Mechanisms of p75 neurotrophin receptor trafficking

By

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Dedicated to my parents Marianne and Joe Hickman

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TABLE OF CONTENTS

Page

LIST OF FIGURES

Figure Page

LIST OF ABBREVIATIONS

ARF – ADP-ribosylation factor
BDNF – brain derived neurotrophic factor
BMP-4 – bone morphogenic protein-4
CaMK – Ca^{2+}/calmodulin-dependent protein kinase
CC1 – coiled-coil domain
CHC – clathrin heavy chain
CTF – carboxyterminal fragment
DAG – diacylglycerol
DAH – dopamine hydroxylase
DCX – doublecortin
DIC – dynein intermediate chain
DIV – days *in vitro*
DLK – dual leucine zipper kinase
DRG – dorsal root ganglia
ECD – extracellular domain
Erk – extracellular signal-regulated kinases
End3 – endothelin 3
GDNF – glial cell-derived neurotrophic factor
GDFRa3 – GDNF family receptor alpha-3
GEF – guanine nucleotide exchange factors
HDCA – histone deacetylase 1
HGF – hepatocyte growth factor
ICD – intracellular domain
IKK – IκB kinase
IP3 – Inositol trisphosphate
ITAM – immunoreceptor tyrosine-based activation motif
JNK – c-Jun N-terminal kinase
OL – oligodendrocyte
MOMP – mitochondrial outer membrane permeability
MVB – multivesicular bodies
NCC – neural crest cells
NF – neurofilament
MS – Multiple Sclerosis
NGF – nerve growth factor
NPC – neural progenitor cells
NSC – neural stem cells
NRAGE – neurotrophin receptor-interacting MAGE homolog
NRG1 – Neurgulin-1
NRIF – neurotrophin receptor interacting factor
Nrp1 – Neuropilin-1
NT3 – neurotrophin 3
NT3 – neurotrophin 4
p75NTR – p75 neurotrophin receptor
PCD – programmed cell death

PIK3 – phosphoinositide 3-kinase
PIP2 – phosphatidylinositol 4,5-bisphosphate
PKC – protein kinase C
PLC-γ - phosphoinositide phospholipase C gamma
PNS – peripheral nervous system
PTEN – phosphatase and TENsin homolog
Sema3A – Semaphotin 3A
SCP – Schwann cell precursors
SCG – superior cervical ganglia
SDF1 – stromal cell-derived factor-1
SGZ – subgranular zone
Shh – sonic hedgehog
TACE – TNFα-converting enzyme
TFD – trophic factor deprivation
TH – tyrosine hydroxylase
TNF – tumor necrosis family
TRAF – TNF receptor-associated factors
V-SVZ – ventricular-subventricular zone
XIAP – X-linked inhibitor of apoptosis protein

1. Introduction

1.1 Sympathetic nervous system development

The development of the nervous system involves the precise control of several biological systems: the generation of new cells, differentiation into diverse cell types, and the dynamic response to external factors at specific developmental windows. The sympathetic nervous system provides a useful model to study the interplay between these systems. The sympathetic nervous system is responsible for the “fight or flight” reaction during emergency or exercise. It innervates organs involved in mediating the physiological output of these activities. For example, a sympathetic response in a human would involve an increase in heart rate and breathing, vasodilation of blood vessels in skeletal muscles to increase oxygen turnover while constricting vessels in the smooth muscle of the gut to slow metabolism and direct energy to high consumption organs. Stimulating sweat glands and dilating pupils are other examples of sympathetic output (McCorry, 2007). Ganglionic sympathetic neurons respond to acetylcholine from pre-ganglionic neurons in the spinal cord. They in turn release norepinephrine to their peripheral targets. One exception is sympathetic innervation of the sweat glands, which is cholinergic. In order to mediate a prolonged and sustained activated sympathetic-like status, the chromaffin cells of the adrenal medulla can release norepinephrine and epinephrine into the blood stream (McCorry, 2007).

The central nervous system, including the brain and spinal cord, originates from the neural tube, which forms as the ectodermal layer of the developing embryo folds in

on itself. The remaining ectoderm will become the skin, while the neural tube develops into central nervous system structures. As the neural tube closes, a population of cells known as neural crest cells (NCCs) detach from the dorsal edge of the closing neural tube and migrate ventrally. These cells are precursors to the entire peripheral nervous system, which includes the sensory neurons of the dorsal root, the autonomic, and enteric nervous systems (Young et al., 2011).

NCC that arise caudal to the fourth and fifth somite of the developing embryo represent the precursors to the dorsal root ganglia and the sympathetic ganglia (Young et al., 2011; Newbern, 2015). NCCs from vagal or sacral regions will go on to generate the enteric nervous system while cranial and sacral NCCs generate portions of the parasympathetic nervous system (Sasselli et al., 2012; Newbern, 2015).

NCCs undergo an early epithelial to mesenchymal transition which differentiates them from the neural tube precursors and gives them a migratory phenotype. This coincides with delamination caused by the down regulation of N-cadherin and an increase in Cadherin 6, which promotes the detachment of NCCs (Clay and Halloran, 2014). This is thought to occur via bone morphogenic protein-4 (BMP4) expressed by the ectoderm (Young et al., 2011). NCCs are then free to migrate ventromedially through or around adjacent somites (Rickmann et al., 1985; Krull, 2001). The first NCCs to delaminate avoid the dense somite tissue and instead follow the intersomitic boundary ventrally toward the dorsal aorta (Thiery et al., 1982; Guillory and Bronner-Fraser, 1986). As the somites differentiate into sclerotomes, chains of NCCs form and migrate ventrally through them (Figure 1.1).

A subpopulation of these cells ceases migrating adjacent to the neural tube and

coalesces to form the medially-located dorsal root ganglia (DRG). The DRG contain sensory neuron cell bodies, which innervate peripheral tissues, and supporting satellite glial cells. The formation of the DRG occurs in two waves of NCC migration. Early migrating cells, defined by their expression of TrkB and TrkC form large-diameter proprioceptors and mechanoreceptors (Newbern, 2015). The downregulation of Runx3 drives differentiation of mechanoreceptors, while continued expression is observed in proprioceptors (Marmigere and Ernfors, 2007). The second wave of sensory neuron precursors consists mainly of small-diameter TrkA- positive nociceptive neurons. The ganglia forms in an inside-out fashion, with the earliest arriving cells residing at the core and subsequent cells proliferating around the perimeter to encapsulate the core (Marmigere and Ernfors, 2007; George et al., 2010; Newbern, 2015). Activity dependent release of brain derived neurotrophic factor (BDNF) from the core DRG neurons drives expression of protocadherin-1 in the outer shell, which promotes the final coalescence of the ganglia (Bononi et al., 2008; Newbern, 2015).

A second population of NCCs continues migrating ventrally past the DRG and coalesces adjacent to the dorsal aorta, forming the sympathetic ganglia chain (Figure 1.1). Within each somite, migrating cells remain in the rostral half. Several diffusible molecules and their cognate receptors have been shown to segregate migrating NCCs as they burrow through the developing somites. These precursors migrate in a chain-like fashion, which is crucial for controlling their direction (Erickson, 1985; Kasemeier-Kulesa et al., 2005). Both attractive and repulsive molecular cues restrict the migrating chains to the rostral half of each somite. When researchers manipulated the embryo to reverse the orientation of the somite, the neurons migrated through the opposite side,

indicating that this migration is mainly controlled by extrinsic cues and is not intrinsically encoded by the cell. There is evidence at early developmental times that precursors are able to reverse their migratory direction, where prospective sympathetic ganglia neurons can migrate dorsally and mature into DRG neurons. After several hours, however, this plasticity is lost (Kasemeier-Kulesa et al., 2005).

Many of the molecular cues that direct this migration and maturation have been determined. EphrinB ligands in the dorsal neural tube first act as repulsive cues to prevent early migrating NCCs from migrating along a dorsolateral route (Santiago and Erickson, 2002). As the somites develop into sclerotomes and the NCCs switch from the intersomitic pathway to migration through the anterior somite, semaphorin 3A (Sema3A) is expressed by the posterior somite to restrict migration of neuropilin-1 (Nrp1) - expressing NCCs (Schwarz et al., 2009). Other studies show that F-spondin expressed in caudal somites can also restrict migration of precursors to the rostral half (Debby-Brafman et al., 1999). In contrast to these repulsive cues, thrombospodin-1 in the rostral region acts as a chemoattractant (Tucker et al., 1999).

Interestingly, the pattern of distinct chains of sympathetic precursors migrating through distinct somite/sclerotomes is lost upon reaching the ventral edge. Before coalescing into discrete ganglia, cells disperse and intermix within a narrow rostral-caudal corridor adjacent to the dorsal aorta. Lineage tracing studies in chick embryos have shown that NCCs originating from several somite layers are found within each mature sympathetic ganglion(Yip, 1986). This final step of gangliogenesis requires the dynamic extension and retraction of filopodia and movement to sort NCCs into discrete ganglia (Kasemeier-Kulesa et al., 2005). The pattern of distinct ganglia associating with

specific somites is restored but not before cells from several somite chains have mixed.

Migrating NCCs in the ventromedial pathway can also differentiate into the adrenal medulla, a portion of the adrenal gland made up of a subpopulation of sympathoadrenal precursors that differentiate into endocrine cells that control the release of systemic hormones from the adrenal gland. These cells receive direct input from spinal cord pre-ganglionic sympathetic precursors, not from the peripheral sympathetic chain. These andrenal cells are produced from a common pool of sympathetic-adrenal precursors that coalesce in the para-aortic region, ventral to the sympathetic ganglia (Saito et al., 2012). Several factors have been implicated in controlling this migration and differentiation. BMPs are well-described regulators of many types of signaling, from cell growth, patterning, and differentiation in development to adult tissue homeostasis and repair (Wang et al., 2014). Ligands BMP-4 and BMP-7 are produced by the dorsal aorta and drive the expression of chemoattractive factors stromal cell–derived factor–1 (SDF1) and Neurgulin-1 (NRG1). Deletion of their respective receptors, CXCR4 and ErbB in NCCs markedly disturbed the cells ability to accumulate in the para-aortic mesenchyme. Further, ectopic expression of BMP inhibitor Noggin in the dorsal aorta eliminated the expression of SDF1 and NRG1, suggesting that BMPs can act directly or indirectly on NCCs. After reaching the para-aortic niche, sympathetic precursors segregate from adrenal medulla chromaffin cells which reside more ventrally. While BMPs, SDF1, and NRG1 are required for the migration of sympathoadrenal precursors to the dorsal aorta, BMPs and NRG1 are also required for the adrenal-sympathetic precursor segregation. At later embryonic stages, only adrenal medulla cells were able to respond to BMPs and NRG1 signals to

segregate from the sympathetic ganglia (Saito et al., 2012).

Sympathetic precursors in both the coalescing ganglia as well as the adrenal medulla can respond directly to BMP-4 from the dorsal aorta, which initiates differentiation into mature sympathetic neurons through the expression of key noradrenergic neuronal genes, tyrosine hydroxylase (TH), dopamine hydroxylase (DBH), and neuronal markers neurofilament 160 (NF160) and SCG10 (Reissmann et al., 1996; Schneider et al., 1999).

While the chain of sympathetic ganglia in the periphery forms from trunk NCCs, the superior cervical ganglia (SCG) forms from NCCs that migrate rostrally from the anterior vagal and lower cervical regions of the neural tube (Rubin, 1985; Durbec et al., 1996). Between embryonic day 11.5 and 14.5 in mice, the undeveloped SCG forms proximal to the C1-C4 vertebrae (Nishino et al., 1999). This rostral migration is mediated through the glial cell-derived neurotrophic factor (GDNF) family member Artemin, which is expressed along blood vessels. In contrast to the more caudal sympathetic chain, loss of Gfra3, a component of the Artemin receptor complex, causes a failure of migration in the SCG precursors (Honma et al., 2002).

NCCs that develop later migrate in a dorsolateral pathway away from the closing neural tube and under the growing ectoderm and will differentiate into pigment-producing melanocytes of the skin. This has been shown to involve EphrinB, which later in development acts as an attractive cue to promote migration into the dorsolateral pathway (Bronner-Fraser, 1986; Santiago and Erickson, 2002).

NCCs are defined, in part by expression of the transcription factor, Sox10. In addition to the various neuronal cell types that derive from NCC precursors, peripheral

glia that reside within ganglia and proximal to nerves also differentiate from the same population of cells. Satellite glia reside within the dorsal root and sympathetic ganglia and provide various supporting roles by ensheathing neuron somas. Schwann cells and their precursors associate with developing peripheral axons to form myelin sheaths, which support long-range nerve transmission (Carney et al., 2006; Newbern, 2015). While neurons lose robust Sox10 expression as they complete their terminal differentiation, peripheral glia maintain expression of Sox10.

Delta/Notch signaling in the DRG is critical for proper gliogenesis. Delta-1, the ligand for the Notch receptor is expressed by early neurons in the ganglia core to promote gliogensis in neighboring undifferentiated NCCs (Newbern, 2015). Notch signaling is thought to act to promote Sox2 expression, which is required to maintain a glial phenotype as well as prevent melanocyte specification (Wakamatsu et al., 2004). Schwann cell precursors (SCPs) respond to NRG1 released from the axon to promote lineage specification and eventually myelination (Dong et al., 1999; Newbern, 2015).

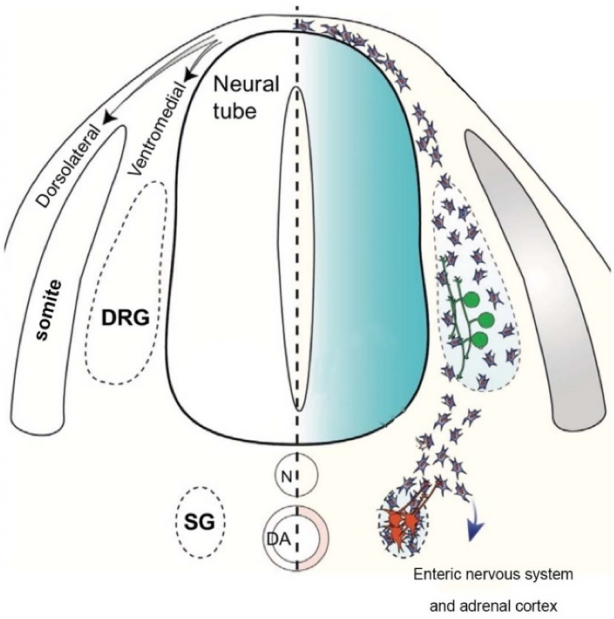

Figure 1.1. Migration of neural crest cells (NCC) from the closing neural tube form the ganglia of the peripheral nervous system. Chains of NCCs (purple) migrate ventromedially towards the dorsal aorta (DA). A subpopulation coalesces into the dorsal root ganglia (DRG, green), while some continue migrating ventrally and coalesce proximal to the DA and form the sympathetic ganglia chain. Additional populations of NCCs will form the enteric nervous system and the chromaffin cells of the adrenal cortex. **Adapted from: Newbern, J. M. (2015). Molecular control of the neural crest and peripheral nervous system development. *Current Topics in Developmental Biology*, *111*, 201–231.**

1.2 Sympathetic target innervation

Once discrete sympathetic ganglia have formed, they have to send long-range projections to target tissues. Much like the initial migration of the sympathetic precursors, this process involves chemical cues and precise regulation of receptors at the extending growth cone. Axon outgrowth of sympathetic neurons in rodents begins in the final days of gestation when the neurons undergo their final mitotic division. Little is known about the signaling that initiates the early sprouting of sympathetic axons (Rubin, 1985) and what promotes their survival prior to reaching their final target tissues.

Hepatocyte growth factor (HGF) is thought to act in an autocrine fashion to promote axon extension, but appears distinct from factors involved in long range outgrowth (Yang et al., 1998; Maina and Klein, 1999). HGF has also been shown to promote survival of a subpopulation of early sympathetic neuroblasts (Maina et al., 1998; Davies, 2009), thus it is possible that HGF can serve as an early survival factor, prior to target innervation.

Several molecules and their receptors have been shown to promote sympathetic axon extension *in vitro* and *in vivo.* Artemin is a member of GDNF family of growth factors and is expressed by the smooth muscle of peripheral blood vessels (Baloh et al., 1998; Nishino et al., 1999; Honma et al., 2002). Artemin induces and promotes sympathetic axon outgrowth *in vitro* and *in vivo*. Deletion of either artemin or its co-receptors GDNF family receptor alpha-3 (GFRa3) or the receptor tyrosine kinase Ret in mice causes developmental abnormalities and only partial innervation of sympathetic targets (Honma et al., 2002; Glebova and Ginty, 2005).

The superior sympathetic ganglia lie proximal to the carotid artery which undergoes a bifurcation early in development. Endothelin 3 (End3) is preferentially expressed in the external branch of the artery, but absent from the internal branch. A subpopulation of SCG neuroblasts express End3 receptor EndrA. End3 promotes axon extension *in vitro* and along the external ceratoid artery *in vivo* (Makita et al., 2008; Davies, 2009).

Neurotrophin 3 (NT3) is the primary trophic factor mediating sympathetic axon extension along peripheral blood vessels (Francis et al., 1999). NT3 is highly expressed by peripheral blood vessels during the developmental window when sympathetic axons

are extending towards their target tissues (Francis et al., 1999). Although NT3 preferentially binds to TrkC, a member of the receptor tyrosine kinase family of neurotrophin receptors, it is not expressed by sympathetic neurons; instead, NT3 signals through the TrkA receptor, which is the receptor for Nerve growth factor (NGF), but can also bind NT3 with low affinity (Birren et al., 1993; ElShamy and Ernfors, 1996; Belliveau et al., 1997a; Kuruvilla et al., 2004). Mice lacking NT3 exhibit a substantial loss in sympathetic neurons (Wyatt et al., 1997); however, axon outgrowth is drastically reduced in *nt3-/-* mice in early development, before the majority of developmental cell death of these neurons occurs, suggesting that observed deficits are due to a loss of axon growth (Kuruvilla et al., 2004). This supports data that shows NT3, while capable of initiating TrkA intracellular signaling at the growth cone, cannot be retrogradely trafficked to the neuron soma to regulate pro-survival gene expression. The molecular basis for this selectivity has not been fully elucidated. It has been shown that NGF, but not NT3, is able to induce TrkA internalization (Kuruvilla et al., 2004). In contrast, Harrington et al. (2011) reported that both NGF and NT3 can induce TrkA internalization, but NT3 dissociates in the acidic environment of the endosome, while NGF remains bound resulting in a Rac1/cofilin-dependent breakdown of the local actin network, freeing the endosome to be retrogradely transported (Harrington et al., 2011). Since NGF-activation of TrkA is required for sympathetic neuron survival, the observed loss of neurons in *nt3*$^{-/-}$ mice is thought to be a secondary consequence of neurons failing to reach NGF-producing targets *in vivo*. Further, the amount of neuron loss is similar in *ngf*$^{-/-}$ and *nt3*$^{-/-}$ ganglia and not increased in double knockout animals (Wyatt et al., 1997; Francis et al., 1999; Davies, 2009).

