The Association of p75NTR and Co-receptors in Hippocampal Neurons and Astrocytes

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A Dissertation submitted to
the

Graduate School-
Newark

Rutgers, The State University of New

Jersey In partial fulfillment of the

requirements for the degree of

Doctor of
Philosophy

Graduate Program in Biological

Science written under the direction

of

Dr. Wilma
Friedman

and approved
by

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Newark, New
Jersey

May
2015

ABSTRACT OF THE
THESIS

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Neurons in the hippocampus are dependent on neurotrophins for survival. Upon injury, proneurotrophins cause hippocampal neurons to undergo apoptosis. These two distinct effects can be mediated by the interaction of p75NTR with different co-receptors. Trk receptors typically signal for survival and the signaling is enhanced in the presence of p75NTR. Apoptotic signaling through proneurotrophins is dependent on the presence of p75NTR and sortilin. The work in this thesis demonstrates that the association of p75NTR and TrkB, and the association of p75NTR with sortilin are both ligand dependent. The association of p75NTR and TrkB is dependent on internalization of the complex and TrkB activation . Sortilin and p75NTR associated both on the cell surface and in intracellular vesicles.

Hippocampal astrocytes also express these receptors but do not signal for death upon proneurotophin treatment and are not dependent on mature neurotrophins for survival. This thesis demonstrates that the interaction of p75NTR and sortilin does not occur on hippocampal astrocytes. TrkB and p75NTR present on hippocampal astrocytes do not increase their association following treatment with

BDNF.

Taken together, the different responses between hippocampal neurons and astrocytes may be partially determined by limiting the interactions of p75NTR and co- receptors on astrocytes.

The data in this thesis demonstrate that the association of p75 with different co-receptors may determine the trafficking of these receptors through intracellular compartments, which is likely to influence intracellular signaling and the functional response of neurons and astrocytes to these factors.

Dedication

The foremost dedication is to my mother, Judith Yarotsky. Without her years of sacrifice and dedication to her children I could not have become the man I am today.

I would also like to dedicate a part of this work to my father, Walter Yarotsky. He gave me my first microscope as a child and kindled my yearning to see a world my own eyes could have never revealed to me.

Acknowledgements

I would like to sincerely thank my research advisor, Dr. Wilma J. Friedman for all the hard work she put in to this project, and more importantly, to me.

I would also like to thank Dr. Nihal Altan-Bonnet, Dr. Haesun Kim, Dr. Cheryl Dreyfus, Dr. Marta Volosin, Dr. Jeffrey D. Kidder, Dr. Jorge Golowasch, Dr. Farzan Nadim, and Dr. Edward Bonder, who have all had a hand in either shaping my research or my intellectual development.

Lastly, I would like to thank everyone else in the Biology department at Rutgers University and NJIT who has helped me through years. This group is too numerous to list but no less valuable to me, thank you.

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List of Abbreviations: AB-

amyloid beta

BDNF- brain derived neurotrophic factor

CNS- central nervous system

CREB- cAMP response binding element

ECD- extracellular domain

EEA1- early endosomal antigen 1

EGFR- epidermal growth factor receptor

LAMP-1- lysosome-associated membrane protein

LTD- long term depression

LTP- long term potentiation

MAG- myelin-associated glycoprotein

MAPK-mitogen-activated protein kinase

NF-KB- nuclear factor KB

NGF- nerve growth factor

NgR- nogo receptor

NRIF-neurotrophin receptor interacting factor

NTRAP-neurotrophic factor receptor-associated protein

NTS1 and 2- neurotensin receptor one and two

NT-3- neurotrophin three

NT-4- neurotrophin four

OMgP- oligodendrocyte myelin glycoprotein

PI3K- phosphoinositide-3 kinase

PLC-y1- phospholipase C y1

TNFR- tumor necrosis factor receptor

I. Introduction

A: The Neurotrophins

The neurotrophin family of trophic factors is composed of nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin 3 (NT-3) and neurotrophin 4 (NT-4) (Levi-Montalcini and Hamburger, 1951; Barde et al., 1982; Hohn et al., 1990; Hallbook et al., 1991). These peptides are first synthesized as precursor "pro-neurotrophins" that can be cleaved by furin and other convertases to yield the "mature" forms of the peptides or they can be secreted without cleavage (Lee et al., 2001; Harrington et al., 2004). The peptide structures have been solved crystallographically and they are generally found to be noncovalently associated homodimers (McDonald and Blundell, 1991; McDonald et al., 1991; Fandl et al., 1994; Robinson et al., 1995; Butte et al., 1998; Robinson et al., 1999).

The central dogma of the neurotrophin hypothesis is that cells secrete limited amounts of neurotrophins in order to cull the population of innervating neurons so as to ensure a balance between the number of contacts made versus the size of the target tissue (Levi-Montalcini and Hamburger, 1951). However, additional functions for these factors were later identified. Their role during development is essential as mice lacking neurotrophins, such as NT-3, show severe sensory and sympathetic deficits (Farinas et al., 1994). Both sensory neurons and sympathetic ganglia can be eliminated during development by injecting an antibody that blocks NGF (Levi-Montalcini and Booker, 1960). Conversely, raising levels of NGF in chick embryos, through the implantation of NGF-secreting cancer cells or salivary glands, increases the number of neurons in the cervical ganglia (Levi-

Montalcini and Hamburger, 1951). The actions of neurotrophins are not limited to neurons that have already made functional contacts (Farinas et al., 1998). Tissues that are in between sensory neurons and their final targets express neurotrophins and these molecules may provide trophic support for axons that have not yet reached their targets (Farinas et al., 1996).

In the central nervous system (CNS), NGF is produced in several areas of the brain by both neurons, under normal conditions, and in astrocytes and microglia following injury (Arendt et al., 1995; Elkabes et al., 1996). Neurotrophins have also been shown to play essential roles once functional connections have already been established. BDNF mRNA levels have been shown to dramatically increase following long-term potentiation (LTP) in the hippocampus and long-term depression (LTD) in the cerebellum, highlighting increases in BDNF during synaptic modifications (Patterson et al., 1992; Yuzaki et al., 1994). In addition to increases in transcription, transfer of BDNF, through exocytosis, from presynaptic to post-synaptic neurons has been demonstrated to occur in an activity dependent manner (Kohara et al., 2001).

B: Functions of neurotrophins in the hippocampus

The role of the hippocampus in the formation of new memories is essential as surgical removal of the area results in the complete loss of new memory consolidation (Scoville and Milner, 1957). The hippocampus is located in the medial temporal lobe and is, in rats, composed of the dentate gyrus, CA1, CA2, CA3, and CA4. The structures of the CA areas contain a dense network of pyramidal neurons and the dentate gyrus contains granule cells.

Neurotrophins and Apoptosis

Observations in retinal neurons and PC12 cells determined that when neurotrophins bound p75NTR, and those individual cells did not express a cognate trk receptor, neurotrophins induced apoptosis (Rabizadeh et al., 1994; Frade et al., 1996). During both development and postnatal refinement of the hippocampus, p75NTR is robustly expressed in neurons (Buck et al., 1988; Lu et al., 1989). Although TrkA is not expressed in the hippocampus, TrkB and TrkC have been found in hippocampal neurons (Ernfors et al., 1992; Ip et al., 1993). Experiments in the hippocampus revealed that all neurotrophins can induce cell death through p75NTR (Friedman, 2000). Neurotrophins signal for cell death by activating the intrinsic apoptotic pathway (Friedman, 2000). The intrinsic apoptotic pathway involves the release of cytochrome-c from the mitochondria where it then interacts with Apaf-1 which ultimately leads to the activation of caspace-9 (Liu et al., 1996). Neurons are produced in excess in the hippocampus and are subsequently culled in the first postnatal week (Gould et al., 1991). In the CA region of the hippocampus neuronal death up to P10 is dependent on p53 a downstream target of p75NTR (Murase et al.)

Proneurotrophins also cause changes in hippocampal neurons. Following seizure, proNGF is upregulated and causes the death of hippocampal neurons (Volosin et al., 2008). Cleavage resistant proBDNF, in contrast, does not kill hippocampal neurons but dramatically reduces cholinergic fibers and spines on hippocampal neurons (Koshimizu et al., 2009b). Interestingly, in contrast to neurons in the hippocampus, basal forebrain neurons do undergo apoptosis when treated with proBDNF (Volosin et al., 2006).

Survival

The dentate gyrus of the hippocampus is unique as it is one of the few areas in the adult brain that generates new neurons throughout life. Regulation of BDNF is likely to play a significant role in the development and maintenance of this and other systems in the hippocampus as many studies have implicated changes in BDNF and proper neuronal function (Nibuya et al., 1995; Kempermann, 2002; Garza et al., 2004). *In vitro*, it has been demonstrated that BDNF is necessary for hippocampal progenitor cells to produce new neurons (Bull and Bartlett, 2005). TrkB knockout mice also show increased cell death in the hippocampus, particularly in the dentate, during the early postnatal culling period (Alcantara et al., 1997). Interestingly, this pro-survival mechanism may not be exclusively driven by trk receptors as p75NTR knockout mice have 40% fewer neuroblasts than wild types (Young et al., 2007). NT-3 mRNA is also elevated in the dentate after neuronal stimulation (Lindvall et al., 1992). Also, mice deficent for NT-3 show fewer neurons and increased glia in the hippocampus (Shimazu et al., 2006).

In vivo studies using TrkB(lox/lox) mice show a marked reduction both dendrite and spine number in adult born neurons indicating that BDNF signaling is required for proper maintenance of hippocampal circuitry throughout life (Bergami et al., 2008). In addition to requiring neurotrophins for proper development, the mature hippocampus maintains other neuronal populations by secreting neurotrophins throughout life. Basal forebrain projections to the hippocampus are dependent on all the members of the neurotrophin family which are each produced in the adult hippocampus (Hefti et al., 1984; Alderson et al., 1990; Friedman et al., 1993; Chen et al., 1997).

Learning and memory are dependent on the rearrangement of synapses in the hippocampus through both long-term potentiation (LTP) and long-term depression (LTD) (Bliss and Collingridge, 1993). TrkB knockout mice and BDNF knockout mice show greatly reduced LTP (Korte et al., 1995; Minichiello et al., 2002). Rescue of LTP in the CA1 of the hippocampus is achieved in BDNF knockouts by adenoviral-mediated re-expression of BDNF (Griesbeck et al., 1996). Most studies involving the role of neurotrophins in LTP and LTD involved knockouts, overexpression of proteins, or unusually high levels of stimulation (McAllister et al., 1999). Interestingly, endogenous levels of BDNF secretion determine both LTP and LTD in the hippocampus as sequestration of BDNF by TrkB-IgG in slice cultures could alter both processes (Aicardi et al., 2004). Proneurotrophins and p75NTR also cause alternations in the hippocampus (Woo et al., 2005). ProBDNF causes LTD in hippocampal neurons in wild-type mice but not p75NTR knockouts (Woo et al., 2005).

C: The p75 Neurotrophin Receptor

The study of neurotrophins and their actions lead to the discovery of receptors capable of carrying out those actions. The first neurotrophin receptor identified that was capable of binding NGF was p75NTR (Chao et al., 1986; Radeke et al., 1987) All members of the neurotrophin family are capable of binding p75NTR (Rodriguez-Tebar et al., 1990; Squinto et al., 1991; Rodriguez-Tebar et al., 1992). As a member of the tumor necrosis factor receptor family (TNFR), p75NTR contains a death domain characteristic of a subclass of the larger super family and binds neurotrophins through an extracellular region containing multiple cysteine repeats (Yan and Chao, 1991; Chao, 1994). The subdivision of death domain containing

TNFR family members is further divided with p75NTR being a sole member of this division (Liepinsh et al., 1997). This is due to p75NTR having a domain that is so divergent from standard type 1 death domains, found on receptors like FAS and $p55^{TNFR}$, that it is classified as a type II death domain (Liepinsh et al., 1997). Although both death domains signal for apoptosis the mechanism by which p75NTR activates apoptosis is divergent from other TNFR family members (Troy et al., 2002). Standard type 1 death domains activate the extrinsic apoptotic pathway via caspace 8 activation, while p75NTR signals for apoptosis through caspace 9 which is involved in the intrinsic apoptotic pathway (Troy et al., 2002).

The lack of enzymatic activity by p75NTR has made a full understanding of its downstream signaling components difficult to fully complete. For many years it was unclear if p75NTR even had signaling capabilities, and it was not until the observation that cell lines containing no other neurotrophin receptors responded to NGF treatment by increasing production of ceramide, that it became clear that the receptor could signal in response to neurotrophins (Dobrowsky et al., 1994). This increase in ceramide production can have diametrically opposed effects depending on cell type. In PC12 cells, p75NTR-induced ceramide production promotes cell survival while, conversely, in oligodendrocytes p75NTR-induced ceramide production leads to cell death (Yoon et al., 1998; Foehr et al., 2000). The opposing effects of either life or death for cells via p75NTR is not merely limited to differing cell types as hippocampal neurons cultured for only one hour show increased neurite outgrowth when treated with high levels of NGF while those same cells will undergo apoptosis when treated with the same level of NGF after five days in culture (Brann et al., 1999; Friedman, 2000). Interestingly, hippocampal neurons maintained in culture for two weeks no longer undergo apoptosis when

exposed tohigh levels of NGF even though p75NTR is still expressed (Koshimizu et al., 2009a). Although the intracellular region of this single transmembrane receptor lacks any catalytic activity, it is phosphorylated or palmitoylated on several intracellular serine, threonine, or cysteine residues where it can then recruit several different adaptor proteins in order to signal (Grob et al., 1985; Barker et al., 1994). These adaptor proteins contain, but are not limited to, NRIF, NRAGE, NADE, TRAF, and RIP-2 (Casademunt et al., 1999; Ye et al., 1999; Mukai et al., 2000; Khursigara et al.,

2001; Salehi et al., 2002). The number of adaptor proteins that p75NTR can recruit, and the fact that many of them have opposing actions, makes characterizingp75NTR signaling pathways difficult to understand.

The activation of p75NTR by NGF in both developing sensory and sympathetic neurons has been shown to support survival by activating the NF-κB transcription factor (Maggirwar et al., 1998; Hamanoue et al., 1999). Upon NGF binding to p75NTR activated NF-κB translocates to the nucleus to activate genes responsible for the suppression of apoptosis and up regulation of neurotrophins which enhance neuronal survival (Maggirwar et al., 1998; Hamanoue et al., 1999; Zaheer et al., 2001).

Indirect evidence for the role of p75NTR in the development and survival of neurons is found in p75NTR knockout mice. Two different strains of p75NTR knockout mice have been generated which lack the ability to bind NGF due to deletions resulting in shortened receptors that each lacks a full extracellular domain (Lee et al., 1992; von Schack et al., 2001). Despite a mild external phenotype both strains are characterized by both a severe reduction in sensory neurons and a reduced volume of peripheral nerves (Lee et al., 1992; von Schack et al., 2001). The

role of NGF-induced apoptotic signaling by p75NTR has been reported in a variety of cell types from rat hippocampal neurons and oligodendrocytes, to chicken retina precursor cells (Casaccia-Bonnefil et al., 1996; Frade et al., 1996; Friedman, 2000). BDNF has also been shown to elicit death via p75NTR in cultured sympathetic neurons (Casaccia-Bonnefil et al., 1996). Neurotrophins binding to p75NTR have been shown to activate apoptosis through several distinct and overlapping pathways. The activation of Jun kinase has been shown to be one of the major pathways activated by p75NTR as neurotrophin binding increases phosphorylation of Jun kinase and the subsequent release of cytochrome c from mitochondria (Casaccia-Bonnefil et al., 1996). Upon release of cytochrome c activation of caspaces 9, 6, and 3 induces cell death in contrast to other classic death receptors (Dechant and Barde, 1997; Martinou et al., 1999; Friedman, 2000; Troy et al., 2002).

Rat neurotrophin receptor p75NTR interacting MAGE homolog (NRAGE) is another important factor in p75NTR induced neuronal cell death (Salehi et al., 2002). In addition to activating the JNK pathway, NRAGE regulates the activity of p53, a regulator of the cell cycle (Wen et al., 2004).

Cleavage of p75NTR by various secretases may, in itself, be another form of p75NTR-induced signaling. The intracellular domain of p75NTR has been shown to be released from the membrane and translocates to the nucleus (Jung et al., 2003; Kanning et al., 2003). This cleavage involves another p75NTR associated protein, neurotrophin receptor interacting factor (NRIF). NRIF has been shown to associate with p75NTR after treatment with both BDNF and proNGF and then translocate to the nucleus promoting apoptosis (Kenchappa et al., 2006; Volosin et al., 2008).

