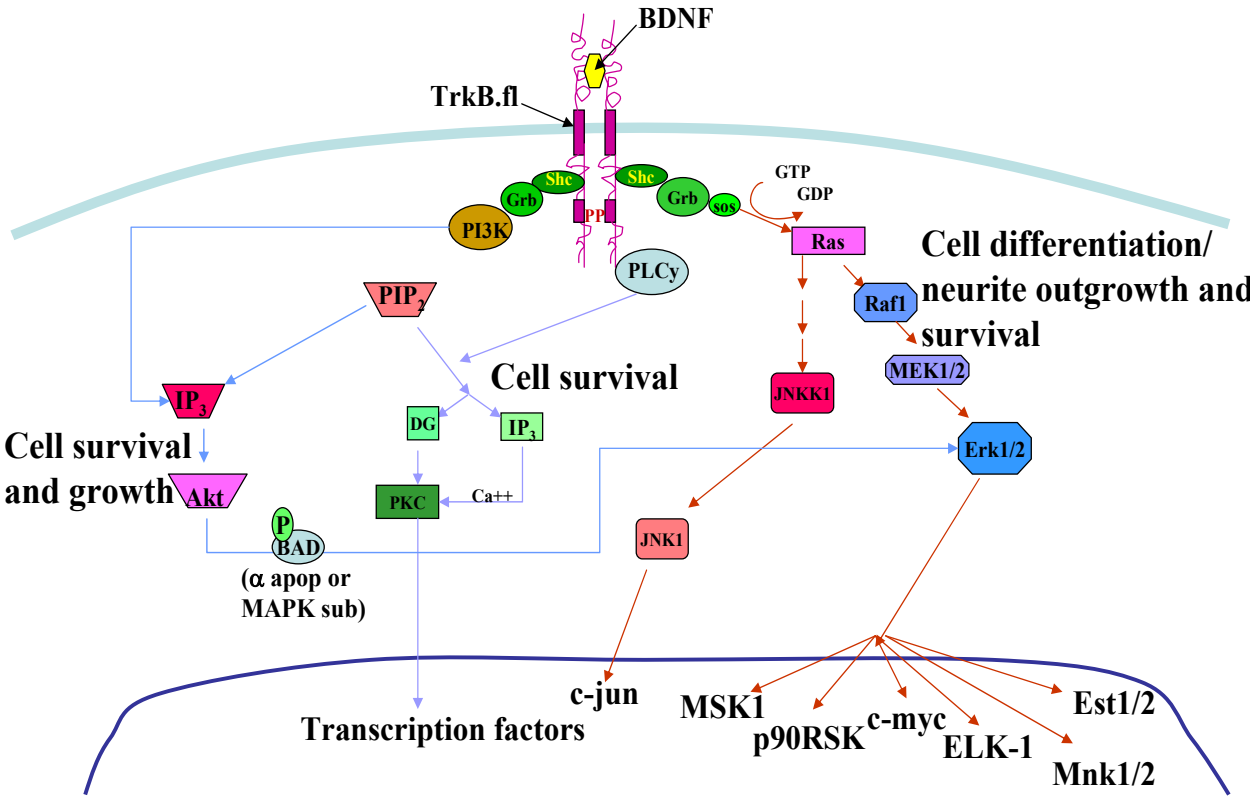
2.2 Intracellular signaling mediated by the full-length trkB receptor

The growth factor effects of BDNF are mediated through the activation of trkB.fl (Nakamura et al., 1996) (Easton et al., 1999) (Atwal et al., 2000) (Patapoutian and Reichardt, 2001). TrkB.fl activation is defined by the induction of autophosphorylation at tyrosines 484, 670, and 674 (McCarty and Feinstein, 1998). These tyrosines comprise the putative kinase loop. Activation of the putative kinase loop induces a conformational change in the receptor structure.

Substrate binding sites are exposed in activated trkB.fl homodimers. Adaptor proteins bind to these substrate binding sites to activate the phosphoinositide-3-kinase (PI3K), phospholipase C-gamma (PLCγ), and ras/mitogen-activated protein kinase (MAPK) intracellular signaling pathways (Figure 4) (McCarty and Feinstein, 1998). TrkB.fl directly binds to and activates PLCγ (Sommerfeld et al., 2000). Both PI3K and ras/MAPK are activated when the Shc protein binds to substrate binding sites on trkB.fl. Signaling pathways also crosstalk with one another. For instance, PI3K activates MAPK/Erk through the adaptor molecule Akt (Atwal et al., 2000). Signaling through these pathways leads to transcription factor activation. Downstream gene expression, such as bcl-2 and cAMP-responsive-element-binding-protein (CREB), stimulate cell survival and differentiation (Riccio et al., 1999).

Figure 4. TrkB.fl activates intracellular signaling pathways.

BDNF activates the trkB.fl homodimer. TrkB.fl activation induces conformational changes in the intracellular domain, and exposes intracellular substrate binding sites. TrkB.fl-mediated cell survival, differentiation, and proliferation are regulated through the PI3K, PLCγ, and MAPK/Erk pathways.

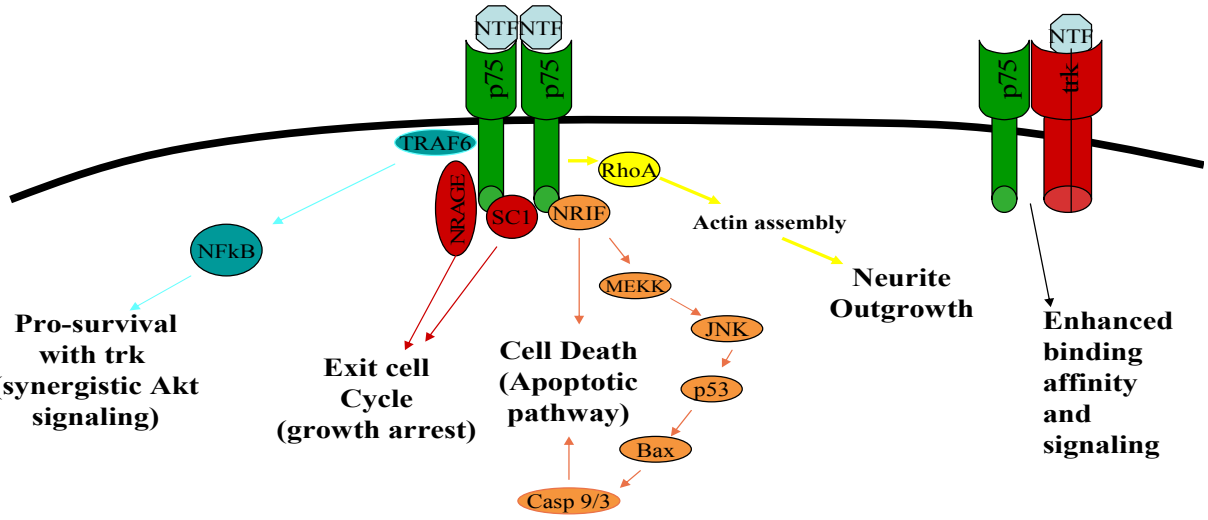


BDNF induces its neurotrophic effects through the activation of the high affinity trkB receptors and by binding to the p75 neurotrophin receptor ($p75^{NTR}$) (Carter and Lewin, 1997). $p75^{NTR}$ is the low affinity neurotrophin receptor that binds to all of the nerve growth factor neurotrophins. It modulates neurotrophin function by: 1) dimerizing with trk receptors to enhance the binding affinity, 2) interacting with trk receptors to enhance signaling, and 3)

initiating apoptotic signaling (for a detailed review, refer to Carter et al., 1997) (Carter and Lewin, 1997). BDNF activation of trkB, p75 ^{NTR}, and trkB/p75 ^{NTR} modifies the survival versus death response (Figure 5).

Figure 5. p75 ^{NTR} receptor-mediated signaling mechanisms (apoptosis Vs. survival).

p75 promotes cell survival by enhancing trk function and promotes cell death by activating the apoptotic signaling pathway.



3. Conclusions

BDNF is a potent neurotrophic factor that can modulate cell survival, proliferation, differentiation, synaptogenesis, and neurotransmission. BDNF functions are mediated by its high affinity tyrosine kinase receptor, trkB. There are two trkB isoforms whose translation is regulated by multiple promoters, polyadenylation sites, and alternate splicing. Traditional signaling cascades, PI3K, PLCγ, and MAPK, are activated through full-length trkB receptor

(trkB.fl) activation. Although the truncated trkB (trkB.tc) receptor is the most abundant in the adult brain, its function remains unclear. *The purpose of this dissertation is to identify the potential roles played by the truncated trkB receptor in the adult nervous system.*

This dissertation will address the following questions:

- Although trkB.tc does not activate any known signaling cascades, does it have an active role in BDNF function?
    - Does trkB.tc regulate BDNF function by mediating its intracellular sorting?
    - If so, is trkB.tc and internalized BDNF sorting as a protein complex?
- Does trkB.tc play a role in the maintenance of neuronal homeostasis in the brain?
    - Does the distribution of trkB.tc change in the PD brain, and if so, does is correspond to regions of Pathology?
- What is the correlation between the normal function of trkB.tc and its changes seen in disease?

2. Rationale and Hypothesis

Neurotrophic factors have been proposed as therapeutic targets in neurodegenerative diseases. Their pluripotent effects require a comprehensive understanding of the regulatory mechanisms involved neurotrophic function selection: survival, proliferation, differentiation, or modulation of neurotransmission. We have addressed one mode of neurotrophin regulation by elucidating the function of the truncated trkB receptor isoform. Understanding the role of the truncated trkB receptor in the normal brain will allow us to better understand the significance of changes in its protein expression and distribution in neurodegenerative diseases like Parkinson's disease.

1. Roles for truncated trkB receptor in the CNS

The role of trkB.fl in BDNF signaling has been largely identified. The role of the truncated receptor is still unclear. Dogma has described the role of the truncated trkB receptor in two ways: 1) as a dominant negative receptor that forms heterodimers with trkB.fl to inhibit its function (Eide et al., 1996) or 2) as a receptor that sequesters BDNF (Biffo et al., 1995). Recently, this dogma has been challenged to include a role for trkB.tc in cell signaling (Baxter et al., 1997) and neurite extension (Yacoubian and Lo, 2000).

Acidic metabolic release is a general means to study signaling cascade activation. TrkB.tc induced kinase-mediated metabolic release in Ltk- cells (Baxter et al., 1997). TrkB.tc signaling was inhibited by trkB.fl co-expression. This cell signaling may induce neurite extension (Yacoubian and Lo, 2000). Yacoubian et al. (2000) transfected trkB.tc and trkB.fl into ferret cortical slices and showed that the receptors mediate different modes of dendritic growth (Yacoubian and Lo, 2000). Over-expression of trkB.fl regulated proximal neurite growth and

overexpression of trkB.tc regulated distal neurite growth. Although these data indicate an active role for trkB.tc in BDNF-mediated signaling, its mechanism of function remains unclear.

2. BDNF in Parkinson's disease

Parkinson's disease (PD) is a debilitating disease, with peak onset in the sixth decade, characterized by rigidity, stooped posture, slowness of movement, resting tremor, and axial instability (Adams et al., 1997). This neurodegenerative disease affects both the motor and limbic systems (Braak and Braak, 2000). Hallmark pathological features in Parkinson's disease include Lewy bodies and neurites in the motor system nuclei and the substantia nigra. Currently, the treatment for Parkinson's disease is mainly limited to dopamine replacement (Dunnett and Bjorklund, 1999). This treatment does not target the underlying mechanisms of the disease, and over time becomes ineffective due to neuronal desensitization, cell death, and is riddled by side effects.

Potential therapeutic targets in Parkinson's disease, and other neurodegenerative diseases, are neurotrophic factors (Bradford et al., 1999). Neurotrophic factors are attractive therapies because they may target the underlying mechanisms of neurodegeneration.

BDNF mRNA and protein are decreased in the PD substantia nigra (Howells et al., 2000) (Mogi et al., 1999) (Parain et al., 1999). Because BDNF mediates cell survival and neuronal differentiation, the decrease in BDNF in the substantia nigra may contribute to the pathogenesis of PD. Furthermore, BDNF may be an excellent therapeutic target due to its potential role in reversing neurodegeneration.

The role of BDNF in the Parkinson substantia nigra is still not clear. Although many studies show that this protein is decreased in PD, there is conflicting evidence whether it induces

cell survival in dopaminergic neurons of the substantia nigra. Parain et al. (1999) showed that there was a substantial decrease in both BDNF-positive and BDNF-negative pigmented neurons in the PD substantia nigra. Because of the decrease in BDNF-positive neurons, Parain et al. (1999) concluded that BDNF does not mediate cell survival in these cells. This is not the only interpretation of these results. Pigmented neurons undergoing neurodegeneration most likely activate BDNF expression, but endogenous levels of BDNF in PD neurons may not be sufficient to induce survival. Consequently, it may be necessary for exogenous BDNF to be supplied to these neurons in order to promote survival.

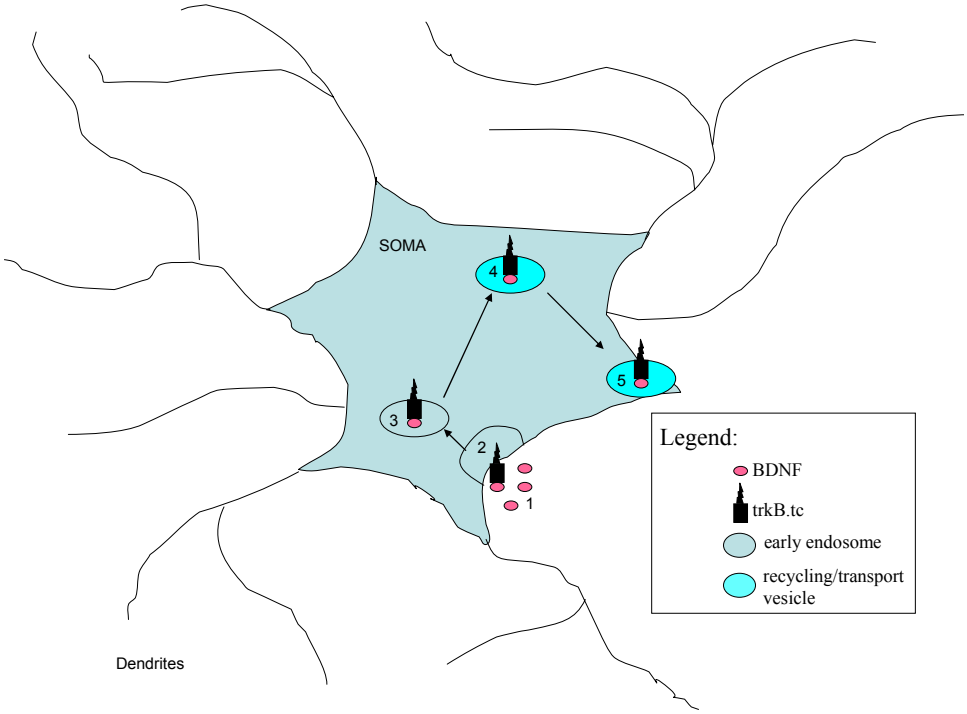
In the dopaminergic progenitor cell line SN4741, BDNF rescued neurons from 1-methyl-4- phenylpyridinium (MPTP) neurotoxicity (Son et al., 1999). MPTP and 6-OHDA are specific toxins for dopaminergic neurons and are used in many animal models of PD. The neuroprotective affects of BDNF in neurons exposed to MPTP toxicity supports the hypothesis that BDNF does indeed protect degenerating dopaminergic neurons. Furthermore, BDNF can reverse the motor deficits caused by 6-OHDA toxicity in *in vivo* models of PD (Klein et al., 1999). BDNF can reverse both the pathological and clinical symptoms in animal models of PD, and therefore may be a potential therapeutic target in PD.

3. Hypothesis

We hypothesize that the truncated trkB receptor mediates the endocytic sorting of internalized BDNF and its subsequent translocation to distal full-length trkB receptors. Specifically, trkB.tc receptors at the plasma membrane bind to and internalize BDNF via receptor mediated endocytosis. The trkB.tc/BDNF complex is sorted to recycling or transport vesicles and then translocated to distal trkB.fl receptors (Figure 6).

Figure 6. Intracellular translocation of internalized BDNF by trkB.tc.

BDNF binds to trkB.tc at the cell surface (1). It is internalized via receptor-mediated endocytosis (2) and sorted to early endosomes (3). The trkB.tc/BDNF complex is then sorted to recycling or transport vesicles (4) where it can later be translocated (5).



The translocation and subsequent release of BDNF by trkB.tc to distal trkB.fl receptors may be necessary for the maintenance of synaptic connections between neuronal populations. For example, BDNF mRNA and proteins are altered in Parksinson's disease. If the distribution and expression of trkB.tc in Parkinson's disease is also altered, this may contribute to impair BDNF function. Specifically, if the transport of trkB.tc is impaired, it can not chaperone the translocation of internalized BDNF to trkB.fl receptors. This would result in the accumulation of BDNF at the soma and its subsequent lysosomal degradation, resulting in aberrant distribution of

BDNF protein. The disruption of BDNF transport in PD may inhibit the availability of BDNF to striatal and nigral neurons and its subsequent neuroprotective effects in these regions.

3. Materials and Methods

1. Receptor-mediated endocytosis of b-BDNF by SH-SY5Y cells

1.1 Biotinylation of rhBDNF

rhBDNF was biotinylated using the FluoReporter Mini-Biotin-XX protein labeling kit as specified in the Molecular Probes protocol. The FluoReporter Mini-Biotin-XX kit will label 0.5-3mg/ml of protein with water soluble biotin-XX sulfosuccinimidyl ester. Biotin is covalently linked to the amines of protein via two aminohexanoic chains, resulting in a 14-atom spacer. This optimizes the binding of the biotin moiety with avidin. For our experiments, 200ul of 0.5mg/ml BDNF solution was added to a reaction tube with 20µl 1M sodium bicarbonate solution. Reactive biotin-XX solution (2µl) was added to the reaction tube and mixed thoroughly. This concentration of biotin-XX yields between 3-8 biotin molecules per labeled protein. The reaction was incubated at room temperature, with constant stirring, for 1.5hours. The BDNF reaction solution was added to a spin column, provided in the kit, and centrifuged at 1100xg for 5min at room temperature. The purification spin yields 70-80% biotinylated BDNF in 250ul PBS that was stored at -80^{o}C in aliquots.

1.2 Differentiation of SH-SY5Y neuroblastoma cells

100mm Primeria Petri dishes were coated with 0.5mg/ml mouse laminin overnight at 37^{o}C. Dishes were washed 3x5minutes in water and stored at -20^{o}C in PBS. 12mm glass coverslips were inserted into each well of a 24-well plate. The coverslips were coated with 5mg/ml polyornithine overnight at 37^{o}C. The coverslips were then washed 3x5minutes in water and coated with 0.5mg/ml mouse laminin overnight at 37^{o}C. Coverslips were washed

3x5minutes in water and then stored at -20^{o}C in PBS. Prior to plating, dishes and coverslipped plates were washed 2x5 minutes in room temperature PBS.

Undifferentiated SH-SY5Y cells (4x10^{4} cells/ml) were plated on laminin coated primeria petri dishes for molecular biology analyses and on polyornithine/laminin coated 12 mm round glass coverslips in 24 well plates for microscopy analyses. At day 0, SH-SY5Y cells were cultured in base media (10% FBS and 1% penicillin/streptomycin in DMEM). Base media or treatment media was used in 24-well plates (500µl) and in 100mm Petri dishes (10mls) for all treatments. There are three stages of SH-SY5Y differentiation: 1) undifferentiated, 2) retinoic acid (RA) differentiation, and 3) fully differentiated (RA/BDNF). Undifferentiated cells were treated with base media in parallel with the treatments for RA and fully differentiated cell treatments. RA differentiated cells were treated with RA (10µM) at days 1, 3, 5, and 7 *in vitro*. For cultures that were RA differentiated, we increased the initial plating concentration to 1x10^{5}cells/ml. RA inhibited cell growth. Therefore, we needed the higher initial cell concentration to yield sufficient cellular material for molecular analysis. This was not necessary in fully differentiated cells, presumably due to an increase in cell proliferation as well as differentiation when BDNF was introduced into the cultures. For fully differentiated cells, cultures were treated with Retinoic acid in the base media at days 1, 3, and 5 *in vitro*. At days 6, 8, and 10 *in vitro*, cultures were treated with 50ng/ml BDNF. For b-BDNF differentiation studies, cultures were treated with 50ng/ml b-BDNF instead of BDNF (refer to Table 1 for treatment schedules).

Table 1. Schedule of differentiation treatments in SH-SY5Y cells.

This table is a representation of the treatment schedule for undifferentiated, RA differentiated, fully differentiated, and b-BDNF fully differentiated SH-SY5Y cultures.