1.3 Cell death in the nervous system

Coincident with the formation of new neurons comes a significant amount of programmed cell death (PCD) during development to ensure proper organ size and innervation. Creating superfluous cells is not unique to the nervous system. PCD has been well described in development as a non-pathological mechanism to ensure proper organ function. For example, PCD has been described as a mechanism to remove damaged and defective spermatocytes (Baum et al., 2005), removing excess skin tissue in the interdigital space to form individual fingers and toes (Merino et al., 1998), and eliminating self-recognizing immune cells to prevent the development of autoimmunity (Krammer, 2000; Buss et al., 2006).

The majority of the developmental apoptosis observed in the sympathetic nervous system occurs shortly after extending axons reach their NGF producing targets (Davies, 2009). The prevalent hypothesis in the field suggests that neurons compete for a limited amount of trophic support. Neurons improperly targeted to regions with low levels of NGF or areas of redundant innervation leads to cell death. This is an active cellular process that requires *de novo* protein synthesis. *In vitro* studies have elucidated the key signaling events during the time course of NGF withdrawal (Kristiansen and Ham, 2014).

NGF withdrawal induces cell death in sympathetic neurons by activating intrinsic apoptotic machinery. After withdrawal, the expression of several BH-3 domain containing proteins increases. Dp5, Bim, Puma, and Bmf are able to inhibit the anti-apoptotic activity of Bcl2, while promoting mitochondrial outer membrane permeability (MOMP) through Bax activation. Bax forms a pore in the mitochondrial membrane

through which cytochrome c is released into the cytosol. Cytoplasmic cytochrome c, along with Apaf-1 and procaspase 9 form the apoptosome, which in turn cleaves and activates caspase 3 (McIlwain et al., 2013). When activated via cleavage, Caspase 3, an “effector” caspase, is free to break down intracellular proteins through proteolysis, which ultimately leads to cell death. c-Jun N-terminal kinase (JNK) activity also increases in sympathetic neurons after NGF withdrawal. JNK phosphorylates and activates the transcription factor c-Jun, which is required for NGF withdrawal-induced cell death(Palmada et al., 2002). c-Jun activation leads to the transcription of pro-apoptotic target genes as well as positive regulation of its own expression. JNK also phosphorylates members of the Bcl2 family to antagonize the anti-apoptotic activity of Bcl2 or BclXl, as well as activating pro-apoptotic members Bim and Bmf (Eilers et al., 2001; Dhanasekaran and Reddy, 2008). Other factors are involved in the precise regulation of this process. For example, X-linked inhibitor of apoptosis protein (XIAP), an endogenous caspase inhibitor expressed in sympathetic neurons, increases in expression during development. XIAP is protective in neurons maintained in NGF against apoptosis, even when cytochrome c is injected directly into the cell (Potts et al., 2003; Kristiansen and Ham, 2014). This mechanism ensures protection of mature neurons, which unlike non-neuronal cells do not turn over throughout the lifetime of the organism. Hallmark phenotypic and morphologic changes indicate when a cell has initiated the apoptotic process, such as cell shrinkage brought about via the breakdown of cytosketelal proteins, chromatin condensation (known as pyknosis), and the breakdown of the nuclear envelope.

To further understand the complex molecular signals that mediate sympathetic

neuron survival and death, a more detailed background on neurotrophin signaling is needed.

1.4 Neurotrophin signaling

The discovery that diffusible factors from neuron target tissues can directly regulate the survival and differentiation of the neurons themselves was a seminal finding in developmental neurobiology. Since the initial findings by Rita Levi-Montalcini, Viktor Hamburger, and Stanley Cohen decades ago (Levi-Montalcini, 1966), an entire family of neurotrophins and their receptors have been characterized. The neurotrophic hypothesis, as postulated by these pioneering researchers, states that target organs secreting limiting amounts of growth factors to developing neurons leads to the considerable death cell observed during peripheral nervous system (PNS) development (Levi-Montalcini, 1966; 1987; Korsching, 1993; Huang and Reichardt, 2001).

The discovery of NGF as an instructive and permissive signal for sensory neuron survival was followed by the discovery of structurally related factors that were released in the periphery. At the time, it was a revolutionary concept that these peripheral organs could control the development of their own neural circuits (Chao, 2003). Brain derived neurotrophic factor (BDNF), neurotrophin 3 (NT3), and neurotrophin 4 (NT4), together with NGF, make up the neurotrophin family of trophic factors, which are required for the proper development of the nervous system. Neurotrophins are synthesized as pro-neurotrophins and are cleaved to produce mature proteins (Chao, 2003). The cleavage is carried about by furin or pro-convertases at highly conserved residues to produce mature, carboxy-terminal neurotrophins which are approximately 12kDa in size and exist in stable, non-covalent dimers (Chao, 2003). However, recent findings have

revealed that pro-neurotrophins can also be released, both during normal function of the nervous system and following injury (Hempstead, 2014).

How these related proteins affect the developing neurons depends on the combination of neurotrophin receptors they express. All four neurotrophins in both their pro- and mature forms bind to the p75 neurotrophin receptor (p75NTR). NGF has a selective affinity for TrkA, BDNF and NT4 for TrkB, and NT3 binds selectively to TrkC. Neurotrophin dimers bind to their respective Trk receptors, which also exist at the cell surface as dimers.

The three Trks represent a family of tyrosine kinase receptors that contains three single-pass type I transmembrane proteins. Their extracellular domains (ECD) are defined by leucine-rich repeats flanked on either side by two cysteine repeats and are highly glycosylated (Deinhardt and Chao, 2014). They interact with ligands using the second of their two immunoglobulin (Ig) domains located adjacent to the transmembrane domain (Wiesmann et al., 1999). Additionally, alternately spliced isoforms generate Trk receptors with different ECDs, intracellular domains (ICD)s and ligand binding affinities (Deinhardt and Chao, 2014).

NGF binds to TrkA with nanomolar (nM) affinity. While TrkA is often called the NGF high affinity receptor, NGF binds to p75NTR with similar affinity (Hempstead et al., 1991; Esposito et al., 2001). However, when TrkA and p75NTR are co-expressed in cells, NGF affinity for TrkA goes up 100-fold (Mahadeo et al., 1994). Interestingly, mutating the extracellular domain of p75NTR, where NGF binds, does not alter this higher affinity interaction. However, mutating p75NTR such that it can no longer form a complex with TrkA attenuates the increased affinity. This complex forms through

interactions between TrkA and p75NTR transmembrane and intracellular domains, but not the ligand-binding ECDs (Esposito et al., 2001). This suggests that p75NTR increases NGF-TrkA activity through an allosteric modulation that creates a higher affinity binding site for NGF (Harrison et al., 2000; Esposito et al., 2001). While TrkC is the preferred receptor for NT3, NT3 is promiscuous and can also bind to TrkA in the absence of p75NTR (Cordon-Cardo et al., 1991; Belliveau et al., 1997b; Kuruvilla et al., 2004). NT3 expression by peripheral blood vessels along with target organ expression of NGF work in concert to ensure proper sympathetic neuron innervation and survival.

Ligand binding to Trk receptors causes dimerization and subsequent trans-activation. The intracellular kinase domain contains key tyrosine residues and phosphorylation of these initiates downstream signaling cascades, including activation of Phosphoinositide phospholipase C gamma (PLC-γ), Phosphoinositide 3-kinase (PIK3), and extracellular signal-regulated kinases (Erk) (Deinhardt and Chao, 2014). Phosphorylation of Trk at the terminal tyrosine recruits and activates PLC-γ, which in turn hydrolyzes Phosphatidylinositol 4,5-bisphosphate (PIP2) into second messenger Inositol trisphosphate (IP3) and diacylglycerol (DAG). IP3 causes a release in intracellular calcium stores (Ca^{2+}), which activates Ca^{2+}-sensing enzymes like Ca^{2+}/calmodulin-dependent protein kinase (CaMK) or calcineurin (Deinhardt and Chao, 2014). Calcineurin activation is also associated with the clatherin-mediated endocytosis of Trks (Scott-Solomon and Kuruvilla, 2018). Additionally, DAG formation can activate protein kinase C (PKC) (Figure 1.2).

Phosphorylation of Trk receptors at the transmembrane-adjacent tyrosine leads to Shc binding, which activates PI3K via Grb1/2. PI3K activation leads to stimulation of

Akt, which promotes survival through phosphorylation and inhibition of pro-apoptotic proteins Bad and caspase 9. Akt can also inhibit cell cycle arrest through phosphorylation of cdk inhibitors p21 and p27 as well as promoting NFκB activity by directly phosphorylating IκB kinase (IKK) (Maggirwar et al., 1998; Mayo and Donner, 2002). Shc association with Trk also recruits and activates Ras, which leads to activation of Raf, which feeds into the MAPK signaling pathway, specifically Erk1/2, to initiate pro-neural differentiation and survival genes through phosphorylating various transcription factors, such as CREB (Reichardt, 2006). Receptor activation can also activate Rho family GTPases like Cdc42 and Rac to control the cytoskeletal dynamics associated with cell motility and axon outgrowth (Reichardt, 2006) (Figure 1.2).

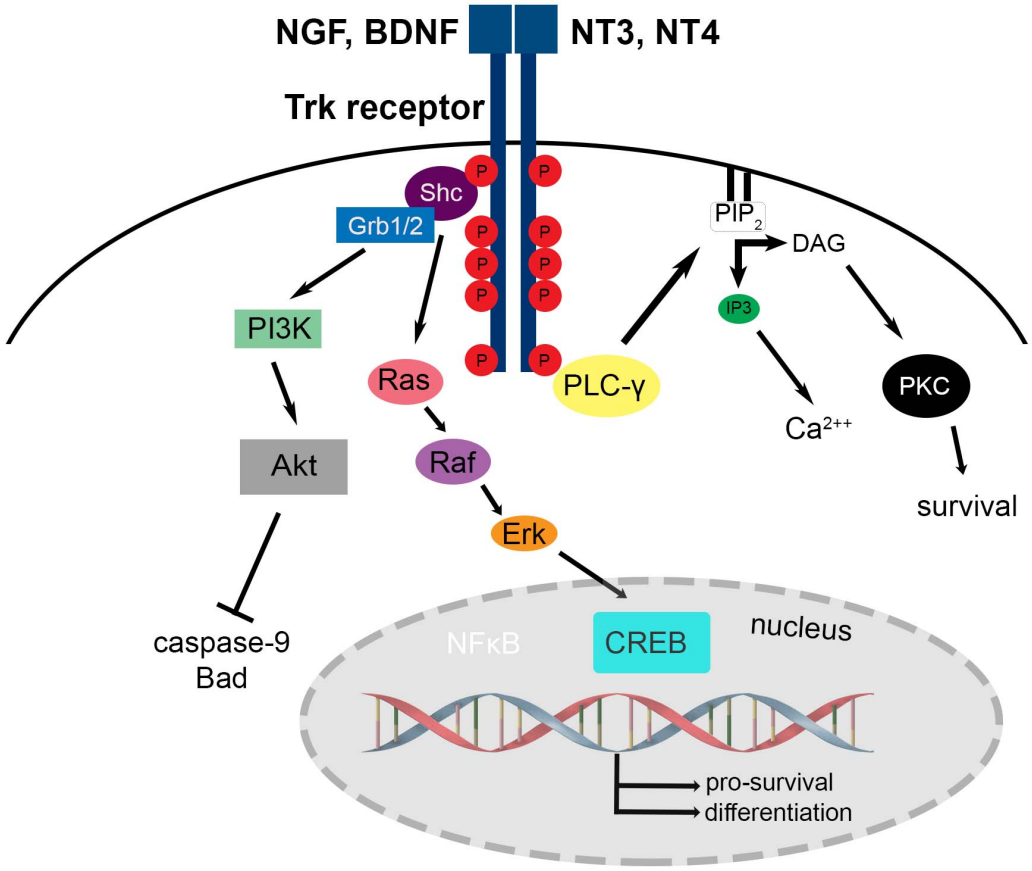

Figure 1.2. Summary of molecular components involved in Trk receptor signaling. Stimulation of Trks by neurotrophins leads to survival signaling through multiple parallel pathways.

Following NGF activation of TrkA, the receptor undergoes internalization and retrogradely trafficked signaling endosome is formed, which, remarkably, contains a vast array of intracellular signaling components (Howe and Mobley, 2005). The continued post-endocytic signaling of NGF results in Rac1/cofilin-dependent breakdown of the local actin network, freeing the endosome to be retrogradely transported (Jaworski, 2007). Endocytosis of Trk receptors and subsequent trafficking from distal axons to the neuronal soma occurs by either canonical clathrin-mediated endocytosis (Howe et al., 2001) or Pincher-mediated micropinocytosis (Shao et al., 2002; Valdez et

al., 2005). Both of these mechanisms require the GTPase dynamin, which is activated through PLCγ (Bodmer et al., 2011; Harrington et al., 2011).

The fate of these early Trk-containing endosomes is complex and may be dependent on the required biological outcome. Studies have localized internalized TrkA with early Rab5 and late Rab7-positive endosomes (Deinhardt et al., 2006). Additionally, several groups have shown that the formation multivesicular bodies are required for retrograde transport (Ye et al., 2018). The translocation of activated Trk receptors from the distal growth cone occurs via dynein, a motor protein that moves along axonal microtubules towards the minus end to the cell soma (Heerssen et al., 2004). In order to recruit dynein to late endocytic vesicles, phosphorylated Trk receptors can recruit adaptor proteins snapin or retrolinkin (Zhou et al., 2012). Additionally, TrkA can directly phosphorylate dynein intermediate chain (DIC) suggesting it can self-regulate its retrograde transport (Mitchell et al., 2012).

Internalization of active TrkA receptors also allows for the signaling to be sustained and amplified, even after exogenous NGF is no longer available. It has been shown that Coronin-1, an endosomal effector protein can extend the TrkA signaling cascade in endosomes by allowing the vesicles to avoid lysosomal fusion and degradation (Scott-Solomon and Kuruvilla, 2018). Arf6 is a member of the ADP-ribosylation factor (ARF) family of proteins, which fall under the Ras superfamily of small GTPases. It has been shown to be involved in membrane trafficking of several receptors. AMPA receptors were reported to be recycled to the cell surface through Arf6 expressing endosomes (Zheng et al., 2015). Additionally, the internalization and recycling of β1 integrin also involves activation of Arf6 (Powelka et al., 2004). Arf6 acts

at several steps in endosome recycling, including clathrin recruitment to endosomes to the recruitment of NM23-H1, a kinase involved in dynamin-dependent vesicle fission (D'Souza-Schorey and Chavrier, 2006).

It was recently shown that TrkA can activate Arf6 to regulate its own recycling (Li et al., 2015). Work presented in this thesis demonstrates that Arf6 is activated by TrkA in sympathetic neurons through a mechanism dependent on PI3K and a member of the cytohesin family of guanine nucleotide exchange factors (GEFs). All 8 of the Arf6 GEFs contain a PH domain, which binds to phosphatidylinositol, and the PH domains of the cytohesins have highest affinity for phosphatidylinositol 3,4,5 triphosphate (PIP3) (Klarlund et al., 1997) a lipid produced by PI3 kinase. Therefore, it is likely that TrkA activation of PI3 kinase induces a local increase in PIP3, which recruits a cytohesin, leading to Arf6 activation. In contrast, Li et al. (2015) reported that TrkA activated Arf6 through a mechanism dependent on Src. However, these mechanisms are not mutually exclusive; for example, the Src family member Fyn was reported to phosphorylate cytohesin-1, resulting in activation of the GEF and, subsequently, Arf6 (Yamauchi et al., 2012). Therefore, TrkA may activate Arf6 through PI3 kinase-dependent recruitment of a cytohesin, followed by stimulating the cytohesin's GEF activity through Src-mediated phosphorylation. Arf6 is therefore crucial to promoting TrkA recycling back to the plasma membrane.

In PC12 cells treated with NGF, TrkA activated Arf6 through its interaction with GGA3 (Li et al., 2015). There are 3 GGA family members (GGA1-3), which are adaptor proteins involved in vesicular trafficking in the trans-Golgi network and in endocytosis (Bonifacino, 2004). NGF treatment activated Arf6 through TrkA and Arf6 recruited

GGA3 to TrkA-containing endocytic vesicles through direct interaction with the cytoplasmic tail of TrkA. Association with GGA3 resulted in reduced TrkA degradation and promoted recycling to the cell surface. Knockdown of GGA3 in PC12 cells resulted in an attenuation of TrkA signaling due to the increased degradation of internalized receptor (Li et al., 2015).

Survival signaling through Trk signaling can be enhanced by the co-expression of p75NTR (Hempstead et al., 1991). However, p75NTR has also been shown to be an important mediator of PCD in sympathetic neurons in order to balance the survival effects of Trk signaling (Bamji et al., 1998).

1.5 p75NTR and the regulation of cell survival and death

All of the neurotrophins, and their pro-forms, can bind to the p75 neurotrophin receptor (p75NTR), which is structurally and functionally quite distinct from Trk receptors. p75NTR is a member of the tumor necrosis family (TNF) of receptors due to similarities in its intracellular domain. Cysteine-rich domains in its extracellular domain interact with neurotrophins (Baldwin et al., 1992), while key conserved residues in the transmembrane aid in receptor dimerization and downstream signaling (Vilar et al., 2009). p75NTR can exist at the plasma membrane as a monomer or dimer, or in complex with other receptors such as the Trks and sortilin, a member of the Vps10p family of sorting receptors.

p75NTR signaling has a wide variety of cellular effects, depending on the specific ligands and co-receptors present and the particular cellular context. p75NTR and TrkA, when co-expressed in cells, can form a high affinity complex capable of binding with high selectivity to NGF, but not NT3 (Hempstead et al., 1991). This interaction promotes

and augments the NGF survival signal through TrkA (Hempstead et al., 1991). P75NTR alone can also mediate survival signaling through TRAF recruitment of upstream NFκB pathway kinases (Khursigara et al., 1999). Downstream activation and nuclear translocation of NFκB subunits p65 and p50 subunits promotes survival signaling (Hayden and Ghosh, 2012).

However, p75NTR has been most investigated due to its ability to function as a cell death receptor. Its ability to stimulate PCD in response to ligand binding has been observed and studied in several biological systems, such as sympathetic, motor, and hippocampal neurons, photoreceptor cells, oligodendrocytes, and Schwann cells. (Casaccia-Bonnefil et al., 1996; Frade et al., 1996; Bamji et al., 1998; Syroid et al., 2000; Linggi et al., 2005; Ceni et al., 2014). Studies in *p75NTR-/-* mice have confirmed its role in developmental apoptosis and eliminating excess neurons in the developing forebrain, trigeminal ganglia, retina, spinal cord, and SCG (Frade et al., 1996; Bamji et al., 1998; Frade and Barde, 1999; Agerman et al., 2000; Naumann et al., 2002). Deletion of p75NTR in these tissues results in a significant increase in neuron number, suggesting it is required for normal developmental cell death.