Neurotrophins and their precursors are not the only ligands known to bind

and activate p75NTR. Amyloid beta (Aβ) is a protein thought to play a significant role in Alzheimer's disease, through its accumulation in plaques in the brain. Aggregated amyloid beta has been shown to activate JNK via p75NTR and induce cell death (Sotthibundhu et al., 2008).

p75NTR and Axon Growth

Injury in the central nervous system (CNS) shows a marked reduction in neuronal ability to regenerate when compared to the peripheral nervous system (PNS). The non-permissive environment of CNS myelin was found to be responsible as PNS myelin grafts allowed regenerating CNS axons to extend far beyond normal repair (Ng and Tang, 2002). Three different proteins are responsible for one of the major systems inhibiting CNS axonal repair: myelin-associated glycoprotein (MAG), Nogo, and oligodendrocyte-myelin glycoprotein (OMgp) (McKerracher et al., 1994; Mukhopadhyay et al., 1994; Chen et al., 2000; Prinjha et al., 2000; GrandPre et al., 2002; Wang et al., 2002). Nogo receptor (NgR) binds to these myelin inhibitory proteins but its lack of intracellular domain ruled it out as a signal transducing receptor for any of these molecules (Fournier et al., 2001). p75NTR acts as the signal transducer as it binds all three inhibitory proteins and immunoprecipitates with NgR (Wang et al., 2002; Wong et al., 2002). The addition of a third receptor lingo-1, leads to the formation of an active inhibitory signaling complex (Mi et al., 2004). Neurite outgrowth inhibition is achieved by the activation of RhoA, via p75NTR, through the displacement of the RhoA inhibitor, Rho-GDI (Yamashita et al., 2002; Yamashita and Tohyama, 2003). The adult nervous system expresses another member of the TNF super family, TROY, which has shown to be a p75NTR functional homolog (Park et al., 2005; Shao et al., 2005). When TROY is expressed in cells that lack p75NTR, exposure to myelin inhibitory

compounds causes an association between TROY and NgR which leads to the activation of RhoA (Park et al., 2005; Shao et al., 2005).

D: The trk family of neurotrophin receptors

Neurotrophins are also capable of binding to the trk family of tyrosine kinase receptors. Trk was originally identified as an oncogene composed of the transmembrane and cytoplasmic kinase containing domains of TrkA joined to a portion of non-muscle tropomyosin which gave the family its name, tropomyosin-related kinase (trk)(Martin-Zanca et al., 1986).

The Trk family contains three members, TrkA, TrkB and TrkC. TrkA binds NGF, NT-3 and NT-4 although the binding of NGF is of much higher affinity, while TrkB binds BDNF and NT-4, and TrkC binds NT-3 (Patapoutian and Reichardt, 2001). All members of the Trk family have an extracellular domain that contains three leucine-rich motifs and a membrane proximal domain that contains two different immunoglobulin-like regions (Urfer et al., 1998). One of the immunoglobulin-like regions is itself divided into two distinct regions, one that binds to a conserved region found in each of the members of the neurotrophin family, and a second that binds to unique sequences found in different family members thereby conferring the specificity of individual Trk receptors for its cognate ligand(s) (Ultsch et al., 1999).

The intracellular region of Trk receptors contain a kinase domain that, upon neurotrophin binding and receptor dimerization, can auto and cross-phosphorylate up to ten highly conserved tyrosine residues located in the kinase domain (Kaplan and Stephens, 1994). Three of these ten tyrosines are involved in maintaining a

regulatory loop while five others, upon phosphorylation, activate the Phosphoinositide-3 Kinase (*PI3K*) signaling pathway which leads to NGF-induced neurite outgrowth and survival (Inagaki et al., 1995). In addition to sites located on the kinase domain, tyrosines in non-catalytic areas also appear to be important for Trk signaling, as residues Y490 and Y785 activate multiple signaling pathways that regulate survival and neuronal growth respectively, mediated by the adapter protein Shc and Phospholipase C-γ1 (PLC-γ1) (Newton, 1995; Brunet et al., 2001).

Low levels of NGF are usually not sufficient to activate TrkA but when cells express both TrkA and p75NTR a high affinity binding site is formed that can enhance NGF-mediated TrkA activation (Hempstead et al., 1991; Mahadeo et al., 1994). The co-expression of both receptors has been shown to increase the association between NGF and TrkA by 25-fold (Mahadeo et al., 1994). The mechanism of this association is unclear. The interaction of the two receptors may or may not depend on ligand binding. A mutant form of NGF that binds to TrkA but not to p75NTR is less efficient at activating TrkA, suggesting that p75NTR modulates the interaction (Ryden et al., 1997).

Interestingly, some models suggest that the association of the two receptors may not require binding of the neurotrophins to p75NTR as high affinity binding sites to NGF can be generated when a mutant p75NTR that lacks a wild type extracellular domain, replaced with the EGF receptor's extracelluar domain (ECD), but retains the transmembrane and cytoplasmic domains (Esposito et al., 2001).

The juxtamembrane domain of p75NTR is the most highly conserved amongdifferent species and this high level of conservation suggests that it likely plays a role in protein-protein interactions (Large et al., 1989).

The binding kinetics for all neurotrophins have been determined but it is still unclear if p75NTR and TrkB or TrkC can form a signaling complex. The interaction of p75NTR and Trks may also be cell type specific as sympathetic neurons with an activated Trk can be protected against p75NTR-mediated pro-NGF induced cell death, but neurons from the basal forebrain, even in the presence of an activated Trk receptor, undergo apoptosis (Teng et al., 2005; Volosin et al., 2006).

E: The Rab family of proteins and endocytosis of neurotrophin receptors

All known cell types internalize molecules via some form of endocytosis. Receptors are often internalized via clathrin giving this internalization route the term "receptor-mediated endocytosis" (Conner and Schmid, 2003). Clathrin coated pits are involved in both constitutive endocytosis, as is the case for transferrin, and in ligand dependent endocytosis in the case of the Epidermal Growth Factor Receptor (EGFR)(Hopkins et al., 1985). Once the membrane-bound receptors are internalized the receptor can be sorted into many distinct compartments, including but not limited to, early endosomes, late endosomes, recycling endosomes and lysosomes (Mukherjee et al., 1997). In order to distinguish among these compartments, certain protein markers are frequently used: Rab5 for early endosomes, Rab7 for late endosomes, and Rab11 for recycling endosomes (Zerial and McBride, 2001). From early endosomes, proteins can be trafficked to recycling endosomes, responsible for reinserting receptors into the plasma membrane , late endosomes, responsible for sorting proteins to other intracellular pathways, or lysosomes which degrade receptors (Jovic et al.). The polarized shape of neurons places spatial demands on trafficking of compartments. In hippocampal neurons, for instance, early endosomal antigen 1 (EEa1), a Rab5 effector, is found only in somatodendritic compartments

and not in axons (Wilson et al., 2000). In contrast, in hippocampal neurons, recycling endosomes, which can return proteins to the plasma membrane throughout the entire cell, are evenly distributed in the soma, axon and dendrites (Prekeris et al., 1999).

NGF was found to be retrogradely transported along axons, and this transport was necessary for the survival of those neurons (Hendry et al., 1974). The retrograde transport of TrkA bound to NGF was necessary for the activation of several different transcription factors including cAMP response element-binding protein (CREB)(Riccio et al., 1997). When p75NTR was found in endocytic vesicles transporting tetanus toxin in living motor neurons it was discovered that NGF shared the same pathway (Lalli and Schiavo 2002). The observation that both Trks and p75NTR could be retrogradely transported following stimulation with NGF suggested that the different receptors were either transported together, used separate trafficking pathways, or both. In addition, the possibility that other members of the Trk family may use similar mechanisms also existed.

Differences between the internalization of the Trk receptors were, indeed, discovered. In sympathetic neurons TrkA, when bound to NGF, was found in long-lived endosomes that traveled from axon terminals to cell somas (Ye et al., 2003). These TrkA positive endosomes were found not to recycle back to the plasma membrane but were instead shuttled to the degradative pathway where they underwent lysosomal degradation (Saxena et al., 2005). When examined in a different cell type, PC12 cells, which express TrkA but not TrkB, TrkA was bound to NGF in endosomes that rapidly recycled back to the plasma membrane, whereas transfected TrkB, bound to BDNF, was found in endosomes that were targeted for

the degradative pathway (Chen et al., 2005a). The movement of TrkA could differ depending on cell type, and two of the Trk family members, TrkA and TrkB, had distinct modes of traveling through the endocytic pathway in the same cell type although the difference could be due to the addition of TrkB in a cell type that doesn't endogenously express the receptor. The method of internalization was also found to be divergent as TrkA positive endosomes were generated by Rac dependent macroendocytosis while TrkB seems to require internalization by clathrin-dependent endocytosis (Valdez et al., 2007; Zheng et al., 2008).

In PC12 cells, TrkA is initially internalized into endosomes containing the early endosome protein Rab22 (Wang et al., 2011). As the endosome trafficks from the distal end of a neurite it recruits neurotrophic factor receptor-associated protein (NTRAP), which is necessary for the activation of CREB, and ultimately Rab7 (Fu et al., 2010). These later stage endosomes contain mitogen-activated protein kinases (MAPKs) and these "signaling endosomes" activate targets of MAPK as the vesicles are retrogradely transported (Fu et al., 2010). The signaling endosomes containing TrkB have been less well characterized. Although NTRAP has been shown to associate with TrkB and TrkC it appears that TrkB may be retrogradely transported by a different mechanism (Fu et al., 2010). In cortical neurons Snapin, a dynien interacting protein that recruits TrkB to the motor protein, is essential for retrograde signaling (Zhou et al., 2012). Due to distinct natures of TrkA and TrkB it is unsurprising that the two receptors would be trafficked in distinct ways. It is interesting to note that neurotrophins that act via the same receptor have been shown to elicit different actions. In sensory neurons TrkA can be activated by both NGF and NT3, however, only NGF activates calcineurin which then marks the

receptor for internalization and retrograde transport, while NT3 does not (Bodmer et al., 2011).

When PC12 cells, which contain both TrkA and p75NTR, were studied with regard to the trafficking of TrkA and p75NTR, endosomes positive for p75NTR were internalized at a rate three times slower than those containing TrkA, and those vesicles accumulated in the cell somas before being shuttled into the recycling pathway (Bronfman et al., 2003). Interestingly, a variant PC12 cell line that only expresses p75NTR internalized more BDNF or NT3 than NGF, indicating that the receptor's internalization rate can vary depending on which neurotrophin it interacts with (Saxena et al., 2004). The internalization of p75NTR after binding NGF also does not appear to be achieved by a single mechanism. In sympathetic neurons p75NTR internalization with NGF can be achieved using both a clathrin-mediated and a lipid raft-mediated route (Hibbert et al., 2006). Interestingly, it appears that at least in some cell types p75NTR may be internalized in the absence of ligands as motor neurons were shown to internalize p75NTR in both the presence and absence of NGF in approximately the same amount (Deinhardt et al., 2007). In contrast to the pathways taken by TrkA:NGF positive endosomes, p75NTR:NGF positive vesicles were found not to overlap with lysosomal marker lysosome–associated membrane protein (LAMP-1) even after three hours of treatment (Deinhardt et al., 2007). Only after very long time points, on the order of twenty hours or more, does one see significant accumulation of p75NTR in lysosomes (Butowt and von Bartheld 2009). Rab5 is a marker for early endosomes and given p75NTRs slow internalization rate it was a surprise to discover that a majority of p75NTR positive endosomes overlapped with Rab5 (87%) even at the, relatively,

late time point of thirty minutes (Gorvel et al., 1991; Deinhardt et al., 2007).

F: Sortilin

The immature precursor forms of neurotrophins, pro-neurotrophins, were hypothesized to be solely responsible for trafficking and secretion of the mature forms (Suter et al., 1991). Binding studies revealed that pro-neurotrophins bound to p75NTR preferentially over TrkA and cells treated with a stable, cleavage resistant form of proNGF underwent apoptosis (Lee et al., 2001). Additional observations suggested that p75NTR may require a co-receptor, as not all p75NTR positive cells underwent apoptosis when treated with proNGF (Lee et al., 2001). ProNGF-induced apoptosis was found to require both p75NTR and a sorting protein named sortilin, as only cells expressing both of these receptors could activate apoptotic pathways in response to pro-NGF (Nykjaer et al., 2004). In addition to pro-NGF, pro-BDNF and pro-NT3 have also been shown to be pro-apoptotic (Teng et al., 2005; Yano et al., 2009).

Although sortilin binds to neurotensin with a high affinity it is not a member of the neurotensin receptor family, as it is a type-1 receptor that contains an intracellular domain that is highly homologous to a sorting region of Vps10P, a carboxypeptidase sorting protein expressed in yeast, and a C-terminus that is structurally homologous to the cytoplasmic domain of insulin growth factor-II receptor (Petersen et al., 1997). Neurotensin receptors one and two, NTS1 and NTS2, differ because they are seven membrane-spanning G-protein coupled receptors (Vincent et al., 1999). The exact role of sortilin in pro-neurotrophin-induced apoptosis and its interaction with p75NTR is not fully understood. Its role as a receptor has only recently been proposed as it was initially studied as a protein

that was responsible for the maturation of pro-peptides in the trans-Golgi network (Mazella, 2001). Most of the receptor pool of sortilin is found on intracellular compartments, only 5-10% is found on the plasma membrane of neurons and approximately 8% in other cell types such as adipocytes (Chabry et al., 1993; Nielsen et al., 1999). The binding of neurotensin to sortilin causes its internalization into signaling endosomes (Munck Petersen et al., 1999). This mechanism for sortilin internalization may also occur when it binds to pro-neurotrophins and may incorporate a p75NTR:sortilin: pro- neurotrophin complex into signaling endosomes. Interestingly, sortilin may play an important role in the sorting of some mature neurotrophins in the secretory pathway. Primary neurons expressing a truncated form of sortilin missort BDNF but not NT-4 from a regulated to a constitutively active secretory pathway (Chen et al., 2005b).

G: Neurotrophins and Astrocytes

Like hippocampal neurons, astrocytes in the hippocampus also express TrkB, TrkC, and p75NTR (Hutton et al., 1992; Wang et al., 1998). Interestingly, despite expressing some of the same receptors, hippocampal neurons and astrocytes show marked differences in their responses to neurotrophins. Like neurons in the hippocampus, astrocytes can also secrete NGF (Hefti et al., 1984; Micera et al., 1998). However, unlike neurons which constantly secrete NGF in order to sustain targets from the basal forebrain, astrocyte secretion of NGF is induced after injury suggesting an autocrine or paracrine function (Oderfeld-Nowak and Bacia, 1994; Goss et al., 1998). Cocultures of neurons and astrocytes have demonstrated that secretion of other members of the neurotrophin family may be secreted for the protection of surrounding neurons. BDNF secretion by astrocytes is markedly

increased when treated with Amyloid beta and that additional BDNF is neuroprotective (Kimura et al., 2006). Astrocytes, unlike neurons, do not undergo apoptosis in response to injury-induced production and secretion of NGF but instead respond by arresting their cell cycle and inhibiting proliferation (Troy et al., 2002; Cragnolini et al., 2009; Cragnolini et al., 2011).

The developmental stage of astrocytes has an effect on their responses to neurotrophins as immature astrocytes show activation of TrkB in response to BDNF but older reactive astrocytes do not (Climent et al., 2000). This may be due to the expression of a truncated form of TrkB which lacks an internal kinase domain being the dominant form of TrkB in non-reactive mature astrocytes (Goutan et al., 1998). Upon injury mature astrocytes up regulate full length TrkB (Goutan et al., 1998). Surprisingly, although reactive astrocytes show decreased TrkB activation, levels of radiolabeled BDNF and NT4 could be detected in intracellular pools (Alderson et al., 2000). Surprisingly, these neurotrophins were detected up to seven hours after their uptake indicating that mature astrocytes internalize neurotrophins and release them in a delayed and time-dependent manner (Alderson et al., 2000). Astrocytes in the dentate gyrus two weeks after kainic acid treatment show marked BDNF immunoreactivity whereas BDNF levels are undetectable in other cell types in the same area (Goutan et al., 1998). This pattern suggests that astrocytes may not activate TrkB in response to neurotrophins following injury but are instead pooling them in intracellular vesicles (Goutan et al., 1998).

II. Research aims:

1. Determine if the association of trkB and p75NTR is dependent on cognate ligand treatment.

The presence of both TrkA and p75NTR causes an increase in the binding of NGF by 25 fold (Mahadeo et al., 1994). A mutant form of NGF that only binds to TrkA shows decreased TrkA activation suggesting that both receptors are necessary for efficient NGF signaling (Ryden and Ibanez, 1997). To date few studies have been done examining the association of p75NTR and the two remaining members of the Trk family, TrkB and TrkC. Does treatment with cognate ligands, BDNF and NT4, cause and association of p75NTR and TrkB?

2. Determine if the association of TrkB and p75NTR can be detected in intracellular vesicles.

Both TrkA and p75NTR have been shown to internalize following ligand treatment (Riccio et al., 1997; Lalli and Schiavo, 2002). Further studies have examined endocytosis of p75NTR and other Trks separately but have not examined if p75NTR and TrkB can be found in the same endosomes. If p75NTR and TrkB associate following ligand treatment do the receptors associate on intracellular vesicles?

3. Determine if the association of sortilin and p75NTR is dependent on cognate ligand treatment.

Sortilin and p75NTR have been shown to interact following treatment with ProNGF (Nykjaer et al., 2004). Previous studies have identified this interaction using transfected protein constructs. Are endogenous levels of p75NTR and sortilin sufficient to cause

association of the two receptors following ligand treatment in primary neuronal cultures?