Differentiation stage	Day 0 *in vitro*	Day 1 *in vitro*	Day 3 *in vitro*	Day 5 *in vitro*	Day 6 *in vitro*	Day 7 *in vitro*	Day 8 *in vitro*	Day 9 *in vitro*	Day 10 *in vitro*	Day 11 *in vitro*	Day 12 *in vitro*
Undifferentiated											
RA differentiated											
Fully Differentiated											
B-BDNF Fully differentiated											

Color legend

- Cultures plates on D0
- Base Media change (control)
- 10μM RA
- 50ng/ml BDNF
- 50ng/ml b-BDNF
- 50ng/ml b-BDNF for organelle and MAPK experiments

1.3 b-BDNF pulse-labeling of differentiated SH-SY5Y cultures

Only fully differentiated cultures were used for b-BDNF pulse-labeling studies. At day 11 *in vitro*, a complete media change was performed on differentiated SH-SY5Y cells with base media. These were treated with 50ng/ml b-BDNF for 10 minutes at 4^{o}C. At this time, the b-BDNF media was aspirated, the cultures were washed 2x1minute base media, and replaced with fresh base media at room temperature. The cultures were harvested at 10, 15, 30, 60, and 120 minutes post-b-BDNF treatment for molecular and microscopy analyses. For molecular analysis,

cultures were washed 3x1minute in PBS. Then, 1ml RIPA buffer (1xPBS, 1% Igepal, 0.5% sodium deoxycholate, 0.1% SDS, 10mg/ml PMSF in isopropanol, aprotinin, and 100mM sodium orthovanadate) was added to each Petri dish. The cells were mechanically removed with a cell scraper and frozen in 0.5ml aliquots at -20^{o}C. For microscopy analysis, the 24-well plates were rinsed 3x1minute in PBS and then fixed in 4% paraformaldehyde in PBS for 15 minutes. The cells were washed 3x5minutes in PBS and then stored at 4^{o}C in PBS.

2. Microscopic analysis of the endocytic sorting of B-BDNF and trkB

2.1 Light microscopy analysis of differentiated SH-SY5Y cultures

We characterized the cellular morphology of undifferentiated, RA-differentiated, BDNF-differentiated, and b-BDNF-differentiated cultures. Phase-contrast images of live SH-SY5Y cultures, of each differentiation stage, were captured with a 40x objective and imaged with a Nikon Eclipse TE200 fluorescent microscope and Spot RT v3.4 image software. A qualitative analysis of cellular morphology was assessed.

2.2 Immunofluorescent labeling and laser confocal microscopy analysis of trkB and B-BDNF in differentiated SH-SY5Y cultures

SH-SY5Y cells were washed in PBS and fixed in 4% paraformaldehyde for 15min. Following fixation, the cells were washed in PBST (0.1% Tween-20 in PBS) and incubated for 30 minutes in 10% normal donkey serum in PBST at 37^{o}C to permeabilize the membrane and block non-specific binding. The primary antibodies were added and incubated for 2 hours at room-temperature. Table 2 lists the primary antibodies used in these experiments. Double and

triple label experiments used a cocktail of both (double-label) or triple labels (triple-label) in a single incubation. This was possible because all antibodies used in these reactions were raised in different species to avoid cross-reaction. Secondary antibodies were added as a cocktail for a half hour at room temperature. In the double-label experiments, the goat secondary antibody to trkB.fl was conjugated to Alexa 488 and the rabbit antibody to trkB.tc was conjugated to Cy3. In triple-label experiments, the secondary antibody to trkB.tc, b-BDNF, and the organellar marker were conjugated to Alexa-488, Cy3, or Alexa-647, respectively and b-BDNF was detected by Cy3-streptavidin. The cells were washed in PBS and mounted using gelvatol (23 g polyvinyl alcohol 2000, 50 ml glycerol, 0.1% sodium azide to 100 ml PBS).

Table 2. List of primary antibodies used in the microscopy and western blot experiments.

Antibody	Target	Species	Source	Cat #	Concentration
TrkB [TK-]	TrkB.tc	Rabbit	Santa Cruz Biotech	sc-119	1:200 IFL, WB
TrkB [794]	TrkB.fl	Goat	Santa Cruz Biotech	sc-12	1:200 IFL, WB
TrkB [H-181]	trkB.tc and trkB.fl	Rabbit	Santa Cruz Biotech	sc-8316	1:200 WB
α-Adaptin	Endosomes	Mouse	ABR	MA3-061	1:500 IFL
AC17 (Lamp-2)	Lysosome	Mouse	E.Rodriugez Boulan, Cornell University	(Nabi and Rodriguez-Boulan, 1993)	1:1000 IFL
Rab4	Recycling endosome	Mouse	BD Transduction Labs	610888	1:500
SMI 52	MAP2	Mouse	Sternberger Monoclonals	SMI 52	1:500 IFL
Pan-NF	Neurofilament	Mouse	Zymed, Inc	18-0171	1:500 IFL
streptavidin, AlexaFluor® 546 conjugate	Biotin-BDNF	NA	Molecular Probes	S11225	1:200

2.3. Metamorph analysis of b-BDNF and trkB distribution within organelles

Samples were imaged with the Olympus Fluoview BX61 confocal microscope and analyzed with the Metamorph Image software (Metamorph Offline Version 6.2r6, 2004, Universal Imaging Corporation, Downingtown, PA). Specifically, four separate experiments were performed with four separate 600x fields and 5 optical sections per stack. There was an increment of 0.5μm between sections of each stack. The first section between each stack was generated from approximately the same z-plane through the cell. This was determined by the distance of the first section from the glass coverslip. Select z-sections were merged to create a 3-D confocal image. We analyzed each z-section to quantify the co-localization of double- and triple- label samples. First, the threshold area of the co-localized fluorescence was determined. Threshold area is the area of fluorescence, in this case double and triple label co-localization, in each Z-section analyzed. We also determined the area of the entire cell. Co-localization threshold area was normalized to total cell area and calculated by dividing "co-localization threshold area" by "total cell area". This value represents the percent co-localization of double- and triple- labels.

The statistical software SPLUS-2000 and Microsoft excel were used for statistical analyses. Microsoft excel was used to generate graphs showing changes in trkB.tc/b-BDNF distribution over time and within organelles. SPLUS-2000 was used to run one-way ANOVA and two-sided t-tests to determined statistical significance between timepoints and organelles. Each experimental condition had an N=20. This sample number was obtained from 4 sections of 5 stacks per experimental condition. Statistical analyses were performed for changes in total, endosomal, lysosomal, and recycling vesicle localization of trkB.tc.

3. Western blot analysis of the endocytic sorting of B-BDNF and trkB

Proteins generated from the b-BDNF treatment experiments were thawed and sonicated to generate protein lysates for western blot analysis. Protein quantity was determined by Bicinchoninic Acid Solution (BCA) protein assay. Specifically, 1 and 3 µl samples of SH-SY5Y lysates were added to the BCA/copper sulfate solution, incubated at 37^{o}C for 1hour, and read on a spectrophotometer at 570nm (λ_1).

Lysates were separated by SDS-PAGE electrophoresis. For each gel, both kaleidoscope and biotinylated protein standards were used. All samples were diluted in 2x sample buffer with 20% 2-mercaptoethanol. Precast 10-20% mini-gels were used with the mini protean 3 electrophoresis system at 125V, 85mA, and 1.5hours. Proteins were transferred from the gel to a PVDF membrane for 1hour at 100V, 250mA, and in cold transfer buffer. The PVDF membrane was blocked with 5% non-fat milk (NFM) for 30minutes. Primary antibody was incubated in 1% NFM in PBST at 4^{o}C, overnight. Secondary antibody was incubated in 1% NFM for 1hour at room temperature. The antibody complex was amplified with ABC complex and detected by chemiluminescence reactions. To control for gel loading variability, we probed each membrane for actin, pMAPK, and trkB.tc proteins.

4. Immunomagnetic isolation of endosomes

Prior to isolation, the Dynabeads were coated with 10µg of donkey-anti-rabbit secondary antibody. The Dynabead/antibody solution was incubated for 16-24hr at 37°C with slow tilt rotation. After incubation, the supernatant is removed and the beads are washed four times: two times in 0.1% normal donkey serum in PBS for 5 minutes at room temperature, one time in 0.2M

Tris (pH8.5) solution for 24 hours at 20°C, and a final wash in 0.1% normal donkey serum in PBS for 5 minutes in room temperature. We used the EEA1 primary antibody for our isolation. 4-8 µg of EEA1 primary antibody/10^7 Dynabeads was added to the Dynalbeads. This solution is incubated for 30 min (or overnight if convenient) at 2-8°C using bidirectional rotation. After incubation, the supernatant is removed and the beads are washed four times in 0.1% normal donkey serum in PBS at 2-8°C. The coated beads were used to isolate early endosomes from differentiated SH-SY5Y cells pulse-chased with biotin-labeled BDNF.

Cells were lysed in Organelle lysis buffer (0 .25M sucrose, 10mM Tris, pH 7.4, 1mM EDTA, 0.1mM PMSF, 1µm/ml aprotinin, and sodium orthovanidate). 100µg of cell lysates were added to 1mg of coated dynalbeads. Mix this solution for 6 hours at 2-8°C with bi-directional rotation. Remove supernatants and wash the beads in Buffer A (2mM EDTA and 5% normal donkey serum in PBS) for 3 x 15 minutes. Resuspend beads in RIPA buffer and sonicate the organelles from the beads. Remove supernatants and collect for western blot analysis.

5. Immunoprecipitation of trkB.tc and b-BDNF

We used the Catch and Release™ Immunprecipitation kit from Upstate Biotechnology to immunoprecipitate trkB.tc proteins from lysates of differentiated SH-SY5Y cells. First, suspend 500µg of lysates in 500µl of 1x Catch and Release Lysis/Wash Buffer. Add 4µg of trkB.tc antibody and 10 µl of Antibody Capture Affinity Ligand to this solution. Incubate at room temperature for 15 minutes, rocking. Transfer this solution to the spin column (with 500µl per spin column). Centrifuge 5-10 minutes at 1,500xg at room temperature. Wash 3 times with 500µl of wash buffer for 3 minutes at 2,000xg. Add 30µl of IP Elution Buffer to spin column and centrifuge for 2 minutes, 500xg. Collect flow-through and use in western blot analysis.

6. Archival human autopsy brain tissues

6.1 Human autopsy control and Parkinson's diseased (PD) brain tissues

Paraffin embedded archival material was obtained from the London Health Science Center, (London, Ontario, Canada), and the Department of Pathology, University of Pittsburgh School of Medicine. Human brain tissues obtained at autopsy were fixed in formalin within 24 hours of death, for 7 days, and then embedded in paraffin. Sections of 4μm thickness were cut and mounted onto microscopic slides (Fisher Scientific, Springfield, NJ). Five PD cases (one familial and four idiopathic; average age: 75.8) and three age- matched control subjects free of neurological disease (average age: 72) were used for this study.

The diagnosis of PD was based on the clinical history and typical histopathological findings including neuronal loss, gliosis, pigmentary incontinence and Lewy body formation in the substantia nigra and locus coeruleus. Neocortical regions were unremarkable. There was no evidence of AD, DLB, progressive supranuclear palsy or corticobasal degeneration. Control patients died of complications following myocardial infarcts and their brain tissue was histologically normal.

6.2 Nova red labeling and light microscopy analysis of trkB in the frontal cortex, striatum, and substantia nigra of control and PD tissues

Tissue sections were baked at 55^{o}C for 30minutes and were deparaffinized 3x10minutes in histoclear. Then, they were rehydrated through a series of decreasing concentrations of alcohol (100, 100, 95, 90, 70%). Endogenous peroxidase was quenched with 3% H_2O_2 in methanol for 10 minutes at room temperature. For antigen retrieval, 0.4% pepsin was added to

the tissue for 20 minutes at 37°C. Tissue sections were incubated with 10% normal donkey serum for 30 minutes at 37°C. The primary antibodies (see table 3) were added for 3 hour, at room temperature. The biotin-conjugated secondary antibodies were added for 30 minutes at room temperature. The biotin complex was amplified with the ABC kit for 30 minutes at room temperature. The color reaction was developed with the Nova Red chromagen for approximately 5 minutes. The slides were coverslipped using aqueous mount. Panels in Figures 22-24 were collected with a 60x objective and inserts in figures were collected with a 100x objective. Images were captured on a Nikon Eclipse TE200 fluorescent microscope with Spot RT v3.4 image software.

Table 3. Antibodies used in paraffin-embedded human autopsy tissues.

Antibody	Target	Species	Company	Concentration
TrkB [tk-]	TrkB.tc	Rabbit	Santa Cruz Biotech	1:200
TrkB [794]	TrkB.fl	Goat	Santa Cruz Biotech	1:200
SMI52	MAP2/ dendrites	Mouse	Sternberger monoclonal	1:500
Pan-neurofilament	Axons	Mouse	Zymed Inc	1:500
Alpha-synuclein	Dystrophic neurons	Mouse	Zymed, Inc	1:500
GFAP	Astrocytes	Guinea Pig	Advanced Immunologicals	1:1000

6.3. Immunofluorescent labeling and laser confocal microscopy analysis of trkB in the striatum and substantia nigra of the PD brain

For immunofluorescence, tissues were deparaffinized and dehydrated as per light microscopy. Following pepsin treatment, tissues were labeled with trkB.tc or trkB.fl antibodies for 2hrs at room temperature. Tissues labeled for trkB (tc or fl) were then double-labeled with an antibody to one of the following proteins: Map2 (microtubule associated protein), pan-neurofilament, TH, glial fibrillary acidic protein (GFAP), Ricinus Communis Agglutinin I (RCA-1), and alpha-synuclein. Fluorescence-conjugated secondary antibodies were used at 1:500 dilution for 30 minutes at room temperature. In all images, trkB.fl is labeled with the Alexa 488 tag and trkB.tc is labeled with a Cy3 tag. Tissues were coverslipped with gelvatol (23 g polyvinyl alcohol 2000, 50 ml glycerol, 0.1% sodium azide to 100 ml PBS) and air-dried overnight at room-temperature. Images were captured on an Olympus Fluoview BX61 confocal microscope. Panels in Figures 25-29 were collected with a 60x objective. A qualitative analysis of images generated by Metamorph software was performed to observe changes in protein distribution.

4. Truncated trkB facilitates the endocytic sorting of internalized BDNF in differentiated SH-SY5Y cells

We hypothesized that trkB.tc facilitates transport of BDNF to distal trkB.fl receptors. To determine if trkB.tc mediates the endocytic sorting of BDNF, we followed the intracellular transport of the trkB.tc/b-BDNF complex in SH-SY5Y cells. Specifically, we studied the 1) internalization of the trkB.tc/b-BDNF complex via receptor-mediated endocytosis and 2) its subsequent sorting to lysosomes or recycling endosomes. We found that trkB.tc and b-BDNF are co-localized within early endosomes during early timepoints and to recycling endosomes at later timepoints post-b-BDNF treatment. Furthermore, trkB.tc/b-BDNF localization was significantly less in lysosomes than in early or recycling endosomes.

1. SH-SY5Y cells differentiate into a neuronal morphotype.

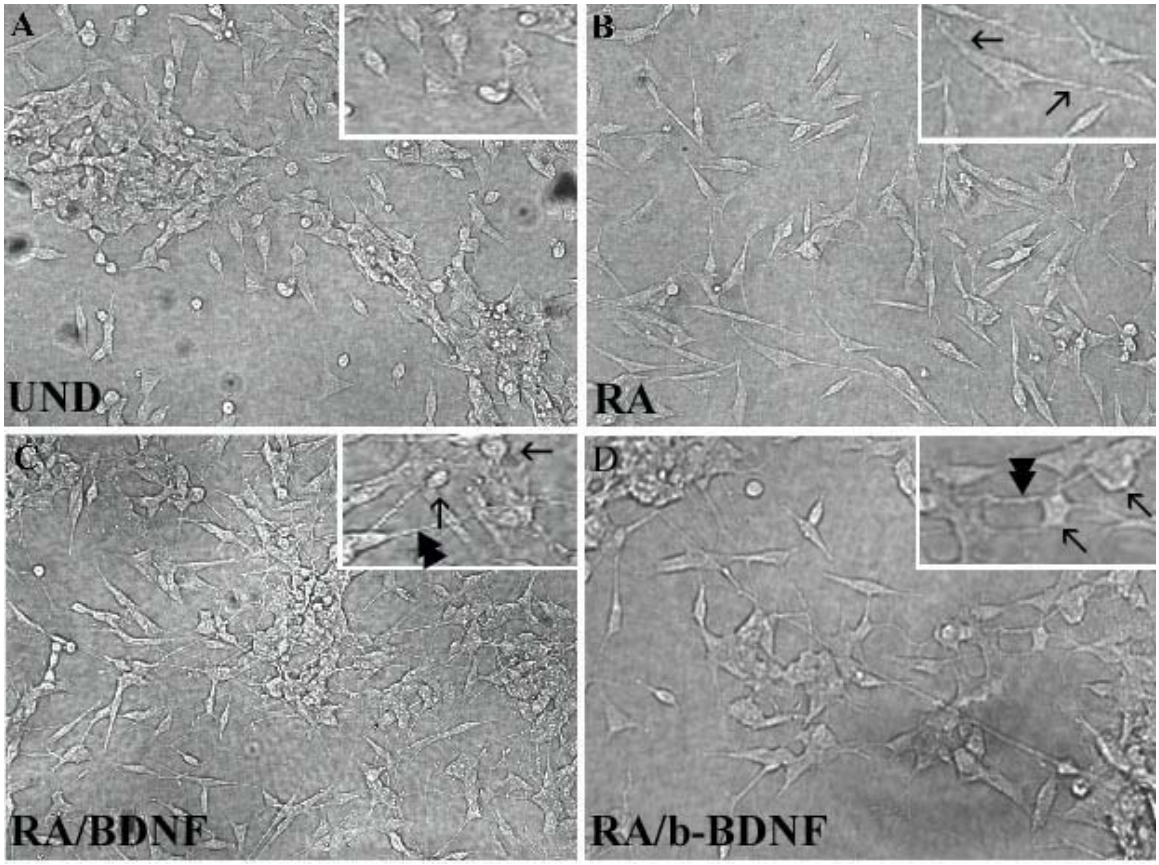
The SH-SY5Y cell-line is derived from neuroblastoma cells. There are three major benefits to using this cell line: 1) they differentiate into a neuronal morphotype by successive incubation with retinoic acid (RA) and BDNF (Encinas et al., 2000) (Simpson et al., 2001), 2) they express trkB receptors and respond to BDNF stimuli (Encinas et al., 2000), and 3) they express dopaminergic proteins and respond to 6-OHDA and MPTP toxicity (Simpson et al., 2001) (Yu and Zuo, 1997). These cells respond to BDNF-stimuli via trkB.fl by activating PLCγ and MAPK (Encinas et al., 2000).

SH-SY5Y cultures were differentiated into a neuronal phenotype by successive incubation with retinoic acid (RA) and BDNF (Figure 7) (Encinas et al., 2000) (Simpson et al., 2001). Undifferentiated SH-SY5Y cells were small and angular (Figure 7a). Retinoic acid

treatment (10uM for 7 days) induced elongation of the cells (Figure 7b) (Simpson et al., 2001). Following 5 days of RA treatment, 50ng/ml of BDNF or b-BDNF was added for 5 days to induce further differentiation. BDNF and b-BDNF treatment yielded cells with a rounded soma and extensive neurites (Figure 7c,d).

Figure 7. BDNF or b-BDNF is necessary to fully differentiate SH-SY5Y cells.

A. Undifferentiated (UND) SH-SY5Y cells are small, angular, non-polarized cells. B. Retinoic acid (RA, 10uM) induces elongation of the soma and extension of neurites (arrows). C. Rentinoic acid followed by 50ng/ml BDNF (RA/BDNF) treatment further differentiates SH-SY5Y cells. The soma are small and rounded (arrows) while the processes (double arrowhead) become more complex. D. Retinoic acid followed by 50ng/ml b-BDNF (RA/b-BDNF) treatment induces a morphotype consistent with RA/BDNF treatment. Biotinylated BDNF can also induce SH-SY5Y differentiation. (Magnification = 40x)



Neuronal polarization was characterized by immunofluorescent labeling of MAP-2 (dendritic) and neurofilament (axonal) proteins (Figure 8). As the SH-SY5Y cell became more differentiated, MAP2 labeling revealed increased dendritic complexity (Figure 8a-d). Dendritic projections were visible in the RA treatment group (Figure 8b), but became most extensive following further differentiation with BDNF or b-BDNF (Figure 8c,d). Neurofilament proteins were negligible in both the untreated and RA treated groups (Figure 8e,f). Cultures further treated with BDNF or b-BDNF extended single elongated axons (Figure 8g,h). Figure 8g emphasizes that each neurofilament-positive cell is associated with only one elongated axon and shows that b-BDNF was able to fully differentiate SH-SY5Y cells. From this point on, we will use the term "differentiated" to refer to SH-SY5Y cells differentiated by sequential RA and BDNF.

To investigate the role of trkB.tc in the endocytic sorting of internalized BDNF, we treated SH-SY5Y cells with biotin-labeled BDNF. Because we were only interested in the sorting of internalized BDNF, we needed to label BDNF with a molecular tag. We used the biotin tag because, unlike radioactive and fluorescent labels, biotin is a very versatile molecule that can be used in a plethora of analytical techniques. In this study, we treated cultures with biotin labeled BDNF (b-BDNF) and used the harvested cultures for both immunofluorescence and western blot analyses. We validated the bioactivity of b-BDNF prior to experimentation. Figures 7 and 8 show that b-BDNF could stimulate differentiation. To further confirm the bioactivity of b-BDNF, we assayed its ability to activate phosphorylated MAPK (pMAPK). Figure 9 shows that b-BDNF stimulates sustained activation of pMAPK in differentiated SH-SY5Y cells from 15 to 60 minutes post-b-BDNF treatment. This is consistent with the time of BDNF-induced pMAPK activation previously described (Meyer-Franke et al., 1998). Therefore,

b-BDNF was able to bind to trkB.fl, induce autophosphorylation of the homodimer, and activate intracellular signals necessary for differentiation to occur.

Figure 8. Retinoic acid (RA) and BDNF differentiation of SH-SY5Y cells yields a neuronal morphotype.