Because p75NTR can bind all of the neurotrophins and their proforms, the specific ligand activating apoptotic signaling depends on the cellular context. For example, in the developing retina, NGF stimulates p75NTR-mediated apoptosis (Frade and Barde, 1999) since there are no TrkA receptor in the retinal cells. In sympathetic neurons, it has been suggested that BDNF serves as the pro-apoptotic ligand (Bamji et al., 1998; Deppmann et al., 2008), although evidence for endogenous BDNF protein activating the receptor in these neurons is lacking.

Pro-apoptotic ligand binding to endogenous p75NTR activates JNK signaling in sympathetic neurons and direct inhibition of JNK kinase activity is sufficient to prevent the induction of apoptosis (Kenchappa et al., 2010). There are 3 JNK genes in mammals and within this family, only deletion of JNK3 in SCGs prevented p75NTR mediated cell death *in vitro* and *in vivo* (Kenchappa et al., 2010). Like other members of the TNF family of receptors, p75NTR can promote intracellular signaling through direct interaction with a number of cytosolic effector proteins. One family of these proteins, aptly named TNF receptor-associated factors (TRAFs), interacts with the intracellular domain of p75NTR. Most TRAFs contain an E3-ubiqutin ligase domain, but instead of targeting its substrates for degradation, the ubiquitin chains added are linked via lysine 63 and promote protein-protein interaction. While TRAFs 1-6 have all been reported to associate with p75NTR, the role of TRAF6 is best understood. Several groups have shown that ligand-mediated p75NTR activation of both JNK and NFκB require TRAF6 association with the receptor (Khursigara et al., 1999; Yeiser et al., 2004). *Traf6-/-* mice fail to activate JNK in response to ligand binding as well as fail to undergo normal developmental cell death (Yeiser et al., 2004).

The activation of JNK by p75NTR has also been reported to require the Neurotrophin receptor interacting factor (NRIF), a zinc finger protein that associates with the intracellular domain of p75NTR (Casademunt et al., 1999). NRIF was discovered to be necessary for the receptor's apoptotic signal (Casademunt et al., 1999) and subsequently shown to be required for BDNF-induced JNK activation (Linggi et al., 2005). Another interacting partner of p75NTR linked to JNK activation in sympathetic neurons is neurotrophin receptor-interacting MAGE homolog (NRAGE) (Salehi et al.,

2000). It is unclear exactly how NRAGE contributes to downstream JNK activation, but overexpression is sufficient to activate JNK and begin the intrinsic apoptotic death pathway in PC12 cells. It likely acts as an adaptor linking TRAF6, NRIF, and p75NTR in some way that allows them to promote apoptotic signaling. Notably, *nrage*$^{-/-}$ neurons are resistant to p75NTR-mediated apoptosis (Salehi et al., 2000; Bertrand et al., 2008).

It has also been suggested that p75NTR can regulate JNK through the production of ceramide, a lipid signaling molecule (Casaccia-Bonnefil et al., 1996). Dobrowsky and colleagues first reported p75NTR activation of this lipid signal in glioma cells (Dobrowsky et al., 1994). They found that p75NTR activation increased intracellular ceramide levels, which resulted in a decrease in cell growth, while direct inhibition of sphingomyelinase in these cells prevented ceramide accumulation and blocked the receptor-mediated inhibition of growth. Chao et al., reported that p75NTR stimulated an increase in ceramide production in oligodendrocytes and this correlated with JNK activation, resulting in apoptosis (Casaccia-Bonnefil et al., 1996). Subsequently, multiple groups have reported p75NTR's ability to stimulate ceramide production in several cell types, including neurons (Culmsee et al., 2002) and Schwann cells (Hirata et al., 2001) through activation of sphingomyelinase.

The proteolysis of p75NTR represents an additional mechanism for signaling to downstream cellular targets. The ECD of p75NTR is first cleaved by the metalloproteinase TNFα-converting enzyme (TACE, also known as ADAM17), releasing an approximately 30 kDa membrane-bound fragment (CTF) (Weskamp et al., 2004). The remaining soluble ectodomain is released into the extracellular space and the CTF is further cleaved proximal to the transmembrane domain by γ-secretase complex,

releasing a soluble ~25 kDa intracellular domain into the cytosol (Jung et al., 2003; Kanning et al., 2003) .

The cleavage of p75NTR has been shown to be necessary for neuronal apoptosis in sympathetic (Kenchappa et al., 2006), and hippocampal neurons (Volosin et al., 2008). The release of p75NTR's ICD enables direct signaling to the nucleus by enabling nuclear translocation of the intracellular binding partner NRIF. Although NRIF has been reported to contribute to the activation of JNK (Linggi et al., 2005; Kenchappa et al., 2010), it is a C2H2-type zinc finger protein that can regulate gene transcription (Korade et al., 2009). Deletion of NRIF (Linggi et al., 2005; Volosin et al., 2008) or blocking its nuclear translocation (Geetha et al., 2005; Kenchappa et al., 2006) prevented p75NTR apoptotic signaling. However, it should be noted that the role of p75NTR proteolysis in signaling appears to depend on the cellular context and is not always required for the induction of cell death (Vicario et al., 2015).

p75NTR can also complex with other receptors to induce apoptosis in response to unprocessed neurotrophins. Pro-neurotrophins can induce PCD by forming a high affinity protein complex with p75NTR and co-receptor Sortilin. Sortilin, and other members of Vps10p family have multifunctional biological roles in protein sorting and trafficking. Proneurotrophins bind to Sortilin via their pro-domain while interacting with p75NTR with the mature domain(Nykjaer et al., 2004). This complex stimulates cell death in cultured sympathetic neurons *in vitro* (Nykjaer et al., 2004; Teng et al., 2005), and mice with a genetic deletion of Sortilin exhibit a reduction in normally occurring, developmental sympathetic neuron apoptosis (Jansen et al., 2007).

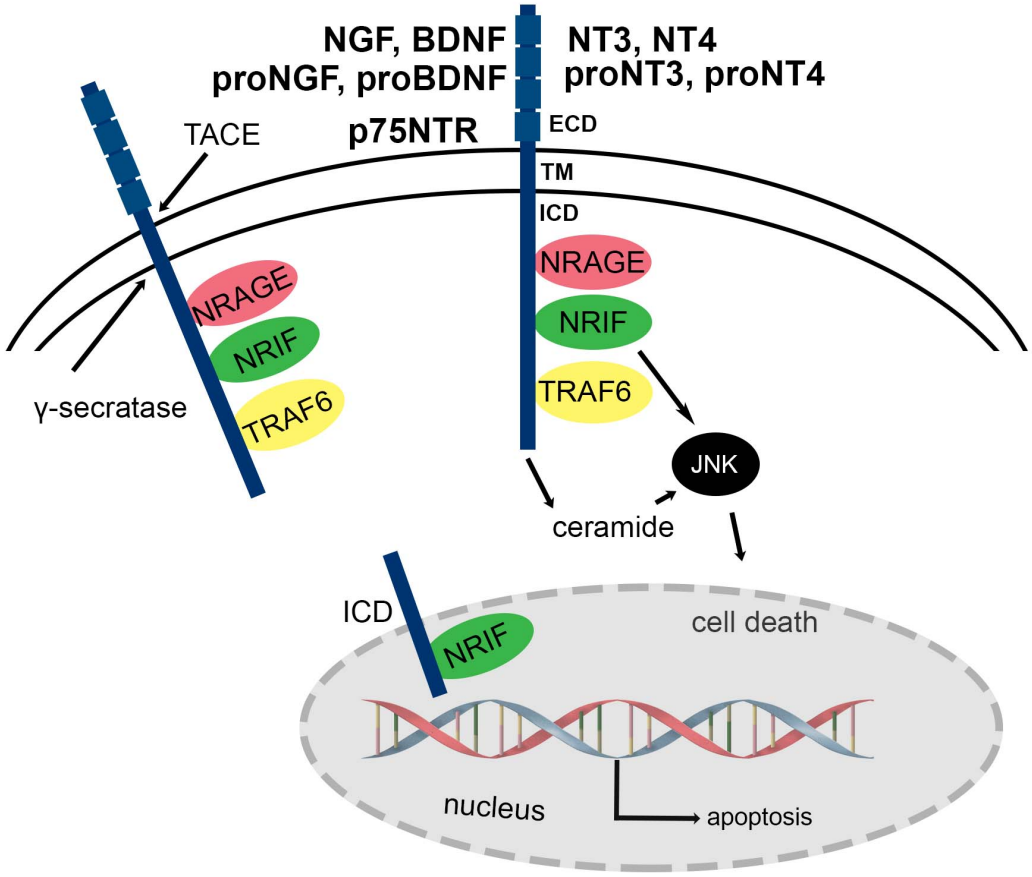

Figure 1.3. Summary of molecular components involved in p75NTR receptor signaling. Stimulation of p75NTR by neurotrophins or pro-neurotrophins leads to cell death signaling. Additionally, cleavage of the ICD can induce apoptotic through transcriptional regulation.

1.6 Anterograde receptor trafficking

While the previous section focused on ligand binding to TrkA and subsequent receptor activation and retrograde signaling, it is also important to understand how neurons regulation the initial insertion of Trk receptors and p75NTR into the plasma membrane. Like most receptors destined for the plasma membrane, Trk receptors are synthesized and delivered to the surface in Golgi-derived vesicles. After synthesis and maturation in ER-Golgi, mature receptors are shuttled directly to the distal axons

through canonical secretory machinery. Trks associate with Sortilin (which also interacts with p75NTR at the surface) and that interaction appears to be required for proper sorting of Trk-containing vesicles from the Golgi (Vaegter et al., 2011). *Sortillin-/-* studies show it is required for proper anterograde transport of the Trk receptors, however its precise role remains unclear. One possibility is that Sortilin can act as a scaffold to link Trk receptor-containing vesicles with anterograde kinesin motors.

In sensory neurons, TrkA is transported in Rab3+ secretory vesicles by plus-end directed motor protein, KIF1A (Tanaka et al., 2016). In other Trk-expressing cell types, several proteins have been studied that may also act as adaptors for the formation of the proper anterograde motor complex. In hippocampal TrkB-positive neurons, a complex consisting of the Rab27B GTPase, Slp1, and a microtubule-binding adapter protein, CRMP-2, links the receptor to Kinesin-1 vesicles for anterograde transport (Arimura et al., 2009). Additionally, members of the JIP family of adaptors appear to be required for proper TrkB trafficking (Scott-Solomon and Kuruvilla, 2018).

1.7 Project goals

Many of the molecular players in Trk receptor trafficking, internalization, recycling, and retrograde transport are well known. In contrast, virtually nothing is known about p75NTR anterograde transport to the cell surface. We are beginning to understand how p75NTR is internalized, but little is known about how it signals back to the cell soma. The main goals of the following studies are aimed at more fully elucidating how p75NTR trafficking to and from the membrane is regulated. Chapter 2 will focus on how p75NTR trafficking enables sympathetic neurons to switch from NT3 responsiveness to target-derived NGF responsiveness. Chapter 3 will investigate the

formation and molecular regulation of the p75NTR retrograde apoptotic signaling complex after NGF withdrawal or ligand binding to the receptor. Specifically, I sought to:

- Determine if exposure to neurotrophins affects the surface expression of p75NTR in neurons
- Define the intracellular signal(s) that is/are required for shuttling p75NTR to the surface
- Determine if p75NTR protein, not yet at the cell surface, is stored intracellularly or directly trafficked to the plasma membrane following synthesis
- Determine how the expression of p75NTR at the neuronal surface influences neurotrophin responsiveness
- Elucidate how p75NTR gets retrogradely trafficked in response to ligand or non-ligand mediated cell death

2. Neurotrophin responsiveness of sympathetic neurons is regulated by rapid mobilization of the p75 receptor to the cell surface through TrkA activation of Arf6

This chapter contains the data from my first author manuscript that has been accepted at the Journal of Neuroscience:

Hickman, FE; Stanley, E; Carter, BD. (2018) Neurotrophin responsiveness of sympathetic neurons is regulated by rapid mobilization of the p75 receptor to the cell surface through TrkA activation of Arf6. *Journal of Neuroscience*

2.1 Abstract

The p75 neurotrophin receptor (p75NTR) plays an integral role in patterning the sympathetic nervous system during development. Initially, p75NTR is expressed at low levels as sympathetic axons project toward their targets, which enables neurotrophin-3 (NT3) to activate TrkA receptors and promote growth. Upon reaching nerve growth factor (NGF) producing tissues, p75NTR is up regulated resulting in formation of TrkA-p75 complexes, which are high affinity binding sites selective for NGF, thereby blunting NT3 signaling. The level of p75NTR expressed on the neuron surface is instrumental in regulating trophic factor response; however, the mechanisms by which p75NTR expression is regulated are poorly understood. Here, we demonstrate a rapid, translation independent increase in surface expression of p75NTR in response to NGF in rat sympathetic neurons. p75NTR was mobilized to the neuron surface from GGA3-postitive vesicles through activation of the GTPase Arf6, which was stimulated by NGF, but not NT3 binding to TrkA. Arf6 activation required PI3 kinase activity and

was prevented by an inhibitor of the cytohesin family of Arf6 GEFs. Overexpression of a constitutively active Arf6 mutant (Q67L) was sufficient to significantly increase surface expression of p75NTR even in the absence of NGF. Functionally, expression of active Arf6 markedly attenuated the ability of NT3 to promote neuronal survival and neurite outgrowth while the NGF response was unaltered. These data suggest that NGF activation of Arf6 through TrkA is critical for the increase in p75NTR surface expression that enables the switch in neurotrophin responsiveness during development in the sympathetic nervous system.

2.2 Introduction

Neurotrophin regulation of the sympathetic nervous system is intricately controlled during embryonic development to maximize the limited supply of trophic support available during neuronal innervation of target tissues. These neurons depend on Nerve growth factor (NGF) binding to its tyrosine kinase receptor, TrkA, for their survival. However, prior to reaching NGF-producing targets, sympathetic axon growth is promoted by neurotrophin-3 (NT3) produced in adjacent blood vessels. Although TrkC is the preferred receptor for NT3, it is not expressed by sympathetic neurons; instead, NT3 signals through TrkA receptors, which can bind NT3 with low affinity (Birren et al., 1993; ElShamy and Ernfors, 1996; Belliveau et al., 1997a; Kuruvilla et al., 2004). Once axons reach NGF-rich regions, TrkA receptors lose responsiveness to NT3 in favor of NGF to promote sympathetic neuron survival and innervation of the target (Benedetti et al., 1993; Brennan et al., 1999; Kuruvilla et al., 2004). This shift in responsiveness has been attributed to an increase in the expression of the neurotrophin receptor p75 (p75NTR), which in turn, forms a complex with TrkA. This p75NTR-TrkA complex functions as a high affinity receptor that is very selective for NGF over NT3 (Hempstead et al., 1991). While it is known that TrkA activation can induce p75NTR transcription, the mechanisms regulating p75NTR trafficking to the cell surface have not been investigated.

The p75NTR is a pleiotropic signaling receptor, capable of promoting survival and growth or apoptosis and degeneration, depending on the expression of its co-receptors and ligands (Kraemer et al., 2014). While p75NTR-TrkA complexes elicit pro-survival and axonal growth signaling selectively in response to

NGF, neurotrophin binding to p75NTR alone or binding of pro-neurotrophins to a complex of p75NTR and members of the Vps10p-domain family of receptors can initiate cell death and axonal degeneration (Nykjaer et al., 2004; Teng et al., 2005). Hence, precise developmental timing of p75NTR expression on the neuronal surface is critical for determining neurotrophin selectivity as well as modulating survival versus apoptotic signaling.

In considering the mechanisms by which target-derived NGF could increase p75NTR trafficking to the plasma membrane, we speculated that the GTP binding protein Arf6 may be involved. Arf6 is one of 6 related Arf GTPases (designated Arf1-6). However, unlike Arf1-5, which are primarily localized to the Golgi and endoplasmic reticulum and regulate vesicular trafficking between these two organelles (Wieland and Hartert, 1999; Volpicelli-Daley et al., 2005), Arf6 is localized to the plasma membrane and endosomal compartments where it plays an integral role in plasma membrane endo- and exocytosis, endocytic recycling, and actin cytoskeleton rearrangement (D'Souza-Schorey and Chavrier, 2006; Hongu and Kanaho, 2014). Moreover, Arf6 was recently reported to promote TrkA recycling following NGF- mediated endocytosis through recruiting the adapter protein, Golgi-localized, λ adaptin-ear-containing ADP ribosylation protein 3 (GGA3) (Li et al., 2015).

The activity of Arf proteins is regulated, in part, by guanine nucleotide exchange factors (GEFs). There are 8 GEFs that can promote Arf6 activation, including cytohesin-1, which was shown to translocate to the plasma membrane of PC12 cells in response to NGF activation of TrkA and the formation of phosphatidylinositol 3,4,5-trisphosphate (PIP3) (Venkateswarlu et al., 1999). Since

Arf6 localizes to the plasma membrane, as well as endosomes, enrichment of cytohesin-1 at the cell surface would be predicted to locally activate Arf6, although this was not investigated. Interestingly, activation of Arf6 by another tyrosine kinase receptor, c-Met, was shown to promote recycling of the β1-integrin receptor and increase its expression at the plasma membrane (Hongu et al., 2015). These results suggest that TrkA activation may similarly increase the expression of p75NTR on the surface of sympathetic neurons through regulation of Arf6.

In this report, we provide evidence for a rapidly occurring NGF-induced increase in p75NTR surface expression in sympathetic neurons, independent of new protein translation, caused by TrkA activation of the GTPase Arf6. Furthermore, we show that Arf6 regulation of p75NTR levels at the plasma membrane alters trophic factor responsiveness of sympathetic neurons.