4. Determine if the association of sortilin and p75NTR is trafficked on intracellular vesicles.

The binding of neurotensin to sortilin causes the receptor to be internalized and incorporated into signaling endosomes (Munck Petersen et al., 1999). Does the association of p75NTR and sortilin also get internalized following ligand treatment?

5. Determine if the association of p75NTR and coreceptors differs between hippocampal neurons and astrocytes.

Astrocytes express both p75NTR and TrkB (Hutton et al., 1992; Wang et al., 1998). Does p75NTR associate with TrkB following treatment with BDNF in hippocampal astrocytes? Unlike neurons, ProNGF does not induce death in hippocampal astrocytes (Cragnolini et al., 2009). Does the association of p75NTR and sortilin differ in hippocampal neurons and astrocytes?

III. Materials and Methods

Cell Culture

E18 rat fetuses were sacrificed with CO_2. The animals were sterilized by soaking in an 80% ethanol bath for 10 minutes after which the brains were removed and each hippocampus was dissected out. Cells were triturated for 5 minutes and plated on 25mm Deckglaser Cover Glasses coverslips (EMS). The coverslips were coated for a minimum of two hours with poly-D-lysine (0.1mg/ml) before being washed twice with distilled water. The coverslips were placed in 35mm culture dishes (TPP). The cells were maintained in 2 ml of 5% heat-inactivated fetal bovine serum medium for 24 hours. Each day following, half of the media was removed and replaced with Serum-Free Medium which consisted of a 1:1 mixture of Eagle's minimal essential medium and Ham's F-12 (Invitrogen) supplemented with glucose (6 mg/ml), putrescine (60 μM), progesterone (20 nM), transferrin (100 μg/ml), selenium (30 nM), penicillin (0.5 units/ml), and streptomycin (0.5 μg/ml) (Sigma)

Treatments and Reagents

Cells were incubated for 5 days at 37°C with 5% CO_2 and 95% humidity. Cells were treated with 50 ng/ml each of NGF, BDNF, NT3, NT4 or 2 ng/ml proNGF. Endocytosis was blocked by either incubating cells on ice for 30 minutes prior to treatment or a 30 minute pretreatment with 50μM Dynasore (Sigma). TrkB phosphorylation was blocked by treatment with 200 nM K252a for 30 minutes prior to treatment.

Immunocytochemistry

Coverslips were fixed by first removing 1 ml of the culture media and adding 1 ml of 4% paraformaldehyde for 10 minutes at room temperature. The mix was then aspirated off and the cells were fixed for an additional 10 minutes in 4% paraformaldehyde. Paraformaldehyde was aspirated off and the dishes were washed three times with quick rinses of PBS. PBS containing 5% Goat serum and 0.3% Triton X was used to block the cells for 1 hour at room temperature on a shaker platform. Staining was performed for 1 hour at room temperature or overnight at 4°C while shaking in PBS containing 0.3% Triton X and 5% Goat Serum with double or triple combinations of the following antibodies: monoclonal mouse or rabbit anti-p75 (Chemicon), anti-$TrkB_{out}$ (gift from Dr. David Kaplan), sheep anti-TrkB(Novus), chicken anti-TrkB (Promega), rabbit anti-SorCS2 (gift from Dr. Barbara Hempstead), mouse anti-EEA1 (BD transduction), rabbit anti-Rab5, Rab7, or Rab11(Cell Signaling Technologies), and rabbit anti-NTR3/Sortilin (Alpha Diagnostics). All primary antibodies were diluted 1:1000. The coverslips were then washed for 20 minutes with PBS at room temperature three times on a shaking platform. Secondary antibodies were diluted 1:1000 in PBS containing 0.3% Triton X and 5% Goat Serum and placed on a shaker platform for 1 hour at room temperature in double or triple combinations of the following Alexa conjugated antibodies: anti-rabbit 488, anti-sheep 488, anti-chicken 488, anti-rabbit 633, anti-rabbit 555, anti-mouse 555 (Invitrogen). An additional three washes were performed with PBS for 20 minutes. Coverslips were mounted onto Fisherbrand microscope slides (Fisher Scientific) using 15 µl of Prolong Gold Anti-Fade Reagent (Invitrogen) and allowed to dry in the dark overnight.

Microscopy and FRET

Coverslips were viewed using an inverted, multi-photon, confocal microscope (Zeiss) and the data was analyzed using LSM FCS software (Ziess). The coverslips were viewed using a

40X oil immersion lense. In each experiment the following configuration was used: all lasers were set to 50%, beam splitters 488/561/633 were used, filters were set to 500-550, 575-

615, 650-710 and all channels were sent through a pinhole of 67 µm. Acceptor photobleaching was used to detect FRET indirectly. Photobleaching of the acceptor was achieved by setting the 555 nm laser channel intensity to 100% and exposing the region of interest to 250 iterations. After the images were acquired the intensity of each channel in the region of interest in addition to a background region was measured by the LSM FCS software and imported to Microsoft Excel (Microsoft). Background corrections were made by subtracting the intensity of each channel in the background region from the intensity of each channel in the region of interest. Once corrected for background the donor channel (488) intensity was examined before and after photobleaching. If the mean fluorescence intensity in the donor channel was 10% higher or greater after photobleaching of the acceptor channel (555) then a FRET event was counted.

Immunoprecipitation

Cells were washed with ice-cold phosphate-buffered saline (PBS) and lysed in lysis buffer (Tris-buffered saline with 0.1% Triton, 60 mM octyl-glucoside, 1 mM PMSF, 10µg/ml aprotinin, 1 µg/ml leupeptin, and 0.5 mM sodium vanadate) and lysed on ice for 30 mins. 200 µg of total protein from hippocampal neuron lysates was incubated with 2 µl of monoclonal antibody 192 IgG (Millipore Bioscience Research Reagents) and incubated

overnight at 4˚C on a rocking platform. Protein G fast flow beads (GE Healthcare) were added and shaken for an additional 2 hrs at 4˚C. Immunoprecipitates were washed three times with lysis buffer and once with water and then subjected to Western blot analysis. The blots were probed with antibodies to TrkB (gift from Dr. David Kaplan), and NTR3/Sortilin (Alpha Diagnostics). Blots were stripped and reprobed with anti-p75 9651.

Statistics

Statistics were performed using Primer of Biostatistics. Standard deviation was calculated and then a two way analysis of variance (ANOVA) was performed. P vaules were determined using multiple comparisons- Bonferroni t-test.

IV. Results

Chapter 1-p75NTR and TrkB

p75NTR and TrkB form a signaling complex

In order to determine whether FRET could be used to measure the association between p75NTR and TrkB in response to BDNF, a time course of BDNF treatment was performed. Hippocampal neurons were treated with 50 ng/ml of BDNF or vehicle control for 0, 5, 15, 30 and 60 minutes. The cells were then fixed with 4% paraformaldehyde and immunostained with antibodies to the extracellular domains of both p75NTR and TrkB. The FRET pair fluorophores used were Alexa 488 and 555. Regions of interest for photobleaching were drawn on cells that were positive for both proteins on the somas. Two images were taken before the acceptor was photobleached and then eight images were taken after photobleaching. Measurements of the fluorescence intensity in both channels were recorded. To correct for background fluorescence a randomly selected region was chosen and the intensity of each channel in that area was subtracted from the region of interest. The increase in intensity in the donor channel was counted as a FRET event if, after acceptor photobleaching, the intensity value rose by more than ten percent. The neurons treated for 30 minutes exhibited the maximal FRET response that was statistically different from control values (Fig 1).

TrkB and p75NTR interact only when exposed to cognate ligands

Both BDNF and NT4 are capable of binding to both TrkB and p75NTR, suggesting that both neurotrophins may induce a signaling complex between the two receptors (Patapoutian and Reichardt, 2001). To examine whether or not cognate ligands for this potential signaling complex induced the association of p75NTR and TrkB, hippocampal neurons were treated with either BDNF or NT4 for 30 minutes. FRET occurred in 63.6% of neurons treated with BDNF and 52.6% of those treated with NT4 (Fig 2).

In contrast, NT3 and NGF both bind to p75NTR but not to TrkB (Patapoutian and Reichardt, 2001). Hippocampal neurons were treated with 50 ng/ml of either NGF or NT3. Neither neurotrophin caused a significant change in the association between p75NTR and TrkB when compared to untreated control neurons indicating that only cognate ligands induce an association of p75NTR and TrkB (Fig 2).

The interaction of p75NTR and TrkB is dependent on TrkB phosphorylation

A previous study using tagged constructs of full length p75NTR and various tagged constructs of different domains of TrkB, overexpressed in A293 cells, showed an interaction between the two receptors (Bibel et al., 1999), however TrkB was expressed at such high levels that the receptors were found to dimerize and cross-phophorylate one another even in the absence of ligand (Bibel et al., 1999). Treatment with an inhibitor of Trk tyrosine kinases, K252a, caused a decrease in the association of the tagged receptors (Bibel et al., 1999). To determine whether association of endogenous p75NTR and TrkB was dependent on TrkB phosphorylation, hippocampal neurons were treated for 30 min with or without

K252a before being treated for 30 minutes with 50 ng/ml of BDNF (Fig 3). Treatment with K252a abolished the interaction of p75NTR and TrkB indicating that the ligand-induced association of p75NTR and TrkB is dependent on the phosphorylation of TrkB. To confirm that K252a inhibited the phosphorylation of TrkB western blot analysis was performed (Fig 4E).

The interaction of p75NTR and TrkB is dependent on internalization

Since radioactive NGF is transported from distal axons to cell somas and NGF injected directly into the cytoplasm is biologically inactive, multiple investigations lead to the discovery that neurotrophins can be endocytosed and retrogradely transported along with their receptors (Heumann et al., 1981; Ure and Campenot, 1997). To test whether the association of p75NTR and TrkB was dependant on internalization, hippocampal neurons were cultured on ice, in order to halt endocytosis, for 30 minutes prior to being treated with 50 ng/ml BDNF for 5, 15, and 30 minutes. Cultures kept under normal incubator conditions were used as a positive control and received the same treatments of BDNF. The cells were then fixed with 4 % PFA and stained against p75NTR and TrkB. Secondary antibodies coupled to Alexa 488 and Alexa 555 were used to make FRET pairs. Neurons cultured at 4° Celsius showed no increase in FRET in all treatments while their incubator cultured counterparts all showed FRET events in line with previous experiments (Fig 5).

Dynasore is a compound that inhibits endocytosis by blocking the ability of dynamin to pinch off endocytic vesicles from the plasma membrane(Macia et al., 2006). To

determine if the previous results were indeed the result of inhibiting endocytosis and not merely the result of slowing the movement of p75NTR and TrkB in the plasma membrane itself, neurons were treated with 50μm of Dynasore for 30 minutes prior to being treated with 50 ng/ml of BDNF for 5 or 15 minutes. Cells left untreated with Dynasore but still treated with BDNF were used as a control. The cells were fixed and stained against p75NTR, TrkB and Rab5. FRET pairs were achieved using Alexa 488 and Alexa 555 while Rab5 was labeled with Alexa 633. Acceptor photobleaching was performed on cell somas and regions of interest were drawn in areas that were triple positive. In cells pretreated with Dynasore, no increases in FRET events were observed compared to controls and cells not treated displayed FRET event increases similar to previous experiments (Fig 11). These two experiments indicate that the association of p75NTR and TrkB is enhanced on intracellular vesicles and that internalization step is necessary for that association.

TrkB and p75NTR interact in early endosomes

EEa1 is a marker for early endosomes as it can be colocalized in endosomes positive for Rab5, another early endosomal marker, but not Rab7 which is a marker of late endosomes (Mu et al., 1995). Hippocampal neurons were treated with BDNF as in previous experiments but were triple stained for p75NTR, TrkB and EEa1. The secondary antibodies coupled to the primary antibodies against p75NTR and TrkB still formed FRET pairs and the secondary antibody coupled to the antibody against EEa1 was far red, Alexa 633, which has no overlap with Alexa 555. During the analysis, the three channels were given artificial colors that, when they overlap, the

pixels displayed a white color. Acceptor photobleaching was carried out on whole cell somas but FRET was investigated in areas that displayed triple labeling before photobleaching. In previous experiments regions of interest were drawn on somas and 50 ng/ml of BDNF typically raised the number of FRET events to approximately 50%. When we looked in areas that were only positive for p75NTR, TrkB and EEa1 after 30 minutes approximately 80% of those areas were positive for FRET, indicating that the receptors specifically localized to the early endosome were associated with each other following BDNF treatment (Fig 6).

Rab5 is also a marker of early endosomes and we investigated if p7NTR and TrkB interact in vesicles positive for all three proteins (Zerial and McBride, 2001). The same treatments and time points were used except for the inclusion of an antibody against Rab5 rather than EEa1. The number of triple labeled vesicles increases over time as the number of triple labeled puncta increased over 30 minutes (Fig10). The number of FRET events between p75NTR and TrkB increased over time with exposure to BDNF and reached a maximum of 70% of triple labeled vesicles displaying FRET(Fig 8). Thus, p75NTR and TrkB associate in early endosomes and that association increases until at least 30 minutes.

TrkB and p75NTR interact in late endosomes

Rab7 is a marker for late endosomes which, in sensory neurons, also contain MAPK and therefore referred to as "signaling endosomes" (Fu et al., 2010). In previous studies, vesicles containing TrkA bound to NGF and positive for Rab7 have been studied while Rab7 positive vesicles containing TrkB bound to BDNF have not

(Riccio et al., 1997). We investigated whether p75NTR and TrkB, when bound to BDNF, can move through the endocytic pathway from early to late endosomes. Hippocampal neurons were treated with 50 ng/ml of BDNF for 15, 30 and 60 minutes. Triple staining against p75NTR, TrkB and Rab7 was performed before acceptor photobleaching. In endosomes positive for all three proteins FRET occurred in approximately 70% of them (Fig 13). TrkB and p75NTR interact in late endosomes positive for Rab7.

TrkB and p75NTR do not interact in recycling endosomes

Rab11 is a marker for recycling endosomes (Mukherjee et al., 1997). We investigated whether p75NTR and TrkB move through the endocytic pathway into the recycling pathway and, if so, whether they continue to interact. Hippocampal neurons were treated with 50 ng/ml of BDNF for 30, 60, 90, 120, 180 and 240 minutes. Interestingly, both receptors were detected in Rab11 positive endosomes after 4 hours (Fig 17), however FRET was not detected in any of the puncta positive for p75NTR, TrkB and Rab11 (Fig 15). When Rab11 positive endosomes were examined for the inclusion of p75NTR or TrkB, but not both, the two proteins also accumulate separately in Rab11 positive endosomes indicating that they may no longer be associating and are being recycled back to the plasma membrane (Fig 17).

Summary

The association of p75NTR and TrkB is highest after 30 minutes of treatment with BDNF. TrkB and p75NTR associate after cognate ligand treatment. Treatment with either BDNF or NT4 showed an increase in the association of p75NTR and TrkB while treatment with NGF and NT3 did not increase the association. The association of p75NTR and TrkB is dependent on TrkB phosphorylation as treatment with K252a prior to BDNF treatment blocked their association. The association of p75NTR and TrkB is dependent on internalization as blocking endocytosis with incubation at 4°C and treatment with Dynasore both decreased the association of p75NTR and TrkB compared to untreated control levels. TrkB and p75NTR are found in early endosomes positive for EEA1 and Rab5 and that association increases up until 30 minutes. The number of Rab5 positive early endosomes that contain p75NTR and TrkB also increases over time following BDNF treatment. Late endosomes containing Rab7 display an increased association between p75NTR and TrkB following BDNF treatment and that association increases over time. Neurons treated with BDNF for up to four hours show no increase in the association of p75NTR and TrkB in recycling endosomes positive for Rab11. Although the number of vesicles containing Rab11, p75NTR, and TrkB increases throughout the four hour treatment, the two receptors do not associate. In addition, vesicles positive for p75NTR and Rab11 or TrkB and Rab11 also increase over time.

Chapter 2-p75NTR and sortilin

Sortilin and p75NTR form a signaling complex in hippocampal neurons

ProNGF, but not NGF, treatment induces an association between p75NTR and sortilin

Previous studies with 293 cells transfected with constructs for both p75NTR and sortilin co-immunoprecipitation studies have demonstrated interactions between the two receptors both with and without proNGF treatment (Nykjaer et al., 2004). Cells that were treated with proNGF prior to immunoprecipitation showed an increase in the association of p75NTR and sortilin over the control constitutive levels suggesting that further interaction can be induced by treatment with an appropriate ligand (Nykjaer et al., 2004). In untreated hippocampal neurons we observed FRET between p75NTR and sortilin only once in seven separate experiments (Fig 18), suggesting that basal association between these two receptors is a rare event. When hippocampal neurons were treated with proNGF, FRET events were observed in 56.8% of the neuronal somas (Fig 18).

NGF was used as a negative control for the induction of a signaling complex between p75NTR and sortilin. The pro region of proNGF has been shown to bind to sortilin and the mature domain binds to p75NTR (Nykjaer et al., 2004). Therefore NGF, lacking the pro domain should not induce an interaction between p75NTR and sortilin. Neither control neurons nor those treated with NGF showed any FRET events, indicating that p75NTR and sortilin, at normal physiological levels in hippocampal neurons, only interact when bound to a cognate ligand (Fig 18).