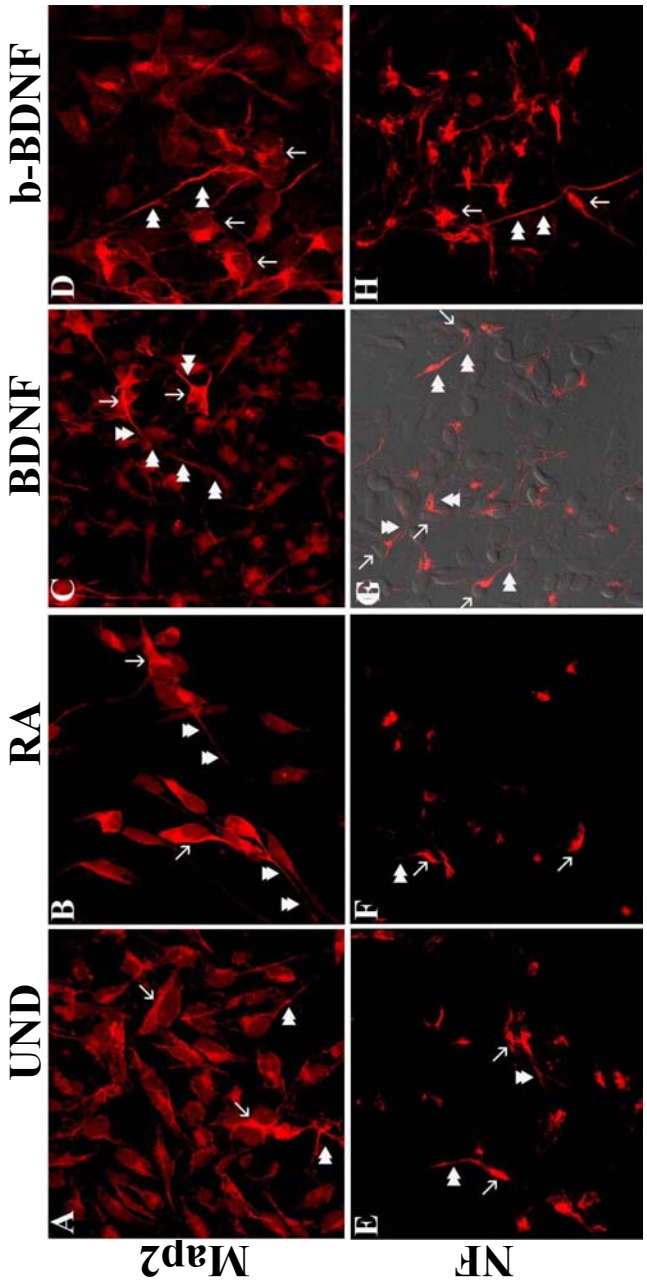
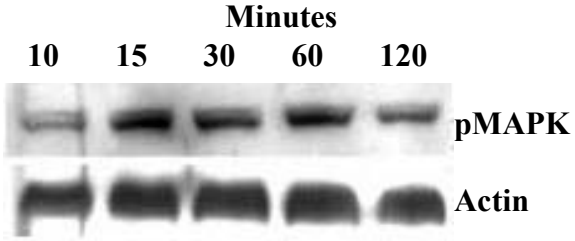


Figure 8. Retinoic acid (RA) and BDNF differentiation of SH-SY5Y cells yields a neuronal morphotype. **A.** Undifferentiated (UND) SH-SY5Y cells are small angular cells (arrows) but a few have short dendritic (Map2+) processes (double arrowhead). **B.** RA induces elongation of the soma (arrowheads) and extension of dendritic processes (double-arrowheads). **C.** RA/BDNF treatment induces the formation of complex dendritic branching (double arrowheads). **D.** Replacing BDNF with b-BDNF does not reduce the complexity of dendritic branching (double-arrowheads). **E.** Neurofilament (NF) proteins are limited to the soma (arrows) and isolated short axons (double-arrowheads) in undifferentiated cells. **F.** RA does not further induce axonal elongation. **G.** RA/BDNF treatment induces the formation of long axonal processes (double-arrowheads). Differentiated cells extend a single neurofilament-positive axon (arrows). **H.** B-BDNF treatment induces neuronal polarization as seen with BDNF treatment. (Magnification = 60x)

Figure 9. b-BDNF treatment of differentiated SH-SY5Y cells activate phosphorylated MAPK.

RA/BDNF differentiated cells treated with b-BDNF show sustained pMAPK activation from 15 to 60 minutes.

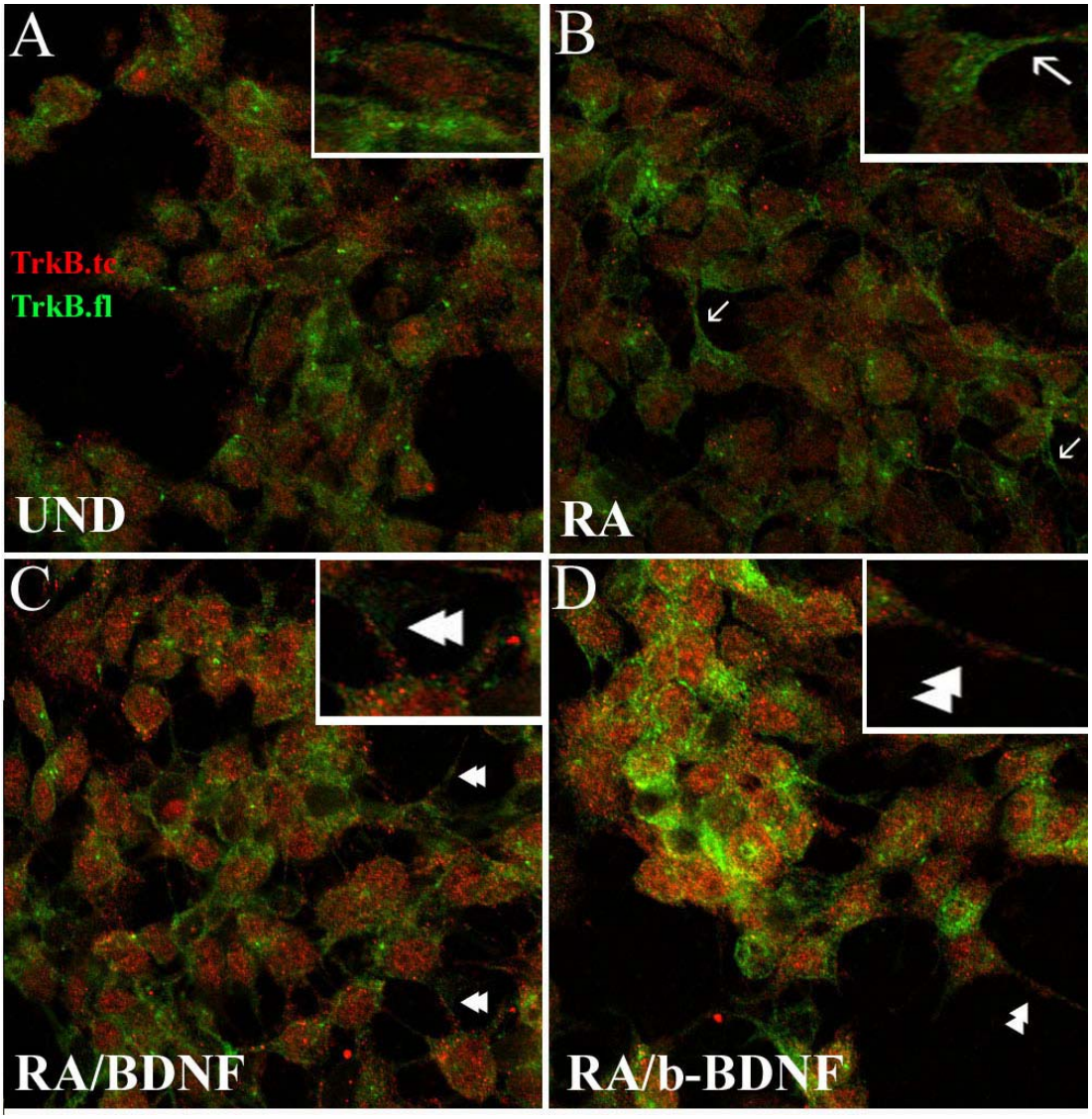


2. TrkB.tc is abundantly expressed in SH-SY5Y cells

We characterized the distribution of trkB.tc and trkB.fl proteins in undifferentiated, RA differentiated, RA/BDNF differentiated, and RA/b-BDNF differentiated SH-SY5Y cells by immunofluorescence laser confocal microscopy (Figure 10). Throughout all differentiation groups, we observe that trkB.tc and trkB.fl proteins are abundant yet differentially distributed within the cell. Although the trkB proteins are widely distributed within the soma, their co-localization is minimal. TrkB.tc is more abundantly distributed throughout the neuron than trkB.fl. In neurites present in RA differentiated cells, trkB.fl protein is widely distributed throughout the somata and proximal neurites (Figure 10b, arrows). Characterization of neuronal polarization seen in Figure 8b indicates that these trkB.fl-positive neurites are dendritic processes. The TrkB.tc isoform is more abundantly expressed in neurites of RA/BDNF and RA/b-BDNF differentiated cells than RA differentiated cells (Figure 10c,d). TrkB proteins localized within the same neurites do not appear to co-localize (Figure 10c,d, arrowheads).

Figure 10. TrkB.tc is differentially distributed from trkB.fl in differentiated SH-SY5Y cells.

A. In undifferentiated SH-SY5Y cells, trkB.tc (red) and trkB.fl (green) are differentially distributed. B. TrkB.fl is localized to dendrites in RA differentiated cells (arrows). C,D. In RA/BDNF and RA/b-BDNF differentiated cells, trkB.tc and trkB.fl are differentially distributed within neurites. Neurites in these cells show minimal co-localization of trkB.tc and trkB.fl (double-arrowheads). Also, trkB.tc protein is more abundantly expressed than trkB.fl. (Magnification = 600x).



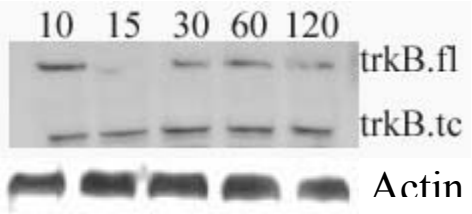
3. TrkB.tc is associated with internalized BDNF

Sommerfield et al. (2000) showed that trkB.fl is downregulated following BDNF binding and internalization, but that trkB.tc is not downregulated in primary cerebellar granule neuronal cultures. To verify that trkB.tc is not degraded following b-BDNF binding and internalization, we looked at the protein expression of trkB.tc in differentiated SH-SY5Y cells following b-BDNF internalization. Western blot data confirm that total amount of trkB.tc protein is abundantly expressed at all timepoints analyzed (Figure 11a) while trkB.fl protein distribution changes over time (Figure 11a). Furthermore, we performed an immunoprecipitation (IP) with an antibody specific for trkB.tc. Western blot analysis revealed that b-BDNF protein was covalently linked with trkB.tc (Figure11b). Unfortunately, the protein generated from the IP was at the border of detection for the western blot assay. Therefore, we could not normalize the amount of protein loaded between lanes. It was necessary to load maximum volume of protein isolates in order to detect any b-BDNF.

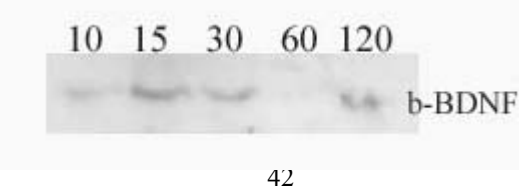
Figure 11. Association of trkB.tc with b-BDNF in differentiated SH-SY5Y cells.

A. Western blot analysis shows that trkB.tc proteins are abundantly expressed at all timepoints (in minutes) analyzed. TrkB.fl expression varies over time. B. Immunoprecipitation lysates show that b-BDNF is associated with trkB.tc.

A.



B.



To further determine if trkB.tc is associated with internalized BDNF following internalization, we studied the cellular distribution of trkB.tc and b-BDNF in differentiated SH-SY5Y cells. Surprisingly, the association of trkB.tc and b-BDNF at 10 minutes (Figure 12a-c) is significantly less than at later timepoints (Figure 12d-o). This phenomenon may be a result of b-BDNF arbitrarily binding to trkB.fl or trkB.tc receptors. This is to be expected as BDNF binds to both trkB.tc and trkB.fl with equal affinity and avidity. The initial increase in trkB.tc/b-BDNF co-localization over time may result from the downregulation of trkB.fl receptors following BDNF stimulation (Sommerfeld et al., 2000). Later, increases in trkB.tc/b-BDNF association over time may be attributed to the endocytic sorting of the trkB.tc/b-BDNF complex. Overall, co-localization of trkB.tc and b-BDNF saturates by 60 minutes and is decreased by 120 minutes (Figure 13). This decrease in co-localization may result from 1) selective degradation of some of the trkB.tc and/or b-BDNF proteins, 2) intracellular release of b-BDNF, or 3) extracellular release of b-BDNF.

Figure 12. TrkB.tc protein (green) expression is abundant and is associated with b-BDNF (red) over all timepoints.

A-C. TrkB.tc co-localization with b-BDNF is first apparent at 10minutes. D-F. At 15 minutes, trkB.tc and b-BDNF co-localization begins to increase. G-I. TrkB.tc and b-BDNF co-localization continues to increase at 30 minutes. J-L. At 60 minutes, trkB.tc/b-BDNF co-localization becomes saturated. M-O. TrkB.tc/b-BDNF co-localization is decreased by 120 minutes.

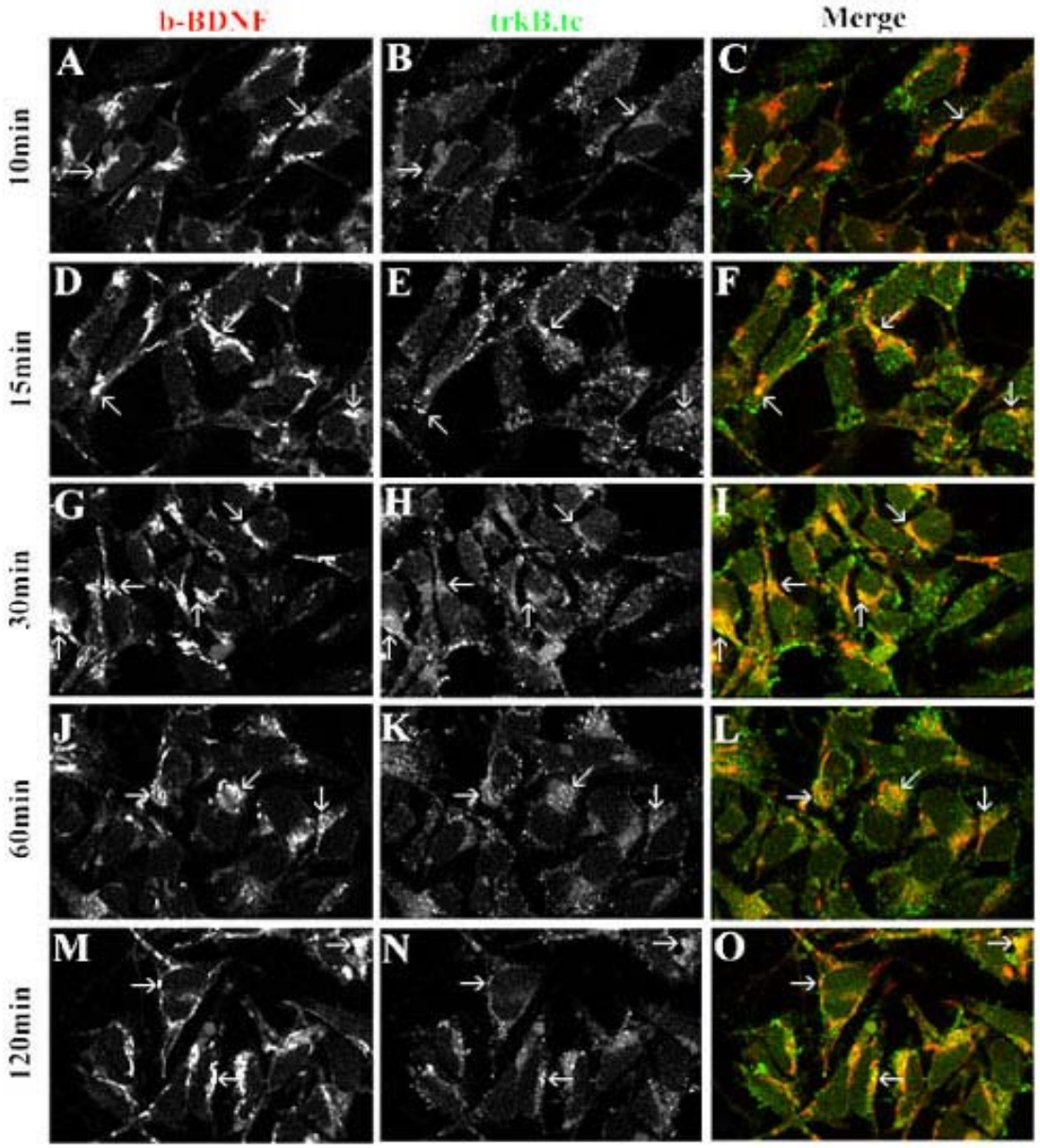
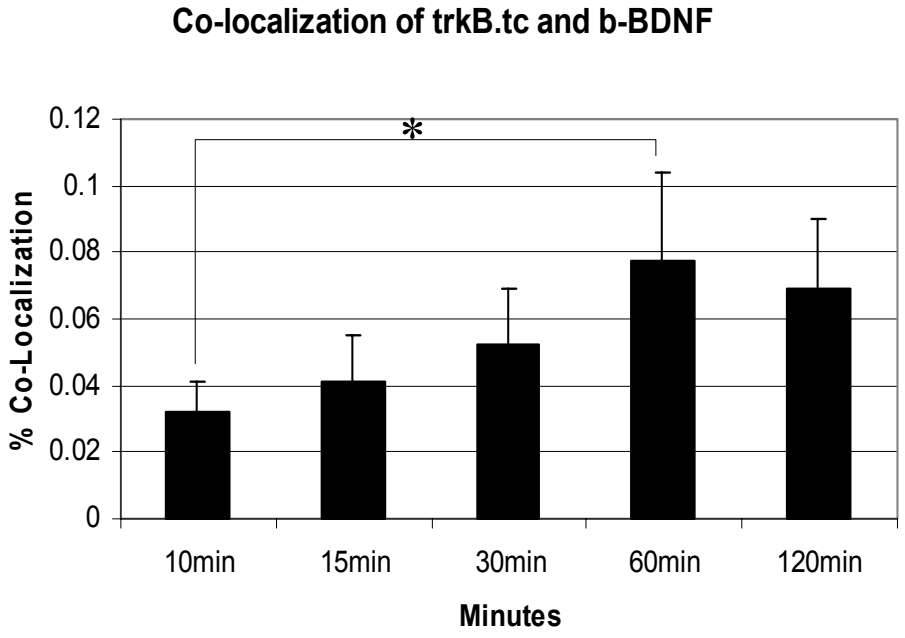


Figure 13. Co-localization of trkB.tc and b-BDNF over time.

The increase in trkB.tc and b-BDNF co-localization is statistically significant between 10 minutes and 60 minutes at $p<0.01$. (n= 4 experiments with four separate 600x fields and five optical sections per stack for each experiment.)