2.3 Methods

Primary Cell Culture – All experiments conducted with tissues derived from animals were approved by the Animal Care and Use Committee at Vanderbilt University. Neurons from the superior cervical ganglia (SCG) were dissected from postnatal day 2-3 Sprague-Dawley or CD1 rats (male and female; Charles River, Wilmington, MA) and dissociated with 0.08% trypsin (Worthington) and 0.3% collagenase (Sigma-Aldrich, St. Louis, MO). For p75NTR-/- studies, C57BL/6 mice or p75NTR-/- (Jackson Laboratories, Bar Harbor, ME) SCGs were dissociated with 0.3% collagenase with 0.1% DNase for 30 minutes, with .0.08% trypsin added for the final 10 minutes. Neurons in single cell suspension were then plated at ~5000 neurons/0.7mm^2 on 4-well chamber slides, 8-well chamber slides (Thermo Fisher Scientific, Waltham, MA), 1 mm glass coverslips, or cell culture dishes coated with

poly-D-lysine (overnight, MP Biomedicals, Santa Ana, CA) and laminin (~2 hrs, Invitrogen, Waltham, MA). Neurons were cultured in Ultraculture media (Lonza, Houston, TX) with 3% fetal bovine serum (Denville Scientific Inc., South Plainfield, NJ), 2 mM L-Glutamine (Invitrogen), 100 units/ml penicillin, and 100 μg/ml streptomycin (Invitrogen) at 37°C in 5% CO_2. Depending on the experiment, one of the following trophic factors or supplements was added to the media to promote neuron survival; 40 ng/ml nerve growth factor (NGF, Harlan Laboratories, Houston, TX), 100 ng/ml neurotrophin-3 (NT3, kindly provided by Regeneron, Tarrytown, NY) or 12.5 mM KCl, unless otherwise specified. To prevent the proliferation of non-neuronal cells in culture, 18 hours after initial plating cells were treated with 10 μM cytosine arabinoside (AraC; Sigma-Aldrich) for 24-36 hours. NGF was removed from neurons 18 hours prior to GTP-activation assays by replacing NGF containing media with media supplemented with 12.5 mM KCl and anti-NGF antibody (0.1 μg/ml; Chemicon, Billerica, MA).

In some experiments, neurons in suspension were electroporated prior to plating using a rat neuron Nucleofactor kit (cat. No. VGP-1003, Amaxa Biosystems, Walkersville, MD) and program G-013. Neurons were electroporated with GFP alone, or GFP and a constitutively active Arf6 (Q67L; Addgene, Cambridge, MA (Furman et al., 2002)), or the Arf6 GAP ACAP1 (Addgene, (Jackson et al., 2000)). Only neurons expressing GFP were used for subsequent analysis. Mouse neurons pooled from postnatal day 4-6 pups were electroporated as above and immediately divided into NGF or NT3 treatment media before plating.

Immunoprecipitation & Western blot analysis – For Arf6 assays, following NGF withdrawal, neurons were treated with NGF (40 ng/ml), NT3 (100 ng/ml), or BDNF (200 ng/ml; generously provided by Regeneron) for indicated times, lysed and subjected to an Arf6 activation assay (Cell Biolabs, San Diego, CA; STA-407-6) per the manufacturer's instructions. Neurons were immunoprecipitated with the provided GGA3 beads, recognizing the GTP-bound Arf6, and Arf6 was detected using a combination of monoclonal (Cell Biolabs, Inc.) and polyclonal (Abcam, Cambridge, UK) antibodies.

For biotinylation experiments, neurons cultured continuously in 12.5 mM KCl were treated for 2 hours with NGF (40 ng/ml), or NGF and the Arf6 GEF inhibitor SecinH3 (50 μM; Abcam) at 37°C, followed by biotinylation of surface proteins using EZ-Link Sulfo-NHS-LC Biotin (Pierce; Rockford, IL) at 4°C. Biotinylated proteins were immunoprecipitated with avidin agarose beads (Pierce), separated by SDS-PAGE, transferred to nitrocellulose and p75NTR was detected with a polyclonal antibody [generated as described previously (Kenchappa et al., 2010)]. The signal was visualized using anti-rabbit peroxidase-conjugated secondary antibody, enhanced chemiluminescence and a BioRad ChemiDOC MP imaging system. The band intensity was quantified using Fiji Software (Schindelin et al., 2012), normalized to total protein in the lysate fraction, and expressed as a percent control of untreated neurons.

Surface expression assays - To determine the mechanisms of NGF-induced p75NTR trafficking, neurons cultured continuously in 12.5 mM KCl were treated with NGF (40 ng/ml), or NGF in combination with one of the following inhibitors:

cycloheximide (CHX, 100 ng/µl; Sigma), K252a (200 nM; Millipore), LY294002 (50 µM; Sigma), or SecinH3 (50 µM; Abcam) for the indicated times. At the conclusion of the time course, live, unpermeabilized cells were placed on ice and stained with an antibody for the extracellular domain of p75NTR (MC192; 1:50; Santa Cruz), sortilin (1:100; graciously supplied by Anders Nykjaer, Aarhus Univeristy; Aarhus, Denmark) or TrkA (sc118; 1:100; Santa Cruz, Dallas, TX) and cholera toxin B conjugated to Alexa 647 (CTB; 1:1000; Invitrogen) in PBS for 90 minutes. Following live staining, neurons were fixed with 4% paraformaldehyde (RT; 25 min). Some neurons were stained with anti-Tuj1 (1:1000; Covance, Princeton, NJ) or anti-neurofilament (1:1000; Millipore), and primary antibodies were visualized with appropriate Alexa Fluor conjugated secondary antibodies (1:1000; Invitrogen). Tuj1 or neurofilament positive cells and GFP electroporated neurons were imaged in a single optical slice using a 63x oil immersion objective on a Leica TCS SP5 confocal microscope equipped with LAS AF software (Leica Microsystems, Inc., Wetzlar, Germany), and surface intensity of individual neurons was determined using Fiji Software, where a standard threshold was applied to each image prior to intensity assessment. The images were analyzed in a blinded manner. Approximately 25-30 neurons per condition were sampled for each experiment. Average surface intensity per condition was normalized and expressed as percent control.

Immunocolocalization analysis –For p75NTR-GGA3 colocalization analysis, rat SCG primary neurons were electroporated with a pEGFP-N1 construct containing GGA3 fused to EGFP (5 ug, generously provided by Dr. Guangyu Wu) and initially

cultured for 18 hours in 12.5 mM KCl prior to the addition of NGF for 2 hours. Fixed, permeabilized neurons were stained for p75NTR (1:50, MC192) and anti-GFP (1:1000, Abcam) and counterstained with neurofilament (NF, 1:1000, Milipore) to mark neurons. Approximately 1 micron sections of GFP+, NF+ neurons were imaged in 0.16 um slices on a Zeiss LSM 880 confocal with Airyscan. Post-processed images were analyzed using the Coloc2 plugin in Fiji and Pearson's R coefficients were computed between GGA3 and p75NTR channels. For further intracellular compartment colocalization analysis, un-electroporated rat SCG neurons were initially cultured in 12.5 mM KCl prior to the addition of NGF for 2 hours. Fixed, permeabilized neurons were stained for p75NTR (1:50, MC192) and Rab11 (1:250, VU57 antibody generously provided by Dr. Jim Goldenring). Colocalization analysis was completed as described above.

Survival assays – Neurons were initially cultured for 2 days in 12.5 mM KCl prior to addition of increasing concentrations of NT3 (2.5-100 ng/ml) or NGF (0.2-40 ng/ml), or removal of KCl entirely (starved condition) for 48 hours. Fixed, permeabilized neurons were stained with anti-Tuj1 and visualized with appropriate Alexa Fluor conjugated secondary antibodies. Slides were imaged using a Zeiss Axioskope 2 fluorescent microscope equipped with an AxioCam MRc5 camera and AxioVision software (Carl Zeiss Microscopy, Oberkochen, Germany), and 10 randomized fields were captured per condition per experiment. Neurons were classified as non-apoptotic or apoptotic by blinded assessment of DAPI nuclear staining, as previously described (Kenchappa et al., 2010).

Neurite outgrowth analysis– Rat sympathetic neurons were electroporated with GFP (1 ug) alone, or GFP and a constitutively active Arf6 (Q67L) (5 ug) and immediately plated into media containing either 40 ng/ml NGF or 100 ng/ml NT3. Neurons were fixed after 16 hours and counterstained for with anti-GFP (1:1000, Abcam) and neurofilament (1:1000, Millipore) to visualize electroporated cells and neurites, respectively. Electroporated neurons were imaged using Nikon Eclipse Ti inverted fluorescent microscope. The longest neurite for each GFP+ neuron was measured in a blinded manner using Fiji software. Mouse wildtype or p75NTR-/- neurons were electroporated with GFP and Arf (Q67L) and immediately plated into media containing either 40 ng/ml NGF or 100 ng/ml NT3. Neurons were fixed after 48 hours and counterstained for with anti-GFP (1:1000, Abcam) and neurofilament (1:1000, Millipore) and analyzed as above.

Statistical Analysis – Data are presented as mean ± standard error of the mean (SEM) and expressed as a percentage of the control mean for each experiment. Means were compiled for each condition and outliers detected using a Grubbs' test were replaced with the average of the means. These data were analyzed using a one-way analysis of variance (ANOVA) with a planned independent samples *t* test post hoc on the means contributing to the significance of the variance. For experiments with only 2 data sets, the results were analyzed by an unpaired student's t-test. Two sample sets were considered significantly different when $p < 0.05$. All statistical analysis was performed and graphed in GraphPad Prism (V 6.0, GraphPad Software, Inc., La Jolla, CA).

2.4 Results

p75NTR surface expression is rapidly up regulated by NGF –Previous studies have shown that sympathetic neurons and neuroblasts treated with NGF increase transcription of p75NTR (Miller et al., 1994; Verdi and Anderson, 1994; Wyatt and Davies, 1995). However, given the time required for transcription, translation and trafficking of p75NTR to the distal axon, and the very rapid pace of early mammalian development, we considered the possibility that NGF could more acutely induce surface p75NTR expression. Here, we introduce a novel, rapid up regulation of surface expressed p75NTR induced by NGF starting as early as 30 minutes and persisting for up to 2 hours, resulting in nearly a two-fold increase over baseline expression [Figure 2.1A-B, $F(3,94) = 10.17$, $p < 0.0001$]. Since sympathetic neurons are dependent on NGF for survival, we initially cultured the neurons in KCl (12.5 mM) to promote neuronal survival in the absence of trophic factor support. Surface expression of p75NTR was imaged in live neurons using an antibody directed to the extracellular domain of p75NTR (red), and Alexa 647 conjugated to cholera toxin B (CTB, blue) to define the cell membrane. Importantly, the NGF-induced increase in surface p75NTR was not blocked by co-administration of the translational inhibitor cycloheximide (CHX), while long-term application (24 hrs) of NGF and CHX prevented the up regulation of p75NTR at the plasma membrane [Figure 2.1A,C, $F(4,25) = 4.338$, $p = 0.0084$]. These results suggest that the rapid increase in surface-expressed p75NTR is sourced from existing intracellular p75NTR, evident in permeabilized neurons immunolabeled with p75NTR (Figure 2.1A'). Translocation of p75NTR from intracellular storage pools to the cell surface was specific for NGF, as NT3 elicited no change in

p75NTR surface expression [Figure 2.1D, $F(2,9) = 4.79$, $P = 0.038$]. Assessment of total p75NTR in permeabilized neurons and sympathetic neuron lysates indicated neither neurotrophin altered total p75NTR expression following an acute, 2 hour treatment (Figure 2.1E-F).

We also investigated the effects of BDNF, which only binds to p75NTR in sympathetic neurons, to determine whether p75NTR up regulation could be a positive feedback system from this receptor. However, acute administration of BDNF did not significantly alter the surface expression of p75NTR within 2 hours of treatment [KCl = 100 ± 12.5 vs. BDNF = 110 ± 20.5, $p=0.96$].

Since the rapid recruitment of p75NTR to the cell surface would be expected to occur first at the nerve terminals, as the sympathetic axons reach NGF-producing target tissues, we tested whether the p75NTR up regulation could also be detected in growth cones. After 2 hours of NGF treatment, there was approximately a 2-fold increase in surface expression of p75NTR, specifically on growth cones; this was unaffected by co-administration of CHX with NGF [Figure 2.1G,H., $F(2, 150) = 1.47$, $p=0.03$] and in agreement to what we detected in the neuron cell bodies.

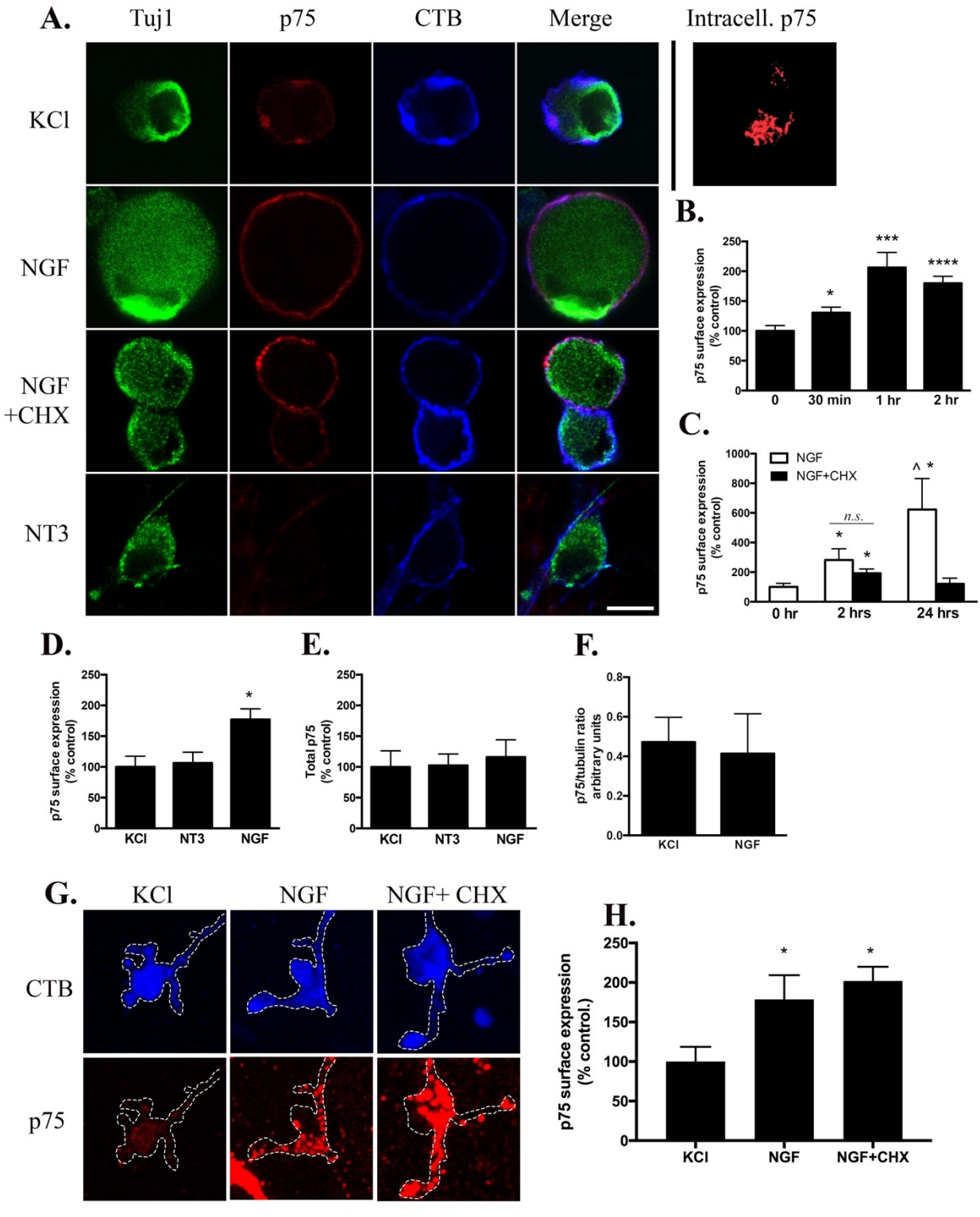
A.
Tuj1
p75
CTB
Merge
Intracell. p75
KCl
NGF
NGF +CHX
NT3
B.
p75 surface expression (% control)
0
30 min
1 hr
2 hr
C.
NGF
NGF+CHX
n.s.
0 hr
2 hrs
24 hrs
D.
p75 surface expression (% control)
KCl
NT3
NGF
E.
Total p75 (% control)
F.
p75/tubulin ratio arbitrary units
G.
KCl
NGF
NGF+ CHX
CTB
p75
H.
p75 surface expression (% control.)
KCl
NGF
NGF+CHX

Figure 2.1. NGF rapidly increases p75NTR surface expression in sympathetic neurons. The surface expression of p75NTR in sympathetic neurons was quantified by immunolabeling live neurons with an antibody to the extracellular domain of the receptor and fluorescently conjugated cholera toxin B (CTB) to define the cell surface, followed by fixation and immunostaining for the neuron specific marker TuJ1 or neurofilament. (**A)** Representative images of p75NTR expression in neurons cultured in KCl (12.5 mM) (white circled neuron, top row), treated for 2 hrs with NGF (40ng/ml) alone or together with cycloheximide (CHX, 100 ng/ml) or treated with NT-3 (100ng/ml). (**A')** Immunostaining for p75NTR in permeabilized neurons cultured in KCl, revealing the presence of intracellular p75NTR. (**B, C)**. The level of p75NTR fluorescence intensity following treatment with NGF for the indicated time was determined by confocal imaging and quantified using Fiji software. (**D)** The expression of p75NTR at the cell surface in response to 2 hrs exposure to NGF was compared to treatment with NT3 (100 ng/ml) by quantification of fluorescence intensity. (**E**) The total level of p75NTR expression was not altered after 2 hrs treatment with NGF or NT3, as quantified after permeabilizing the neurons and immunolabeling the receptor. (**F**) Western blot analysis of total neuronal p75NTR expression with or without 2 hr NGF treatment. The signal was quantified and normalized to tubulin. (n=3; * $p \leq 0.05$, *** $p \leq 0.001$ compared to 0 NGF; ^ $p \leq 0.05$ compared to 24 hours NGF; scale bar = 10 μm). **(G)** Representative images of sympathetic neuron growth cones pre-treated for 1 hr with 10 μM CHX followed by KCl, NGF, or NGF + CHX for 2 hrs before staining for p75NTR in unpermeabilized conditions. Cholera toxin B (CTB) was used to show the growth cone membrane. **(H)** Quantification of p75NTR surface intensity in pixels per area of growth cone (n=3; * $p < 0.05$).

Interestingly, the induction of p75NTR surface expression by NGF appeared specific for neurons, as satellite glial cells (which do not express TrkA receptors) maintained high levels of surface p75NTR even in the absence of neurotrophic support (Figure 2.2A). To determine whether the effects of NGF on receptor trafficking were specific to p75NTR, we assessed TrkA (Figure 2.2B) and sortilin

(Figure 2.2C) surface expression within the first 2 hours of treatment but observed no significant alterations. Cycloheximide also did not have a significant effect on the expression of these receptors within this time frame. These data indicate that NGF induces a rapid, translation-independent increase in surface expression specifically of the p75NTR in sympathetic neurons.