The interaction of sortilin and p75NTR occurs earlier than p75NTR and TrkB

Previous studies have demonstrated an interaction between p75NTR and sortilin by immunoprecipitation (Nykjaer et al., 2004). The timing and duration of the interaction remain unknown. Hippocampal neurons were treated with 10 ng/ml of proNGF for 5, 15, 30 and 60 minutes. Within 5 minutes the number of neurons positive for FRET increased from 10% to 40% and steadily increased until 60% of those treated for 60 minutes were positive for an interaction between p75NTR and sortilin (Fig 19).

The interaction of p75NTR and sortilin is not dependant on internalization

Our previous results have demonstrated that the interaction of p75NTR and TrkB is dependent on internalization (Fig 5). Signaling endosomes containing p75NTR and sortilin have not been demonstrated so we examined whether the interaction of p75NTR and sortilin requires endocytosis. Hippocampal neurons were cultured on ice for 30 minutes before treating them with 10 ng/ml of proNGF for 5, 15 or 30 minutes and compared with cells cultured with the same treatments but kept at 37°C. Unlike p75NTR and TrkB, the neurons cultured on ice showed an interaction between p75NTR and sortilin as early as 5 minutes and this interaction was maintained for up to 30 minutes (Fig 21). Since culturing cells on ice inhibits endocytosis it appears that the interaction of p75NTR and sortilin can occur on the plasma membrane.

Sortilin and p75NTR can be found interacting on early endosomes

Hippocampal neurons were treated with 10 ng/ml of proNGF and were immunostained for p75NTR, sortilin and EEa1. Alexa 488 and 555 were used to create a FRET pair and Alexa 633 was directed against EEa1. Areas positive for all three proteins were targeted as regions of interest. FRET was detected in approximately 40% of neurons after 5 minutes of proNGF treatment. FRET was maintained through 15 minutes and 30 minutes where after 30 minutes approximately 60 % of triple labeled puncta were positive for FRET (Fig 20).

Using another marker for early endosomes, we also investigated whether p75NTR and sortilin could interact in Rab5 positive vesicles. Neurons were treated with 10 ng/ml of ProNGF for 5, 15, and 30 minutes. Immunostaining for p75NTR, sortilin, and Rab5 was performed and regions of interest positive for all three proteins were targeted for photobleaching. Interestingly, as early as 5 minutes FRET between p75NTR and sortilin was detected in Rab5 positive endosomes (Fig 22). This percentage of puncta showing FRET remained constant for all time points measured suggesting that the interaction of p75NTR and sortilin in Rab5 positive endosomes does not increase over the time points measured.

p75NTR also interacts with a sortilin homolog

Sortilin-related VPS10 domain-containing receptor 2 (SorCS2) is a member of the VPS10 domain containing receptor family that is expressed in the developing mouse brain (Rezgaoui et al., 2001). Previous experiments using over expressed p75NTR

and SorCS2 demonstrated that the two proteins form a complex when treated with proNGF (Deinhardt et al., 2011). We investigated whether p75NTR and SorCS2 could form a complex in primary neurons with endogenous protein levels. Hippocampal neurons were treated with 10 ng/ml of proNGF for 1, 5, 15, and 30 minutes. FRET pairs were formed using Alexa 488 and 555 conjugated to secondary antibodies targeted against primary antibodies against p75NTR and SorCS2. FRET was observed between p75NTR and SorCS2 at both the 1 minute and 5 minute time points but not at subsequent time points (Fig 23). This result suggests that the interaction of p75NTR and SorCS2 may be transient and rapidly degraded in developing hippocampal neurons.

Summary

Sortilin and p75NTR associate when treated with cognate ligands but not noncognate ligands. The association between p75NTR and sortilin increases over time when treated with proNGF. Unlike the association of p75NTR and TrkB, the interaction of p75NTR and sortilin is not dependent on internalization but instead can increase over time on the plasma membrane and occurs much earlier. Although the interaction between sortilin and p75NTR is not dependent on internalization the receptors interact in early endosomes positive for EEA1 and early endosomes positive for Rab5. A homolog of sortilin, SorCS2, also associated with p75NTR following ProNGF treatment but differs from the association between sortilin and p75NTR as it is not maintained past five minutes.

Chapter 3: p75NTR and Coreceptors in Hippocampal Astrocytes

Hippocampal astrocytes express p75NTR and coreceptors

In order to determine if hippocampal astrocytes expressed p75NTR and some of its coreceptors, astrocytes were treated with cognate ligands and stained against both receptors. Hippocampal astrocytes were treated with 50 ng/ml of BDNF for 30 minutes before being fixed with 4 % PFA for 10 minutes. Cells were stained with antibodies against p75 and TrkB and visualized with secondary antibodies coupled to Alexa 488 and 555. (Fig 25A) Hippocampal astrocytes were treated with 10 ng/ml of proNGF for 30 minutes before being fixed with 4 % PFA for 10 minutes. Cells were stained with antibodies against p75NTR and sortilin and visulaized with secondary antibodies coupled to Alexa 488 and 555 (Fig 25B).

TrkB and p75NTR show no increased association after ligand treatment

Astrocytes express TrkB and p75NTR but it is unclear if the two receptors can form a signaling complex in response to cognate ligand treatment (Hutton et al., 1992; Wang et al., 1998).

Hippocampal astrocytes were treated with 50ng/ml of BDNF for 30 minutes. The cells were fixed with 4 % PFA for 10 minutes before blocking and staining against p75NTR and TrkB. FRET pairs were made with secondary anitbodies coupled to Alexa 488 and 555. Photobleaching was performed on neuronal somas. No increases in FRET events were observed following ligand treatment indicating that p75NTR and TrkB do not associate in hippocampal astrocytes (Fig 26).

Sortilin and p75NTR show no increase in association following cognate ligand treatment

To determine if p75NTR and sortilin associate in hippocampal astrocytes cells were treated with 2ng/ml of proNGF for 30 minutes. The cells were fixed with 4 % PFA for 10 minutes before blocking and staining against p75NTR and sortilin. FRET pairs were made with secondary anitbodies coupled to Alexa 488 and 555. Photobleaching was performed on neuronal somas. In both control and proNGF treated cells no FRET could be detected between p75NTR and sortilin (Fig 26). This indicates that even in the presence of a cognate ligand hippocampal astrocytes do not form a signaling complex between p75NTR and sortilin.

Summary

The association of p75NTR and coreceptors differs depending on cell type. P75NTR and TrkB show an increased association when treated with cognate ligands on hippocampal neurons but not hippocampal astrocytes. Hippocampal neurons show a basal level of association between p75NTR and sortilin but not in hippocampal astrocytes. Treatment with a cognate ligand also fails to induce an association between p75NTR and sortilin in hippocampal astrocytes. These findings demonstrate that the differences between responses of hippocampal neurons and astrocytes may, in part, be due to differing levels of association between p75NTR and its coreceptors.

V. Discussion

Although originally identified as growth factors, neurotrophins are involved in a myriad of processes. Neurotrophins support diverse processes such as: survival and apoptosis, differentiation and migration, maintenance of neuronal architecture and degeneration of functional connections, in addition to long-term potentiation and long-term depression (Kaplan and Miller, 2000; Aicardi et al., 2004; Hempstead, 2006; Jansen et al., 2007). The Trk family of receptors, upon neurotrophin binding, support survival and differentiation through the activation of the PI3 kinase and MAP kinase pathways (Patapoutian and Reichardt, 2001). Neurotrophins can also bind to p75NTR to support the survival of several cell types by activating NF-κB (Maggirwar et al., 1998; Hamanoue et al., 1999). Conversely, NGF-induced apoptosis via p75NTR has also been reported in a variety of cell types (Casaccia-Bonnefil et al., 1996; Frade et al., 1996; Friedman, 2000).

The elongated shape of neurons presents these cells with a unique problem. Signals that neurons receive at either axon or dendritic terminals have to travel greater distances than those in other cell types. In sympathetic neurons the retrograde transport of TrkA bound to NGF is necessary to signal for survival (Hendry et al., 1974; Riccio et al., 1997). NGF and p75NTR are also retrogradely transported in neurons (Lalli and Schiavo, 2002). The internalization rates of p75NTR:NGF and TrkA:NGF endosomes have been studied and found to be divergent (Bronfman et al., 2003; Deinhardt et al., 2007). NGF, bound to TrkA, can be internalized and incorporated into signaling endosomes and moved into lysosomes upon reaching the cell soma (Deinhardt et al., 2007; Fu et al., 2010). NGF bound to p75NTR, however, is not only internalized at a slower rate but does not appear in lysosomes until almost a day after treatment (Bronfman et al., 2003; Deinhardt et al., 2007). Trk positive endosomes that also contain p75NTR have not been studied and the possibility exists that

the incorporation of all three molecules into endosomes may alter their signaling and degradation.

Proneurotrophins also display varied and diverse roles in various cell types. ProNGF binds to p75NTR and sortilin and causes cell death in hippocampal neurons (Nykjaer et al., 2004; Volosin et al., 2008). ProBDNF elicits the death of basal forebrain neurons but has a role in facilitating long term depression in the hippocampus (Woo et al., 2005; Volosin et al., 2006).

The roles of pro and mature neurotrophins and their various and contradictory functions indicate that not only must their levels be tightly controlled but the receptors they bind to and activate must also play a role in their functions. Astrocytes in the hippocampus secrete neurotrophins and express p75NTR, TrkB, and TrkC (Hefti et al., 1984; Hutton et al., 1992; Micera et al., 1998; Wang et al., 1998). Unlike hippocampal neurons, astrocytes do not undergo apoptosis when treated with high levels of neurotrophins but instead exit the cell cycle and cease proliferation (Troy et al., 2002; Cragnolini et al., 2009; Cragnolini et al., 2011). Astrocytes treated with neurotrophins have been shown to sequester those molecules in intracellular pools yet do not display increased Trk activation like neurons (Alderson et al., 2000). Since a majority of TrkB present on astrocytes is the truncated isoform that lacks an intracellular kinase domain it is possible that this isoform binds BDNF and internalizes it in order to release BDNF at a later time (Goutan et al., 1998).

Because of the essential role that the hippocampus plays in memory consolidation coupled with its reliance on neurotrophins and neurotrophin receptors it is of vital importance to fully characterize the mechanisms by which these factors regulate development, death, and maintenance of its neurons and astrocytes throughout life. These studies demonstrate that the differences between the associations of p75NTR and TrkB, and

p75NTR and sortilin may be regulated both at the cell surface and in intracellular vesicles, which may impact the downstream signaling and function mediated by these receptors. Differences between hippocampal neurons and hippocampal astrocytes to pro and mature neurotrophins may be found in the availability of those molecules to bind to and activate various receptors.

The interaction of p75NTR and TrkB is dependent on cognate ligands

Previous studies have shown that when p75NTR is expressed on the same cell as TrkA a high affinity binding site for NGF is formed (Hempstead et al., 1991; Mahadeo et al., 1994). The binding kinetics for other Trks and their cognate ligands have been determined but the role p75NTR may play in modifying those interactions have not conclusively demonstrated that p75NTR forms a complex with TrkB or TrkC. Our data demonstrate that p75NTR and TrkB associate when treated with either of the cognate ligands, BDNF or NT-4, but not non-cognate ligands, NGF and NT-3. This association also increases over time, peaking at 30 minutes when examining whole cell somas.

The high affinity binding sites generated by both p75NTR and TrkA also increase the activation of TrkA (Hempstead et al., 1991; Mahadeo et al., 1994). Studies using TrkA constructs that lacked a functional kinase domain showed that even in the presence of p75NTR treatment with NGF failed to demonstrate high affinity binding (Esposito et al., 2001), indicating that interaction of the extracellular domains alone is insufficient to generate high affinity binding, and suggesting that activation of the kinase domain in TrkA is necessary for p75NTR and TrkA to interact and form a binding site. To test if the activation of TrkB by BDNF was necessary for the increased association of p75NTR and TrkB we used the tyrosine kinase inhibitor K252a to prevent the activation of TrkB. When TrkB activation was inhibited the association of p75NTR and TrkB was abolished, indicating that the

phosphorylation of TrkB is necessary for the increased interaction.

Both p75NTR and TrkB have been shown independently to internalize following neurotrophin binding via clathrin coated pits (Hibbert et al., 2006; Valdez et al., 2007; Zheng et al., 2008). To test whether internalization of TrkB and p75NTR is required for their association we blocked endocytosis by culturing hippocampal neurons on ice before BDNF treatment. When compared to neurons kept at 37°C, those on ice showed no increase in the association of p75NTR and TrkB following BDNF treatment. Although culturing neurons on ice does indeed inhibit endocytosis it also increases the viscosity of the plasma membrane. This more rigid membrane may have been a possible explanation for the decrease in association of p75NTR and TrkB. To test whether the reduced association was due to membrane dynamics or whether the association of p75NTR and TrkB is indeed dependent on internalization, we used a pharmacological inhibitor of endocytosis, Dynasore, which blocks dynamin-mediated pinching off of endosomes from the plasma membrane. BDNF treatment following Dynasore failed to increase the association of p75NTR and TrkB over control levels. This indicates that blocking endocytosis with either cold or a pharmacological inhibitor blocks an increase in the association of p75NTR and TrkB following BDNF treatment.

Having established that the association of p75NTR and TrkB is dependent on internalization we next turned to examining which particular endosomal pathway the receptor complex traveled through. Our studies concentrated on neuronal somas rather than undifferentiated neurites. EEa1 is a marker for early endosomes and is found in the somas of hippocampal neurons (Wilson et al., 2000). We had previously established thirty minutes as the optimum time point to look for an association between p75NTR and TrkB following ligand treatment. Those experiments examined regions on somas that were

chosen at random. Here we examined areas in the soma that were only positive for p75NTR, TrkB, and EEa1. When examining these early endosomes, an increase in p75NTR and TrkB association can be detected after five minutes of BDNF treatment and that association increased in subsequent time points, and was maximal at thirty minutes.

Rab5 is another marker for early endosomes but is found on those that can then be shuttled to various other endocytic pathways including recycling back to the plasma membrane or to lysosomal degradation (Zerial and McBride, 2001). Because our previous data had shown that p75NTR and TrkB associated in early endosomes positive for EEa1 we examined whether this complex is also found in Rab5-positive early endosomes. Treatment with BDNF showed an increase in the association of p75NTR and TrkB in Rab5 positive vesicles. The increased association reached a maximum at thirty minutes and the number of puncta positive for p75NTR, TrkB, and Rab5 also increased over thirty minutes which indicates that the p75NTR:TrkB complex is continually being internalized and moved into Rab5 positive vesicles following BDNF treatment.

Our data have indicated that p75NTR and TrkB associate in Rab5 positive endosomes. From there the complex can be shuttled back to the plasma membrane, moved to Rab7 positive late endosomes, or fused to lysosomes (Mukherjee et al., 1997). In PC12 cells, TrkA and NGF are found in Rab7 positive endosomes, so called "signaling endosomes" that contain activated MAPKs that signal as the vesicle is retrogradely transported (Fu et al., 2010). Following a sixty minute treatment with BDNF, in order to allow the endosomes to move through different pathways, p75NTR and TrkB were found to associate in seventy percent of Rab7 positive endosomes. These data show that like TrkA, TrkB can also be incorporated into Rab7 positive endosomes.

Following retrograde transport to the somas of PC12 cells, TrkA:NGF containing

endosomes are moved to recycling endosomes positive for Rab11 and rapidly returned to the plasma membrane (Chen et al., 2005a). In addition, p75NTR positive endosomes are also recycled back to the plasma membrane in PC12 cells (Bronfman et al., 2003). In hippocampal neurons we examined if the p75NTR:TrkB complex could be found in recycling endosomes. After a four hour treatment with BDNF no increase in the association of p75NTR and TrkB was detected in Rab11 positive endosomes. Interestingly, the number of puncta positive for p75NTR, TrkB, and Rab11 increased over the four hour treatment, however, the association of p75NTR and TrkB did not. It is possible that at this point in the endocytic pathway the two receptors are being separated and recycled at different rates (Model 2). By analogy, TrkA recycles to the plasma membrane at a much faster rate than p75NTR (Bronfman et al., 2003). Our data show an increase in both TrkB-Rab11 positive puncta and p75NTR-Rab11 positive puncta indicating that the two receptors may be shuttled back to the plasma membrane in separate endosomes, however endosomes positive for p75NTR, TrkB, and Rab11 were also detected, although no FRET was observed in these endosomes.