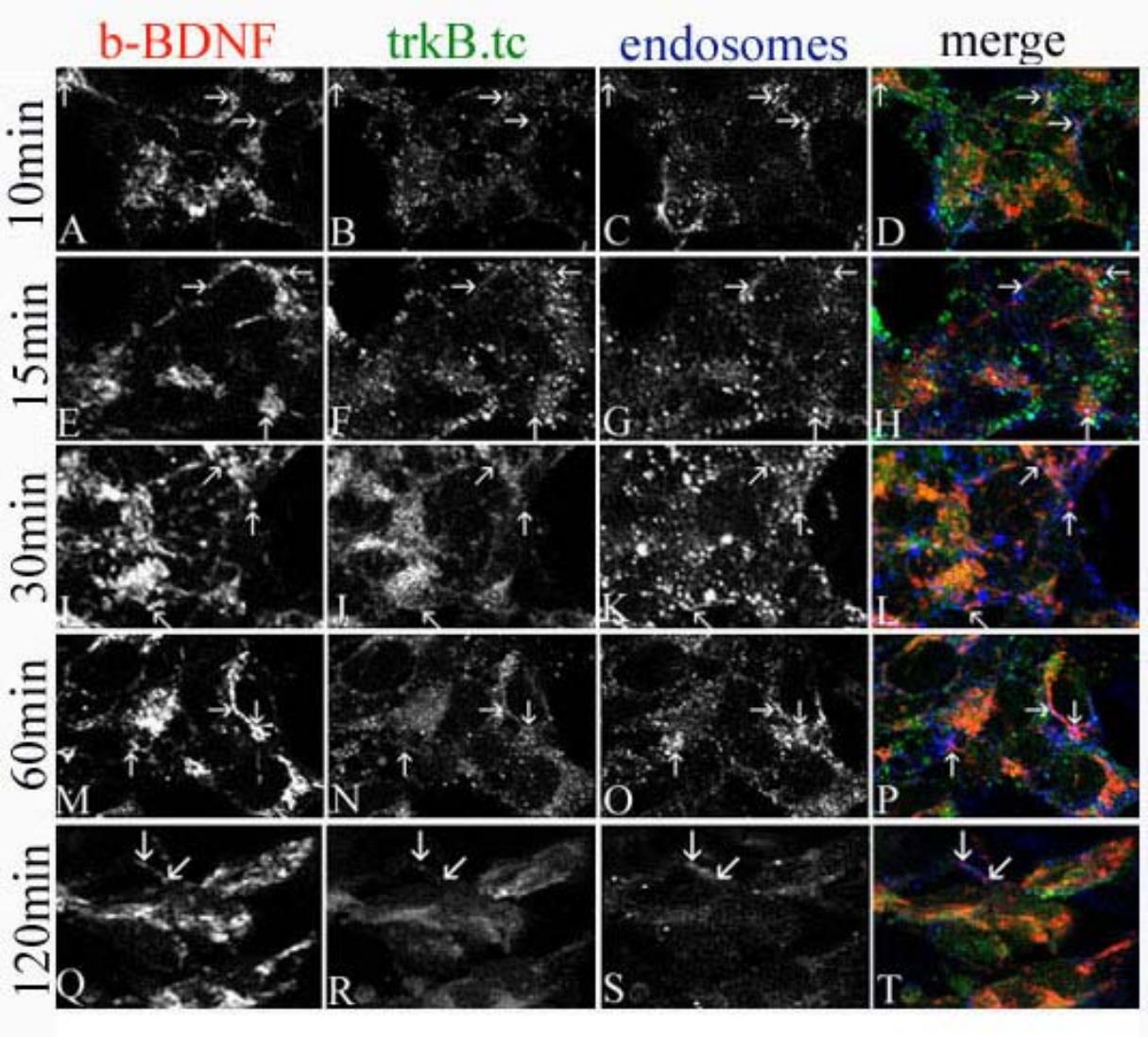
4. Localization of trkB.tc and internalized BDNF to early endosomes

BDNF can bind to both trkB.fl and trkB.tc. TrkB.fl can be activated by BDNF without internalization occurring (Atwal et al., 2000) (Nakamura et al., 1996) (Easton et al., 1999). There is no known signaling cascade activated by BDNF binding to trkB.tc. We treated differentiated SH-SY5Y cells with b-BDNF to elucidate the endocytic sorting pathway of internalized BDNF and its association with the truncated trkB receptor. We observed the co-localization of trkB.tc and b-BDNF with endocytic organelles (α-adaptin positive organelles) by triple-label immunofluorescence laser confocal microscopy (IFLCM). There were five

timepoints analyzed: 10, 15, 30, 60, and 120 minutes post-b-BDNF treatment of differentiated SH-SY5Y cultures.

Figure 14. Sorting of trkB.tc (green) and b-BDNF (red) to early endosomes (blue) following b-BDNF treatment of differentiated SH-SY5Y cells.

A-D. At 10 minutes, there is only minimal localization of the trkB.tc/b-BDNF complex within endosomes (arrows). E-H. By 15 minutes, trkB.tc and b-BDNF are co-localization to endosomes is increased from 10 minutes (arrows). I-L. and M-P. The co-localization of trkB.tc and b-BDNF to endosomes (arrows) is observed as far as 60 minutes post-b-BDNF treatment. Q-T. By 120 minutes, there is a decrease in trkB.tc/b-BDNF localization to endosomes (arrows). (Magnification of fluorescence = 60x)

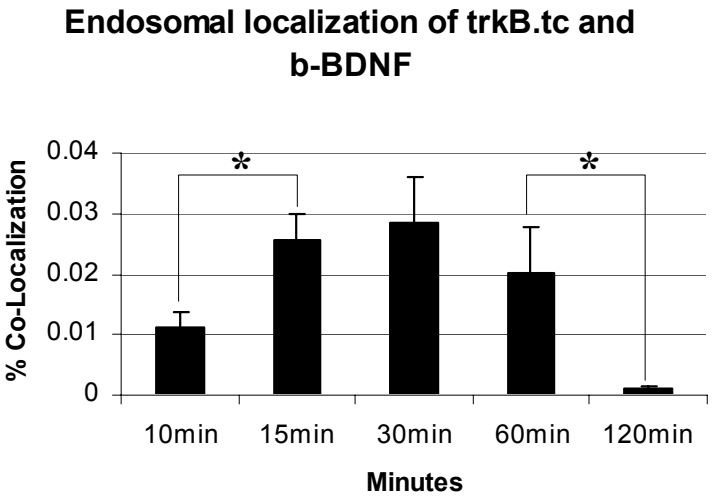


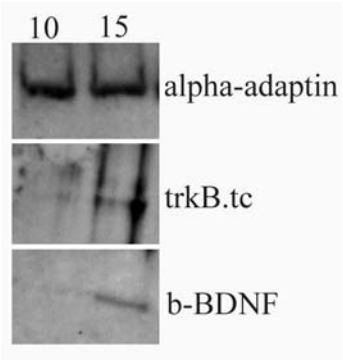
B-BDNF can be internalized and sorted to early endosomes by the truncated trkB receptor (Figure 14). Early endosomal localization of trkB.tc and b-BDNF does not occur in abundance until 15 minutes after addition of b-BDNF (Figure 14e-h) and this increase is statistically significant at $p<0.001$ (Figure 15A). TrkB.tc and b-BDNF were localized within early endosomes isolated from differentiated SH-SY5Y cells at 15 minutes post-internalization (Figure 15B). The initial delay in endocytic sorting of trkB.tc/b-BDNF may result from the sequestering of intracellular cues necessary for receptor-mediated endocytosis to occur. Once internalization occurs, there is a sustained level of trkB.tc/b-BDNF complex within early endosomes as late as 60 minutes (Figure 14m-p). By 120 minutes, trkB.tc and b-BDNF are only minimally localized to early endosomes (Figure 14q-t). The observed changes in endosomal distribution of trkB.tc and b-BDNF between 60 minutes and 120 minutes are statistically significant at $p<0.001$ (Figure 15A). The decline in trkB.tc/b-BDNF localization to early endosomes by 120 minutes indicates that the proteins have been sorted to other organelles or degraded.

Figure 15. Endosomal localization of trkB.tc and b-BDNF.

A. Both the increase in trkB.tc/b-BDNF localization to endosomes between 10 to 15minutes and decrease between 60 and 120 minutes are statistically significant at $p<0.01$. (n= 4 experiments with four separate 600x fields and 5 optical sections per stack for each experiment.) B. Western blot analysis of endosomes isolated from differentiated SH-SY5Y cells. Early endosomes were isolated by positive selection with the EEA1 antibody. These endosomes express alpha-adaptin and contain the trkB.tc and b-BDNF proteins.

A. **B.**





5. Localization of trkB.tc and internalized BDNF to recycling endosomes

Now that we have demonstrated trkB.tc and b-BDNF packaged together within early endosomes, we wanted to determine if trkB.tc and b-BDNF are further sorted as a protein complex, or if the proteins are sorted independently of one another. If trkB.tc facilitates b-BDNF translocation, the TrkB.tc/b-BDNF complex would be transported to recycling endosomes. For this analysis, we determined the co-localization of trkB.tc and b-BDNF to recycling endosomes. The percent co-localization of trkB.tc/b-BDNF to recycling endosomes shows a trend to increase over time (Figure 16). By 15 minutes, trkB.tc and b-BDNF have been sorted from early

endosomes to recycling endosomes (Rab4-positive organelles) (Figure 17e-h). The significant decrease in trkB.tc/b-BDNF co-localization at 30 minutes (Figure 16, 17i-l) can be attributed to one of the following processes: 1) the proteins in these recycling endosomes have been sorted back to the proximal plasma membrane, 2) proteins sorted to recycling endosomes at this early stage are dissociated and no longer transported as a complex, or 3) these proteins are being sorted to lysosomes by 30 minutes. By 60 minutes (Figure 17m-p), there is a surge in trkB.tc/b-BDNF co-localization to recycling endosomes. This increase can be attributed to proteins internalized at later timepoints and subsequently sorted to the recycling endosomes. Vesicles containing trkB.tc/b-BDNF at later timepoints may translocate the complex to the plasma membrane (Figure 17q-t).

6. Localization of trkB.tc and internalized BDNF to lysosomes

Finally, we determined whether trkB.tc and b-BDNF were sorted to lysosomes as a protein complex. The percent co-localization of trkB.tc and b-BDNF to lysosomes in differentiated SH-SY5Y cells was analyzed (Figure 18). Although we could find trkB.tc and b-BDNF co-localized to lysosomes at all timepoints, their distribution to lysosomes at 10 minutes was negligible (Figure 19a-d). We identified two peaks of lysosomal localization of the trkB.tc/b-BDNF complex. The first occurred at 15 minutes (Figure 19e-h). This very early sorting of trkB.tc and b-BDNF to lysosomes occurred due to the overloading of the system with b-BDNF. The abundance of b-BDNF added to the system most likely saturated the trkB.tc receptors, and hence pushed a portion of the receptor-ligand complex directly to the lysosome. Although this did occur, we must note that this influx of lysosomal sorting was still significantly less than the sorting of the proteins to other organelles. The second peak of lysosomal sorting

occurred at 120 minutes (Figure 19q-t). This peak occurred at a time when we expected lysosomal localization of the proteins, but lysosomal sorting was still significantly less than the sorting of the proteins to the other organelles.

Figure 16. Recycling endosome localization of trkB.tc and b-BDNF.

TrkB.tc and b-BDNF are localized to recycling endosome at all timepoints. Localization to these vesicles is widespread at 120 minutes. There is a decrease in trkB.tc/b-BDNF localization to recycling endosome at 30 minutes that is statistically significant at $p<0.01$. (n= 4 experiments with four separate 600x fields and 5 optical sections per stack for each experiment.)

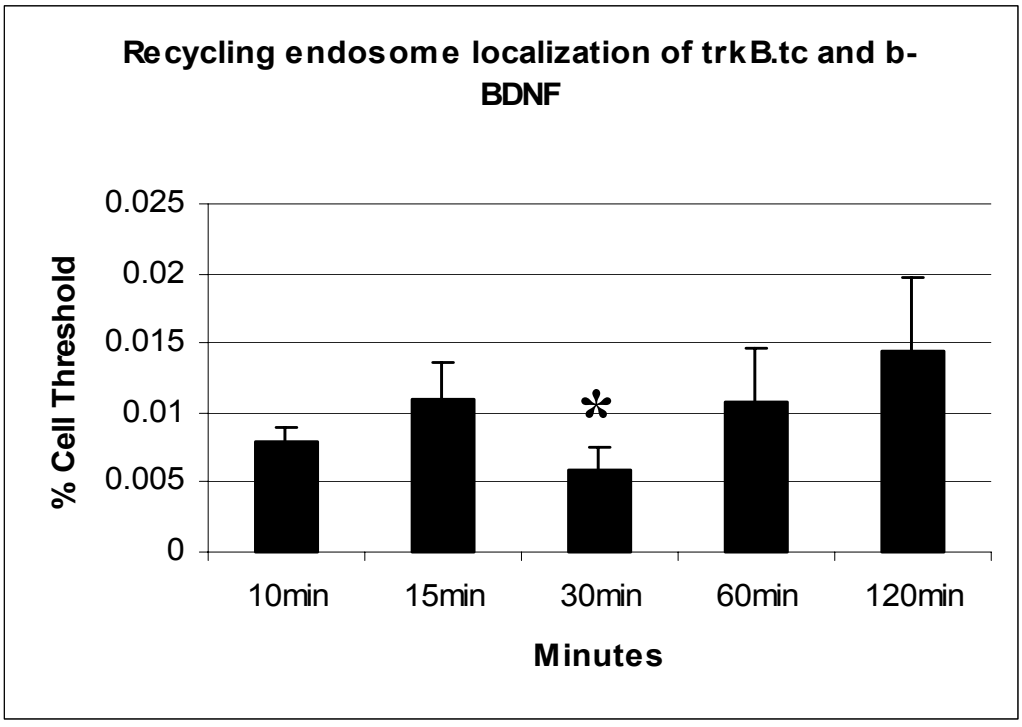


Figure 17. Recycling endosome (blue) localization of trkB.tc (green) and b-BDNF (red) following b-BDNF treatment of differentiated SH-SY5Y cells.

A-D. At 10 minutes, there is only minimal localization of trkB.tc/b-BDNF to recycling endosomes (arrows). E-H. At 15 minutes, you see an upregulation in trkB.tc/b-BDNF co-localization to recycling endosomes (arrows). I-L and M-P. The co-localization of trkB.tc and b-BDNF to recycling endosomes (arrows) is maintained at 30 and 60 minutes post-b-BDNF treatment. Q-T. TrkB.tc and b-BDNF co-localization to recycling endosomes is maintained at 120 minutes. (Magnification of fluorescence = 60x)

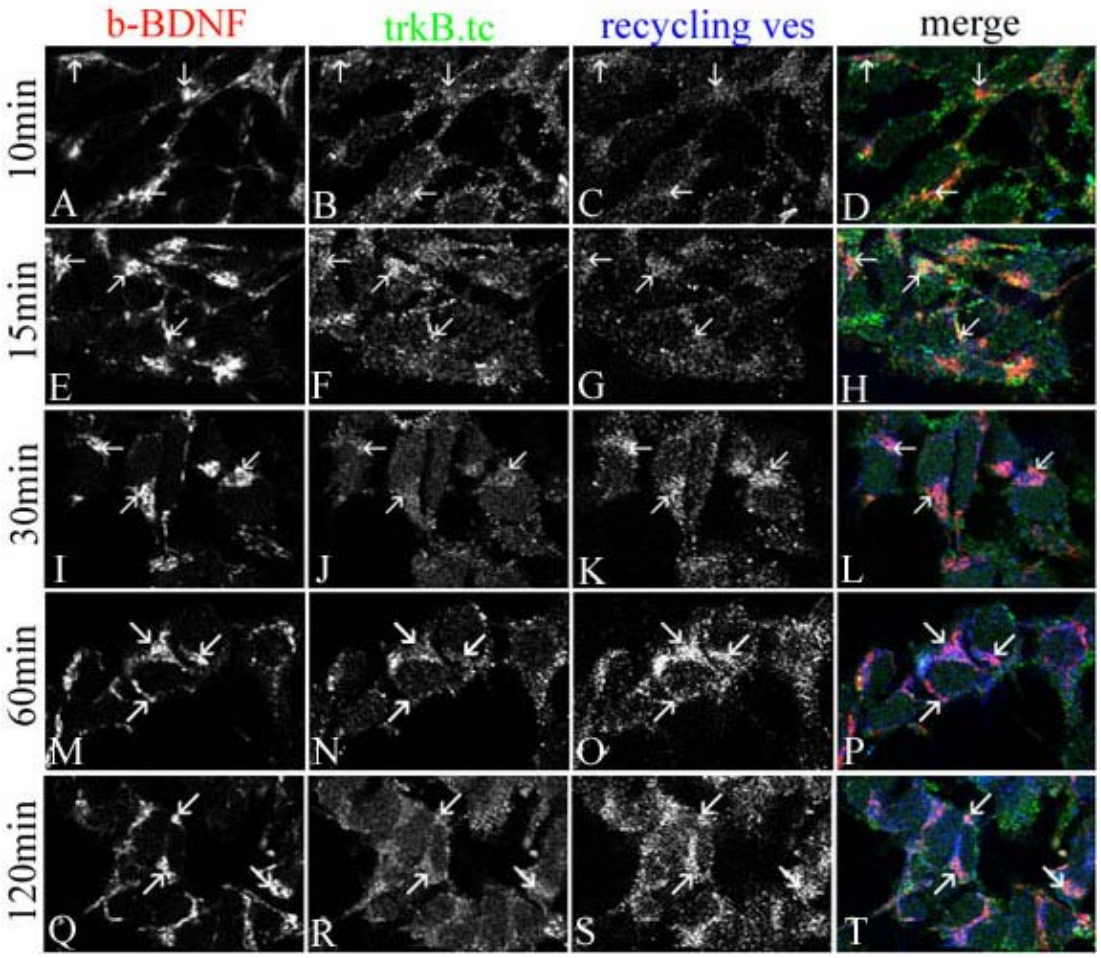


Figure 18. Lysosomal localization of trkB.tc and b-BDNF.

TrkB.tc/b-BDNF localization to lysosomes peaks at 15 and 120 minutes post-b-BDNF treatment. These peaks are significant at $p<0.01$. (n= 4 experiments with four separate 600x fields and 5 optical sections per stack for each experiment.)

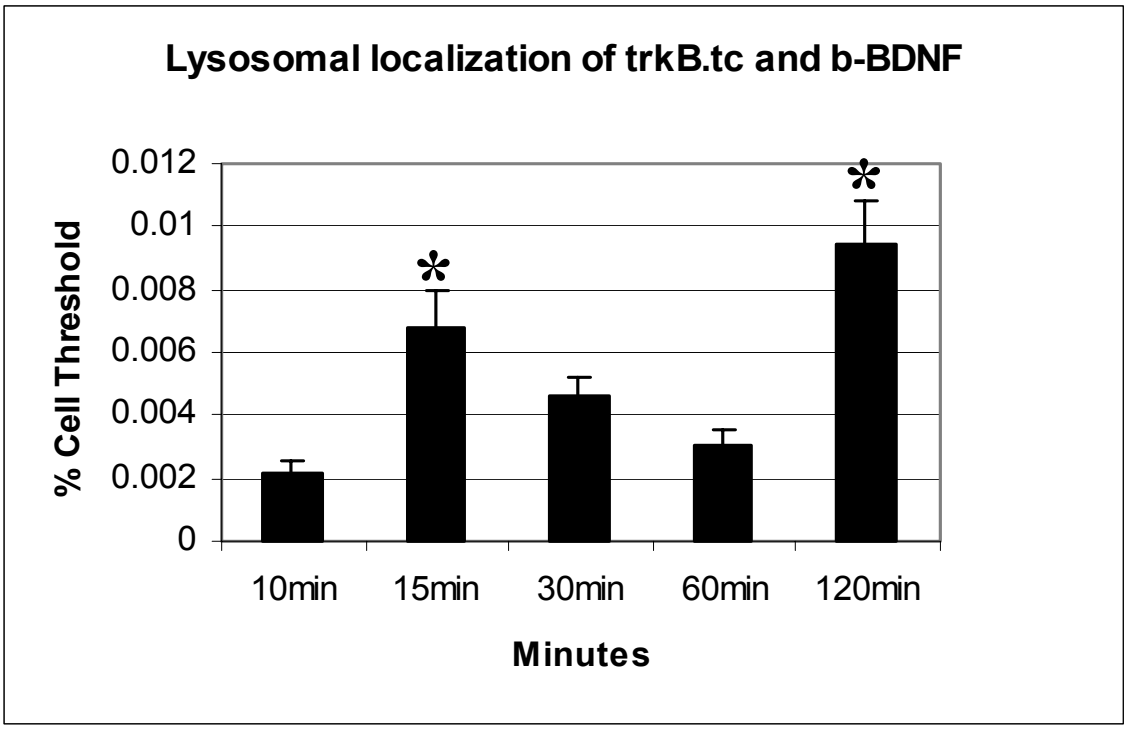
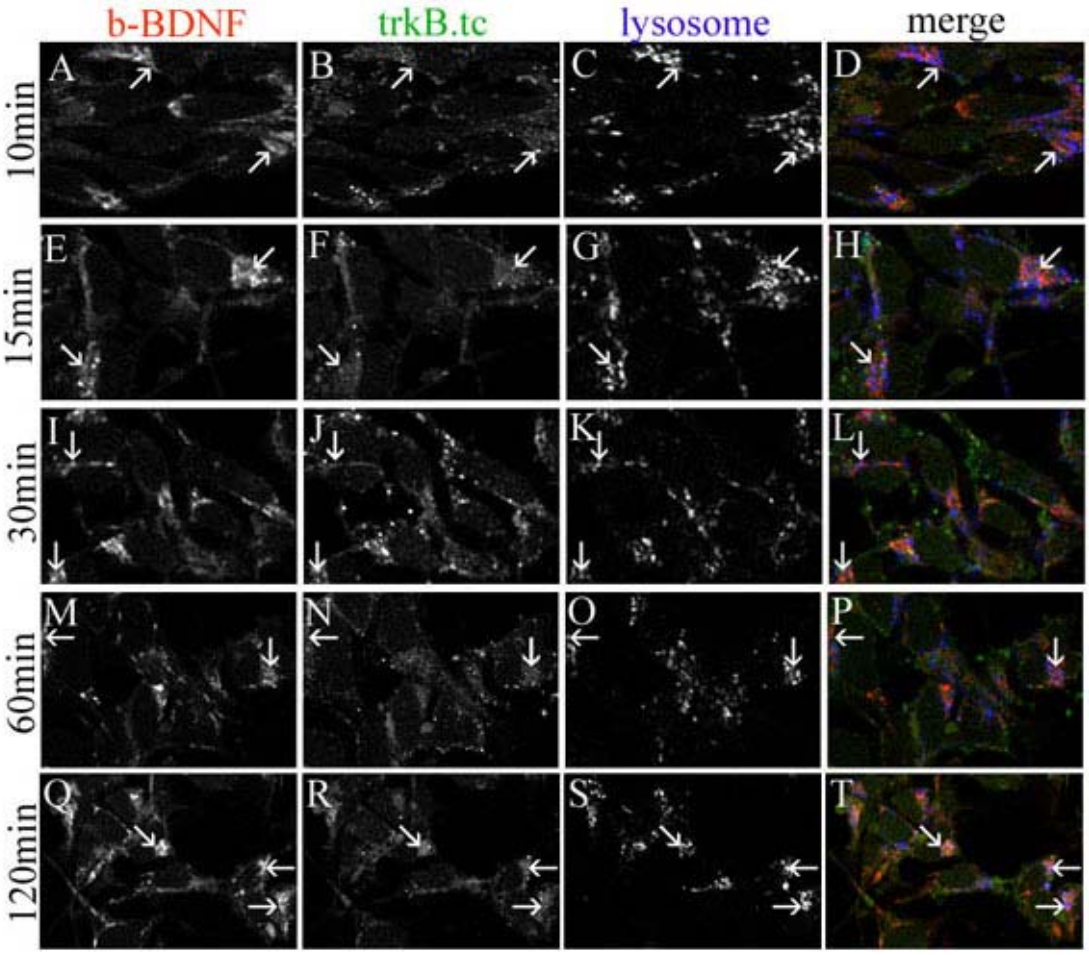


Figure 19. Lysosomal (blue) localization of trkB.tc (green) and b-BDNF (red) following b-BDNF treatment of differentiated SH-SY5Y cells.