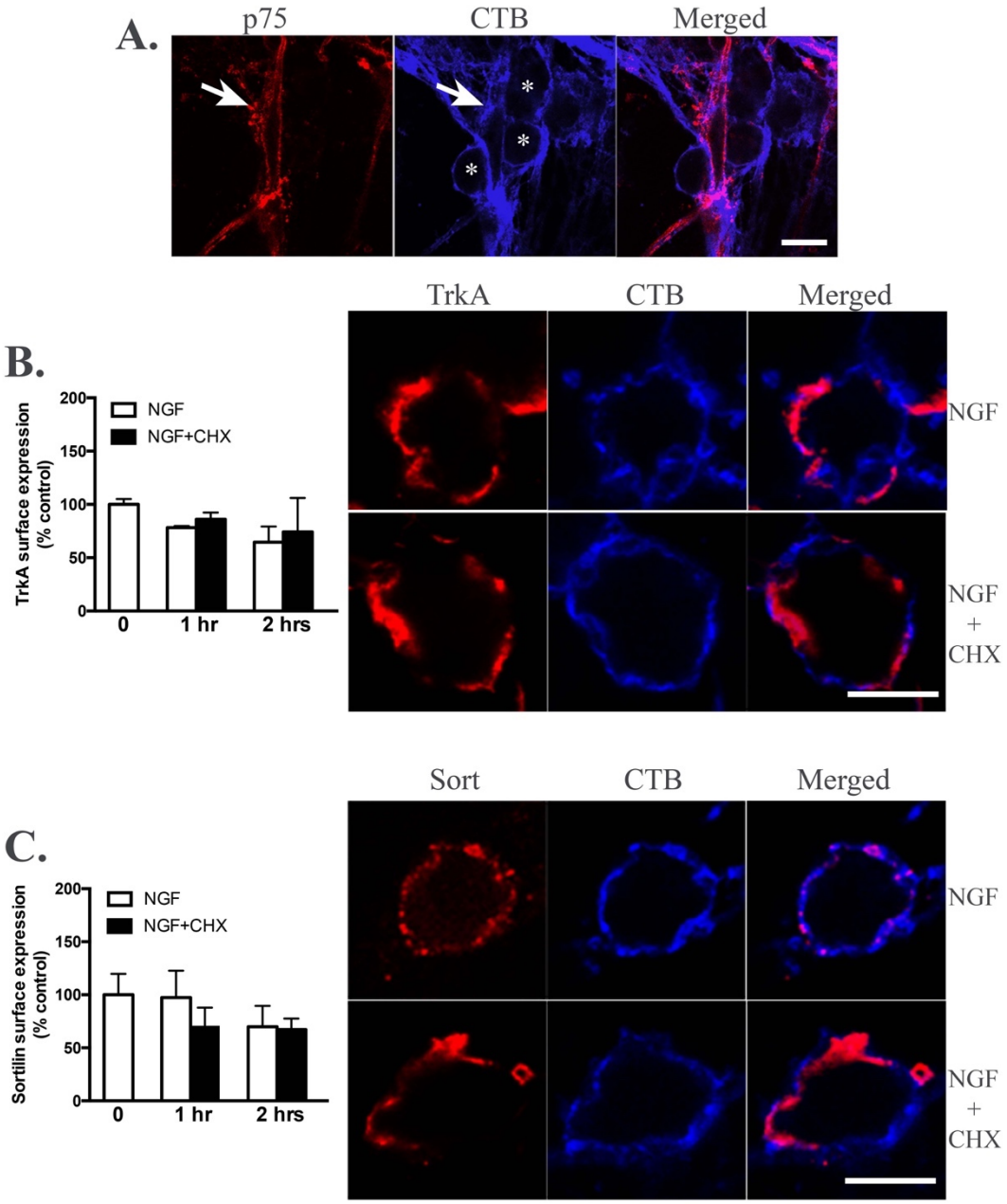

Figure 2.2. Rapid regulation of neurotrophin receptor expression by NGF is specific for neuronal p75NTR. **(A)** Expression of p75NTR on the surface of satellite glial cells present in cultures of sympathetic neurons grown in KCl (12.5 mM) was detected by immunolabeling p75NTR in live cells with an antibody to the extracellular domain of the receptor. The cell surface was defined using fluorescently labeled cholera toxin (CTB). The glial cells maintain robust p75NTR surface expression (white arrow) while sympathetic neurons in the same culture exhibit very low expression of p75NTR on the cell surface (white asterisks). The surface level of TrkA (**B**) or sortilin (**C**) following treatment with NGF (40 ng/ml) with or without CHX (100 ng/ml) for 0, 1 or 2 hrs was determined by immunostaining live neurons with an antibody to the extracellular domain of each receptor. The level of receptor at the cell surface was determined by confocal imaging and fluorescence intensity quantified using Fiji software (representative images are shown on the right). There was no significant change in the surface expression of TrkA or sortilin following NGF treatment, with or without CHX (n= 2-3, scale bars = 10 μm).

NGF increases p75NTR surface expression by activation of Arf6 – A growing body of literature has pointed to the GTPase Arf6 as a pivotal regulator of vesicular trafficking and receptor recycling (D'Souza-Schorey and Chavrier, 2006), including the neurotrophin receptor TrkA (Li et al., 2015); therefore, we considered the possibility that NGF may induce p75NTR shuttling to the plasma membrane through activation of Arf6. To determine if NGF stimulates Arf6 in sympathetic neurons, we applied NGF acutely (0-30 minutes) and assessed activation by pull-down of the GTP-bound form of Arf6 using GGA3-coupled beads (Figure 3A). Peak activation of the GTPase occurred after 15 minutes of NGF treatment and began to abate after 30 minutes [$F(3,14) = 5.272$, $P = 0.012$]. Similar kinetics of

Arf6 activation were recently reported for NGF in PC12 cells (Li et al., 2015). Importantly, the activation detected in the sympathetic neurons was selective for NGF, as 100 ng/ml NT3 or BDNF were insufficient to elicit GTP-binding of Arf6 [Figure 2.3B, $F(3,10) = 4.684$, $P = 0.027$]. Since BDNF only binds to p75NTR, and not p75NTR in complex with TrkA, in sympathetic neurons, this result indicates that the receptor does not activate Arf6 to regulate its own expression. NT3 is able to bind TrkA and can promote survival of the neurons at this concentration ((Belliveau et al., 1997a); Figure 2.6); however, it was unable to activate Arf6, which correlates with its inability to increase p75NTR at the cell surface (Figure 2.1D).

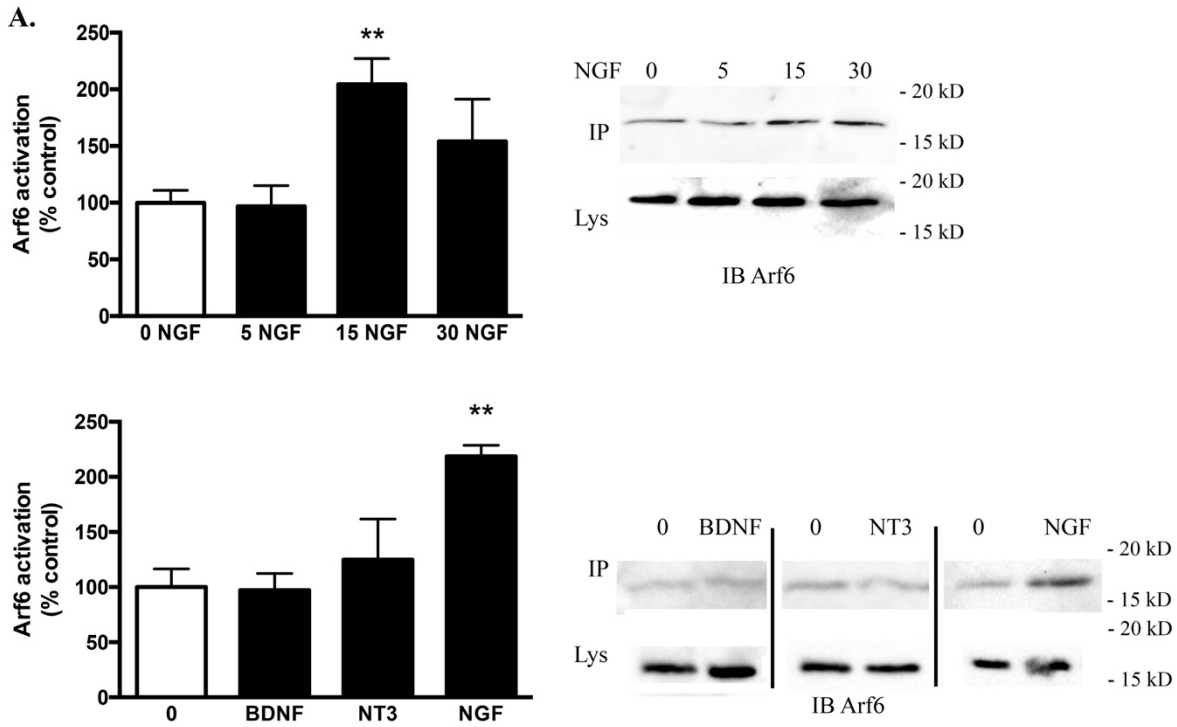

Figure 2.3. NGF induces activation of Arf6. **(A)** NGF was withdrawn from sympathetic neurons and replaced with KCl for ~18 hours, then neurons were exposed to NGF (40 ng/ml) for the indicated time, lysed and the activated Arf6 determined by pull down of the GTP bound form with GGA3-couple beads and western blot for Arf6 (IP). The lysates were also blotted for total Arf6 (Lys) and

the ratio of the active to total used to quantify the fraction of active Arf6, which was expressed as a percent of neurotrophin naïve control. NGF induced a significant increase in GTP-bound Arf6 after 15 minutes that began to abate after 30 minutes. (**B)** The activation of Arf6 in the neurons was specific for NGF, as 15 min treatment with BDNF (100 ng/ml) or NT3 (100 ng/ml) did not alter the activity of Arf6 (n = 3-5; ** $p \leq 0.01$ compared to 0).

To directly evaluate the ability of Arf6 to regulate p75NTR surface expression, sympathetic neurons were electroporated with the constitutively active (CA) Arf6 mutant, Q67L and p75NTR surface expression was assessed using confocal microscopy (Figure 2.4). Neurons electroporated with CA Arf6 displayed a significant increase in p75NTR surface expression in the absence of NGF [100 ± 18 vs. 234 ± 45, $p = 0.026$]. Even when the neurons were continuously exposed to NGF, CA Arf6 was able to further up regulate p75NTR [100 ± 13 vs. 176 ± 30, $p = 0.032$]. Conversely, when we electroporated neurons with the Arf6 GAP, ACAP1, which effectively inhibits Arf6 by inducing conversion to the GDP-bound state, there was a significant reduction in surface expressed p75NTR, even when cultured in NGF-containing media [100 ± 9 vs. 70 ± 10, $p = 0.048$]. These data demonstrate that sustained activation or inactivation of Arf6 is sufficient to regulate the level of accessible p75NTR at the neuronal surface.

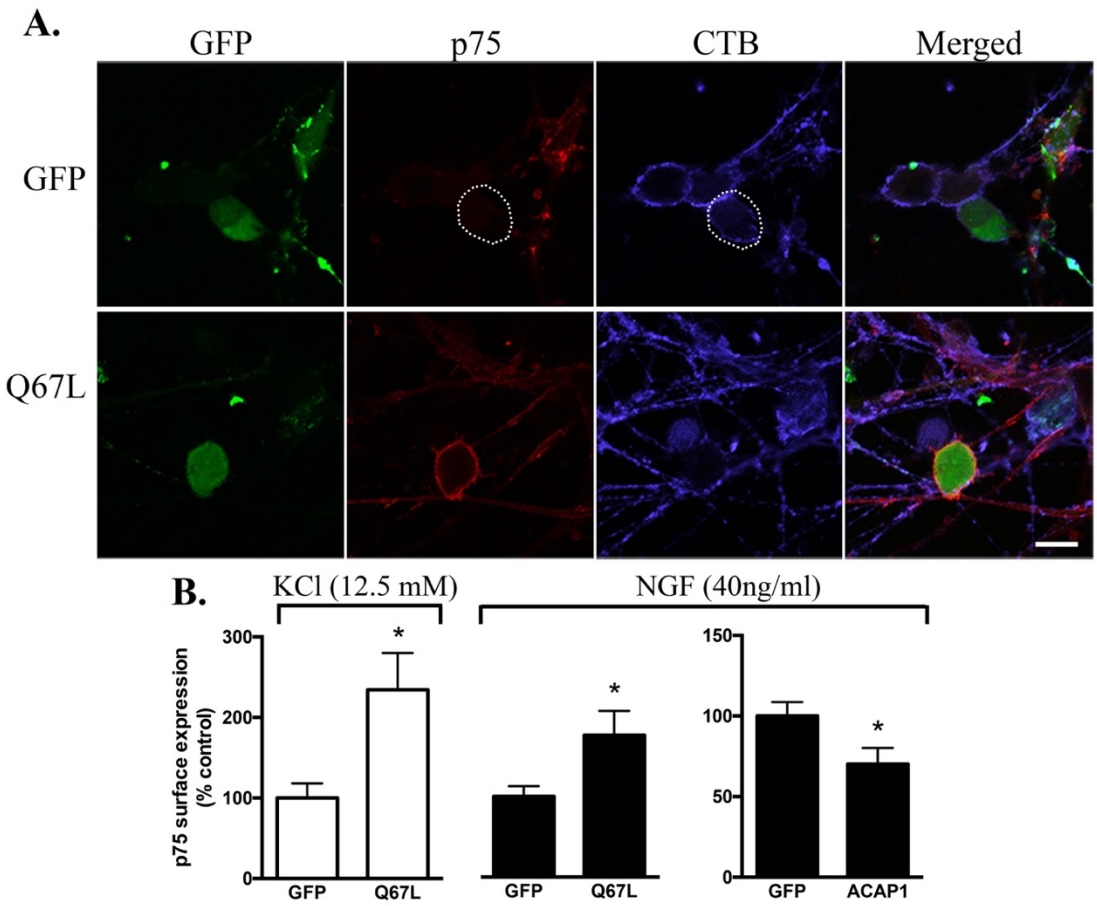

Figure 2.4. Arf6 regulates p75NTR surface expression. Sympathetic neurons were electroporated with a plasmid expressing GFP (top) or GFP and Q67L Arf6 (bottom) or the Arf6 GAP, ACAP1, then cultured with KCl or NGF. After 3 days, the surface expression of p75NTR in sympathetic neurons was quantified by immunolabeling live neurons with an antibody to the extracellular domain of the receptor, followed by fixation, confocal imaging and measurement of fluorescence intensity using Fiji software. To define the surface of the cells, fluorescently labeled cholera toxin (CTB) was added to live neurons during immunostaining. (**A**) Representative images of p75NTR expression on the cell surface in neurons cultured in KCl after electroporation with a vector expressing GFP or GFP and Q67L Arf6. Note the absence of p75NTR in GFP controls (white circle), but increased surface expression in the presence of Q67L Arf6. (**B**) Quantification of p75NTR surface expression in neurons cultured with KCl (white bars) or 40 ng/ml NGF (black bars). The signal is expressed as a percentage relative to neurons expressing GFP alone (n = 5-7; * $p \leq 0.05$ compared to GFP electroporated controls, scale bar = 10 μm).

NGF increases surface p75NTR through a TrkA-PI3 kinase-Arf6 pathway – In sympathetic neurons, NGF binds to TrkA receptors to elicit pro-survival signaling through well-characterized signaling cascades, including activation of phosphoinositide 3-kinase (PI3K) (Deinhardt and Chao, 2014). Interestingly, it was previously reported that NGF stimulated recruitment of the Arf6 GEF cytohesin-1 to the plasma membrane through activation of PI3K in PC12 cells (Venkateswarlu et al., 1999). Therefore, we hypothesized that NGF-induced p75NTR surface expression requires TrkA-mediated activation of PI3K, leading to stimulation of Arf6. To test our hypothesis, we first acutely treated sympathetic neurons with NGF, or NGF combined with the Trk inhibitor K252a and measured the level of p75NTR at the plasma membrane. As predicted, inhibition of TrkA prevented the NGF-induced increase in surface p75NTR [Figure 2.5A, $F(6,49) = 2.468$, $P = 0.035$]. To determine whether PI3K was required for the effects of NGF, we used the selective PI3K inhibitor LY294002. Neurons treated with NGF in the presence of LY294002 failed to up regulate p75NTR [Figure 2.5B, $F(6,28) = 4.66$, $P = 0.0021$]. In contrast, an inhibitor of the ERK pathway (U0126, 10 μM) had no effect on p75NTR trafficking [NGF alone increased p75NTR surface expression $71.01 \pm 2.47\%$, and NGF+U0126 increased it $66.32 \pm 29.78\%$, $p = 0.90$]. Finally, to further establish a role for Arf6 in the effects of NGF on p75NTR expression, we tested SecinH3, which inhibits the cytohesin family of Arf6 GEFs. When SecinH3 was added together with NGF, there was no increase in the levels of surface p75NTR [Figure 2.5C, $F(6,28) = 5.343$, $p = 0.0009$].

To provide a complimentary biochemical assessment to the up regulation of

p75NTR by NGF, we used surface biotinylation assays. Sympathetic neurons were cultured in KCl, then treated 2 hours with NGF in the presence or absence of SecinH3. The neurons were then placed on ice and surface proteins biotinylated. The biotinylated proteins were pulled down from neuronal lysates with streptavidin beads and blotted for p75NTR. It is likely that the basal level of surface p75NTR, in the absence of NGF, is overestimated in this assay since there are some glial cells present in the cultures (despite treatment with AraC), which constitutively express p75NTR at the cell surface (Fig. 2A). Nevertheless, corroborating our imaging results, NGF induced a significant increase in the level of p75NTR at the cell surface, which was inhibited by SecinH3 [Figure 2.5D, $F(2,6) = 6.574$, $p = 0.038$]. Collectively, this data supports a TrkA-mediated, PI3K, dependent rapid regulation of p75NTR trafficking by the GTPase Arf6.