Sortilin and p75NTR associate with cognate ligand treatment

Previous studies have shown that in PC12 cells proNGF is bound by a signaling complex consisting of p75NTR and sortilin (Nykjaer et al., 2004). Further studies have demonstrated that neurons in the brain can also respond to proneurotrophins resulting in neuronal cell death (Volosin et al., 2006). To confirm that proNGF could induce the association of p75NTR and sortilin we treated cultured hippocampal neurons with proNGF and NGF. ProNGF, the cognate ligand of the p75NTR:sortilin complex, increased the association of the receptors while NGF did not. ProNGF binds to p75NTR preferentially over TrkA and that association is rapid (Lee et al., 2001). Our data support the rapid

association of p75NTR and sortilin as we have measured their interaction as early as five minutes after treatment with proNGF. The association also appears to be stable as it increases up to sixty minutes following proNGF treatment. Sortilin was originally identified as a sorting protein and a receptor capable of binding neurotensin (Mazella et al., 1998). When sortilin binds neurotensin it is internalized and, given our previous data, we next examined whether p75NTR and sortilin are internalized after ligand binding (Munck Petersen et al., 1999). Sortilin and p75NTR can be found in early endosomes positive for EEa1 and Rab5, indicating that the receptors can be internalized. Interestingly, in contrast to the ligand induced association of p75NTR and TrkB following ligand treatment, p75NTR and sortilin do not require internalization to increase their association following ligand binding (Model 2). Neurons cultured on ice prior to treatment with proNGF showed an increase in the association of p75NTR and sortilin over untreated controls but those values are achieved rapidly and do not increase over time. Interestingly, neurons cultured on ice showed similar increases in the association of p75NTR and sortilin when compared to neurons treated at 37°C. This result indicates that p75NTR and sortilin can rapidly associate on the plasma membrane and the complex does not need to be internalized in order to increase its association.

The sortilin homolog, SorCS2, also shows an increase in its association with p75NTR following treatment with proNGF. Like sortilin, SorCS2, shows rapid association with p75NTR after one minute, however, in contrast to sortilin the association with SorCS2 disappeared at 15 minutes. This may indicate that trafficking and signaling of sortilin homologs are controlled through different pathways than sortilin itself.

The association of p75NTR and sortilin can be easily explained as proneurotrophin binding is dependent on both receptors being present (Nykjaer et al., 2004). The association

of p75NTR and TrkB is a more complex issue as both receptors are known to bind and signal independent from one another. We have shown that p75NTR and TrkB associate with each other after cognate ligand treatment, that the complex is internalized, and moves through parts of the endosomal pathway. The functional relevance of this association is less clear. During development p75NTR is present on neurons but is down regulated following hippocampal maturation (Buck et al., 1988; Lu et al., 1989). Following injury in the hippocampus, p75NTR is upregulated (Volosin et al., 2008). Levels of both mature and proneurotrophins are also increased as a consequence of injury in the hippocampus (Oderfeld-Nowak and Bacia, 1994; Goss et al., 1998; Volosin et al., 2008). Following injury hippocampal neurons are in an environment where they are susceptible to death from both high levels of mature neurotrophins and proneurotrophins. Removing receptors from the plasma membrane is not an option as they still require survival signals from neurotrophins (Eilers et al., 2001). When p75NTR and TrkA are both expressed in the plasma membrane high affinity binding sites for NGF are generated (Hempstead et al., 1991; Mahadeo et al., 1994). Expression of p75NTR and TrkB on hippocampal neurons may serve the same purpose. The neurons of the injured hippocampus are competing for survival factors and those exhibiting high affinity binding sites would survive in an environment where survival signals were limited. The internalization of the complex could also serve a similar purpose. In PC12 cells TrkA bound to NGF is rapidly recycled back to the plasma membrane(Chen et al., 2005a). When p75NTR binds NGF in PC12 cells the rate of internalization is slower and significant levels of endosomes accumulate in the cell somas before being shuttled back to the membrane (Bronfman et al., 2003). Endosomes containing p75NTR and NGF are significantly longer lived than those containing TrkA and NGF, and can remain in internal pools on the order of a day before being degraded or recycled (Deinhardt et al., 2007; Butowt and von Bartheld, 2009). The presence of p75NTR

also increases the activation of TrkA as mutant forms of NGF that are incapable of binding p75NTR show less activated TrkA (Ryden et al., 1997).

The purpose of the association of p75NTR and TrkB in hippocampal neurons

Previous studies have demonstrated that up regulation of p75NTR is pro-apoptotic (Volosin et al., 2006; Volosin et al., 2008). Although it can signal for cell death, it may not always. Perhaps, in some cell types, the association of p75NTR and TrkB can be neuroprotective. Hippocampal neurons may re-express p75NTR to prolong survival signaling. If p75NTR is present in endosomes positive for TrkB it may confer similar benefits for survival signaling. Not only would signaling endosomes last longer, taking advantage of the limited survival signaling environment, but the signal would, itself, be stronger if p75NTR has similar effects on TrkB.

Astrocytes and p75NTR with coreceptors

The differing responses of hippocampal neurons and hippocampal astrocytes to both pro and mature neurotrophins provides an ideal system to examine how two cell types that express similar proteins can tailor their responses to similar treatments. Hippocampal neurons and astrocytes both express sortilin and p75NTR but astrocytes do not undergo apoptosis when challenged with proNGF(Hutton et al., 1992; Wang et al., 1998; Troy et al., 2002; Cragnolini et al., 2009). Our lab failed to find any association between p75NTR and sortilin in astrocytes. Most cell types express a majority of the total pool of sortilin in intracellular vesicles so astrocytes may restrict sortilin from the plasma membrane to levels that are too low to bind proNGF (Chabry et al., 1993; Nielsen et al., 1999).

Developing astrocytes respond to BDNF by increasing the activation of TrkB but mature astrocytes do not (Climent et al., 2000). Following injury in the mature brain BDNF

can once again activate TrkB (Goutan et al., 1998). This curious finding is the result of astrocytes mainly expressing a truncated form of TrkB that lacks an intracellular kinase domain (Goutan et al., 1998). Our investigation into the association of p75NTR and TrkB in hippocampal astrocytes failed to show an increase in their association following treatment with BDNF. The antibodies used in this study are all targeted to the extracellular portion of each receptor so we were not able to determine if the astrocytes we examined were expressing full length or truncated TrkB. Given our results in hippocampal neurons it is intriguing to speculate that the astrocytes we examined were mainly expressing truncated TrkB. Astrocytes have been shown to upregulate the secretion of neurotrophins following injury (Oderfeld-Nowak and Bacia, 1994; Goss et al., 1998). In addition, mature astrocytes show decreased TrkB activation in response to BDNF and, instead of activating survival signaling pathways, pool BDNF and NT4 in intracellular vesicles (Alderson et al., 2000). Truncated trks are incapable of activating classical tyrosine kinase pathways due to their lack of any catalytic domain but have a completely intact neurotrophin binding extracellular domain (Esteban et al., 2006). This altered ability of truncated trk receptors to activate similar pathways to those in neurons, may explain the difference in neurotrophin signaling between the two cell types.

Our data also show no functional interaction between p75NTR and sortilin. Following injury in the hippocampus there is a marked increase in the production and release of proneurotrophins and mature neurotrophins (Oderfeld-Nowak and Bacia, 1994; Goss et al., 1998; Volosin et al., 2008). Astrocytes may retain sortilin in intracellular vesicles to avoid a functional interaction between sortilin and p75NTR. Previous studies have determined that most of a cell's total sortilin is located on intracellular vesicles and between five and 10 percent is found on the plasma membrane (Chabry et al., 1993; Nielsen et al.,

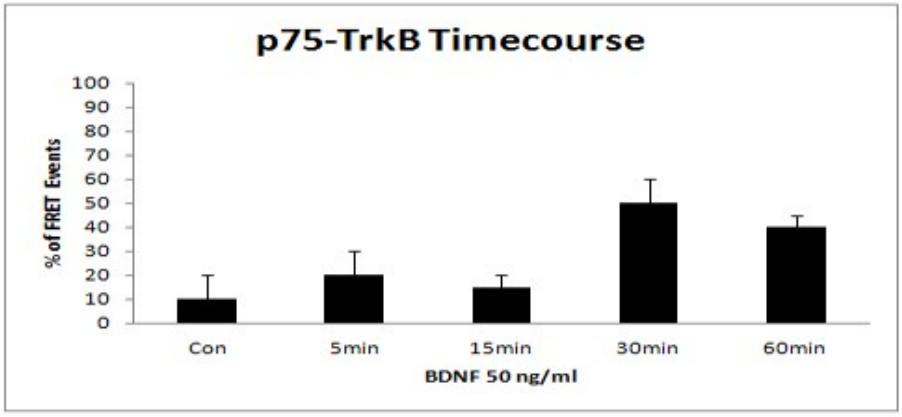
1999). We detected sortilin in hippocampal astrocytes but the cells we examined were permeabilized. The lack of association between p75NTR and sortilin in hippocampal astrocytes may be due to lack of sortilin on the plasma membrane. Perhaps hippocampal astrocytes restrict sortilin intracellular vesicles in order to protect themselves from proneurotrophin-induced apoptosis following injury.

VI. Future Directions

As a confirmation of the conclusion that the activation of TrkB is necessary for the interaction of p75NTR and TrkB, a mutant TrkB receptor that lacks tyrosine kinase activity could be constructed. If this mutant receptor failed to show increased association of p75NTR following BDNF treatment one could conclude that the association is not only ligand dependent but activation dependent as well to confirm the results obtained with K252a. If one could determine the site(s) of the interaction between p75NTR and TrkB a mutant of either receptor lacking the ability to interact with its partner could help determine if the functional interaction is necessary for internalization. Knocking down endogenous levels of p75NTR using siRNA would allow one to measure the movement of TrkB:BDNF containing endosomes. The hippocampus also contains endogenous cells that do not express TrkB and one could also examine the trafficking of only p75NTR with BDNF. One could then compare the movement of those endosomes, through the endosomal pathway, and compare those measurements against TrkB:p75NTR:BDNF positive endosomes to evaluate the possible alteration of p75NTR on TrkB tracking. Fluorescent constructs for both p75NTR and TrkB would allow one to make observations in live cells and evaluate the movements of the receptors both together and separately. Determining the isoform of TrkB that is present in hippocampal astrocytes in culture could help answer whether the differences between neurons and astrocytes, in regards to neurotrophin

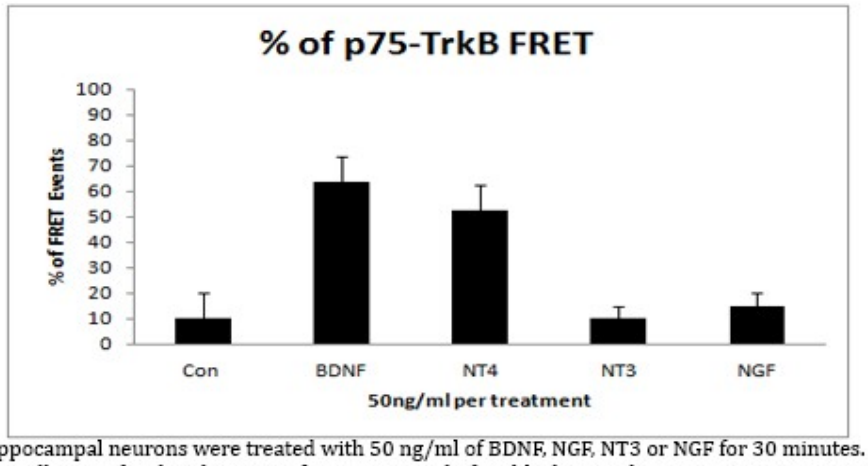
signaling, are due to differences in their receptors. In addition, using biotin labeling or cell fractionation experiments one could determine the level of sortilin expressed on the plasma membrane and intracellular vesicles. Those experiments could help answer if astrocytes restrict sortilin to the interior of the cell in order to prevent an association between sortilin and p75NTR.. Altogether, these studies would provide greater insight into the mechanisms and functional consequences governing the association of p75NTR association with different co-receptors.

Figure 1



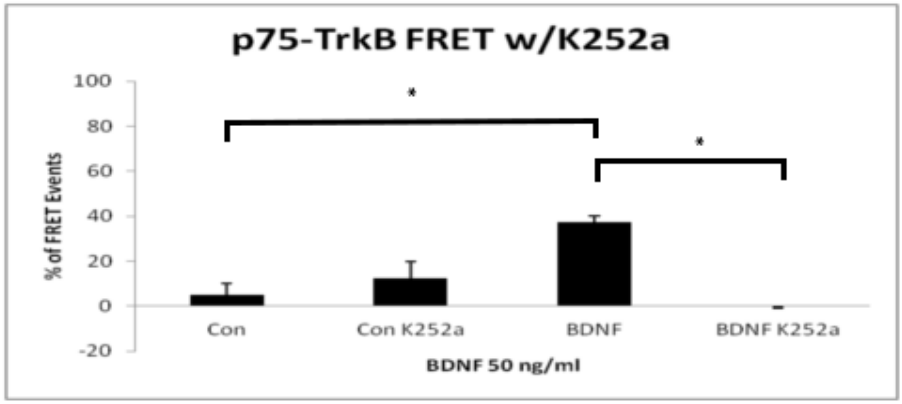
Hippocampal neurons were treated with 50ng/ml of BDNF for 5, 15, 30 and 60 minutes. The cells were fixed with 4 % PFA for 10 minutes before blocking and staining against p75 and TrkB. FRET pairs were made with secondary anitbodies coupled to Alexa 488 and 555. Photobleaching was performed on neuronal somas.

Figure 2



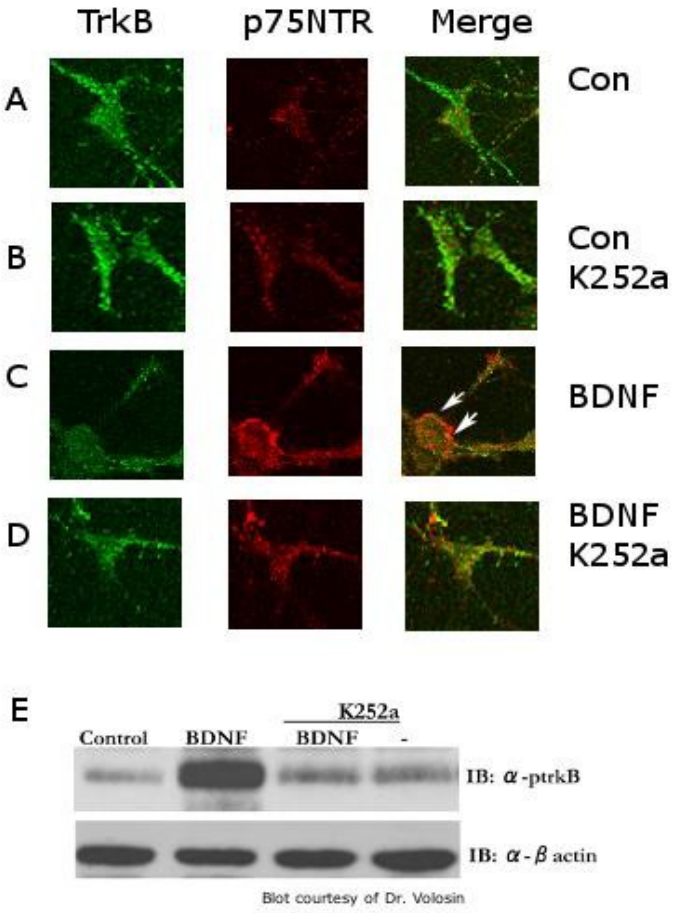
Hippocampal neurons were treated with 50 ng/ml of BDNF, NGF, NT3 or NGF for 30 minutes. The cells were fixed with 4% PFA for 10 minutes before blocking and staining against p75 and TrkB. FRET pairs were made with secondary antibodies coupled to Alexa 488 and 555. Photo-bleaching was performed on neuronal somas.

Figure 3



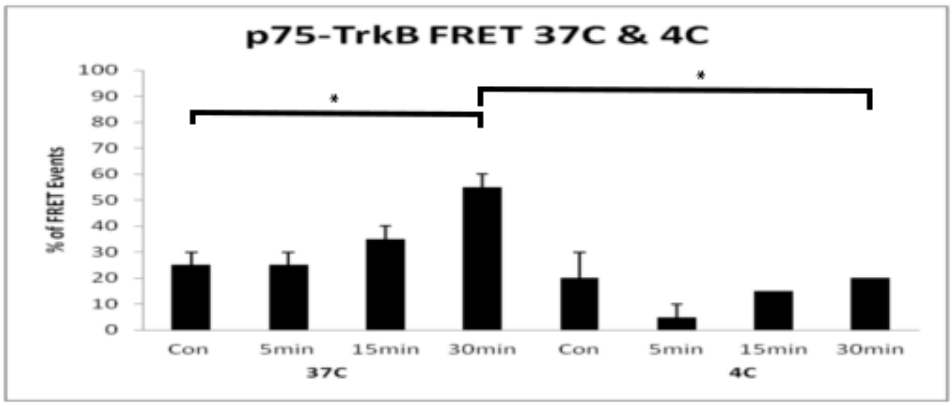
Hippocampal neurons were treated with 50ng/ml of BDNF for 30 minutes with and without a 30 minute pretreatment with k252a (200nm). The cells were fixed with 4 % PFA for 10 minutes before blocking and staining against p75 and TrkB. FRET pairs were made with secondary anitbodies coupled to Alexa 488 and 555. Photobleaching was performed on neuronal somas. Ten neurons were used per treatment and the experiment was performed in triplicate.