A-D. At 10 minutes, trkB.tc and b-BDNF co-to lysosomes is minimal (arrows). E-H. At 15 minutes, there is an increase in trkB.tc/b-BDNF localization to lysosomes (arrows). I-L and M-P. TrkB.tc/b-BDNF localization to lysosomes (arrows) is decreased at 30 and 60 minutes. Q-T. There is a peak in trkB.tc/b-BDNF localization to lysosomes (arrows) at 120 minutes. (Magnification of fluorescence = 60x).

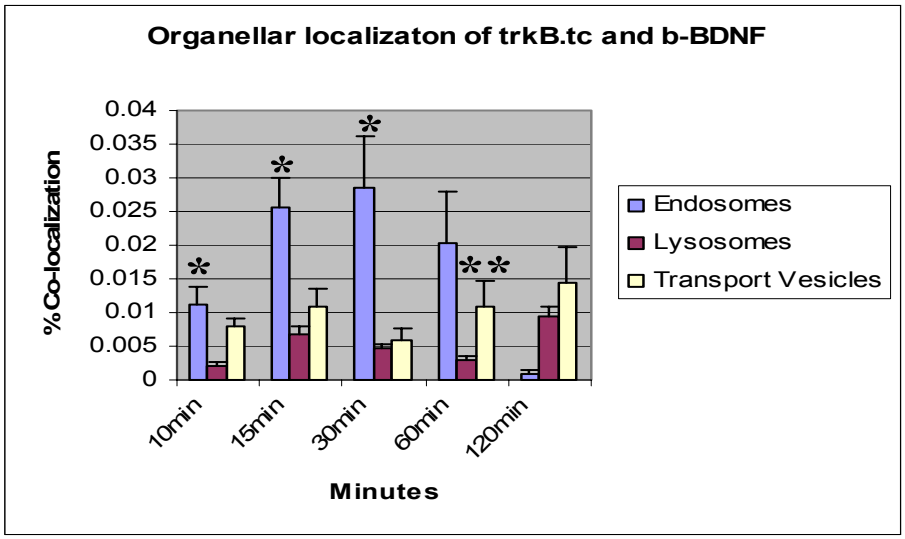


7. The endocytic sorting pathway of trkB.tc and b-BDNF

To summarize the ligand-receptor endocytic sorting pathway we compared the intracellular distribution of co-localized trkB.tc and b-BDNF over time (Figure 20). Immediately following b-BDNF internalization, trkB.tc/b-BDNF co-localization is primarily found within early endosomes (Figure 20, asterisks). Over time, trkB.tc/b-BDNF co-localization shifts to the recycling endosomes and lysosomes, although there is significantly more distribution in the recycling endosomes at 60 minutes (figure 20, double asterisks). By 60 minutes, the ratio of trkB.tc/b-BDNF complex localized to recycling endosomes versus lysosomes is about 3:1 (Figure 20, double-asterisks). By 120 minutes, we expect that the trkB.tc/b-BDNF complex is dissociated. This would allow for b-BDNF to be released into the cytoplasm to activate trkB.fl receptors.

Figure 20. Organellar localization of trkB.tc and b-BDNF.

Immediately following b-BDNF internalization, trkB.tc/b-BDNF co-localization is primarily found within endosomes. Over time, trkB.tc/b-BDNF co-localization is predominantly found within recycling/transport vesicles. TrkB.tc/b-BDNF localization to lysosomes is at its peak at 120 minutes post-b-BDNF treatment. (Asterisks denote statistical significance at $p<0.01$; n= 4 experiments with four separate 600x fields and 5 optical sections per stack for each experiment.)



8. Conclusions

TrkB.fl is a signaling molecule that also mediates retrograde transport of BDNF (Neet and Campenot, 2001) (Ginty and Segal, 2002). The role of the truncated trkB receptor in the signaling and transport of BDNF in mature neurons is not well understood. We have shown that trkB.tc can not only sequester BDNF, but it appears to act as a chaperone protein to drive the endocytic sorting of internalized BDNF. Therefore, trkB.tc internalizes BDNF via receptor-mediated endocytosis, transports BDNF to recycling endosomes and lysosomes, and potentially translocates BDNF to distal trkB.fl receptors. We have shown that the trkB.tc protein was more abundant than trkB.fl in differentiated SH-SY5Y cells and that there is minimal co-localization between trkB.tc and trkB.fl throughout differentiation (Figure 10). These data support previous evidence that trkB.tc is the predominant isoform in the adult brain (Ohira et al., 1999) and our hypothesis that the primary function of the trkB.tc receptor in the differentiated neuron is distinctive from the function of trkB.fl.

8.1 TrkB.fl and $p75^{NTR}$ mediate retrograde transport of BDNF

The retrograde transport of neurotrophins can be mediated by 1) their high affinity full-length trk receptor and 2) their low affinity $p75^{NTR}$ receptor (Neet and Campenot, 2001) (Ginty and Segal, 2002) (Butowt and von Bartheld, 2003) (Bronfman et al., 2003). One model of trk-mediated retrograde transport of neurotrophins is the Signaling Endosome Model proposed by Ginty et al. (2002). In this model, neurotrophins bind to trk receptors at the pre-synaptic terminal and are internalized via receptor-mediated endocytosis. Both the trk receptors and their neurotrophin are sorted to signaling vesicles. Vesicular trk is associated with the vesicle membrane and the neurotrophin is found within the lumen. The main feature of this model is the

maintenance of the trk/neurotrophin complex in the signaling vesicles throughout their retrograde transport. We propose a novel role for the truncated trkB receptor: somal truncated trkB receptors are packaged into signaling vesicles with BDNF and anterogradely transported to the pre-synaptic terminal. As in the Signaling Endosome Model, we propose that the trk receptor and the neurotrophin are transported as a complex.

Axonal neurotrophin transport is also mediated by the $p75^{NTR}$ receptor. Bronfman et al. (2003) showed that NGF is internalized by p75 and sorted to recycling endosomes in the somata. Furthermore, p75 is involved in the anterograde transport of neurotrophins (Butowt and von Bartheld, 2003). Our study showed that BDNF is internalized with trkB.tc and sorted to recycling endosomes in the somata. Their localization to neurites suggests that trkB.tc may also be involved in the axonal transport of BDNF. Furthermore, p75 may interact with the truncated trkB receptor to enhance the transport of BDNF.

8.2 Alternative splicing regulates ligand transport

There is preliminary evidence that indicates a direct link between alternative splicing of receptors and the differential sorting of their splice variant (Jaskolski et al., 2004) (Francesconi and Duvoisin, 2002) (Mu et al., 2003). Glutamate receptors (GluR) have many receptor subunits that are generated from alternative gene splicing (Jaskolski et al., 2004). These receptor subunits are differentially sorted to the ER or plasma membrane. Furthermore, there are motifs specific to each group 1 metabotropic glutamate receptor isoform (mGluR1) that drive axonal versus dendritic sorting (Francesconi and Duvoisin, 2002). NMDA receptor subunits (NR1) are also a product of alternative splicing. Mu et al. (2003) showed that NR1 subunits are alternatively spliced to regulate their activity-dependent ER export.

Alternative splicing also directs the synthesis of the truncated and full-length trkB receptor isoforms. Our data show that trkB.tc is more abundant and differentially distributed than trkB.fl. There are distinct roles for trkB.tc and trkB.fl in the transport of BDNF: trkB.tc facilitates endocytic sorting and potentially anterograde transport and trkB.fl mediates retrograde transport. Therefore, alternative splicing of trkB is one mechanism regulating BDNF transport.

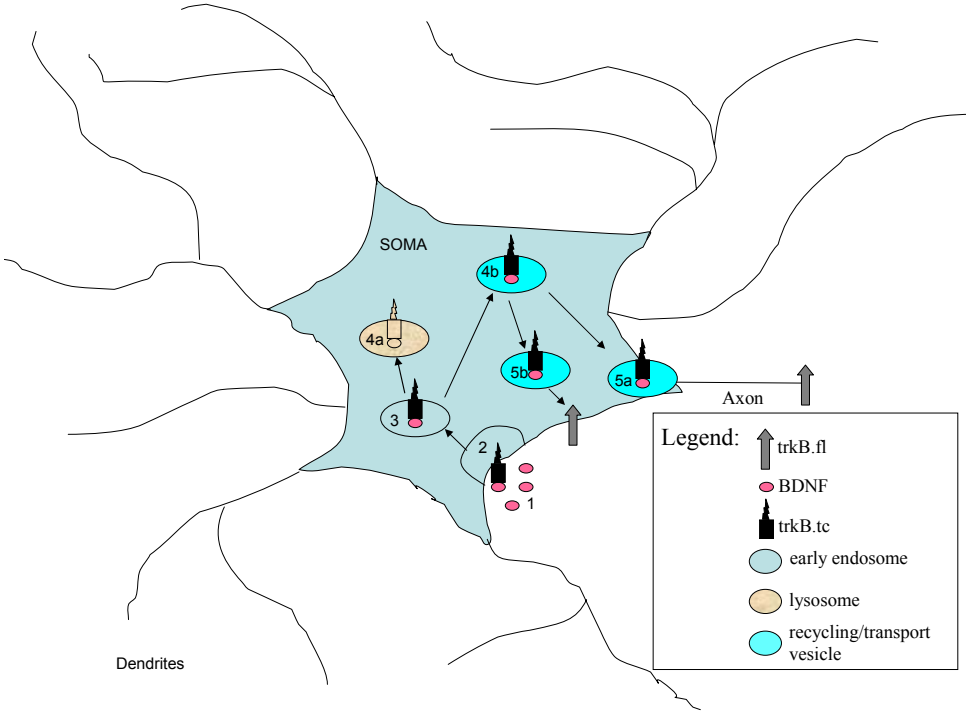
8.3 TrkB.tc facilitates the endocytic sorting of BDNF

We showed that trkB.tc facilitates the endocytic sorting of BDNF. Specifically, BDNF binds to trkB.tc at the cell surface, is internalized via receptor-mediated endocytosis, and sorted to early endosomes as a protein complex (Figure 21). Once in the endosome, trkB.tc and BDNF can be further sorted as a protein complex to recycling endosomes or to lysosomes (Figure 21). Our results suggest that trkB.tc and b-BDNF are sorted as a complex to early endosomes, recycling endosomes, and lysosomes. Neuritic localization of trkB.tc and b-BDNF supports our hypothesis that trkB.tc further facilitates the axonal transport of BDNF to distal trkB.fl receptors. Once localized to recycling endosomes, it is possible for the trkB.tc receptor to be sorted to axonal transport vesicles where it can present BDNF to trkB.fl receptors distal to site of internalization. We hypothesize that this phenomenon may occur between and/or within cells in different regions of the brain. For instance, BDNF may be secreted by cells in region A, internalized by trkB.tc receptors on dendrites or somata, and then be transported to axon terminals in region B of the brain. This would allow trkB.fl receptors to be activated by this internalized BDNF and to subsequently induce neurotrophic affects in that region. By showing

that trkB.tc facilitates the endocytic sorting of internalized BDNF, our data suggest a novel role for the truncated trkB receptor in differentiated neurons of the central nervous system.

Figure 21. Model: Endocytic sorting pathway of trkB.tc and internalized BDNF.

BDNF binds to trkB.tc at the cell surface (1) and is internalized via receptor-mediated endocytosis (2). The receptor-ligand complex is then sorted to early endosomes (3). From here, the receptor-ligand complex can be sorted to recycling/transport vesicles (4b) or lysosomes (4a). Once at the in the transport vesicle, BDNF can be released to proximal (5b) or distal (5a) trkB.fl receptors.



The distal transport of BDNF is essential for the proper maintenance of neuronal connections between brain regions. In many neurodegenerative diseases, like PD, there is a decrease in BDNF mRNA and proteins (Howells et al., 2000) (Mogi et al., 1999). There is evidence that trkB.tc proteins are upregulated in neurites of striatal and nigral neurons in PD

(Chapter 5). TrkB.tc may facilitate the movement of BDNF to sites where BDNF is depleted, allowing BDNF to rescue the target neurons from injury. The role of trkB.tc in BDNF transport may be an attractive mechanism to exploit in the development of therapeutic strategies for neurodegenerative diseases.

5. TrkB protein distribution is altered in Parkinson's disease (PD)

The distribution of trkB proteins in the Parkinson's brain has not been previously addressed. We hypothesize that, along with BDNF proteins, trkB protein distribution and expression is also altered in the substantia nigra of PD brains. To determine if there are disease related changes in trkB.tc and trkB.fl proteins in PD, we performed immunohistochemical and double-label immunofluorescence microscopy of truncated and full-length trkB in autopsy tissues from PD and age-matched control brains. TrkB receptor distribution is altered in the PD brain. In PD, trkB.tc is increased in axons of striatal neurons and dendrites of substantia nigra *pars compacta* (pc) neurons, whereas trkB.fl is localized to dendrites in striatal neurons and axons in nigral neurons. Our results indicate that changes in the normal distribution of trkB.tc are a neuronal response to PD. We found that trkB.tc and trkB.fl are differentially distributed in PD brains and the levels of expression are regionally altered, compared to controls.

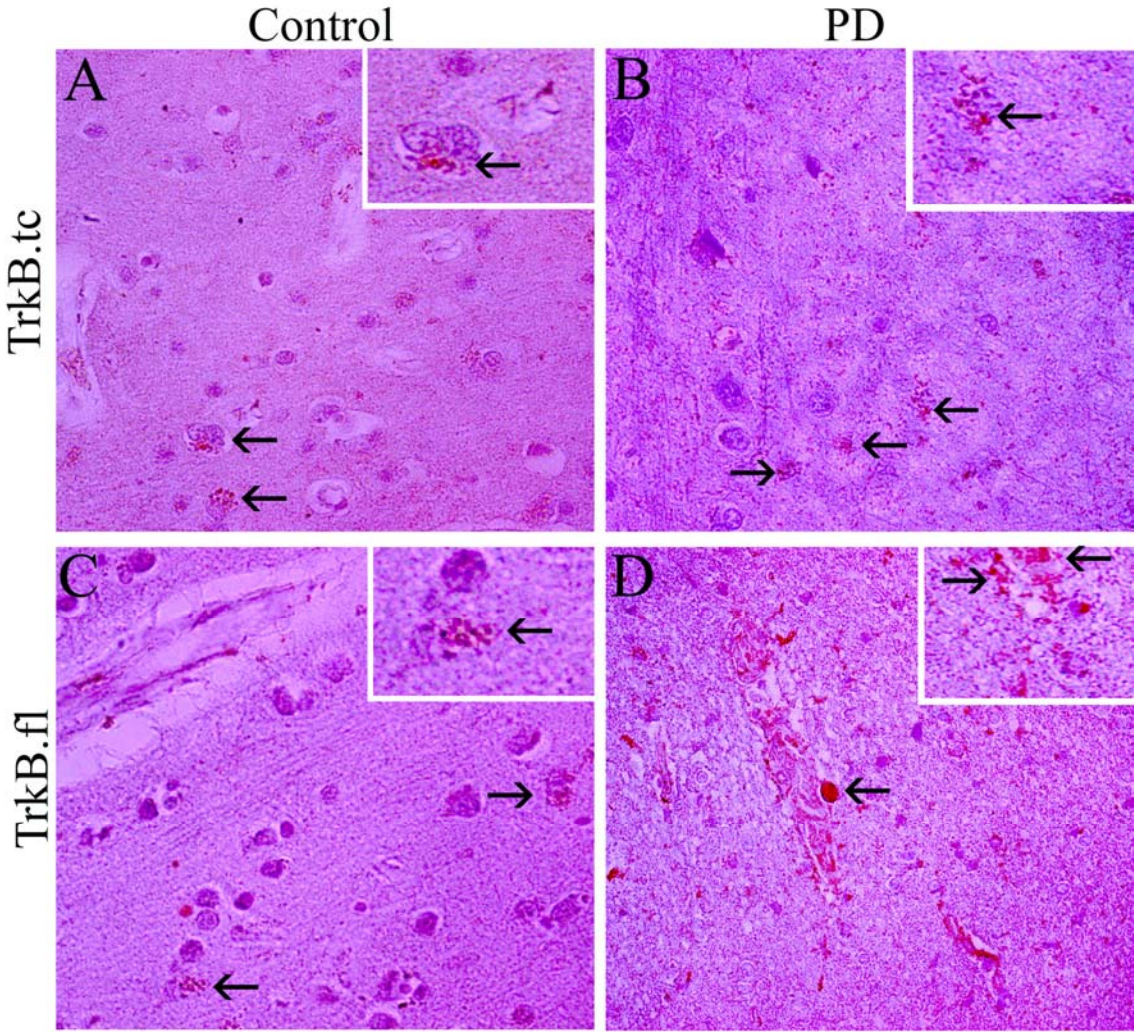
1. Differential distribution of the truncated and full-length trkB isoforms in the normal and PD brain

Previous studies have shown that BDNF protein distribution is disrupted in Parkinson's disease (Howells et al., 2000) (Mogi et al., 1999). We found that, in addition to changes in BDNF expression, there are also changes in trkB protein expression and distribution in PD. We performed an immunohistochemical analysis on control and PD frontal cortex, striatum, and substantia nigra *pars compacta*(pc) autopsy tissues to determine the effects of PD on trkB.tc and trkB.fl protein distribution.

Although the frontal cortex (Figure 22) is not directly targeted in PD, it does receive indirect input from the substantia nigra$_{pc}$, via the striatum. Therefore, we analyzed the differential distribution of trkB.tc and trkB.fl in control and PD frontal cortex autopsy tissues. TrkB.tc labeling (Figure 22a) in the control frontal cortex is punctate, distributed throughout the parenchyma, and is infrequently found in the somata. The distribution of trkB.tc is not altered in the PD frontal cortex (Figure 22b). TrkB.fl cellular distribution is different than trkB.tc. TrkB.fl is primarily found in the somata and is not widely distributed throughout the parenchyma (Figure 22c). This distribution pattern is consistent throughout the PD frontal cortex, except in perivascular regions where trkB.fl is upregulated (Figure 22d). Leakage of mononuclear cells through the blood-brain barrier in PD (Figure 22d, arrows) may attribute to the appearance of perivascular trkB.fl in the frontal cortex.

Figure 22. Distribution of trkB.tc and trkB.fl in the control and PD frontal cortex.

In the control frontal cortex (A), trkB.tc labeling is punctate and widespread throughout the parenchyma. Its distribution is also found in aggregates around the somata (arrows, see insert). This distribution is not altered in the PD brain (B). TrkB.fl is mostly associated with neuronal somata (arrows, see insert) in the control frontal cortex (C). In PD, there is an upregulation of trkB.fl seen in perivascular regions (arrows, see insert) (D).

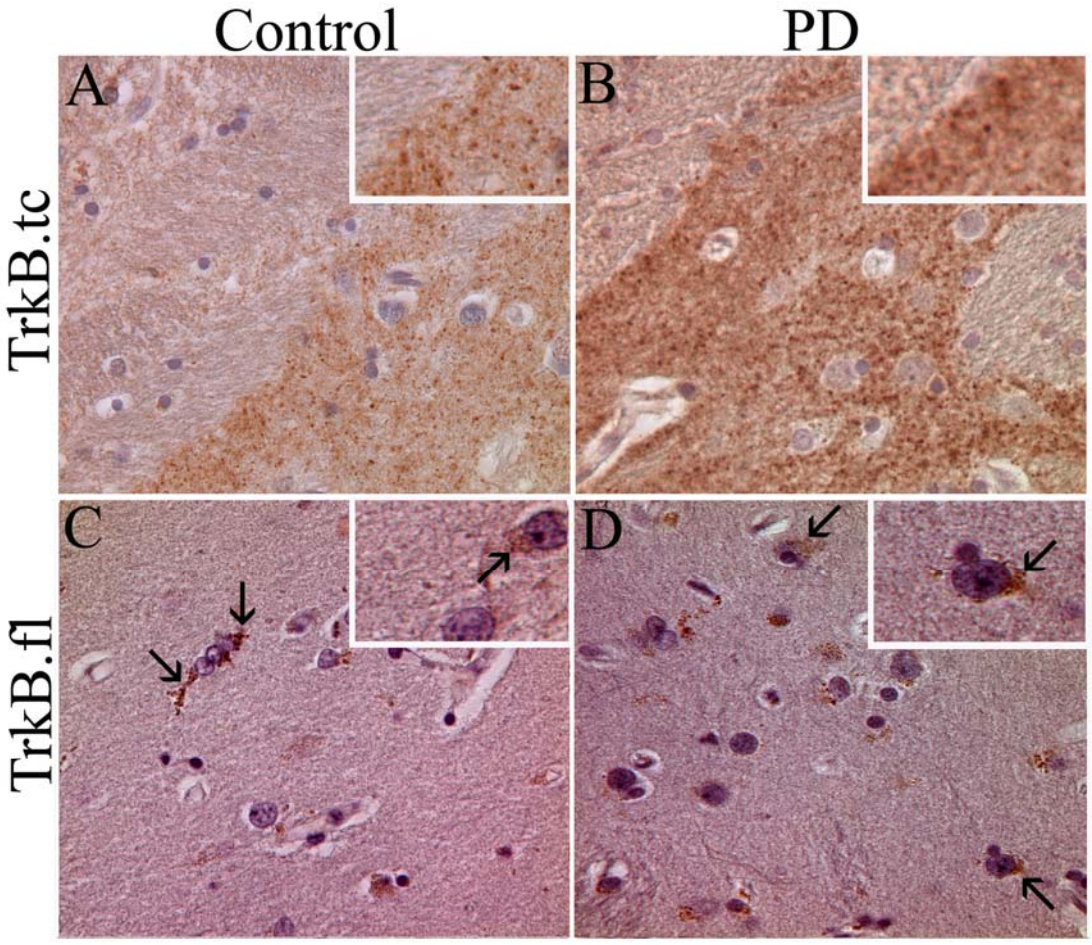


The striatum receives direct input from the substantia $nigra_{pc}$ via the pyramidal, dopaminergic neurons targeted for cell death in PD. We analyzed the changes in striatal trkB distribution and expression in PD and control. In the control striatum, punctate trkB.tc labeling

is abundant throughout the region (Figure 23a). The distribution pattern of trkB.tc is not altered in PD, but is upregulated (Figure 23b). Conversely, protein expression of trkB.fl is limited in the striatum and localized to the somata of neurons (Figure 23b). In PD, trkB.fl distribution in neurons is not altered (Figure 23d).