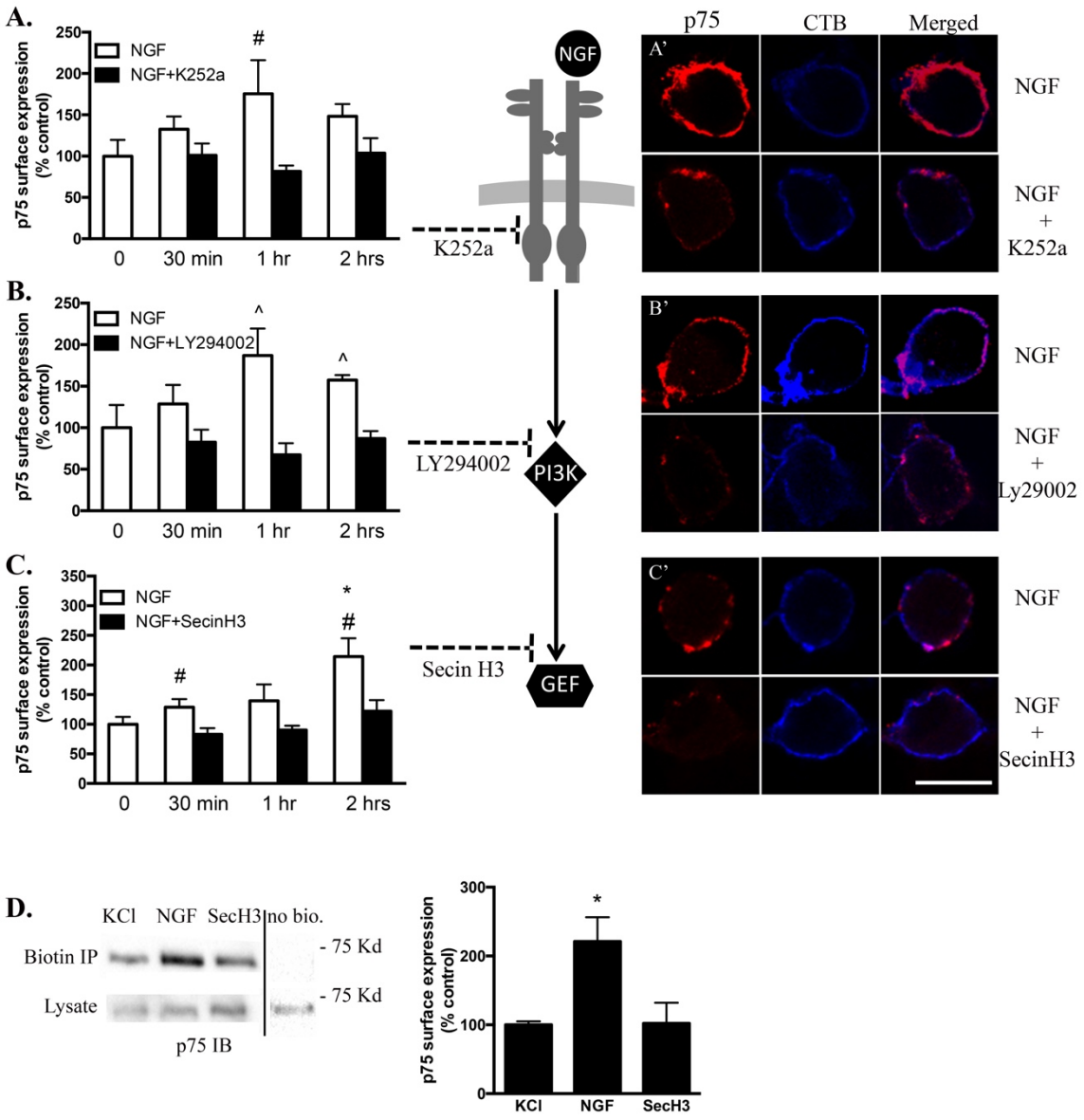

Figure 2.5. NGF up regulates p75NTR surface expression through a TrkA-PI3 kinase-Arf6 pathway. Sympathetic neurons were treated with 40 ng/ml NGF for the indicated time in the absence or presence of (**A**) the Trk inhibitor K252a (200nM), (**B**) the PI3 kinase inhibitor LY294002 (50 μM), or (**C**) the Arf6 GEF inhibitor SecinH3 (50 μM). The level of p75NTR surface expression was evaluated by immunostaining in unpermeabilized cells and, after fixation, the fluorescence intensity was visualized by confocal imaging and quantified using Fiji software and expressed as a percentage relative to neurons cultured in KCl in the absence of NGF. To define the surface of the

cells, fluorescently labeled cholera toxin (CTB) was added to live neurons during immunostaining (representative images are shown on the right). (**D**) Cell surface p75NTR was also quantified by biotinylation assay. Neurons were cultured in KCl, then treated with NGF with or without SecinH3 (SecH3, 50 μM) for 2 hrs, then placed at 4°C and the surface proteins biotinylated. The neurons were then lysed and biotinylated proteins precipitated with streptavidin beads and immunoblotted for p75NTR. To control for nonspecific pull down of p75NTR, the biotinylation reagent was omitted in one sample (no bio). The band intensity was quantified and normalized to the level of p75NTR in the lysate and expressed as a percent of KCl control. (n = 3-8; * $p \leq 0.05$ compared to control; # $p \leq 0.05$, ^ $p \leq 0.001$ compared to time-paired inhibitors.)

NGF mobilizes a pool of p75NTR in GGA3 vesicles – NGF was recently shown to activate Arf6 in PC12 cells, which promoted TrkA recycling through association with GGA3 (Li et al, 2015). Therefore, we considered the possibility that p75NTR could also be recruited to GGA3 containing vesicles and shuttled to the plasma membrane with TrkA. Surprisingly, however, a substantial pool of the p75NTR was found to pre-exist in GGA3 positive vesicles, prior to any NGF treatment. In contrast to TrkA, NGF induced a significant reduction in the colocalization of p75NTR and GGA3 [Figure 2.6A-C, Pearson's coefficient for KCl alone: 0.47 ± 0.03 vs 0.28 ± 0.05 for 2 hour NGF treatment, $p = 0.028$]. These results suggest that the neurons store rapidly accessible p75NTR in GGA3+ vesicles prior to reaching their targets and that upon exposure to NGF, TrkA activates Arf6 to recruit GGA3 from these vesicles and enable p75NTR to traffic to the cell surface.

In addition to the p75NTR localized to GGA3+ vesicles, there was an additional pool of intracellular receptor that was not associated with GGA3. Although some of this was likely newly translated p75NTR, we also considered the

possibility that there could be a readily accessible pool of p75NTR localized to recycling vesicles. Bronfman and colleagues recently demonstrated that p75NTR traffics to Rab11+ recycling endosomes following NGF-induced internalization in PC12 cells (Escudero et al., 2014). Due to the role of Rab11 in membrane recycling and the known association of Rabs with Arf6 (Zerial and McBride, 2001; Hutagalung and Novick, 2011; Hongu and Kanaho, 2014), we hypothesized that Rab11+ vesicles could be additional storage vesicles for intracellular p75NTR. However, upon assessing the internal distribution of p75NTR and Rab11, we found only a small fraction of p75NTR co-expressed with Rab11+ endosomes, but that did not significantly change upon NGF treatment (Figure 2.6D). These data indicate that there is a specific pool of p75NTR in GGA3+ vesicles that can be rapidly recruited to the plasma membrane upon NGF activation of TrkA.

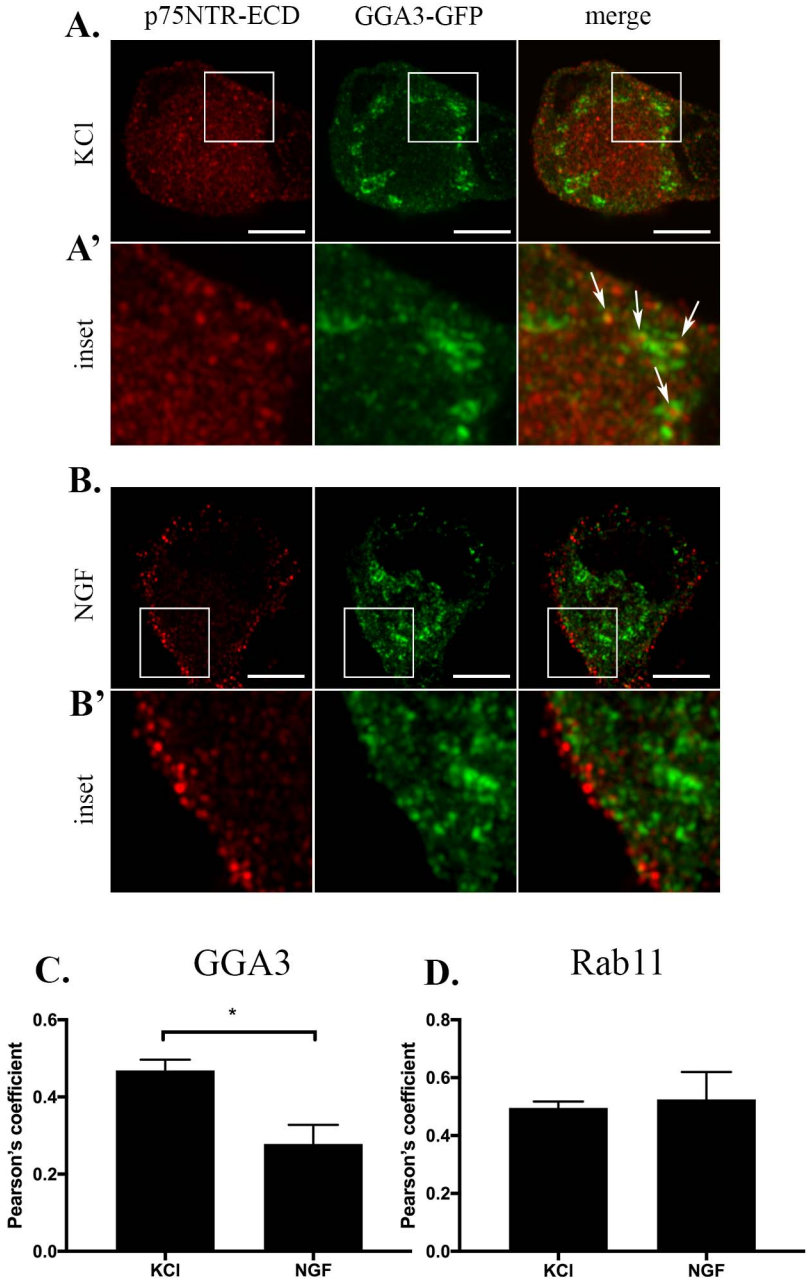

Figure 2.6. The fraction of intracellular p75NTR colocalized with GGA3 is reduced following NGF treatment. Neurons were electroporated with a plasmid expressing GGA3 fused to GFP and maintained in KCl for 16 hrs. Neurons were then either incubated in 40 ng/ml NGF for 2 hrs or kept in KCl (12.5 mM). High resolution z-stacks of GFP+, NF+ neurons were acquired using Zeiss 880 LSM with Airyscan **(A, A')** Representative images of a neuron maintained in KCl and showing p75NTR (red) colocalized with internal GGA3-GFP (green). Arrows are pointing to regions of

colocalization adjacent to the membrane (inset). **(B, B')** This colocalization decreases upon NGF treatment. While GGA3 puncta are still visible adjacent to the membrane, most p75NTR has translocated to the surface. The graphs depict quantification of Pearson's r coefficient for p75NTR colocalization with GGA3 **(C)** or Rab11 **(D)** from each treatment (n = 3 independent experiments; * $p < 0.05$). Scale bar represents 5 μm.

NT3-mediated neuronal survival and neurite outgrowth is inhibited by activated Arf6 – Previous studies have indicated that p75NTR expression modulates the responsiveness of TrkA to NT3; for example, NT3 is more potent in activating TrkA and promoting neurite growth and survival in neurons from p75NTR-/- mice (Lee et al., 1994; Kuruvilla et al., 2004). Furthermore, p75NTR expression is markedly increased as sympathetic neurons innervate their NGF-producing target fields, which has been suggested to shift the responsiveness of the neurons from NT3 to NGF (Kuruvilla et al., 2004). Therefore, we hypothesized that the up regulation of p75NTR surface expression induced by activation of Arf6 would attenuate the ability of the neurons to respond to NT3. To test this hypothesis, we electroporated sympathetic neurons with GFP, or the CA mutant of Arf6 and performed neurotrophin survival assays. Neurotrophin naïve neurons were treated with increasing concentrations of NGF or NT3 for 48 hours and neuron survival was determined by evaluation of DAPI stained nuclei. While electroporated neurons exhibited no alterations in NGF-mediated survival compared to un-electroporated controls (Figure 2.7A), neurons expressing the Q67L CA Arf6 construct show no trophic response to NT3 compared to both un-electroporated controls and GFP-

expressing neurons [Figure 2.7B, $F(10,36) = 4.255$, $p = 0.006$].

Since NT3 is best characterized as promoting neurite outgrowth during development of the sympathetic nervous system (Kuruvilla et al., 2004), we also investigated the effects of NT3 in neurons expressing CA Arf6. Sympathetic neurons electroporated with GFP only or GFP and CA Arf6 were treated with NGF or NT3 for 18 hours, then the longest neurite from each GFP+ cell was measured. The outgrowth from neurons expressing GFP only was not different in those treated with NGF compared to NT3. However, in neurons expressing the CA Arf6 construct, NT3-induced neurite outgrowth was significantly less than NGF-induced outgrowth. Similarly, NT3 promoted longer neurites in neurons expressing GFP only, as control, relative to those expressing CA Arf6 [Figure 2.7C-D, $F(3,12) = 1.4$, $p = 0.011$]. To confirm that this change in neurotrophin responsiveness is due to the increased availability of p75NTR at the plasma membrane, we repeated this experiment using *p75NTR*$^{-/-}$ mice. Compared to their wildtype controls, p75NTR knockout neurons expressing CA Arf6 exhibited significantly longer neurites in response to NT3 [Figure 2.7E, $F(3,8) = 7.2$, $p = 0.011$]. Taken together, these results indicate that Arf6 activation induces up regulation of surface p75NTR, which shifts the responsiveness of the neurons, such that they no longer respond to NT3.

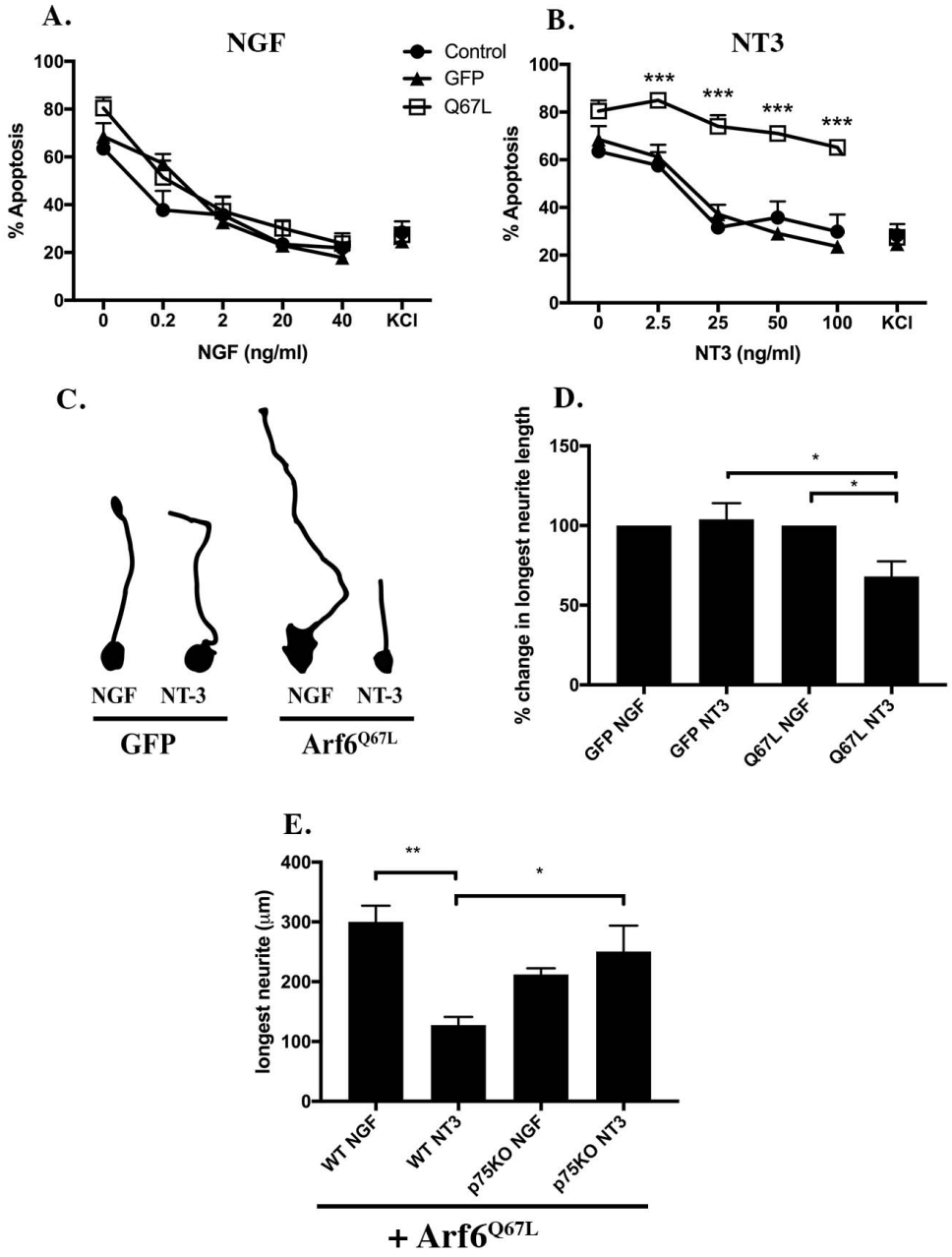

Figure 2.7. Constitutively active Arf6 alters NT3, but not NGF, mediated survival and neurite outgrowth of sympathetic neurons. Sympathetic neurons were electroporated with a plasmid expressing GFP (triangles) or GFP and Q67L Arf6 (square) or untreated (circle), then cultured with KCl for 2 days. The neurons were then treated with the indicated concentration of (**A**) NGF or (**B**) NT3 or maintained in KCl. After 48 hrs, the neurons were fixed, immunostained for TuJ1 to mark neurons, and the nuclei labeled with DAPI. The percent of apoptotic neurons was determined

based on the DAPI staining (n = 3, *** $p \leq 0.01$). Neurite outgrowth was analyzed after 16 hrs of neurotrophin treatment and neurofilament (NF) immunostaining was used to mark neurites. The longest neurite from each GFP+ cell was measured. **(C)** Representative traces of neurite outgrowth from each condition and **(D)** quantification of GFP+ cells in each condition (n =3 independent experiments, one-way ANOVA between treatments * $p < 0.05$, Turkey's multiple comparisons GFP NT3 vs. Q67L NT3: * $p < 0.05$; Q67L NGF vs. Q67L NT3: * $p < 0.05$). **(E)** Sympathetic neurons from wildtype or p75NTR-/- mice were electroporated with Q67L Arf and GFP and immediately plated in either NGF or NT3 for 48 hrs before fixing. The longest neurite from each GFP+, NF+ cell was measured (n = 3 independent experiments, one-way ANOVA between treatments * $p < 0.05$, Turkey's multiple comparisons WT NGF vs. WT NT3: ** $p < 0.01$; WT NT3 vs. p75KO NT3: * $p < 0.05$).

2.5 Discussion

During development of the sympathetic nervous system, NGF increases the expression of p75NTR, which is thought to play a key role in shifting the responsiveness of TrkA from NT3 to NGF. However, the mechanisms by which p75NTR expression is regulated and trafficked to the cell surface were not known. In this report, we reveal a novel, rapid, NGF-induced up regulation of surface p75NTR through a TrkA- PI3 kinase- Arf6 pathway in sympathetic neurons. Notably, the acute increase in surface p75NTR following exposure to NGF was from previously existing pools and not the result of newly synthesized receptor, as treatment with CHX did not prevent p75NTR up regulation. Functionally, our results demonstrate that the rapid increase in surface expression of p75NTR shifts the neurotrophic response of sympathetic neurons, blunting the effectiveness of NT3.