Figure 4



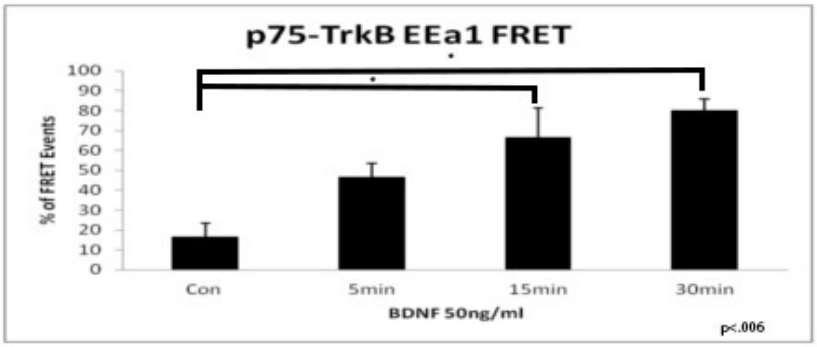
A. Control neurons. B. Control neurons treated with K252a (200nm). C. Neurons treated with 50 ng/ml of BDNF. D. Neurons treated with K252a for 30 minutes before a 30 minute treatment with 50 ng/ml of BDNF. The cells were fixed with 4% PFA for 10 minutes before being blocked and stained against p75 and TrkB. Secondary antibodies coupled to Alexa 488 and 555 were used to detect overlapping staining. E. Control hippocampal neurons were treated with and without K252a for 30 minutes. Hippocampal neurons were treated with and without K252a for 30 minutes before a 30 minute treatment with 50 ng/ml of BDNF. Phospho-TrkB was detected by western blot analysis and actin was probed as a control.

Figure 5



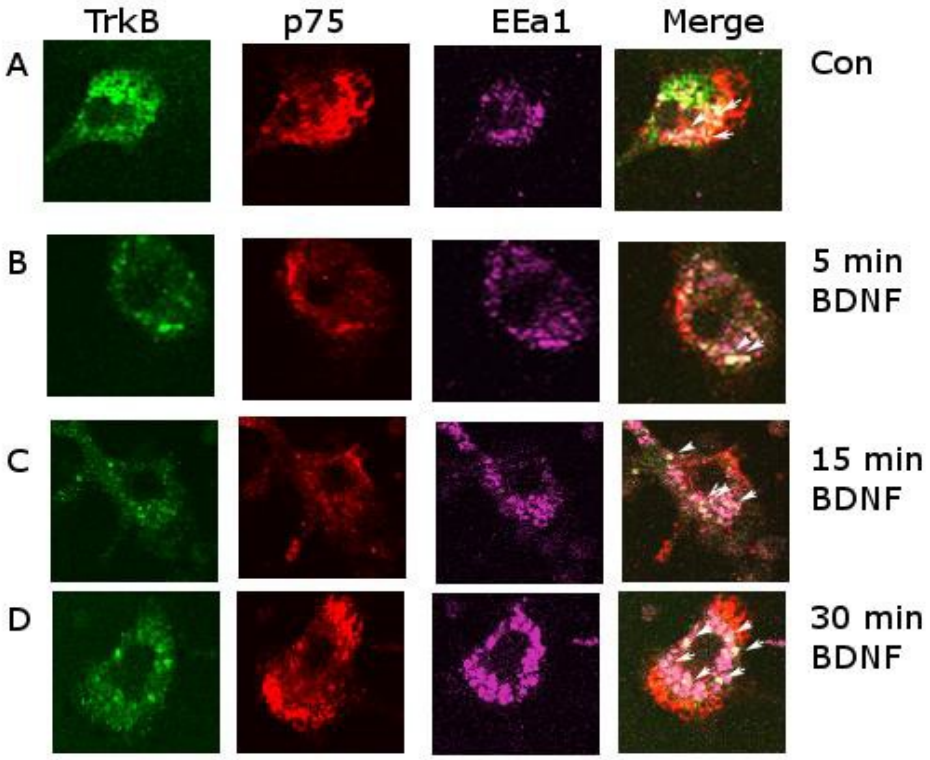
Hippocampal neurons were treated with 50ng/ml of BDNF for 5, 15, and 30 minutes at 37 C or 4 C. The cells were fixed with 4 % PFA for 10 minutes before blocking and staining against p75 and TrkB. FRET pairs were made with secondary anitbodies coupled to Alexa 488 and 555. Photobleaching was performed on neuronal somas. Ten neurons were selected for each treatment snd the experiment was performed in triplicate.

Figure 6



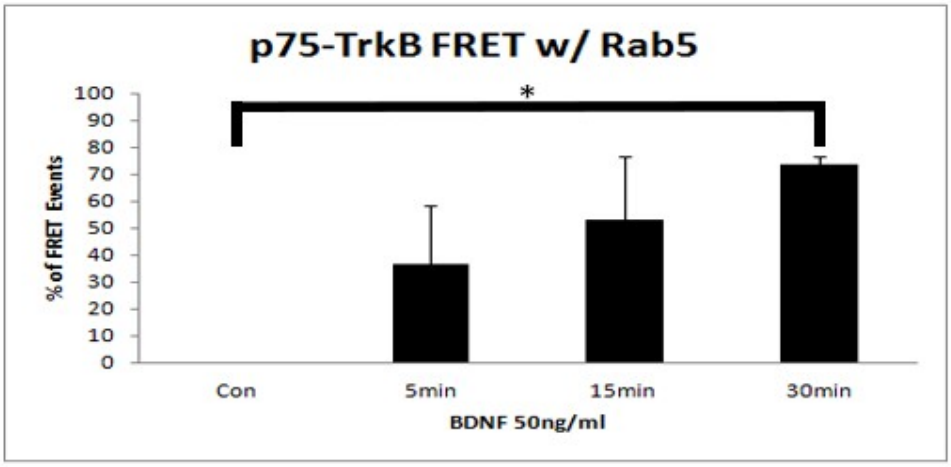
Hippocampal neurons were treated with 50 ng/ml of BDNF for 5, 15 and 30 minutes. The cells were fixed with 4% PFA for 10 minutes before being blocked and stained against p75, TrkB and EEa1. FRET pairs were made with secondary antibodies coupled to Alexa 488 and 555. EEa1 was visualized using a secondary antibody coupled to Alexa 633. Photobleaching was performed on neuronal somas. Ten neurons were selected for each treatment and the experiment was performed in triplicate.

Figure 7



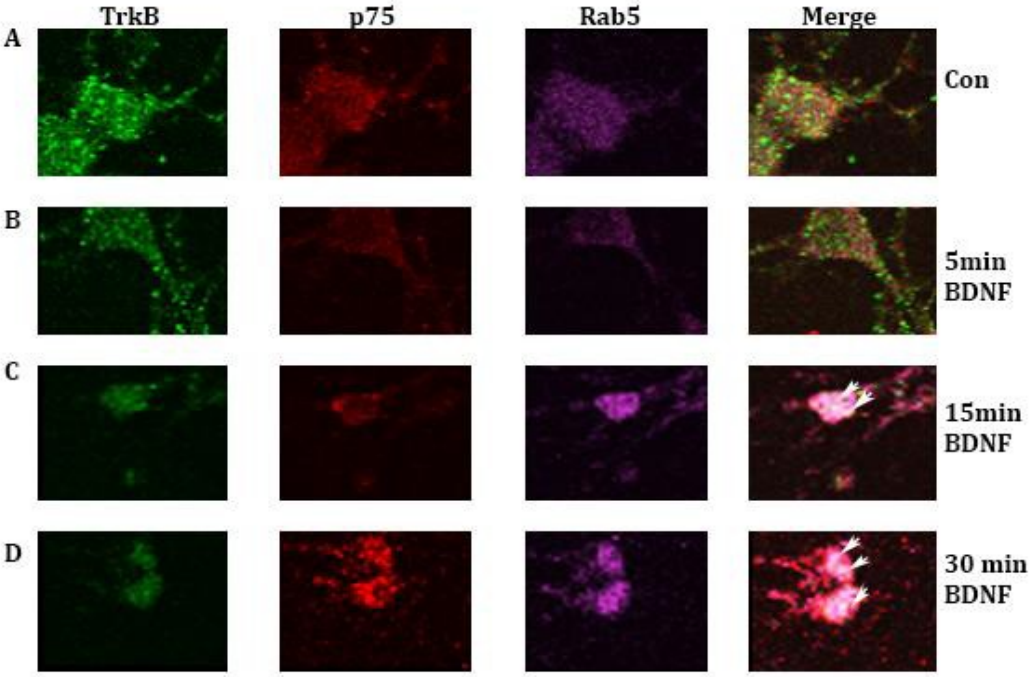
Hippocampal neurons were treated with 50 ng/ml of BDNF and then fixed with 4% PFA for 10 minutes before blocking and staining against p75, TrkB and EEa1. FRET pairs were formed for p75 and TrkB with secondary antibodies coupled to Alexa 488 and 555. Triple staining with EEa1 was viusalized by using a secondary antibody coupled to Alexa 633. Arrows indicate areas of triple labeling. A. Control neurons. B. Neurons treated with 50 ng/ml of BDNF for 5 minutes. C. Neurons treated with 50 ng/ml BDNF for 15 minutes. D. Neurons treated with 50 ng/ml of BDNF for 30 minutes.

Figure 8



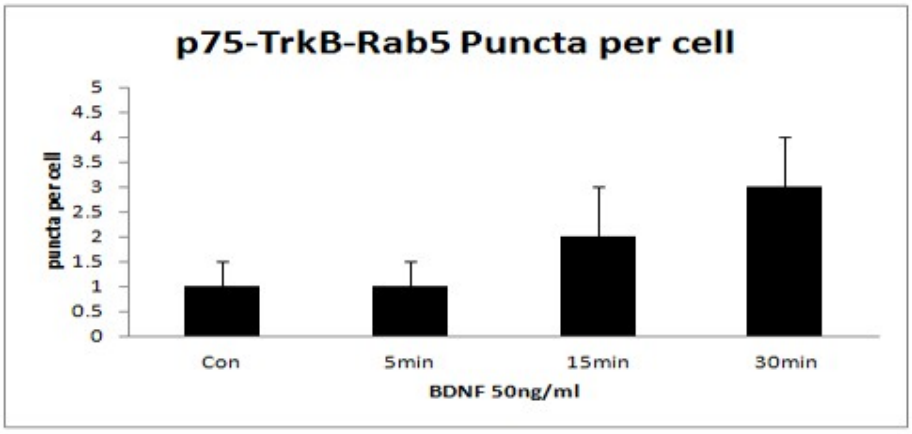
Hippocampal neurons were treated with 50ng/ml of BDNF, for 5, 15, and 30 minutes. The cells were fixed with 4 % PFA for 10 minutes before blocking and stained against p75, TrkB and Rab5. FRET pairs were made with secondary anitbodies coupled to Alexa 488 and 555. Rab5 was visualized using a secondary antibody coupled to Alexa 633. Photobleaching was performed on neuronal somas in areas positive for triple stainig.

Figure 9



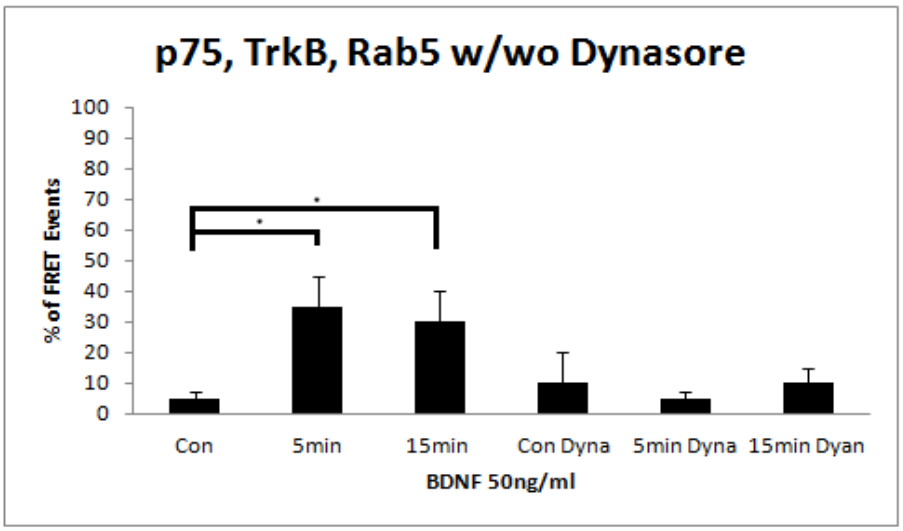
Hippocampal neurons were treated with 50ng/ml of BDNF, for 5, 15, and 30 minutes. The cells were fixed with 4 % PFA for 10 minutes before blocking and stained against p75, TrkB and Rab5. FRET pairs were made with secondary anitbodies coupled to Alexa 488 and 555. Rab5 was visualized using a secondary antibody coupled to Alexa 633. Photobleaching was performed on neuronal somas in areas triple labeled for p75, TrkB and Rab5. A. Control Neurons stained for p75, TrkB and EEa1. B. Neurons treated with 50 ng/ml BDNF for15 minutes and stained for p75, TrkB and Rab5. C. Neurons treated with 50 ng/ml of BDNF for 15 minutes and stained for p75, TrkB and Rab5 D. Neurons treated with 50 ng/ml of BDNF for 30 minutes and stained for p75, TrkB and Rab5.

Figure 10



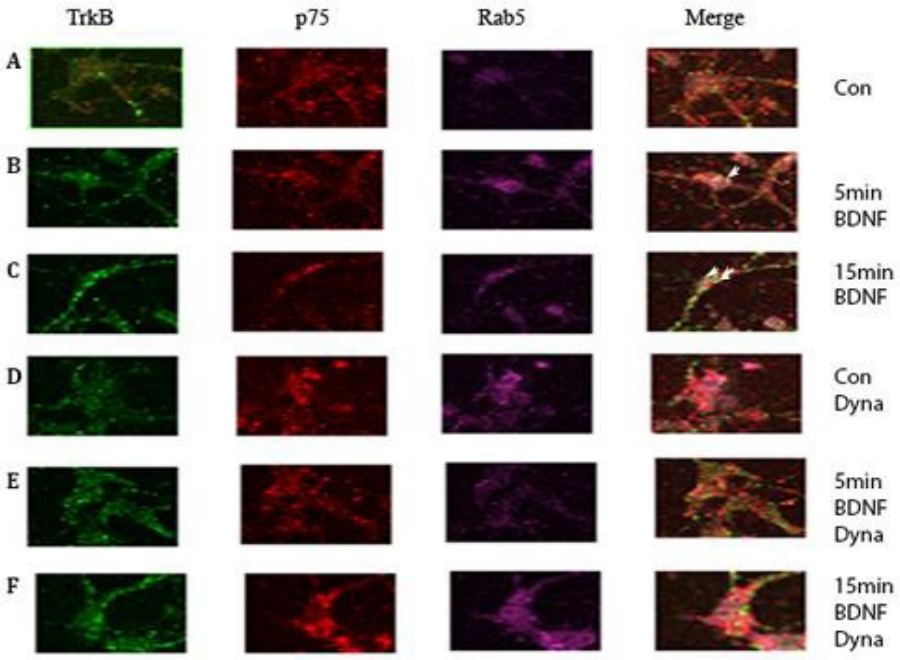
Hippocampal neurons were treated with 50ng/ml of BDNF, for 5, 15, and 30 minutes. The cells were fixed with 4 % PFA for 10 minutes before blocking and stained against p75, TrkB and Rab5. FRET pairs were made with secondary anitbodies coupled to Alexa 488 and 555. Rab5 was visualized using a secondary antibody coupled to Alexa 633. Areas positive for p75, TrkB and Rab5 were counted in all cells in a field. Puncta per cell were determined by dividing the total triple positive areas divided by the total number of cells per field.

Figure 11



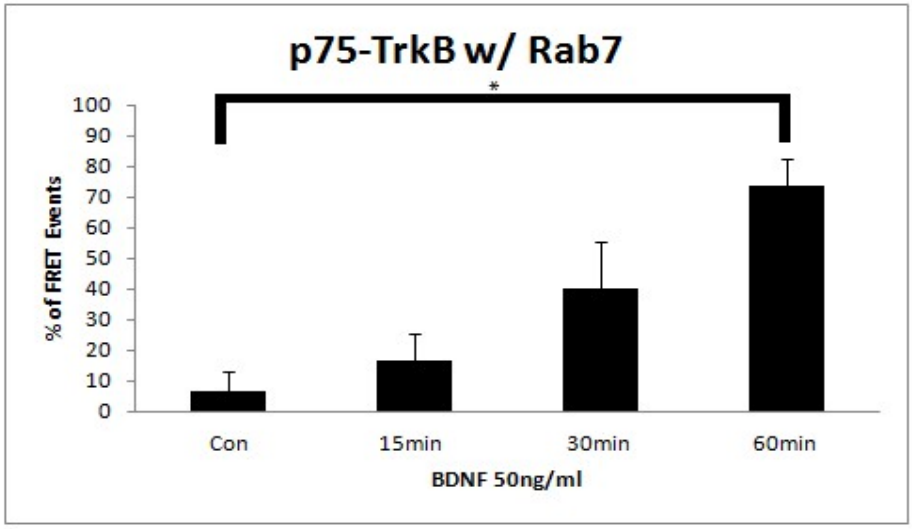
Hippocampal neurons were treated with 50ng/ml of BDNF, for 5 or 15 minutes with and without a 30 minute pretreatents with 50 um Dynasore The cells were fixed with 4 % PFA for 10 minutes before blocking and stained against p75, TrkB and Rab5. FRET pairs were made with secondary anitbodies coupled to Alexa 488 and 555. Rab5 was visualized using a secondary antibody coupled to Alexa 633. Photobleaching was performed on neuronal somas.