Figure 23. Distribution of trkB.tc and trkB.fl in the control and PD striatum.

In the control striatum, trkB.tc is punctate and abundant (A). This distribution pattern is not altered in PD, but overall trkB.tc proteins are upregulated (B). TrkB.fl labeling is minimal in the striatum, but punctate and localized to the soma and proximal neurites in select neurons (C, arrows). In PD, no noticeable differences are seen in neuronal distribution of trkB.fl (D, arrows).



The control substantia nigra$_{pc}$ is rich in pyramidal neurons which express both trkB.fl and trkB.tc proteins (Figure 24a and c). TrkB.tc is abundant in the somata of these neurons as well as distributed within their neurites (Figure 24a). In PD, the pyramidal neurons are degenerating, becoming small and crenated prior to cell death. This decrease in cell size may contribute to the overall decrease observed in somal trkB.tc in PD (Figure 24b). Decreases in somal trkB.tc may also result from increased protein transport from the soma to the neurites. TrkB.fl proteins are evenly distributed throughout the pyramidal cell and are localized to both the soma and its neurites in control sections (Figure 24c). In PD, trkB.fl proteins are concentrated in the neuronal somata and are no longer abundant throughout the neurites (Figure 24d). TrkB.fl distribution within glial cells is addressed in subsequent figures.

2. Axonal and dendritic localization of trkB in PD nigra-striatal neurons

To characterize the neuronal distribution of the trkB isoforms observed in PD, we analyzed axonal and dendritic localization of trkB.tc (Figure 25) and trkB.fl (Figure 26). Axonal and dendritic distributions were determined by co-localization of trkB with neurofilament and map2 proteins, respectively. Our analysis focused on the pyramidal neurons in the substantia nigra$_{pc}$ and their targets in the striatum.

In the control striatum, trkB.tc protein distribution was punctate and proximal, but not localized to, dendritic processes (Figure 25a). The punctate labeling of trkB.tc is localized to axonal projections throughout the control striatum (Figure 25c). This distribution pattern is preserved in PD while overall axonal trkB.tc protein is upregulated (Figure 25b and d). Furthermore, trkB.tc protein was localized to the somata of control (Figure 25a), but not PD neurons (Figure 25b). Axons in the substantia nigra$_{pc}$ do not express trkB.tc proteins (Figure 25g

and h). Instead, trkB.tc is primarily localized to neuronal dendrites in both control (Figure 25e) and PD (Figure 25f).

Figure 24. Distribution of trkB.tc and trkB.fl in the control and PD substantia nigra(pc).

In the control substantia nigra$_{pc}$, trkB.tc is abundantly distributed throughout the somata (arrows, see insert) and neurites (double arrowhead) (A). In PD, trkB.tc is reduced in the somata (arrows, insert) and abundant in the neurites (double arrowhead, B). TrkB.fl is also widely distributed throughout the somata (arrows, see insert) and neurites (double arrowhead) in the control (C), but is more localized in PD (D). In PD, trkB.fl is densely clustered in the somata (arrow) (D).

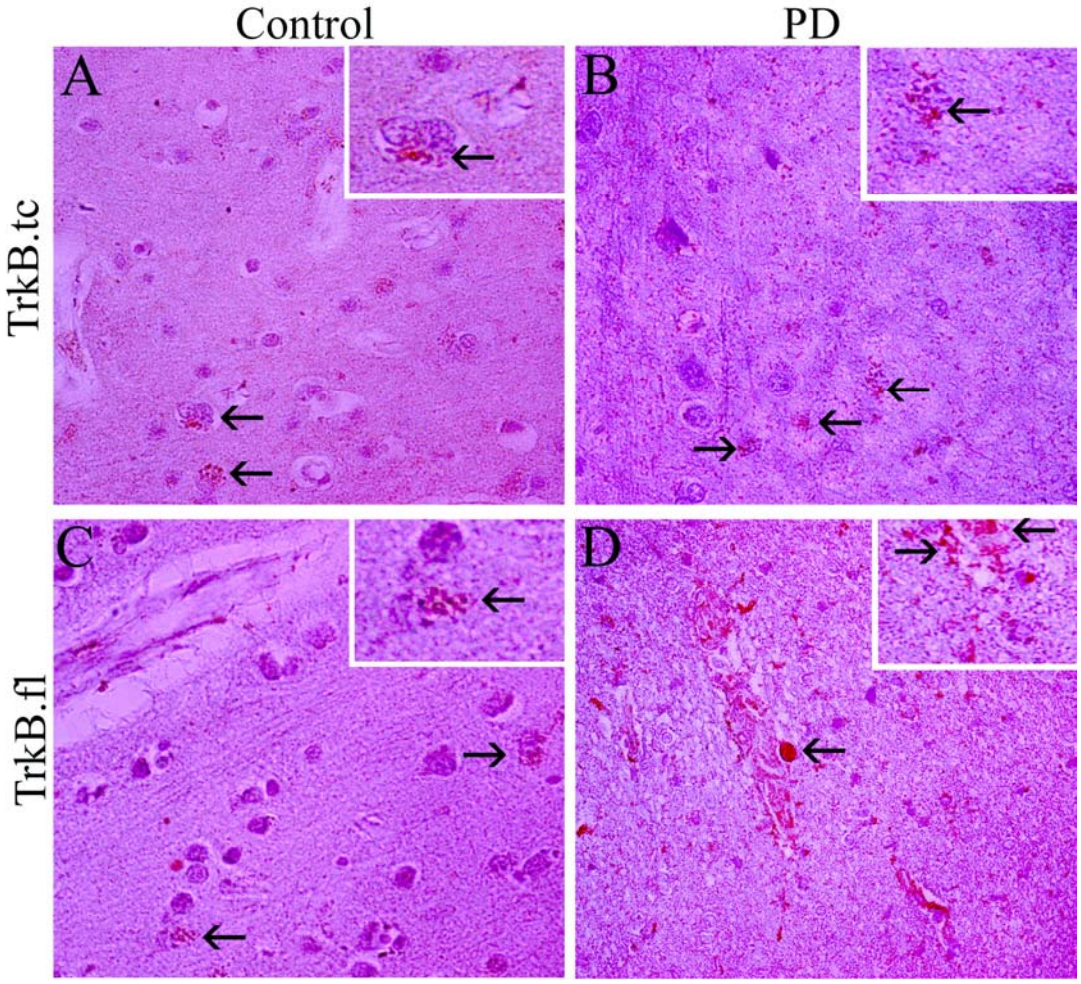


Figure 25(a-d). Neuronal localization of trkB.tc in PD striatum.

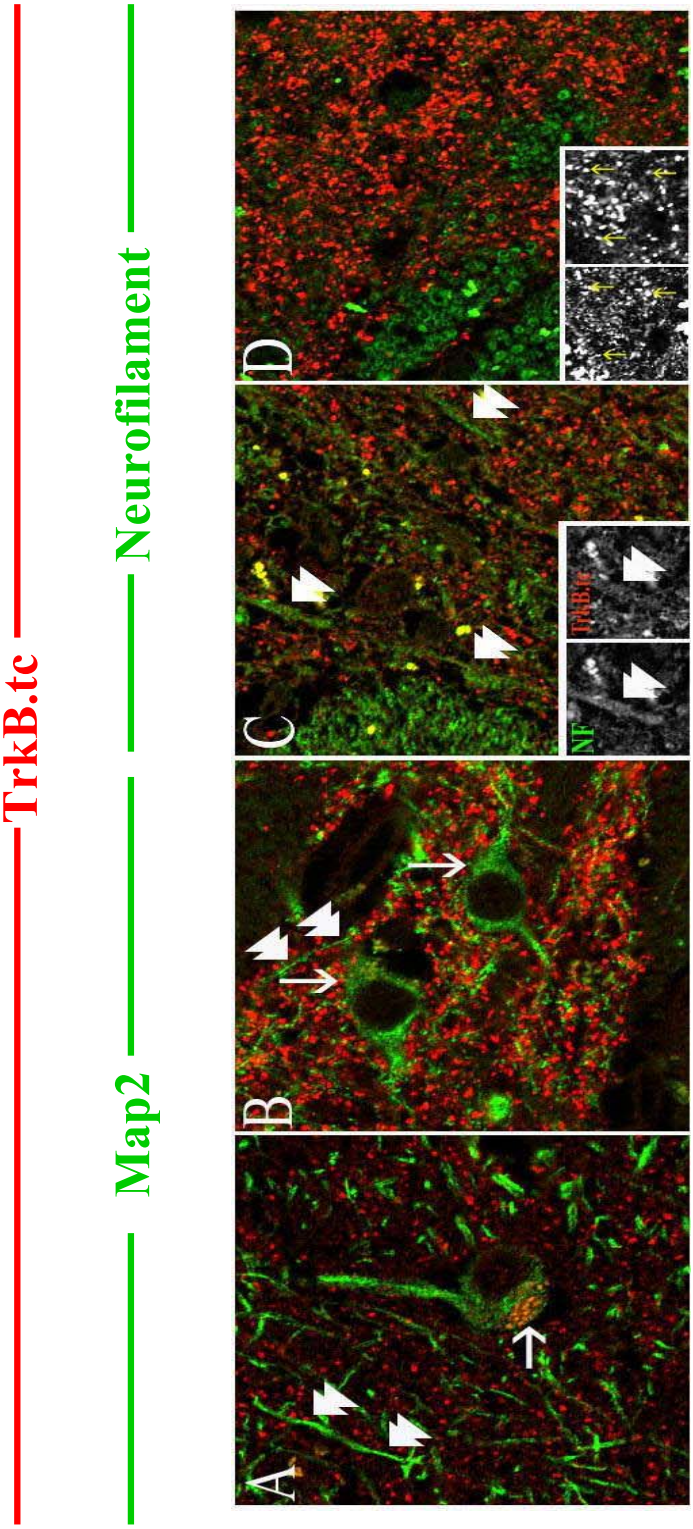


Figure 25(a-d). Neuronal localization of trkB.tc in PD striatum.
In the control striatum, trkB.tc labeling (red) is punctate and distributed about, but not localized to, Map2-positive dendrites (green, double-arrowheads) (A). This distribution is preserved in the PD striatum, but is markedly increased (B, double arrowheads). Somal trkB.tc is decreased in the PD striatum (B, arrow) compared to controls (A, arrow). Punctate trkB.tc labeling is found to be associated with neurofilament-positive axons (green) in the control (C, doublearrowheads) and PD (D, arrows in inserts) striatum.

Figure 25 (e-h). Neuronal localization of trkB.tc in PD substantia nigra.

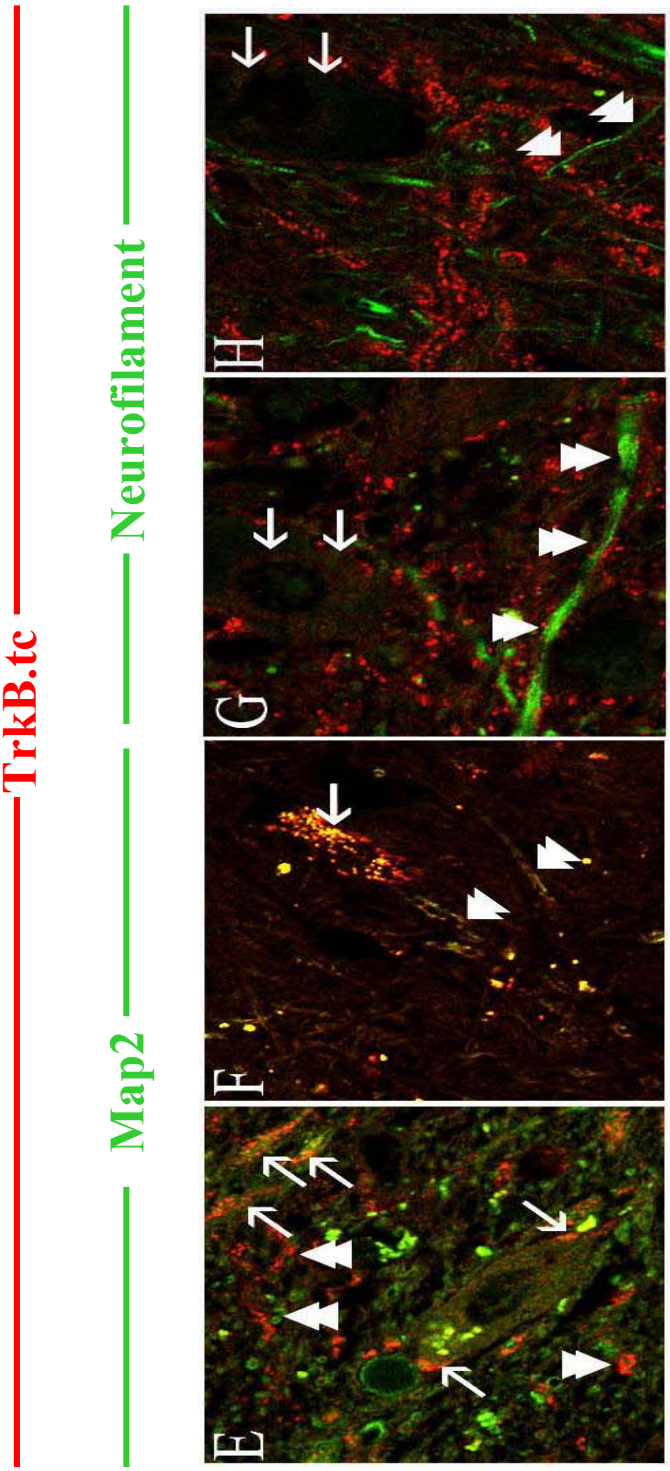


Figure 25(e-h). Neuronal localization of trkB.tc in PD substantia nigra.
In the substantia nigrapc, trkB.tc is localized to Map2-positive dendrites and somata in control (E, arrows) and PD (F, arrows and arrowheads). Not all nigral Map2-positive dendrites express trkB.tc proteins in the control tissues (E, double-arrowheads). Most neurofilament-positive axons (double-arrowheads) in the substantia $nigra_{pc}$ are in close proximity to, but do not co-localize with trkB.tc in control (G) and PD (H) neurons. In the PD substantia nigrapc (H), there is a decrease in somal trkB.tc protein expression compared to controls (G).

Although trkB.fl proteins are not as abundant as trkB.tc in the striatum, trkB.fl proteins are localized to neuronal somata in control and PD neurons (Figure 26a and b). Axons in this region do not express trkB.fl proteins (Figure 26c and d). In the substantia $nigra_{pc}$, trkB.fl is localized to somata in control and PD neurons, but is decreased in PD (Figure 26e and f). TrkB.fl proteins are localized to nigral axons in control and PD neurons, but are increased in PD (Figure 26g and h). Map2-labeling of the substantia $nigra_{pc}$ shows that trkB.fl protein is localized to proximal dendrites in PD (Figure 26f).

3. TrkB distribution in degenerating neurons

To determine if trkB distribution was associated with degenerating neurons, we analyzed the distribution of trkB.tc and trkB.fl with alpha-synuclein-positive neurons in control and PD tissues. Alpha-synuclein is a marker for dystrophic neurons. Dystrophic neurons are found in both the striatum and substantia $nigra_{pc}$ in PD, but are most abundant in the substantia $nigra_{pc}$ (Figure 27 and 28). For this study, we analyzed the distribution of trkB to both dystrophic somata and neurites.

In all control tissues, including the striatum and substantia $nigra_{pc}$, alpha-synuclein-positive dystrophic somata or neurites were lacking. Isolated dystrophic neurites found in the PD striatum express trkB.fl proteins (Figure 27b). These dystrophic neurons appear in localized areas of the striatum and are not widespread. In the PD substantia $nigra_{pc}$, trkB.fl proteins are localized to dystrophic somata and are abundant in dystrophic neurites (Figure 27d). The dystrophic somata and neurites in the striatum and substantia $nigra_{pc}$ do not express trkB.tc proteins (Figure 28a and c). The abundance of trkB.tc protein in neurites of the substantia $nigra_{pc}$ results in trkB.tc-positive neurites in close proximity to dystrophic neurites (Figure 28d).

Figure 26 (a-d). Neuronal localization of trkB.fl in the PD striatum.

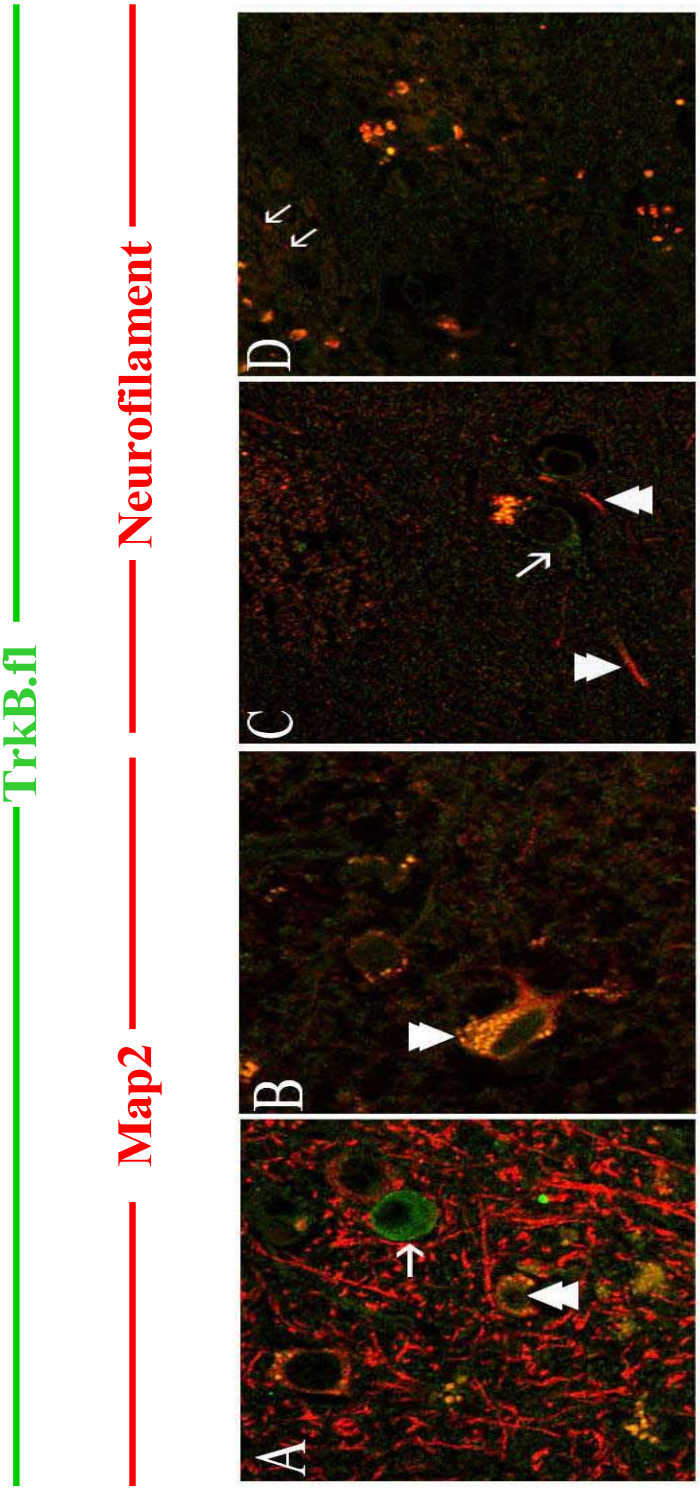


Figure 26 (a-d). Neuronal localization of trkB.fl in PD in striatum. TrkB.fl (green) is localized to MAP2-positive (red) somata (double-arrowheads) and in the striatum of control (A) and PD (B) neurons. Neurofilament labeling (green) of the striatum shows that trkB.fl localization is not axonal in control (C, double-arrowhead) or PD (D, arrows) neurons. TrkB.fl found in the soma of control striatal neurons (C, arrow).