Interestingly, this regulatory mechanism was specific for p75NTR, as neither TrkA or sortilin surface expression was altered under the same conditions. However, Ascano et al reported that TrkA can be rapidly inserted into the growth cones within minutes of NGF treatment (Ascaño et al., 2009). In their study, minimal surface TrkA was detected when NGF was removed and the neurons kept alive by inhibiting caspases. In contrast, we found considerable surface TrkA under neurotrophin naïve conditions, when the neurons were maintained in mildly depolarizing conditions with KCl. Therefore, it is possible that depolarization could induce up regulation of TrkA, similar to what has been reported for TrkB (Meyer-Franke et al., 1998; Du et al., 2000), which will be an interesting topic for future studies.

Our study reveals a novel role for Arf6 in regulating the transport of p75NTR to the plasma membrane; however, this GTP binding protein has been shown to modulate the surface presentation of a number of other receptors, often promoting recycling. For example, AMPA receptors were reported to be recycled to the cell surface through Arf6 expressing endosomes (Zheng et al., 2015). Similarly, the internalization and recycling of β1 integrin involves Arf6. Notably, in HeLa cells treated with serum (Powelka et al., 2004) or endothelial cells exposed to hepatocyte growth factor (Hongu and Kanaho, 2014), there was an in increase in □1 integrin receptor surface expression through Arf6-dependent receptor recycling. Of particular relevance here, Arf6 was recently shown to promote TrkA recycling in PC12 cells treated with NGF through the recruitment of GGA3 (Li et al., 2015). There are 3 GGA family members (GGA1-3), which are adaptor proteins involved in vesicular trafficking in the trans-Golgi network and in

endocytosis (Bonifacino, 2004). NGF induced a direct association between TrkA and GGA3, which reduced TrkA degradation and promoted recycling to the cell surface (Li et al., 2015). We did not detect a change in the total surface TrkA expression within 2 hrs of NGF treatment, suggesting that the existing TrkA at the plasma membrane is efficiently recycled in response to NGF, in agreement with that report. In contrast, the expression of p75NTR at the cell surface was significantly up regulated. This increase in p75NTR at the plasma membrane cannot be explained by receptor recycling since there was minimal expression of p75NTR at the surface in the absence of NGF. Instead, our results suggest that Arf6 activation promotes the shuttling of p75NTR from pre-existing intracellular stores, which (to our knowledge) would be the first example of Arf6 mediating such trafficking.

The up regulation of p75NTR from rapidly accessible storage vesicles appears to occur through a somewhat different mechanism than its neurotrophin-induced recycling. Several groups reported p75NTR internalization via clathrin-dependent and independent mechanisms (Bronfman et al., 2003; Saxena et al., 2004; Deinhardt et al., 2007). Internalization was followed by shuttling through Rab5+ early endosomes to Rab7+ endosomes for axonal retrograde transport in motor neurons (Deinhardt et al., 2006) and in PC12 cells, p75NTR was recycled via Rab11+ endosomes or exocytosed from multivesicular bodies (Escudero et al., 2014). However, our data suggests that while a small fraction of internally stored p75NTR does overlap with Rab11+ vesicles, short-term NGF treatment did not alter this pool of receptor (Figure 2.6D). In contrast, we found a portion of internal p75NTR that colocalized with GGA3 in the absence of NGF. Following NGF

exposure, much of the colocalization between p75NTR and GGA3 was lost, suggesting that TrkA internalization, which results in GGA3 association (Li et al., 2015), recruits GGA3 from p75NTR-containing vesicles, enabling p75NTR to traffic to the cell surface. Nevertheless, since only a fraction of the internal p75NTR colocalized with GGA3, we cannot rule out the possibility that Arf6 activation also mobilizes p75NTR from additional types of vesicles to the cell surface.

How activation of Arf6 and/or removal of GGA3 would induce transport of p75NTR to the cell surface is not known. Downstream effectors of Arf6 include Rac1, PIP5 kinase and phospholipase D, which can stimulate PIP5 kinase through production of phosphatidic acid (Jaworski, 2007). Activation of PIP5 kinase results in production of the signaling molecule phosphatidylinositol 4,5-bisphosphate (PIP2), which has been shown to promote plasma membrane association of vesicles containing β1-integrin through interaction of PIP2 with components of the exocyst complex (Thapa and Anderson, 2012). Similarly, Arf6-mediated membrane recycling to regions of plasma membrane expansion was reported to involve interaction between Arf6 and the Sec10 component of the exocyst complex (Prigent et al., 2003). It will be of interest to determine whether p75NTR trafficking to the cell surface involves the exocyst complex in future studies.

The increase in p75NTR surface expression and the activation of Arf6 was selective for NGF, NT3 did not have any effect on surface p75NTR up regulation (Figure 2.1D) or GTP binding, even at 100 ng/ml (Figure 2.3B). The molecular basis for this selectivity is not known; however, it was previously reported that NGF, but not NT3, is able to induce TrkA internalization (Kuruvilla et al., 2004). The endocytosis of TrkA was shown to occur via NGF-selective stimulation

phospholipase C leading to calcineurin-dependent dynamin1 activation (Bodmer et al., 2011). In contrast, Harrington et al (2011) reported that both NGF and NT3 induce TrkA internalization, but in the acidic environment of the endosome, NT3 dissociates while NGF remains bound (Harrington et al., 2011). The post-endocytic signaling of NGF resulted in Rac1/cofilin-dependent breakdown of the local actin network, freeing the endosome to be retrogradely transported. Interestingly, Arf6 can also lead to Rac1 activation (Santy and Casanova, 2001), suggesting that the effects of TrkA on the cytoskeleton may involve Arf6. Nevertheless, both studies demonstrate that NGF mediates internalization and continuous TrkA signaling, while NT3 is only able to activate TrkA at the cell surface. Therefore, we suggest that the activation of Arf6 by NGF most likely occurs during or following TrkA internalization.

Our findings indicate that TrkA stimulates Arf6 through a mechanism dependent on PI3 kinase and a member of the cytohesin family of GEFs (Figure 2.5). All 8 of the Arf6 GEFs contain a PH domain, which binds to phosphatidylinositol and the PH domains of the cytohesins have highest affinity for phosphatidylinositol 3,4,5 triphosphate (PIP3) (Klarlund et al., 1997) a lipid produced by PI3 kinase. Therefore, it is likely that TrkA activation of PI3 kinase induces a local increase in PIP3, which recruits a cytohesin, leading to Arf6 activation. In contrast, Li et al (2015) reported that TrkA activated Arf6 through a mechanism dependent on Src. However, these mechanisms are not mutually exclusive; for example, the Src family member Fyn was reported to phosphorylate cytohesin-1, resulting in activation of the GEF and, subsequently, Arf6 (Yamauchi et al., 2012). Therefore, TrkA may activate Arf6 through PI3 kinase-dependent

recruitment of a cytohesin, followed by stimulating the cytohesin's GEF activity through Src-mediated phosphorylation.

The ability of NGF to up regulate p75NTR surface expression through the TrkA-Arf6 pathway allows for the critical coordination of sympathetic neuron response to neurotrophins during development. The activation of p75NTR on its own or in association with Vps10p-domain receptor proteins (e.g. sortilin) can induce apoptosis of sympathetic neurons when stimulated by BDNF (Bamji et al., 1998; Palmada et al., 2002) or the pro-forms of neurotrophins (Nykjaer et al., 2004; Teng et al., 2005). Indeed, $p75NTR^{-/-}$ mice have both an increase in SCG neuron number and a decrease in apoptosis (Bamji et al., 1998; Brennan et al., 1999; Kuruvilla et al., 2004), reflecting the importance of p75NTR's cell death signal during development. The pro-apoptotic effects of p75NTR have been shown to be critical for balancing the pro-survival effects of TrkA signaling, in order to establish the proper number of neurons during sympathetic development (Deppmann et al., 2008).

In contrast to its pro-apoptotic function, p75NTR can also associate with TrkA to form a high affinity complex for NGF that promotes neuronal survival, differentiation and neurite growth (Ceni et al., 2014). In the absence of p75NTR, NT3 can bind TrkA and promote axon growth (Benedetti et al., 1993; Belliveau et al., 1997; Kuruvilla et al., 2004), expression of neuronal genes such as tyrosine hydroxylase (Belliveau et al., 1997a; Andres et al., 2008), and survival (Davies et al., 1995; Belliveau et al., 1997a; Francis et al., 1999). This differential response of TrkA to NT3, regulated by the expression of p75NTR, is critical for proper target innervation during development of the sympathetic nervous system (Kuruvilla et

al., 2004). In addition, the up regulation of p75NTR in response to NGF is a very useful mechanism for limiting p75NTR-mediated apoptosis in early stages of development. If p75NTR was expressed on the cell surface, as the neurons are growing toward their targets in response to NT3, then NT3 would be restricted from binding TrkA and could, potentially, induce apoptosis through interaction with p75NTR alone.

While we hypothesize that the increase in surface p75NTR expression leads to the formation of high-affinity binding sites, thereby strengthening TrkA survival signaling. An alternate interpretation of the effects of p75NTR up regulation would be that p75NTR binds NT3 and acts as a sink to prevent binding to TrkA. However, given that the affinity for NGF and NT3 binding to p75NTR is nearly identical (Dechant et al., 1994) and we only see a change in the responses to NT3 when p75NTR surface expression is increased, not NGF, it is more likely that TrkA is more selective for NGF in the presence of p75NTR.

Given the functional dichotomy of the p75NTR in regulating neuronal survival and apoptosis, a better understanding of the mechanisms that mediate p75NTR surface expression provides key insight not only into the fine tuning of the developing sympathetic nervous system, but also the aberrant cell death linked to p75NTR in multiple neurodegenerative disorders.

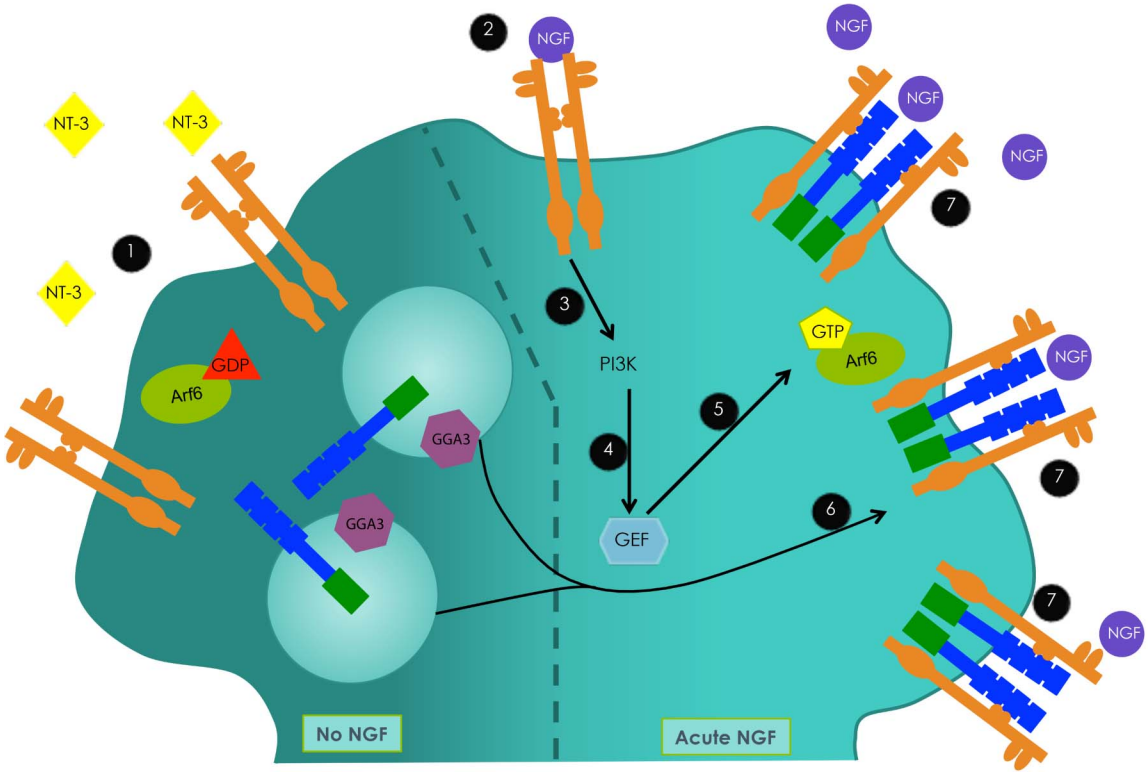

Figure 2.8. Schematic of rapid NGF regulation of p75NTR trafficking by TrkA activation of Arf6. During axonal growth, sympathetic neurons respond to NT3 through binding to TrkA receptors (**1**, orange receptors) prior to reaching NGF-producing target organs. During this time, Arf6 is largely inactive (GDP-bound), and the p75NTR produced is stored in intracellular pools. Once sympathetic axons reach NGF-producing targets, NGF binding to TrkA receptors (**2**) initiates activation of PI3K (**3**), which recruits Arf6 GEFs that stimulate GTP binding of Arf6 (**4-5**). In its active, GTP-bound state, Arf6 initiates transport of p75NTR (blue receptors) from GGA3 containing vesicles to the cell surface (**6**) and promotes of the formation of TrkA-p75NTR complexes (**7**). The high-affinity TrkA-p75 complexes are selective for NGF and NT3 is no longer able to activate TrkA, and sympathetic neurons become dependent on NGF for survival.

3. p75NTR is retrogradely transported following NGF withdrawal or BDNF treatment through a mechanism dependent on deacetylation of the dynactin subunit p150Glued

This chapter primarily contains the data that I contributed to a manuscript that has been submitted for publication:

Pathak, A; Stanley, EM; Hickman, FE; Wallace, N; Brewer, B; Li, D; Gluska, S; Perlson, E; Fuhrmann, S; Akassoglou, K; Bronfman, F; Casaccia, P; Burnette, D; Carter, BD. (2018) Retrograde degenerative signaling mediated by the p75 neurotrophin receptor requires $p150^{Glued}$ deacetylation by axonal HDAC1.

3.1 Abstract

During development, neurons undergo apoptosis if they do not receive adequate trophic support from tissues they innervate or when detrimental factors activate the p75 neurotrophin receptor (p75NTR) at their axon ends. Trophic factor deprivation (TFD) or activation of p75NTR in distal axons results in a retrograde degenerative signal. However, the nature of this signal and the regulation of its transport is poorly understood. Here, we identify p75NTR intracellular domain (ICD) and histone deacetylase 1 (HDAC1) as part of a retrograde pro-apoptotic signal generated in response to TFD or ligand binding to p75NTR in sympathetic neurons. We report an unconventional function of HDAC1 in retrograde transport of a degenerative signal and its constitutive presence in sympathetic axons. HDAC1 deacetylates dynactin subunit $p150^{Glued}$, which enhances its interaction with dynein. These findings define p75NTR ICD as a retrograde degenerative signal and reveal $p150^{Glued}$ deacetylation as a novel mechanism regulating axonal transport.

3.2 Introduction

The balance between neuronal survival and degeneration in the peripheral nervous system is regulated by the limited availability of neurotrophins. The neurotrophins mediate survival and differentiation through binding to the Trk family of tyrosine kinase receptors (Deinhardt and Chao, 2014). All neurotrophins can also bind to the p75 neurotrophin receptor (p75NTR), a member of the TNF receptor superfamily, which can have distinct effects, including mediating axonal degeneration and apoptosis (Ceni et al., 2014; Kraemer et al., 2014). p75NTR can bind all of the neurotrophins and their proforms, the specific ligand activating apoptotic signaling depends on the cellular context. Pro-apoptotic ligand binding to endogenous p75NTR activates JNK signaling in sympathetic neurons and direct inhibition of JNK kinase activity is sufficient to prevent the induction of apoptosis (Kenchappa et al., 2010). The cleavage of p75NTR has been reported to be necessary for neuronal apoptosis in sympathetic neurons (Kenchappa et al., 2006). The release of p75NTR's ICD enables direct signaling to the nucleus by enabling nuclear translocation of the intracellular binding partner NRIF (Linggi et al., 2005; Kenchappa et al., 2010).

Neurotrophins are produced by the target tissues and signal to receptors on the distal axons must be efficiently conveyed back to the cell body. The mechanisms of retrograde survival signaling have been well described in sympathetic and sensory neurons that rely on nerve growth factor (NGF) for survival (Wu et al., 2009a; Harrington and Ginty, 2013; Yamashita and Kuruvilla, 2016). Loss of trophic support leads to a reduction in pro-survival signals reaching

the soma. However, there is growing evidence that trophic factor withdrawal also results in a retrograde pro-apoptotic signal (Mok et al., 2009; Ghosh et al., 2011; Simon et al., 2016). When NGF was withdrawn from distal axons of sympathetic neurons, an increase in phosphorylated c-Jun was observed in cell somas. When microtubule stability was interrupted through treatment with colchicine, this increase in c-Jun was blocked, suggesting retrograde transport is required for apoptosis (Mok et al., 2009). Blocking axonal activation of the stress-induced dual leucine zipper kinase (DLK) or c-Jun N-terminal kinase (JNK), also prevented sensory neuron apoptosis following withdrawal (Ghosh et al., 2011; Simon et al., 2016). The molecular components of this retrograde signaling are yet to be defined.

Retrograde axonal transport is driven by the microtubule minus-end directed motor protein dynein, consisting of a dimer of heavy chains and additional intermediate and light chains. Dynein associates with the activator complex dynactin, which is composed of more than 20 proteins, including the largest subunit $p150^{Glued}$. A dimer of $p150^{Glued}$ binds to the dynein intermediate chain through its coiled-coil domain (CC1) and is required for the formation of this complex (Karki and Holzbaur, 1995; King and Schroer, 2000; McKenney et al., 2014). Formation of this complex and how it is regulated is still an active area of investigation.

Surprisingly, several histone deacetylases (HDACs) have recently been shown to influence axonal transport. While best characterized for their role in regulating gene expression by altering histone configuration, several HDACs can translocate from the nucleus and modify other targets (Cho and Cavalli, 2014).

HDAC1 was recently shown to translocate from the nucleus into the axons of cortical and hippocampal neurons following glutamate and TNF treatment, where it associated with kinesins. This association disrupted mitochondrial transport, which led to axon degeneration (Kim et al., 2010).

In this study, we identify the intracellular domain (ICD) of p75NTR as a retrograde pro-apoptotic signal initiated through both ligand and non-ligand activation of 75NTR in sympathetic neurons. Further, we reveal a novel role for HDAC1 in retrograde signaling through regulating the formation of the dynactin-dynein complex.