Figure 12



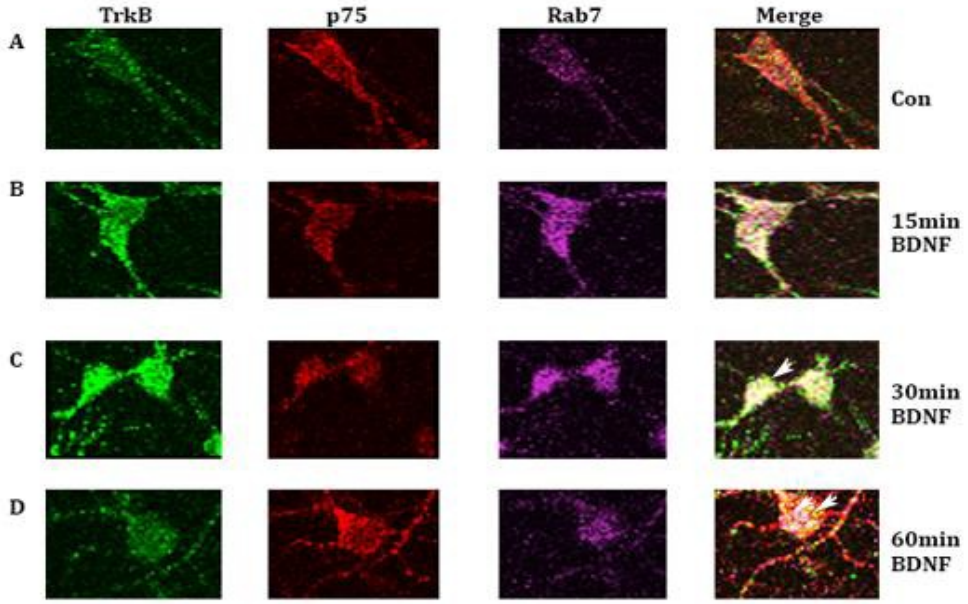
Hippocampal neurons were treated with or without 50 um Dynasore for 30 minutes prior to treatment with 50 ng/ml of BDNF for 5 or 15 minutes. The cells were fixed with 4% PFA for 10 minutes before blocking and staining against p75, TrkB and Rab5. FRET pairs were made with secondary antibodies coupled to Alexa 488 and 555. Rab5 was visualized using a secondary antibody coupled to Alexa 633.
A. Control Neurons stained for p75, TrkB and Rab5. B. Neurons treated with 50 ng/ml BDNF for 5 minutes and stained for p75, TrkB and Rab5. C. Neurons treated with 50 ng/ml BDNF for 15 minutes and stained for p75, TrkB and Rab5. D. Control neurons treated with 50 um of Dynasore for 30 minutes and stained for p75, TrkB and Rab5. E. Neurons treated with 50 um of Dynasore for 30 minutes prior to treatment with 50 ng/ml BDNF for 5 minutes and stained for p75, TrkB and Rab5. F. Neurons treated with 50 um of Dynasore for 30 minutes prior to treatment with 50 ng/ml BDNF for 15 minutes and stained for p75, TrkB and Rab5.

Figure 13



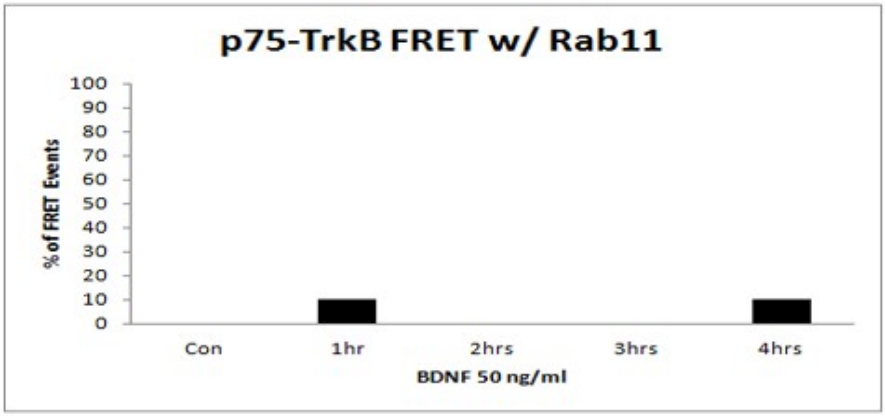
Hippocampal neurons were treated with 50 ng/ml of BDNF for 15, 30 and 60 minutes. The cells were fixed with 4% PFA for 10 minutes before being blocked and stained against p75, TrkB and Rab7. FRET pairs were made with secondary antibodies coupled to Alexa 488 and 555. Rab7 was visualized using a secondary antibody coupled to Alexa 633. Photobleaching was performed on neuronal somas. Ten neurons were selected for each treatment and the experiment was performed in triplicate

Figure 14



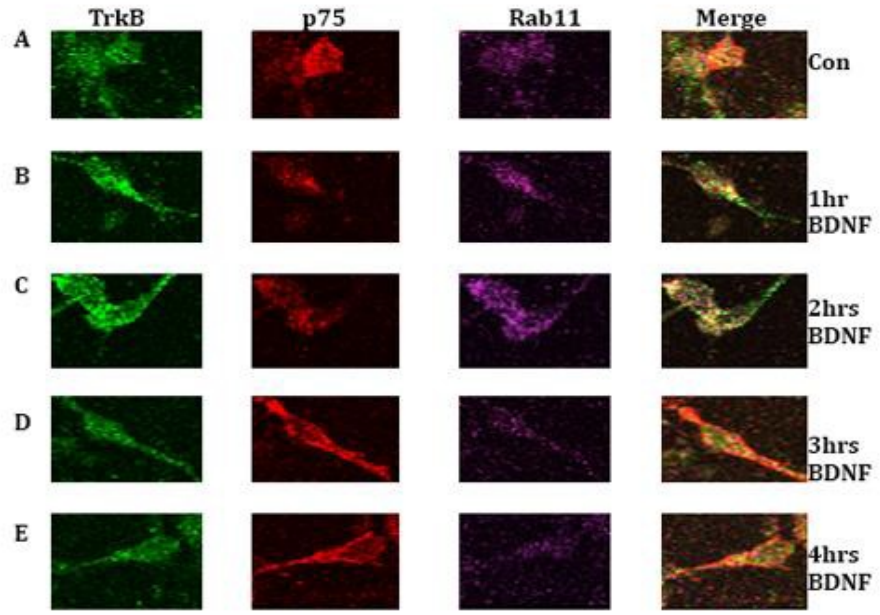
Hippocampal neurons were treated with 50ng/ml of BDNF, for 15, 30, and 60 minutes. The cells were fixed with 4 % PFA for 10 minutes before blocking and stained against p75, TrkB and Rab7. FRET pairs were made with secondary anitbodies coupled to Alexa 488 and 555. Rab7 was visualized using a secondary antibody coupled to Alexa 633. Photobleaching was performed on neuronal somas in areas positive for p75, TrkB and Rab7. A. Control Neurons stained for p75, TrkB and Rab7. B. Neurons treated with 50 ng/ml BDNF for15 minutes and stained for p75, TrkB and Rab7. C. Neurons treated with 50 ng/ml of BDNF for 30 minutes and stained for p75, TrkB and Rab7 D. Neurons treated with 50 ng/ml of BDNF for 60 minutes and stained for p75, TrkB and Rab7.

Figure 15



Hippocampal neurons were treated with 50 ng/ml of BDNF for 1hr, 2hrs, 3hrs and 4hrs. The cells were fixed with 4% PFA for 10 minutes before being blocked and stained against p75, TrkB and Rab11. FRET pairs were made with secondary antibodies coupled to Alexa 488 and 555. Rab11 was visualized using a secondary antibody coupled to Alexa 633. Photobleaching was performed on neuronal somas in areas positive for p75, TrkB and Rab11. Ten neurons were selected for each treatment and the experiment was performed in triplicate.

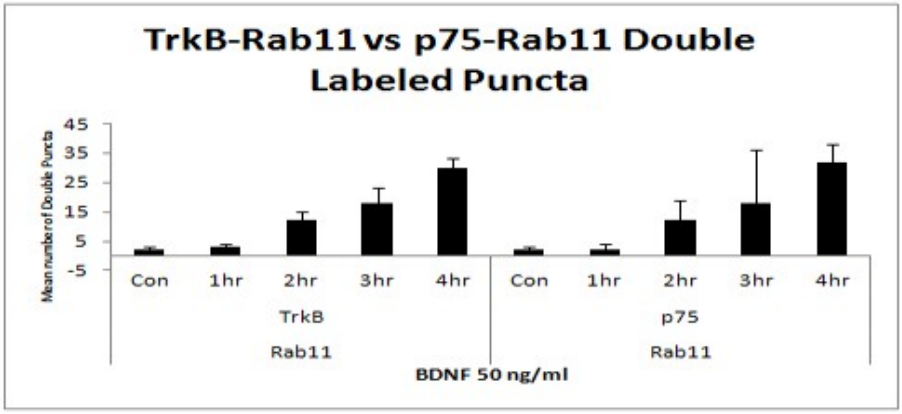
Figure 16



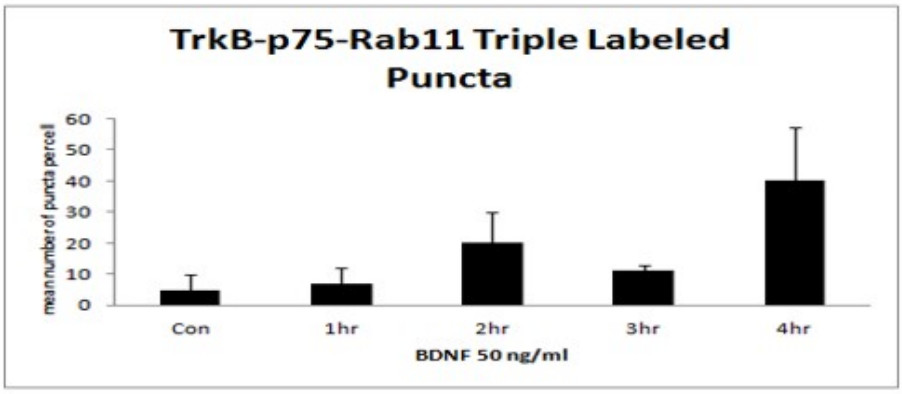
Hippocampal neurons were treated with 50ng/ml of BDNF, for 1, 2, 3, and 4 hours. The cells were fixed with 4 % PFA for 10 minutes before blocking and stained against p75, TrkB and Rab11. FRET pairs were made with secondary anitbodies coupled to Alexa 488 and 555. Rab11 was visualized using a secondary antibody coupled to Alexa 633. Photobleaching was performed on neuronal somas in areas positive for p75, TrkB and Rab11. A. Control Neurons stained for p75, TrkB and Rab11. B. Neurons treated with 50 ng/ml BDNF for1 hour and stained for p75, TrkB and Rab11. C. Neurons treated with 50 ng/ml of BDNF for 2 hours and stained for p75, TrkB and Rab11 D. Neurons treated with 50 ng/ml of BDNF for 3 hours and stained for p75, TrkB and Rab11. E. Neurons were treated with 50 ng/ml of BDNF for 4 hours and stained for p75, TrkB and Rab11.

Figure 17

A

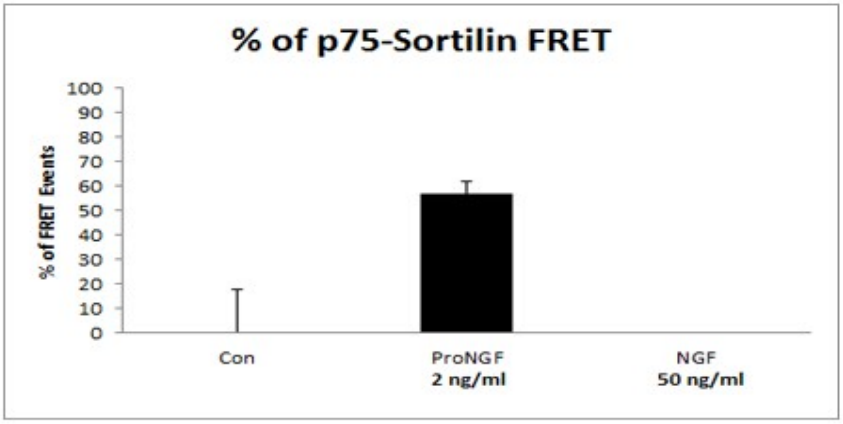


B



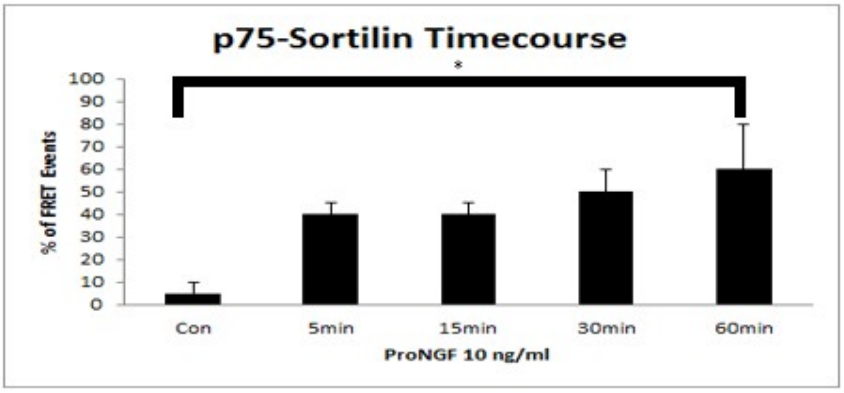
A Hippocampal neurons were treated with 50ng/ml of BDNF, for 1, 2, 3, and 4 hours. The cells were fixed with 4 % PFA for 10 minutes before blocking and stained against p75, TrkB and Rab11. TrkB and Rab11 positive puncta were averaged and compared to p75 and Rab11 positive puncta. B Hippocampal neurons were treated with 50ng/ml of BDNF, for 1, 2, 3, and 4 hours. The cells were fixed with 4 % PFA for 10 minutes before blocking and stained against p75, TrkB and Rab11. FRET pairs were made with secondary anitbodies coupled to Alexa 488 and 555. Rab5 was visualized using a secondary antibody coupled to Alexa 633. Triple labeled puncta were counted and averaged by the number of neurons per field.

Figure 18



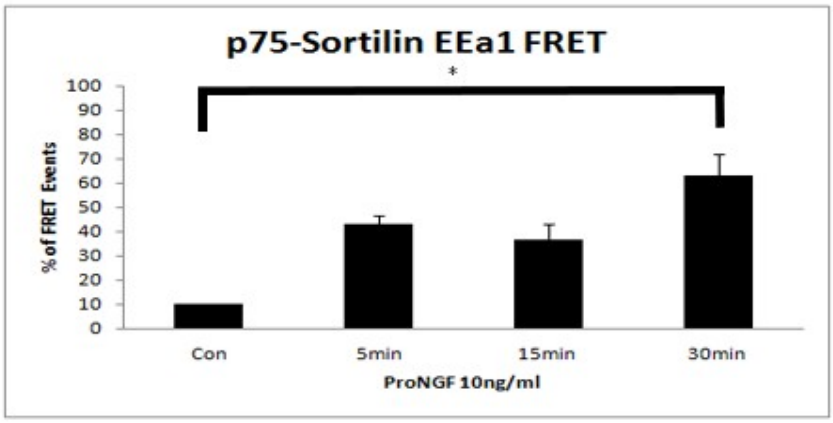
Hippocampal neurons were treated with 50ng/ml of NGF or 2 ng/ml of ProNGF for 30 minutes. The cells were fixed with 4 % PFA for 10 minutes before blocking and staining against p75 and sortilin. FRET pairs were made with secondary anitbodies coupled to Alexa 488 and 555. Photobleaching was performed on neuronal somas.

Figure 19



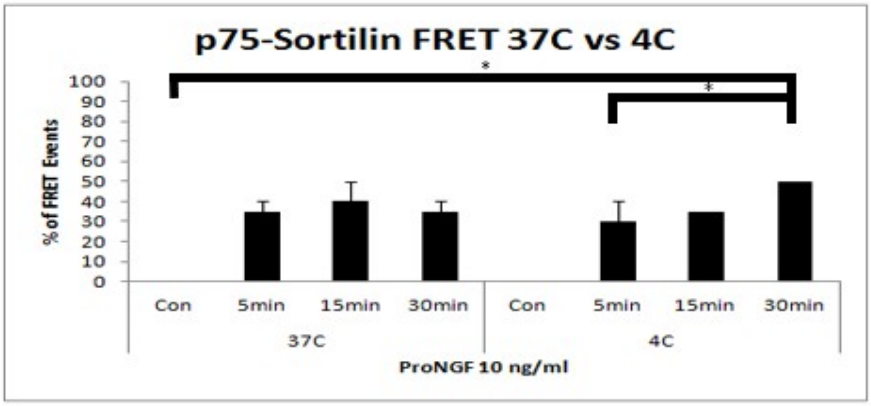
Hippocampal neurons were treated with 10 ng/ml of ProNGF for 5, 15, 30 and 60 minutes. The cells were fixed with 4% PFA for 10 minutes before being blocked and stained against p75 and sortilin. FRET pairs were made with secondary antibodies coupled to Alexa 488 and 555. Photobleaching was performed on neuronal somas.