Figure 26(e-h). Neuronal localization of trkB.fl in the PD substantia nigra$_{pc}$

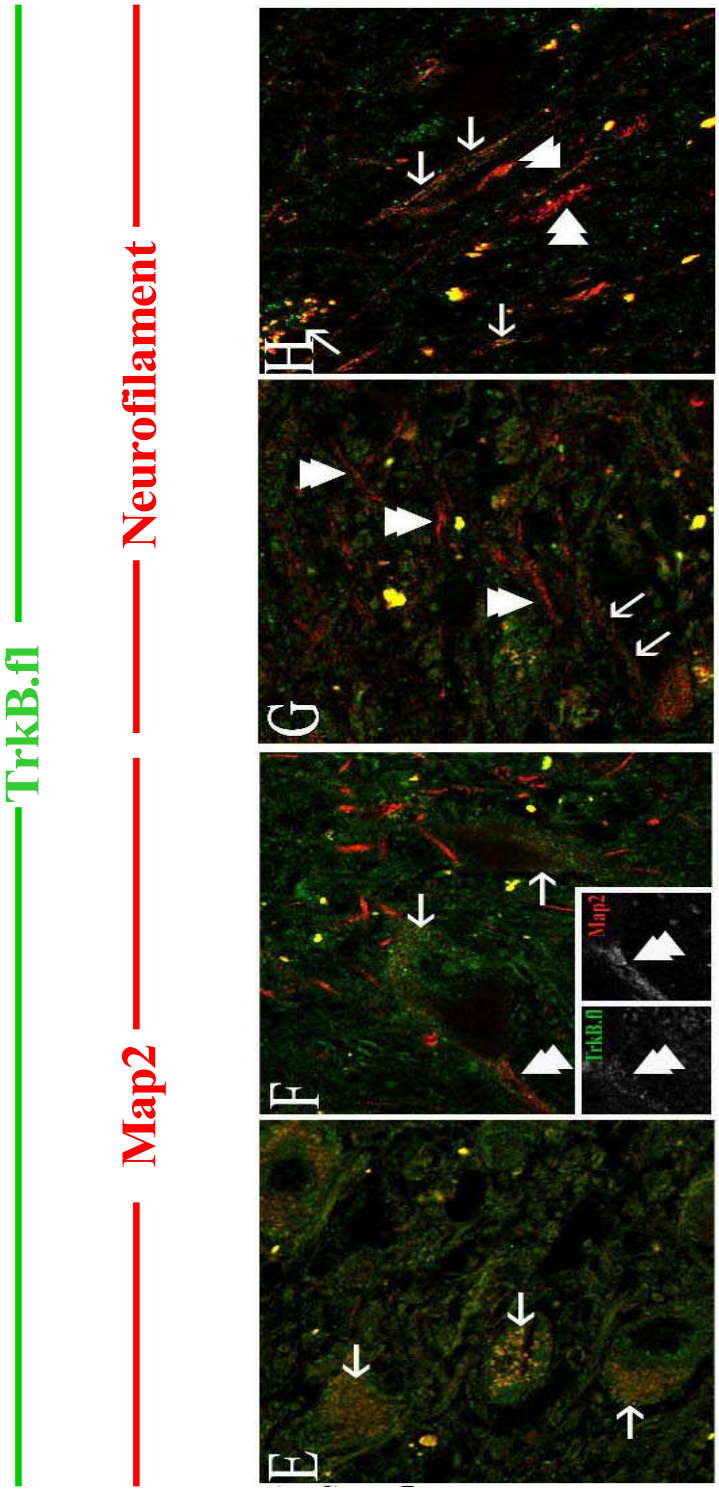


Figure 26(e-h). Neuronal localization of trkB.fl in PD in striatum.
In the substantia nigrapc, trkB.fl proteins are found in the somata of control (E, arrows) and PD (F, arrows) neurons. In PD, trkB.fl is localized to proximal dendrites of nigral neurons (F, double-arrowheads and inserts). In the control substantia nigrapc (G, arrows), there are few axons that express trkB.fl proteins. In the PD substantia nigrapc (H), there is an increase in axonal trkB.fl (arrows). Double-arrowheads denote trkB.fl-negative axons (G, H).

Figure 27. TrkB.fl distribution in degenerating neurons.

Dystrophic neurons were not abundant in control striatum (A) and substantia nigrapc (C). TrkB.fl (green) is found localized to dystrophic (red) neurites (double-arrowheads) in the PD striatum (B), although they are not abundant or widespread. There is a much greater number of dystrophic neurites (double-arrowheads) and somata (arrows) in the PD substantia nigra$_{pc}$ (D).

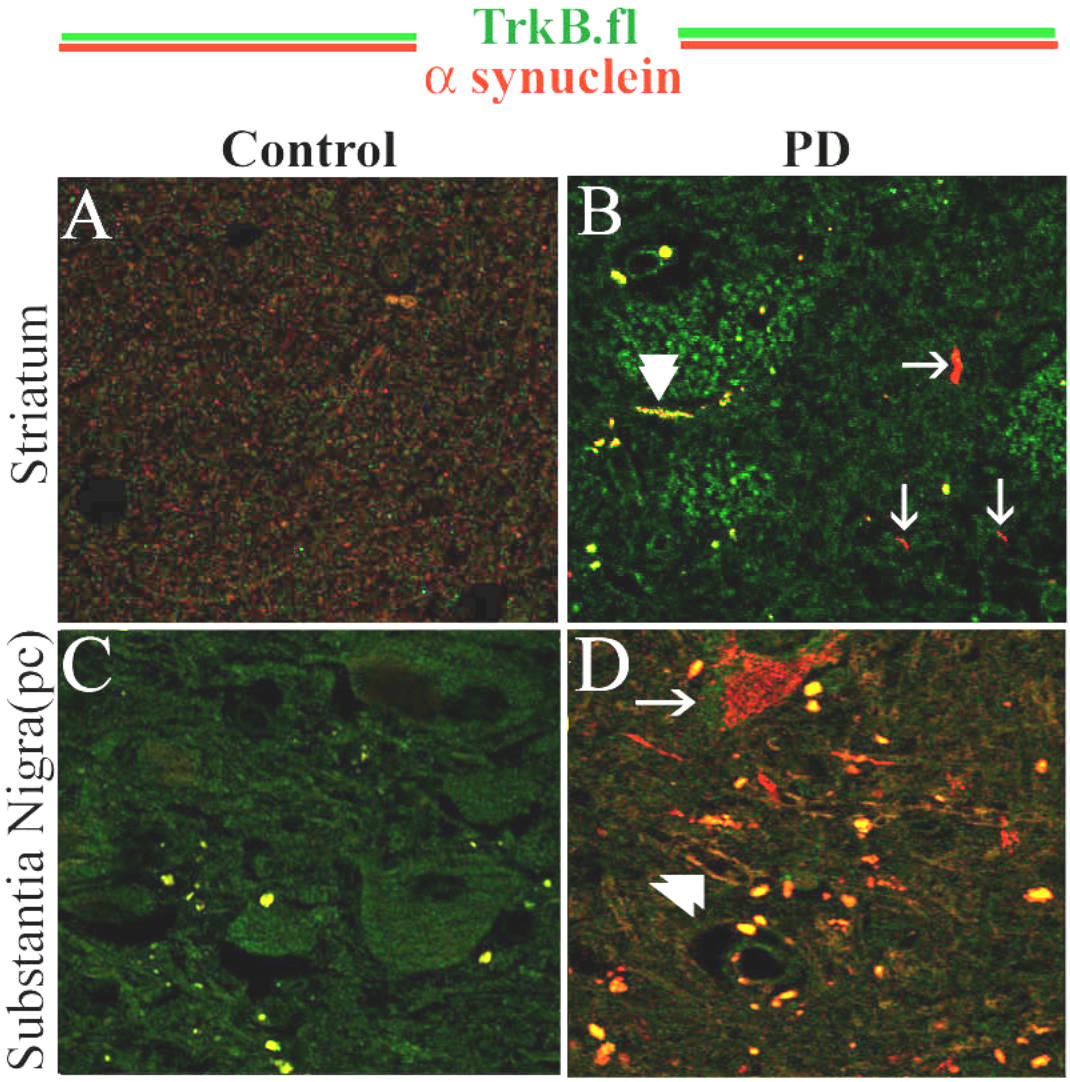
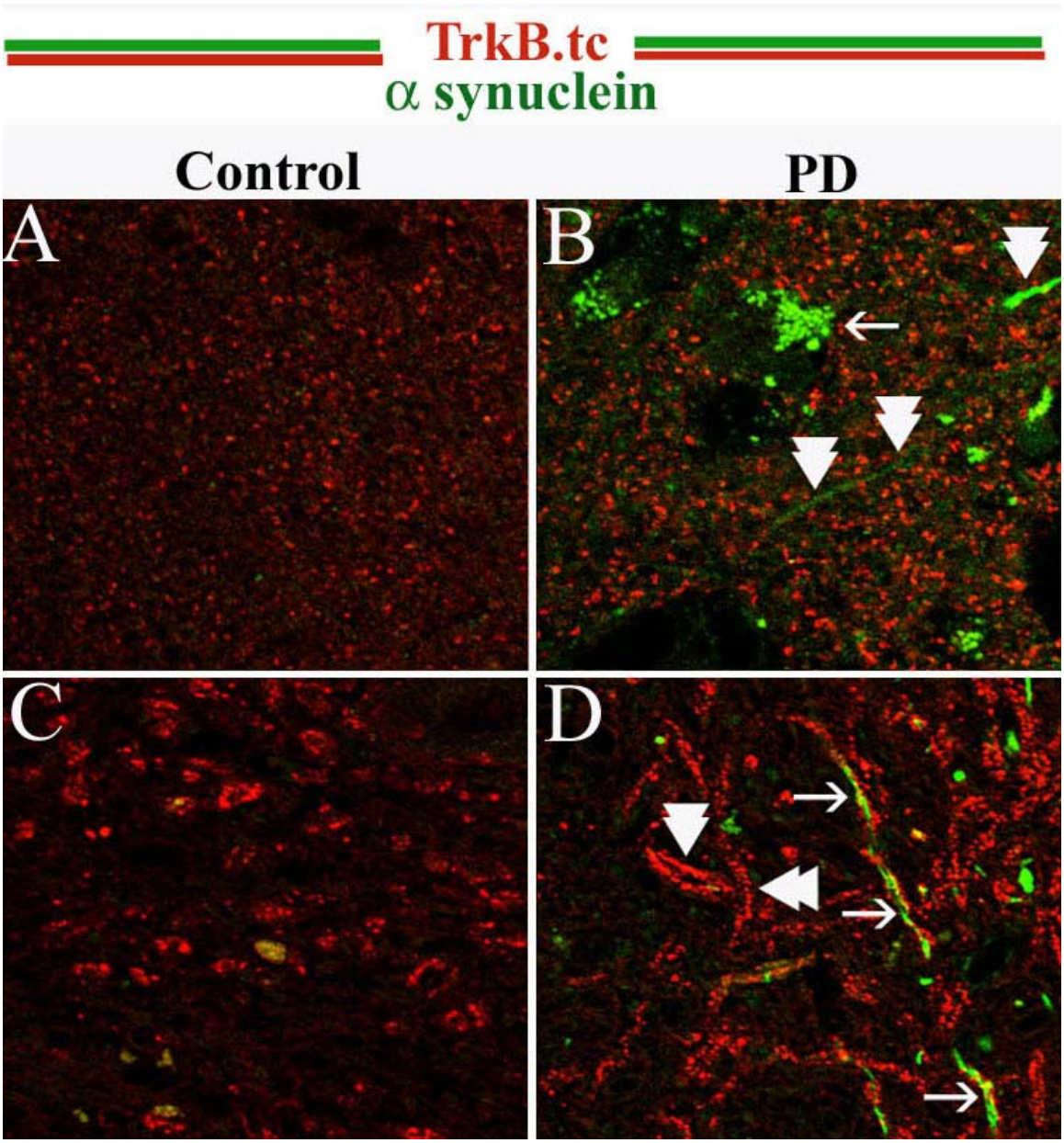


Figure 28. TrkB.tc distribution in degenerating neurons.

Dystrophic neurons were not abundant in control striatum (A) and substantia nigrapc (C). In the PD striatum (B), trkB.tc is not localized to dystrophic neurites (double-arrowheads) or somata (arrows). But, trkB.tc is found in proximity to dystrophic neurites (arrows) in the PD substantia nigra$_{pc}$ (D). There is an abundance of trkB.tc-positive neurites in regions of pathology (D, double-arrowheads).



4. Association of trkB proteins with gliosis in PD

To study to role of gliosis in trkB protein distribution and expression, we looked at the co-localization of trkB.tc and trkB.fl to GFAP, a marker for astrocytes. The most striking difference in the localization of trkB.tc and trkB.fl to regions of gliosis is that trkB.tc is not localized to glial cells in control or PD tissues (Figure 29b and f). TrkB.fl is localized to control (Figure 29c and g) and PD (Figure 28d and h) astrocytes in the striatum and substantia $nigra_{pc}$. There was no difference in the localization of trkB.fl to astrocytes between control and PD tissues. The expression of trkB.fl on glial cells is not an unexpected finding, as BDNF is known to be expressed and internalize by glial cells in the CNS (Batchelor et al., 1999) (Alderson et al., 2000).

Figure 29. Association of trkB proteins with gliosis in PD.

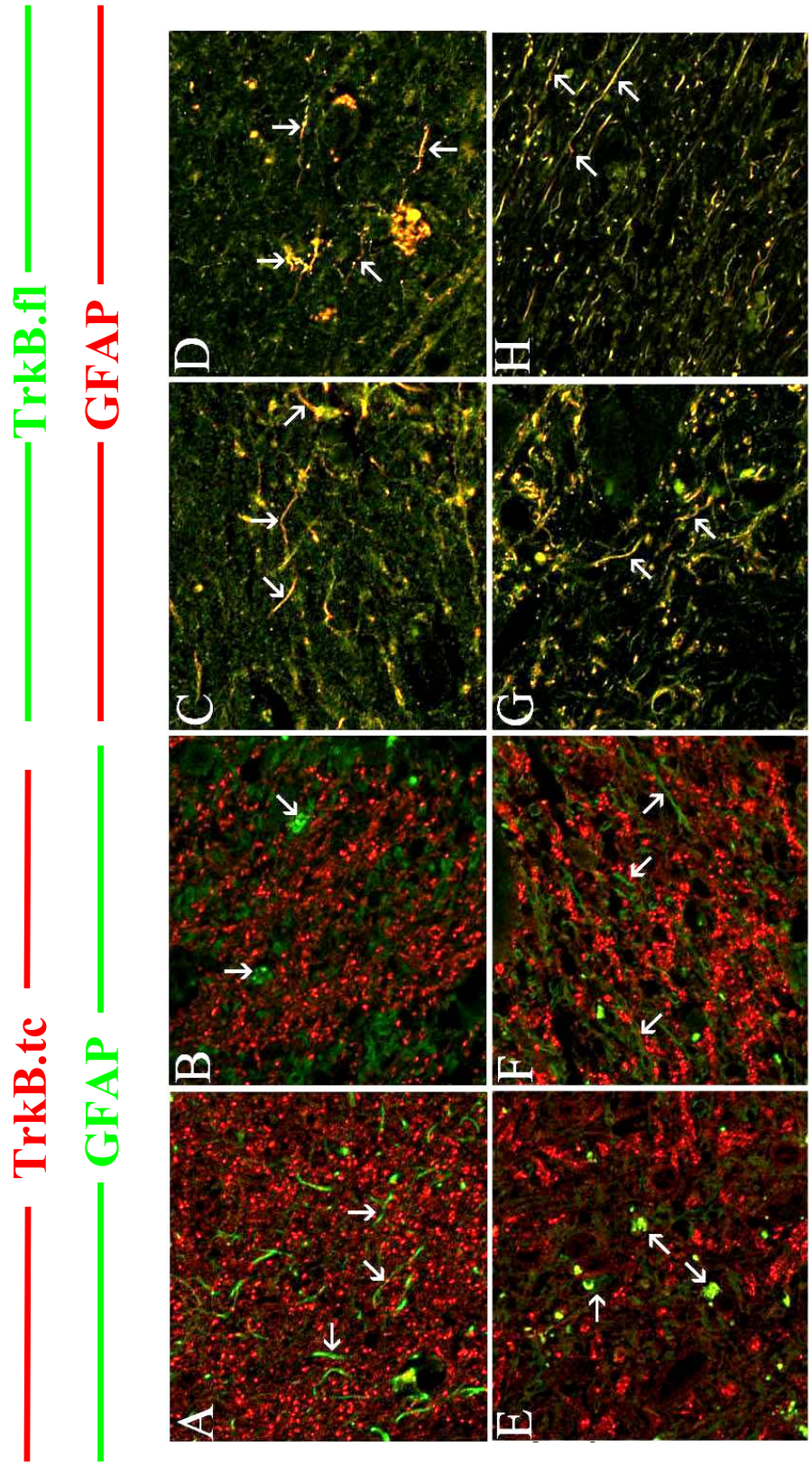


Figure 29. Association of trkB proteins with gliosis in PD
TrkB.tc (red) proteins are not localized to astrocytes (green) in the control (A, arrows) or PD (C, arrows) striatum. This is also the case in the control (E, arrows) and PD (F, arrows) substantia nigrapc. TrkB.fl (green) is localized to astrocytes (red) of both the control (C, arrows) and PD (D, arrows) striatum. Localization of trkB.fl to astrocytes is also observed in the control (G, arrows) and PD (H, arrows) substantia nigrapc.

5. Conclusions

Parkinson's disease is a debilitating motor disorder that affects 1% of the North American population over the age of 65 (Adams et al., 1997). Although we are beginning to understand some of the genetic and molecular factors in the disease process, there is no single known cause. Studying the underlying mechanisms of neurodegeneration will reveal therapeutic targets for the treatment of many neurodegenerative diseases, including PD.

BDNF is a potent neurotrophic factor that protects or rescues distressed dopaminergic neurons (Hagg, 1998) (Son et al., 1999). It had been shown that BDNF mRNA and protein expression are altered in the PD substantia nigra *pars compacta* (Howells et al., 2000) (Mogi et al., 1999). Although this is not the only region affected in PD, it is one of the major targets of the disease, with over 60% of the substantia $nigra_{pc}$ dopaminergic neurons dying in PD compared to normal age-matched controls (Adams et al., 1997). The decrease in nigral BDNF may contribute to the abundant cell death observed in this region. We hypothesized that in addition to the decrease in BDNF proteins, there is also an alteration in the expression of trkB proteins in PD autopsy tissues. Changes in normal trkB distribution in the PD brain may be a neuronal response to neurodegeneration. Furthermore, trkB has two receptor isoforms: a full-length receptor capable of activating intracellular signaling pathways (McCarty and Feinstein, 1998) (Nakamura et al., 1996) (Patapoutian and Reichardt, 2001) and a truncated receptor that may have a role in the intracellular transport of internalized BDNF. We analyzed changes in the differential distribution of each receptor isoform to better understand how altered trkB function can affect BDNF activities. The principal findings of this paper show that trkB.tc and trkB.fl are differentially distributed in the control and PD brains. Changes in the distribution of both trkB isoforms are seen in PD and correspond to regions of pathology (Table 4).

Table 4. Distribution of trkB.tc and trkB.fl in the control and PD striatum and substantia nigrapc.

Presence of labeling in specified region is indicated by 'YES' or 'NO'.

STRIATUM

	DENDRITES	SOMA	AXONS	DYSTOPHIC NEURONS	GLIA
CONTROL					
TrkB.tc	NO	YES	YES	NO	NO
TrkB.fl	NO	YES	NO	NO	YES
PD					
TrkB.tc	NO	NO	YES	NO	NO
TrkB.fl	NO	YES	NO	YES	YES

SUBSTANTIA NIGRApc

	DENDRITES	SOMA	AXONS	DYSTOPHIC NEURONS	GLIA
CONTROL					
TrkB.tc	YES	YES	NO	NO	NO
TrkB.fl	NO	YES	YES	YES	NO
PD					
TrkB.tc	YES	YES	NO	NO	NO
TrkB.fl	YES	YES	YES	YES	YES

TrkB.fl can be activated by intracellular or extracellular BDNF. For extracellular BDNF to regulate cell survival and differentiation, it must bind to a trkB.fl receptor on the cell surface. Binding of BDNF to the trkB.fl homodimer induces autophosphorylation of this dimer and activates downstream signaling cascades. Heterodimerization of trkB.fl and trkB.tc does not activate intracellular signaling cascades. Our data show that trkB.fl does not change its distribution pattern in the striatum between control and PD tissues, but does so in the substantia

nigra$_{pc}$. In the PD substantia nigra$_{pc}$, trkB.fl proteins are upregulated in nigral axons. Furthermore, trkB.fl is also found localized to dystrophic neurons PD. These data suggest that the increase in trkB.fl may actually occur to heighten the sensitivity of the cell to BDNF stimulation. Watson et al. (1999) studied the mechanism of retrograde BDNF signaling in dorsal root ganglion cells. They hypothesized that signaling occurs through retrograde transport of trkB.fl or by signal propagation induced by trkB.fl activation and endocytosis. TrkB.fl was internalized at the axon, retrogradely transported as a complex with BDNF, and activated the nuclear protein pCREB (Watson et al., 1999). Therefore, the increase in trkB.fl protein distribution to axons, observed in our study, may indicate a signaling response to induce cell survival.