Figure 20



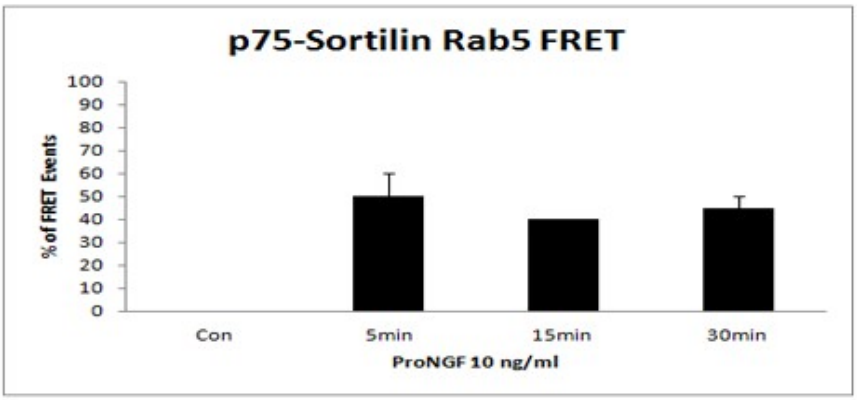
Hippocampal neurons were treated with 10 ng/ml of ProNGF for 5, 15 and 30 minutes. The cells were fixed with 4% PFA for 10 minutes before blocking and staining against p75, sortilin and EEa1. FRET pairs were made with secondary antibodies coupled to Alexa 488 and 555. EEa1 was visualized using a secondary antibody coupled to Alexa 633. Photobleaching was performed on neuronal somas in areas positive for p75, sortilin and EEa1. Ten neurons per treatment were measured and the experiments were performed in triplicate.

Figure 21



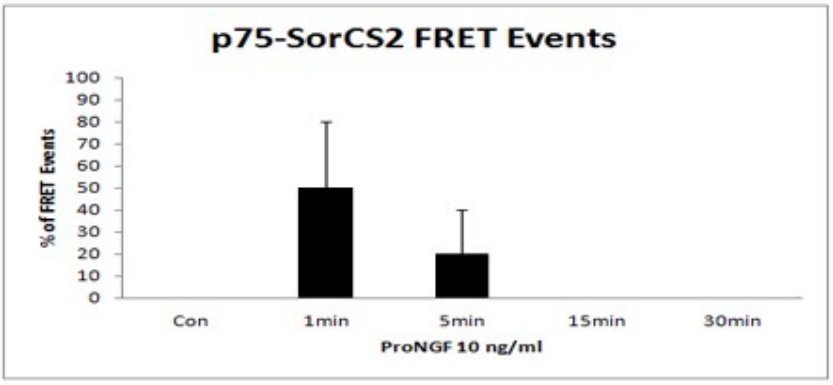
Hippocampal neurons were treated with 10 ng/ml of ProNGF for 5, 15 and 30 minutes at 37C or 4C. The cells were fixed with 4% PFA for 10 minutes before blocking and staining against p75 and sortilin. FRET pairs were made with secondary antibodies coupled to Alexa 488 and 555. Photobleaching was performed on neuronal somas in areas positive for p75 and sortilin. A minimum of 10 neurons per treatment were chosen and the experiment was performed in triplicate.

Figure 22



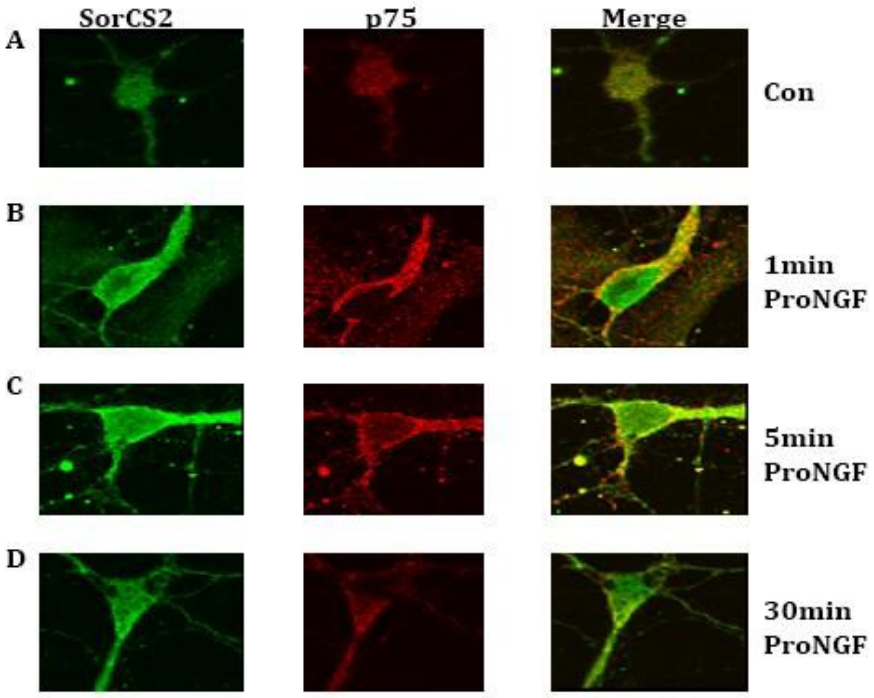
Hippocampal neurons were treated with 10 ng/ml of ProNGF for 5, 15 and 30 minutes. The cells were fixed with 4% PFA for 10 minutes before blocking and staining against p75, sortilin and Rab5 FRET pairs were made with secondary antibodies coupled to Alexa 488 and 555. Rab5 was visualized using a secondary antibody coupled to Alexa 633. Photobleaching was performed on neuronal somas in areas positive for p75, sortilin and Rab5. Ten neurons per treatment were measured and the experiments were performed in triplicate.

Figure 23



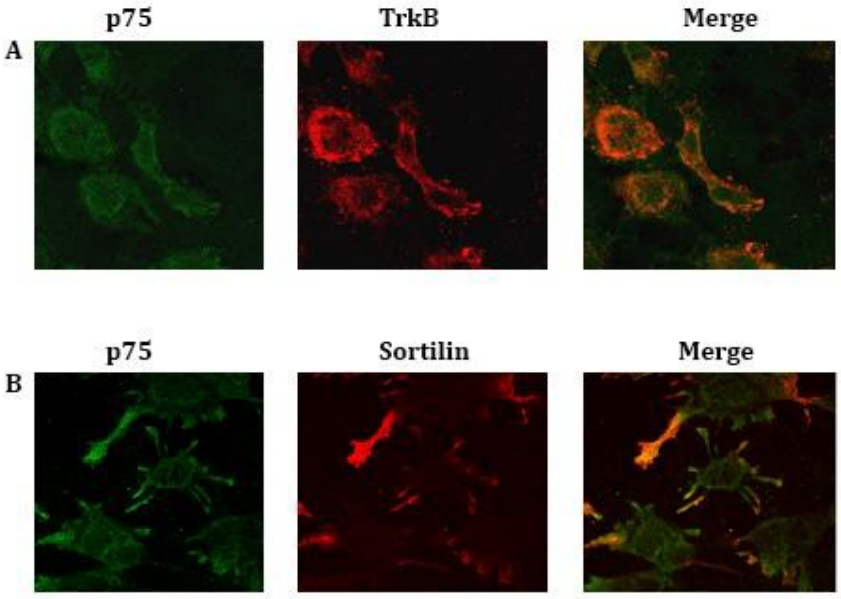
Hippocampal neurons were treated with 10ng/ml of ProNGF, for 1, 5, 15 and 30 minutes. The cells were fixed with 4 % PFA for 10 minutes before blocking and stained against p75 and SorCS2. FRET pairs were made with secondary anitbodies coupled to Alexa 488 and 555. Photobleaching was performed on neuronal somas in areas positive for both p75 and SorCS2. A minimum of 10 neurons per treatment were chosen and the experiment was performed in triplicate.

Figure 24



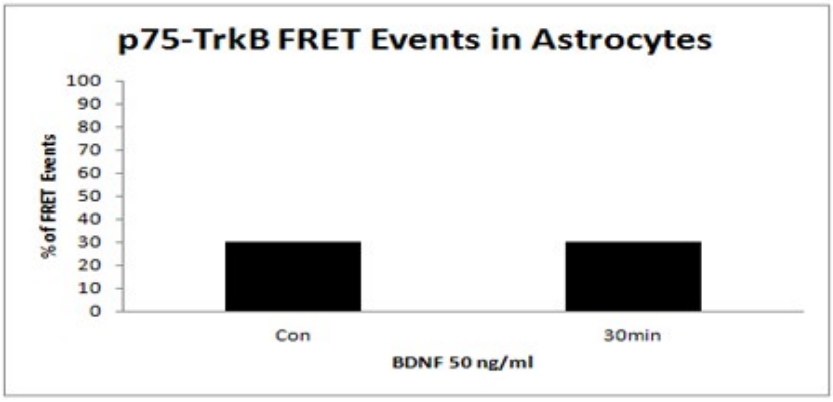
Hippocampal neurons were treated with 10ng/ml of ProNGF, for 1, 5 and 30 minutes. The cells were fixed with 4 % PFA for 10 minutes before blocking and stained against p75 and SorCS2. FRET pairs were made with secondary anitbodies coupled to Alexa 488 and 555. A. Control Neurons stained for p75 and SorCS2. B. Neurons treated with 10 ng/ml of ProNGF for 1 minute and stained for p75 and SorCS2. C. Neurons treated with 10 ng/ml of ProNGF for 5 minutes and stained for p75 and SorCS2. D. Neurons treated with 10 ng/ml of ProNGF for 30 minutes and stained for p75 and SorCS2.

Figure 25



A. Hippocampal astrocytes were treated with 50 ng/ml of BDNF for 30 minutes before being fixed with 4 % PFA for 10 minutes. Cells were stained with antibodies against p75 and TrkB and visulaized with secondary antibodies coupled to Alexa 488 and 555.
B. Hippocampal astrocytes were treated with 10 ng/ml of ProNGF for 30 minutes before being fixed with 4 % PFA for 10 minutes. Cells were stained with antibodies against p75 and sortilin and visulaized with secondary antibodies coupled to Alexa 488 and 555.

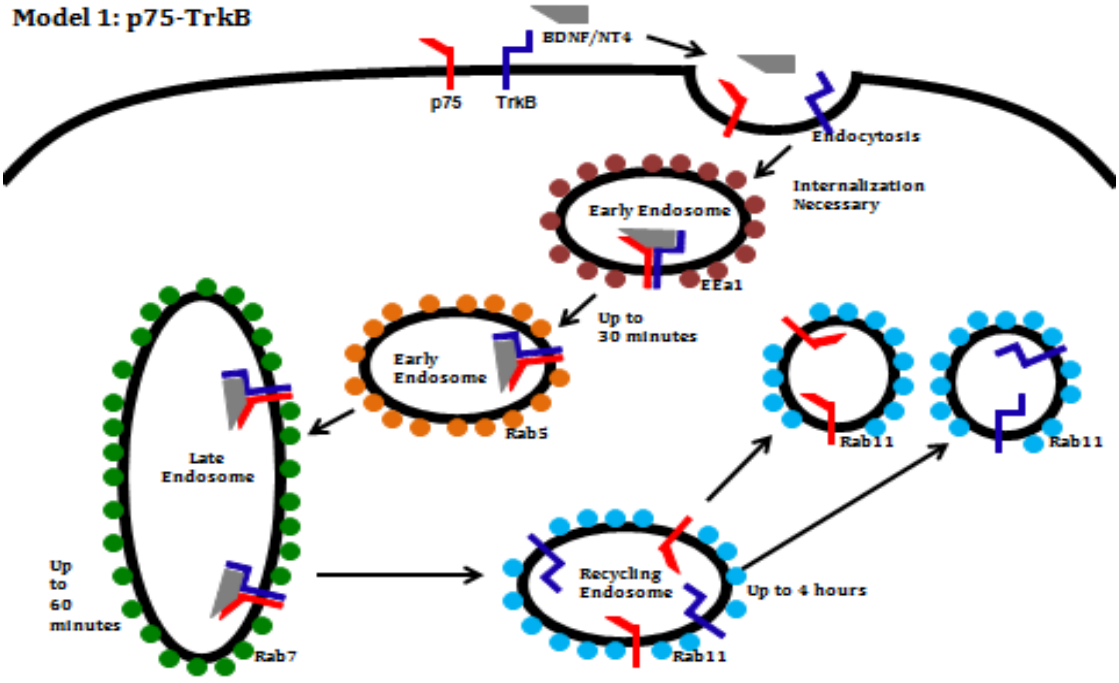
Figure 26

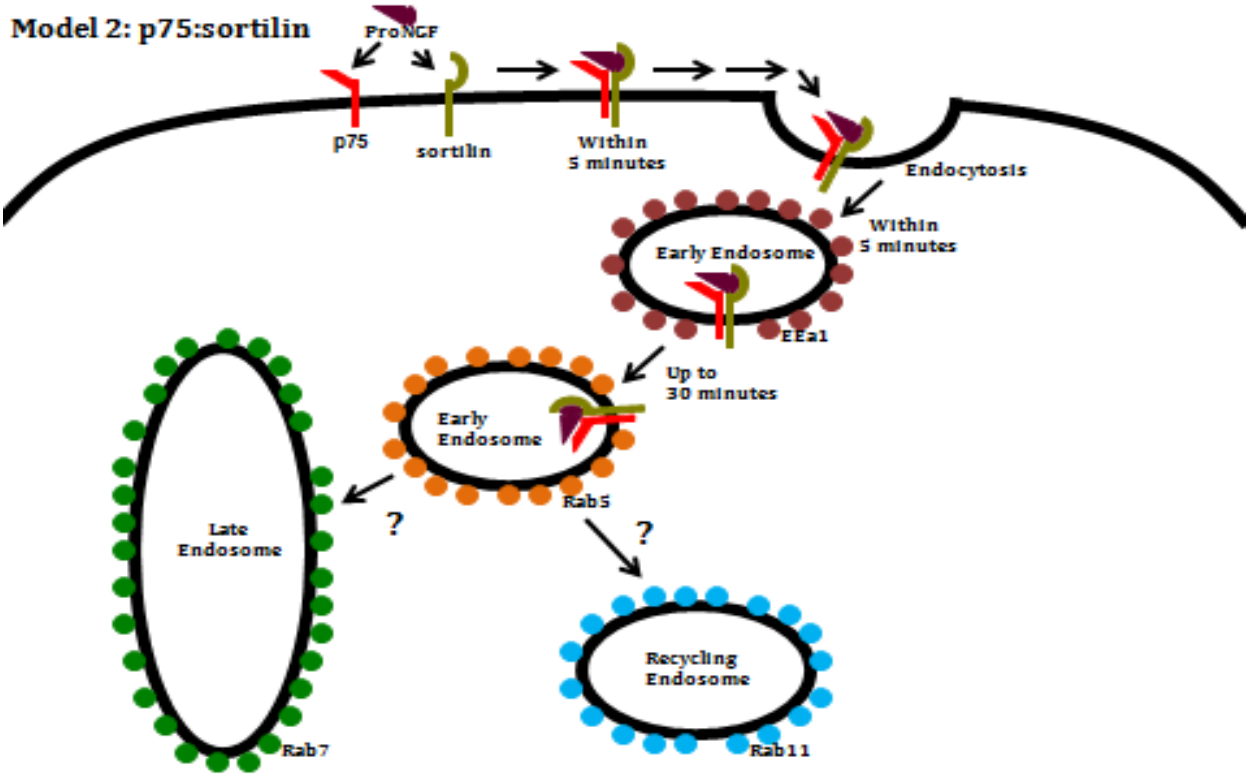


Hippocampal astrocytes were treated with 50 ng/ml of BDNF for 30 minutes. The cells were fixed with 4% PFA for 10 minutes before blocking and staining against p75 and TrkB. FRET pairs were made with secondary antibodies coupled to Alexa 488 and 555. Photobleaching was performed on neuronal somas. Ten astrocytes per treatment were used and the experiment was performed in triplicate.

Figure 27

Hippocampal astrocytes were treated with 2 ng/ml of ProNGF for 30 minutes. The cells were fixed with 4% PFA for 10 minutes before blocking and staining against p75 and sortilin. FRET pairs were made with secondary antibodies coupled to Alexa 488 and 555. Photobleaching was performed on neuronal somas. Ten astrocytes per treatment were used and the experiment was performed in triplicate.


Model 1: p75-TrkB
BDNF/NT4
p75
TrkB
Endocytosis
Internalization
Necessary
Early Endosome
EEa1
Up to
30 minutes
Early
Endosome
Rab5
Late
Endosome
Up
to
60
minutes
Rab7
Recycling
Endosome
Rab11
Up to 4 hours
Rab11
Rab11


Model 2: p75:sortilin
ProNGF
p75
sortilin
Within
5 minutes
Endocytosis
Within
5 minutes
Early Endosome
EEa1
Up to
30 minutes
Early
Endosome
Rab5
?
?
Late
Endosome
Rab7
Recycling
Endosome
Rab11

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