The role of trkB.tc in the normal CNS is not clear. It has been suggested that the truncated receptor acts either as a dominant negative protein that inhibits trkB.fl function or a protein that sequesters BDNF (Biffo et al., 1995) (Barettino et al., 1999). Recently, evidence has shown that trkB.tc may mediate the intracellular transport of internalized BDNF. We showed trkB.tc changes its protein expression in PD. The most significant differences in trkB.tc distribution were as follows: 1) it is decreased in the somata of pigmented neurons in the substantia nigra of PD cases and 2) increased in their axonal projections to the striatum. The axonal accumulation of trkB.tc occurs because it is either transported away from the soma or because it is synthesized in these neurites. Interestingly, trkB.tc was absent from the degenerating neurons positive for α-synuclein suggesting at least two possible scenarios: 1) the increased trkB.tc expression and redistribution to the dopaminergic axonal projections to the striatum is an early event, preceding the α-synuclein aggregates or 2) the higher levels of trkB.tc in some axons could indicate neuroprotection and possibly regeneration. In summary, we

interpret our results as indicating that trkB.tc may be an important mediator and modulator of the BDNF response to neuronal injury in PD. This hypothesis was confirmed by in vitro studies in our laboratory (Chapter 4).

6. Discussion

The goal of this dissertation was to identify a novel role for the truncated trkB receptor in mature neurons of the CNS. Specifically, the purpose of these experiments was to: 1) elucidate the intracellular sorting of BDNF facilitated by trkB.tc, 2) to determine if trkB.tc distribution is altered in Parkinson's disease, and 3) to correlate the changes in trkB.tc distribution in PD and its role in the intracellular sorting of BDNF. The primary results of these experiments are as follows: 1) trkB.tc facilitates the intracellular sorting of internalized BDNF to recycling endosomes, 2) trkB.tc proteins are altered in PD and are associated with regions of pathology, and 3) the altered distribution of trkB.tc in PD suggests increased transport of trkB.tc and potentially that of its ligand, BDNF. Therefore, changes in trkB.tc protein distribution are a neuronal response to neurodegeneration.

1. A novel role for trkB.tc

1.1 TrkB.tc facilitates the intracellular sorting of internalized BDNF to recycling endosomes.

The trkB receptors, trkB.tc and trkB.fl, are products of alternative splicing. They bind to their high affinity ligand, BDNF, with equal affinity and avidity because their extracellular domains are 100% homologous (Klein et al., 1990). Previous studies have indicated a role for trkB.tc in BDNF sequestering during development (Biffo et al., 1995). The mechanism of sequestration includes the binding of BDNF to trkB.tc receptors on non-neuronal tissues, regulating the availability of BDNF to neuronal tissues throughout development. Unlike trkB.fl, trkB.tc receptors are not downregulated in response to BDNF stimuli (Sommerfeld et al., 2000).

This suggests that trkB.tc either does not respond to BDNF or, conversely, that its response to BDNF is sustained over time. TrkB.tc is more abundant in the mature brain than trkB.fl (Ohira et al., 1999). Therefore, we hypothesize that the response of trkB.tc to BDNF stimuli is sustained over time and, more importantly, plays an active role in BDNF signal regulation. Because trkB.tc lacks the catalytic signaling domain found in trkB.fl, we hypothesized that trkB.tc acts as a chaperone protein and facilitates the intracellular sorting of internalized BDNF.

We have demonstrated that trkB.tc binds to BDNF at the cell surface. The trkB.tc/BDNF complex is then internalized via receptor-mediated endocytosis and sorted to early endosomes. From here, the trkB.tc/BDNF complex is sorted to both recycling endosomes and lysosomes. Lysosomal degradation of the trkB.tc/BDNF complex is minimal and only one-third of that sorted to recycling endosomes. The sorting of trkB.tc/BDNF complex to recycling/transport vesicles indicates novel roles for the truncated trkB receptor in neurons: 1) trkB.tc chaperones the intracellular transport of internalized BDNF; 2) this complex is sorted to recycling endosomes that can translocate trkB.tc/BDNF to proximal trkB.fl receptors; and 3) the sorting of trkB.tc/BDNF to recycling endosomes, as opposed to lysosomes, provides an environment where the complex can be further transported to distal trkB.fl receptors. *The trkB.tc-mediated selective sorting of BDNF to trkB.fl receptors indicates a novel mechanism for regulating BDNF signaling.*

1.2. The significance of trkB.tc-mediated presentation of internalized BDNF to distal trkB.fl receptors

BDNF is a neuronal growth factor that also modulates synaptogenesis and neurotransmission (Balkowiec et al., 2000) (Pozzo-Miller et al., 1999). Previous studies have

shown that BDNF can act at the synapse, directly influencing its protein distribution and synaptic morphology (Carter et al., 2002) (Bradley and Sporns, 1999) (Tyler and Pozzo-Miller, 2001). We propose that trkB.tc facilitates the sorting of BDNF to recycling endosomes and it also drives the anterograde transport of BDNF.

There are two possible mechanisms for the trkB.tc mediated anterograde transport of BDNF following their sorting to recycling endosomes: 1) intracellular cues initiate the sorting of only BDNF to axonal vesicles and 2) trkB.tc further chaperones the transport of BDNF to axonal vesicles, maintaining the trkB.tc/BDNF complex. The anterograde transport of BDNF will present BDNF to trkB.fl receptors in the presynaptic terminal. Here, BDNF can activate these receptors to modify the synapse. Increases in synaptic vesicle docking and exocytosis will enhance the synaptic signal. BDNF can be released into the synaptic cleft by activity-dependent exocytosis and subsequently bind to trkB.fl receptors on the post-synaptic membrane. The stimulation of pre- and post-synaptic trkB.fl receptors can influence the strength of the synapse and even its long-term potentiation. Therefore, trkB.tc may regulate synaptogenesis and neurotransmission by regulating the availability of internalized BDNF to synaptic trkB.fl receptors. Furthermore, BDNF could also induce its growth factor effects on the post-synaptic cell.

1.3 Future Directions: Anterograde transport of trkB.tc/BDNF

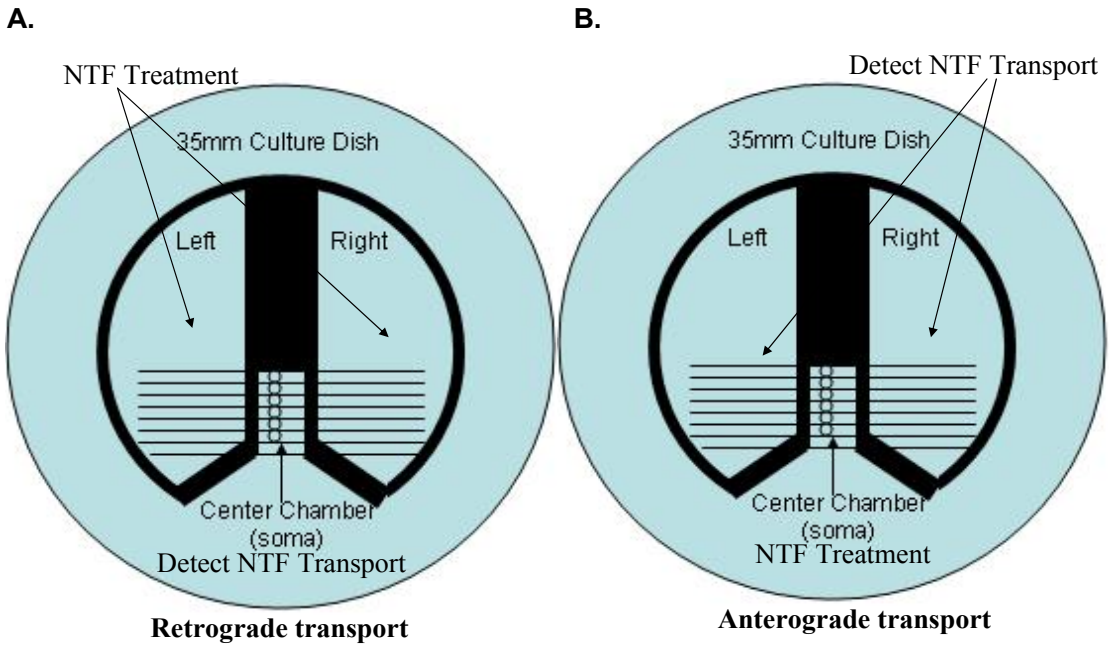
1.3.1 Campenot chambers: a model of anterograde BDNF transport.

The Campenot chamber is a valuable tool that has been used for decades to study retrograde transport of nerve growth factor and trkA (Campenot, 1977) (Senger and Campenot, 1997) (Ure and Campenot, 1997). These chambers create a means for neuronal cell bodies and

their processes to be exposed to different cellular environments (Campenot, 1979). For example, in retrograde studies, the neuritic chamber is exposed to a neurotrophin, but the somata are only exposed to media. Retrograde transport is measured by the amount of neurotrophins localized to the somal chamber (Figure 30). Studies using compartmentalized cultures have addressed the role of adaptor proteins, such as PLCγ, in NGF retrograde signaling (Kuruvilla et al., 2000). Recently, this culture system has been expanded to study the retrograde signaling and transport of trkB.fl and BDNF in dorsal root ganglion cultures (Watson et al., 1999) (Bhattacharyya et al., 2002).

Figure 30. Campenot chamber.

A. Treatment model to study the retrograde transport of neurotrophins. The outer chambers (neurites) are exposed to labeled neurotrophin. The center chamber (somata) is analyzed for transported neurotrophin. B. Treatment model to study the anterograde transport of neurotrophins. The center chamber (somata) is exposed to neurotrophin. The outer chamber (neurites) is analyzed for transported neurotrophin.



The Campenot culture system is ideal to study the anterograde transport of trkB.tc and BDNF. Instead of treating the neurites with BDNF, the somal chamber could be treated with BDNF. DRGs are sensory neurons that respond to BDNF stimuli. It would be necessary to use dorsal root ganglion (DRG) cultures in these studies instead of a neuronal cell line because their neuritic extension is more robust. To investigate the anterograde transport of internalized BDNF, the center (somal) chamber of the Campenot cultures would be treated with biotin-BDNF and the subsequent distribution of b-BDNF to the outer chambers would confirm its anterograde transport. The presence of axonal trkB.tc/b-BDNF complex would be assessed by immunofluorescence laser confocal microscopy. Co-localization of trkB.tc/b-BDNF would indicate whether trkB.tc chaperones anterograde transport of internalized BDNF. Manipulation of these cultures with inhibitors of protein transport, tyrosine kinase activity, and trkB.tc binding to BDNF could further elucidate the mechanisms involved in trkB.tc-mediated transport and presentation of BDNF to distal trkB.fl receptors.

1.3.2 Inhibiting trkB.tc function: knock-out mice versus siRNA.

Evidence provided in this dissertation suggests that trkB.tc facilitates the intracellular sorting of internalized BDNF. Unlike trkB.fl, there is no known inhibitor of trkB.tc function, as we are just beginning to understand its role in the CNS. Therefore, it would be attractive to address the transport of BDNF in mice lacking the trkB.tc receptor.

Knock-out mice are a valuable system to study loss-of-function by deleting a gene for the protein of interest. There are, however, some inherent problems with this system, especially when studying the role of trkB.tc in BDNF transport. First, the trkB gene produces two RNA products due to alternative splicing: the truncated and the full-length trkB receptors. TrkB

knockout mice are deficient in both trkB.tc and trkB.fl proteins. Therefore, changes observed in these mice can not be attributed solely to either receptor isoform.

One means to address this problem is to generate cultures from trkB knock-out mice and to then transfect these cultures with trkB.tc RNA. If the transfection is efficient, these cultures will contain cells that only express trkB.tc proteins. This experimental design would allow us to study the trkB.tc protein independent of trkB.fl. Conversely, we could transfect trkB.fl into the knock-out cultures and observe changes in BDNF transport compared to control cells (from normal mice). This system would provide a means to study trkB.tc loss-of-function.

Recently, the field of small-interfering RNAs (siRNAs) has evolved. siRNAs are sequence specific and act post-translationally. These RNA species can be generated against a specific protein, such as trkB.tc. To study the role of trkB.tc in BDNF transport, siRNAs would be used to inhibit the translation of only trkB.tc, resulting in the inhibition of trkB.tc protein expression (Bass, 2001) (Elbashir et al., 2001). The resulting cells would still express trkB.fl. This system would be a valuable tool in the study of trkB.tc function.

2. TrkB.tc protein distribution is altered in PD

Altered trkB.tc protein distribution and expression in PD may regulate BDNF function. Preliminary studies of human autopsy striatal and nigral tissues showed that trkB.tc was: 1) more abundant, 2) differentially distributed, and 3) upregulated in regions of pathology. A detailed analysis of trkB.tc in neuronal and glial cells in regions of PD pathology showed that trkB.tc is not found on glial cells, but is upregulated in neurites, especially in striatal axons. Furthermore, trkB.tc is decreased in the somata of pigmented neurons. One hypothesis explaining this finding is that the anterograde transport of trkB.tc is upregulated in the PD

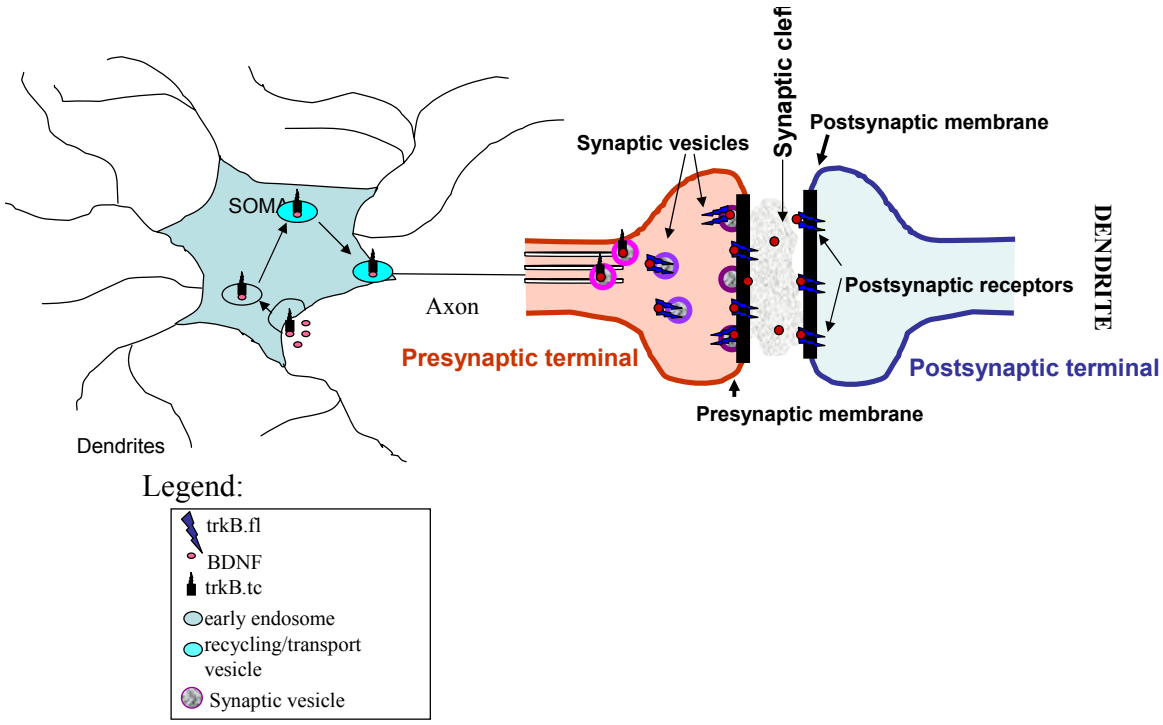
striatum. (The analysis of the changes in trkB.tc distribution in PD is explained in detailed chapter 5).

2.1 Does the aberrant distribution of trkB.tc in PD implicate altered intracellular transport?

Our initial hypothesis stated that aberrant trkB.tc protein distribution in PD contributed to impaired BDNF function. Surprisingly, the observed decrease in somal and increase in axonal trkB.tc in the PD striatum suggests that the cell may be actually reacting to injury by increasing its transport of trkB.tc, and subsequently BDNF. The increased anterograde transport of BDNF and trkB.tc could sensitize the cell to heighten its response to the growth factor effects of BDNF. To sensitize the cell, trkB.tc facilitates the anterograde transport of BDNF to synaptic trkB.fl receptors. Transported BDNF could be released by trkB.tc to activate trkB.fl receptors in the pre- or post- synaptic terminal, strengthening the synapse and potentially protecting the post-synaptic cell from degeneration (Figure 31).

Figure 31. Neuronal response to disease.

TrkB.tc facilitates the anterograde transport of BDNF to the synapse. BDNF is released into the pre-synaptic terminal where it can bind to trkB.fl receptors. BDNF can also be released into the synaptic cleft to bind to post-synaptic trkB.fl receptors.



2.2. Future Directions: TrkB.tc in the neurotrophin response to neurodegeneration

The human autopsy studies performed in this dissertation do not directly address the function of altered trkB.tc protein distribution in PD. *In vivo* and *in vitro* models of PD are seminal to the elucidation of the role of trkB.tc transport in PD.

To determine if trkB.tc is involved in the anterograde transport of BDNF in the normal striatum and substantia nigra, siRNAs specific to trkB.tc could be injected into these regions.

siRNAs would be designed to optimize entry into the cell and resist enzymatic degradation. In the cytoplasm, siRNAs would prevent protein translation of trkB.tc. This will result in a regional knockout of trkB.tc. Following trkB.tc knockout, biotin labeled BDNF would be injected into the striatum or substantia nigra (the region of trkB.tc knockout). The percent of biotin-labeled BDNF in the ipsilateral and contralateral target regions would be determined to elucidate the role of trkB.tc in the anterograde transport of BDNF.

The role of trkB.tc in the PD brain could be determined by lesioning the substantia nigra with MPTP toxins in the primate model of PD. The resulting death of dopamingergic neurons mimics the pathological environment of PD in this region. Biotin labeled BDNF would be injected into the lesioned substantia nigra and its transport would be determined by immunohistochemical analysis. If there were an increase in axonal trkB.tc and BDNF, as well as co-localization of the two proteins, we would conclude that trkB.tc and BDNF are increased in PD to rescue the neuron from injury.

For the endosomal sorting studies described in this dissertation, we used SH-SY5Y cultures. These cultures are a practical cell line to use in an *in vitro* model of PD. Not only do these cells develop into polarized dopaminergic neuronal cells, they are also sensitive to 6-OHDA toxicity {Yu, 1997 #279}. These cells can be manipulated to study the transport of trkB.tc and BDNF in response to neuronal toxicity.

3. Clinical Implications

BDNF is implicated as a potential therapeutic target in many neurodegenerative diseases, including Parksinson's disease and Alzheimer's disease, because of its potent neurotrophic effects (Bradford et al., 1999) (Ferrer et al., 1999). Not only are BDNF proteins and mRNA

decreased in neurodegenerative diseases, but its expression is also decreased in dying neurons of the aged brain (Croll et al., 1998).

1. Role of trkB in the therapeutic administration of BDNF

Replenishing BDNF to regions of pathology could rescue neurons from injury. The clinical use of BDNF is plagued by complications related to its systemic administration. BDNF does not diffuse across the blood-brain barrier (BBB). It is necessary for BDNF to be bound to a transport molecule, like transferrin, in order for it to enter the brain (Mufson et al., 1999). Once across the BBB, BDNF protein randomly diffuses throughout the brain. Therefore, the therapeutic target region does not receive adequate amounts of BDNF to promote survival. Systemic administration is further complicated by side effects, such as weight loss (Mufson et al., 1999).

Recent evidence has implicated trkB in the intracellular transport of BDNF. TrkB.fl receptors mediate the retrograde transport of BDNF (Ginty and Segal, 2002) and trkB.tc potentially facilitates the anterograde transport of BDNF. Transport properties of the trkB receptors can be exploited to develop new strategies for BDNF administration. We need to determine which domains of the BDNF protein are necessary to induce only trkB-mediated transport. From this data, a synthetic peptide can be generated. Consequently, target directed transport of BDNF would be driven by trkB.tc or trkB.fl receptors.

Systemic administration of the BDNF peptide would not be sufficient to induce target-derived transport of the peptide. Instead, it would be necessary to inject the peptide into a specific region of the brain. For instance, in PD, the peptide could be injected into the striatum, where it would be transported to the substantia nigra(pc), cortex, and limbic regions (Mufson et

al., 1999). The trophic effects of BDNF would benefit all of these regions. If the desired transport of the peptide was to be unidirectional, the generated synthetic peptide could include fragments of trkB.tc or trkB.fl protein. The amino acid sequences within trkB.tc and trkB.fl that drive intracellular transport are not known. But, once determined, a chimeric synthetic peptide could be generation. This chimera would include the BDNF sequences necessary induce survival and the trkB sequence necessary to facilitate unidirectional transport.

2. Early markers of neurodegeneration

The early detection of degenerating neurons would facilitate the treatment of many neurodegenerative diseases. BDNF and its trkB receptor expression are altered in neurodegenerative diseases and in the aging brain (Croll et al., 1998) (Ferrer et al., 1999) (Howells et al., 2000) (Mogi et al., 1999) (Chapter 5). The neuronal response to degenerative diseases and normal aging is defined by: decrease in BDNF protein, decrease in trkB.fl protein, and increase in trkB.tc protein. Therefore, the pattern of combined changes in BDNF, trkB.fl, and trkB.tc proteins may be a novel early marker of neurodegeneration. Brain imaging analysis of these changes may provide a new diagnostic tool in the early detection of neurodegeneration.

4. Conclusions

Our experiments were designed to address a novel role for the truncated trkB receptor in mature neurons of the nervous system. We have shown that trkB.tc facilitates the endocytic sorting of internalized BDNF to recycling endosomes and that trkB.tc protein distribution is altered in Parkinson's disease. Aberrant trkB.tc distribution in PD suggests that the changes in

trkB.tc are part of the cells response to disease. We have shown that TrkB.tc is more than just a molecule that forms non-functional heterodimers, but that it has a unique and active role in BDNF function.

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