Project goals

My specific goals for this project were to determine (1) if p75NTR activation in axons increased the interaction between dynein and $p150^{Glued}$ (a subunit of dynactin) and (2) whether p75NTR cleavage was necessary for cell death after NGF withdrawal and BDNF binding. To investigate the requirement for p75NTR cleavage in apoptosis, I generated two p75NTR mutants and tested their ability to block receptor cleavage *in vitro*. These mutants allowed us to demonstrate that the retrograde pro-apoptotic signal is mediated through p75NTR cleavage and ICD transport to the soma.

3.3 Methods

p75NTR site directed mutagenesis

The mCherry-p75NTR-GFP plasmid was obtained from Dr. Katarina Akassoglou (UCSF) . Crucial for TACE cleavage (Underwood et al., 2008), amino acid 234 was mutated from a Valine (V) to aspartic acid (D) or to asparagine (N) using Pfu

Ultra polymerase and the following primers:

V234DF: 5'-AGCTCCCAGCCTGATGTGACCCGCGGCA-3'

V234DR: 5'-TGCCGCGGGTCACATCAGGCTGGGAGCT-3'

D243NF: 5'-GCAGCTCCCAGCCTAATGTGACCCGCGGC-3'

D243NR: 5'-GCCGCGGGTCACATTAGGCTGGGAGCTGC-3'

1μg of template DNA was used and PCR was run under the following conditions followed by a 1 hour incubation with DpnI to digest template: 94°C for 5 minute, 18 cycles of 94°C for 30 seconds, 60°C for 1 minute, 72°C for 7 minutes, followed by 72°C for 10 minutes. Positive mutations were confirmed through sequencing.

p75NTR cleavage validation

To validate that our mutant p75NTR constructs were indeed non-cleavable, 15μg DNA was transfected into HEK293-T cells using calcium chloride on 10cm^2 tissue culture plates. DMEM containing 10% FBS and 1% Pen/Strep was used as the culture media. The following day, each plate was divided into 3 10cm^2 plates for subsequent treatments. After reaching approximately 70% confluence, all plates were treated with 10μM Z-Leu-Leu-Leucinal (*ZLLLH*, a proteasome *inhibitor) for 1 hour. Where indicated, 10μM TAPI (TACE inhibitor) was added simultaneously.* PKC-activator phorbol 12-myristate 13-acetate (*PMA,) was added to cells at a concentration of 1μM for 1 hour to induce p75NTR cleavage. After treatment, cells were lysed on ice in RIPA buffer and 50μg of sample was run on a 8% SDS PAGE acrylamide gel and transferred to a nitrocellulose membrane. Anti-GFP (1:1000) antibody was used to visualize full length p75NTR, the CTF, and ICD as they are*

all tagged to GFP. The signal was visualized using anti-rabbit peroxidase-conjugated secondary antibody and enhanced with chemiluminescence and a BioRad ChemiDOC MP imaging system.

Primary Cell Culture – All experiments were conducted with tissues derived from animals were approved by the Animal Care and Use Committee at Vanderbilt University. Neurons from the superior cervical ganglia (SCG) were dissected from postnatal day 2-3 Sprague-Dawley or CD1 rats (male and female; Charles River, Wilmington, MA) and dissociated with 0.08% trypsin (Worthington) and 0.3% collagenase (Sigma-Aldrich, St. Louis, MO). Neurons in single cell suspension were then plated on 60mm^2 cell culture dishes coated with poly-D-lysine (overnight, MP Biomedicals, Santa Ana, CA) and laminin (~2 hrs, Invitrogen, Waltham, MA). Neurons were cultured in Ultraculture media (Lonza, Houston, TX) with 3% fetal bovine serum (Denville Scientific Inc., South Plainfield, NJ), 2 mM L-Glutamine (Invitrogen), 100 units/ml penicillin, and 100 μg/ml streptomycin (Invitrogen) at 37°C in 5% CO_2. SCG neurons were cultured in 40 ng/ml nerve growth factor (NGF, Harlan Laboratories, Houston, TX) for 18 hours. To prevent the proliferation of non-neuronal cells in culture, 18 hours after initial plating cells were treated with 10 μM cytosine arabinoside (AraC; Sigma-Aldrich) for 36 hours. For the eventual BDNF-treated cells, NGF was removed from neurons 18 hours prior to treatment by rinsing the cells twice with media lacking NGF, then adding media supplemented with 12.5 mM KCl and anti-NGF antibody (0.1 μg/ml; Chemicon, Billerica, MA). Neurons were then treated with 200ng/ml BDNF and anti-NGF or kept in 40ng/ml NGF with the addition of 5uM MS275 for 48 hours.

Immunoprecipitation

Neurons were lysed on ice in RIPA buffer (50mM Tris (pH 8), 150mM NaCl, 1% NP40, 0.5% deoxycholate, 0.1% SDS), sonicated to cleave DNA, then insoluble material pelleted at 16000g for 30 min. Immunoprecipitations (IPs) were carried out with the supernatant, using 250ug total protein incubated overnight at 4°C with 2μl antibody to the dynein intermediate chain (DIC), followed 1-hour incubation with 50/50 mixture of Protein A/G in Tris-buffered saline (TBS). Samples were spun at 1300g 3 times and washed with 1X RIPA buffer before resuspension in SDS sample buffer and boiled for 5 minutes. IP samples were run at 120V on a 7.5% SDS PAGE acrylamide gel with 25ug of sample lysate. Gels were transferred to nitrocellulose at constant 350mA for 90 minutes and the membrane blocked with 5% milk in TBS with 0.1% Tween. Blots were incubated overnight at 4°C with an antibody to $p150^{Glued}$ (1:1000 in TBST, product info). The signal was visualized using anti-rabbit peroxidase-conjugated secondary antibody and enhanced with chemiluminescence and a BioRad ChemiDOC MP imaging system. The $p150^{Glued}$ band intensity was quantified using Fiji Software (Schindelin et al., 2012), normalized to total the amount of DIC pulled down and total protein in the lysate fraction.

3.4 Results

Mutating p75NTR V234 blocks TACE and γ-secretase induced cleavage

To investigate the ability of p75NTR to initiate a retrograde apoptotic signal, sympathetic neurons were cultured in microfluidic devices, which separate the cell bodies from the distal axons and prevent diffusion between the compartments. The neurons were initially plated in NGF to promote survival and axon outgrowth. To investigate the effects of selective p75NTR activation, we removed NGF, which binds to TrkA and p75NTR, we removed NGF and kept the neurons alive with mildly depolarizing conditions by adding 12.5 mM KCl. When BDNF was added to the distal axons only to stimulate p75NTR, cell death was observed in sympathetic neuron soma, indicating that the apoptotic signal was conveyed from the axon to the cell body. To determine where p75NTR processing is required in this polarized cell death signaling, we used the γ-secretase inhibitor, DAPT. We found that p75NTR-mediated apoptosis was blocked by addition of DAPT to the distal axons, but not when added to cell somas (Figure 3.1). These data suggest that local proteolysis of p75NTR is required for BDNF-induced cell death.

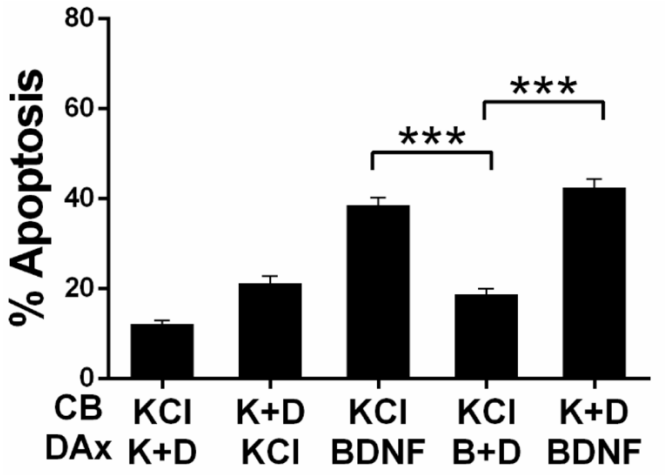

Figure 3.1. Inhibition of axonal γ-secretase prevents p75NTR-mediated apoptosis. Sympathetic neurons were cultured in microfluidics chambers and cell bodies (CB) and distal axons (DAx) were treated with 12.5 mM KCl (K) or 200 ng/ml BDNF (B) +/- the γ-secretase inhibitor, 250 nM DAPT (D), as indicated. After 48 hrs, pyknotic nuclei were quantified in neurons fixed and stained for TUJ1 and DAPI. The bars depict the means ± SEM for n=3-5; ***, $p < 0.001$, student's t-test with Welch correction among indicated groups. **Figure courtesy of Amrita Pathak.**

To directly assess axonal p75NTR cleavage in response to BDNF, we electroporated a dual-tagged p75NTR reporter construct into the neurons, containing mCherry on the N-terminus and GFP on the C-terminus. This mCherry-p75NTR-GFP reporter was previously utilized to investigate the localization of the cleaved receptor products in astrocytes and can differentiate between uncleaved, full length p75NTR (yellow), cleaved p75NTR ICD (green) and extracellular domain (ECD) (red) (Schachtrup et al., 2015). After electroporation, the neurons were allowed to recover in mass cultures with NGF, then switched to KCl with or without BDNF for 2 hrs and the number of mCherry, GFP or overlapping puncta in the axons was quantified. BDNF significantly increased the number of GFP+ only

(ICD) puncta, suggesting p75NTR cleavage within the axons (Figure 3.2A-C).

Since trophic factor deprivation (TFD) also initiates a retrograde degenerative signal (Mok et al., 2009; Ghosh et al., 2011; Simon et al., 2016), we investigated the possibility that NGF withdrawal could stimulate local p75NTR proteolysis. NGF was withdrawn and the axons were live imaged after 14 hours, which we reasoned would be long enough to induce p75NTR cleavage, but prior to the neurons committing to apoptosis (Johnson and Deckwerth, 1993). There was a significant decrease in full length receptor after NGF withdrawal and a corresponding increase in the fraction of GFP+ only puncta (Figure 3.2D, E), indicating that TFD can induce the cleavage of p75NTR in axons.

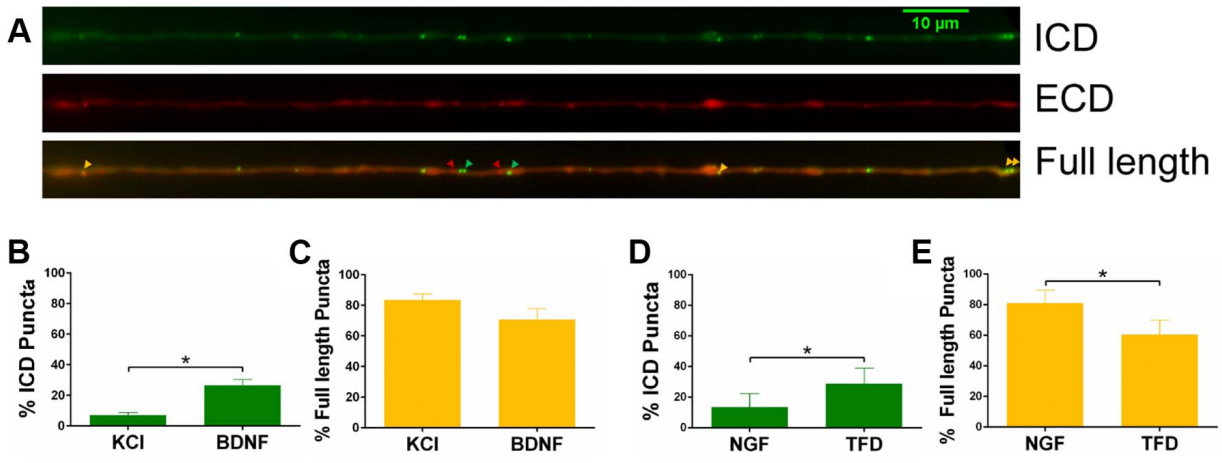

Figure 3.2 – BNDF and TFF induce retrograde transport for p75NTR-ICD A. Representative image of mCherry-p75-GFP in sympathetic neurons showing full length p75NTR (yellow arrowheads), the released ECD (red arrowheads) and the liberated ICD (green arrowheads) in axons. B and C. Quantification of the number of p75NTR ICD only (green) and full length p75NTR (yellow) puncta in axons after treatment with KCl (control) or BDNF for 2 hrs. Depicted are the means ± SEM from at

least 3 different experiments (KCl, n= 266 and BDNF, n=255 events); ∗, $p < 0.05$, student's t-test. D and E. Quantification of the number of p75NTR ICD only (green) and full length p75NTR (yellow) puncta under control conditions and after trophic factor deprivation (TFD) for 14 hours. Depicted are the means ± SEM from 3 different experiments (NGF, n= 212 and TFD, n= 383 events); ∗, $p < 0.05$, student's t-test. **Figure courtesy of Amrita Pathak.**

Although treatment of the axons with DAPT blocked neuronal death (Figure 3.1), DAPT inhibits the cleavage of all γ-secretase substrates. Therefore, we investigated whether cleavage specifically of p75NTR was necessary for the apoptotic signal. We generated a V243N and a V243D mutation in the extracellular, juxtamembrane region of the mCherry-p75NTR-GFP construct. The V243N mutation introduces a glycosylation site that prevents the receptor from undergoing proteolysis by TACE, which is a prerequisite for γ-secretase cleavage (Underwood et al., 2008). We confirmed that the V243N mutation blocked p75NTR cleavage (Figure 3.3). Similarly, we also found that the V243D mutation blocked proteolysis of the receptor (Figure 3.3), although the mechanism is not clear. The PMA induced cleavage in wildtype p75NTR was verified by cotreatment with TACE inhibitor TAPI, which also resulted in a reduction in cleavage (Figure 3.3). Importantly, expressing either of these mutant constructs in sympathetic neurons significantly attenuated both BDNF (Figure 3.4A), and NGF-withdrawal induced apoptosis (Figure 3.4B), supporting the hypothesize that p75NTR cleavage AND retrograde transport of the ICD are required for apoptosis.

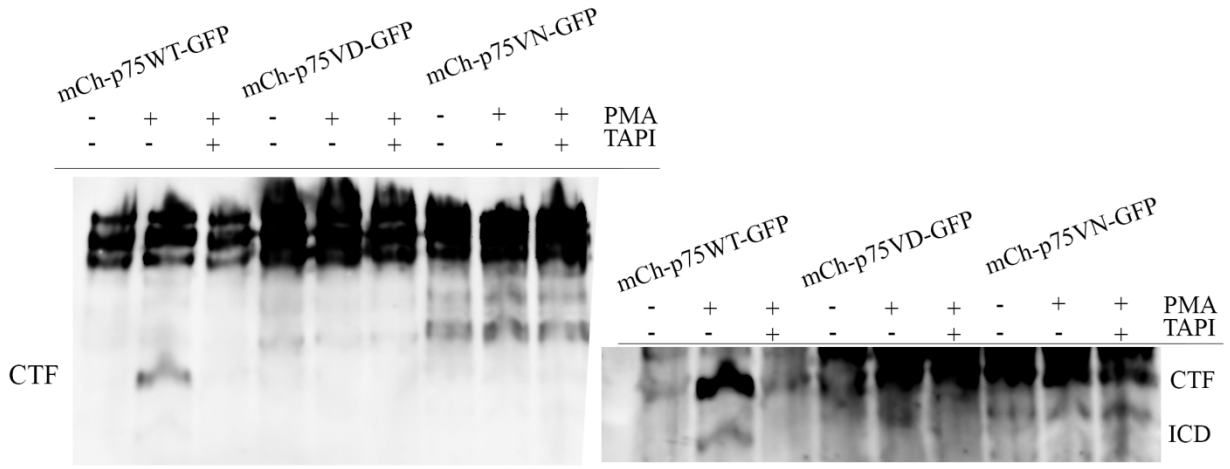

Figure 3.3. p75NRT mutants V243N and V243D show a reduction in PMA-induced cleavage. Western blot showing the cleavage of mcherry-p75-GFP wildtype (mCh-p75WT-GFP), V243D mutant (mch-p75VD-GFP) and V243N mutant (mch-p75-GFP) transfected Hek293 cells treated with PMA and/or TAPI as indicated. Shown here are the full length, c-terminal fragment (CTF) and ICD of p75NTR. On the right is a darker exposure of the Western blot shown to the left, for better visualization of ICD band.

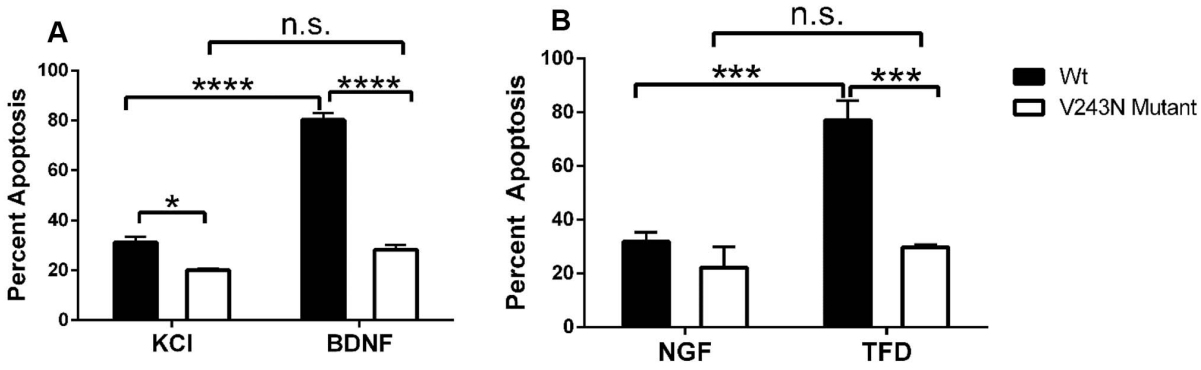

Figure 3.4. Expression of cleavage resistant V243N mutant p75 prevent neuronal apoptosis induced by BDNF (A) or trophic factor deprivation (TFD) (B). Sympathetic neurons were electroporated with either wildtype or V243N mutant mCherry-p75-GFP construct and cultured in NGF. The neurons were then treated with KCl +/- 200 ng/ml BDNF, maintained in 20ng/ml NGF or NGF was removed. After 48 hrs, pyknotic nuclei were quantified in neurons fixed and stained for

TUJ1 and DAPI. The bars depict the means ± SEM for n=3-5; ∗, p< 0.05, ∗∗∗, p < 0.001, ∗∗∗ ∗, p < 0.0001 2-way ANOVA with a Sidak's and Tukey's multiple comparisons test among indicated groups.

Deacetylation of p150^{Glued} enhances its interaction with dynein

Class I HDACs, including HDAC1, primarily localize to the nucleus (Cho and Cavalli, 2014); however, it was recently reported that HDAC1 can translocate into axons following injury (Kim et al., 2010). We hypothesized that HDAC1 also plays a role in p75NTR-mediated cell death. The presence of HDAC1 in uninjured sympathetic axons was confirmed in both cultured SCG neurons and *in vivo* in isolated nerves (data not shown). Notably, inhibition of HDAC1 using the selective inhibitor, MS275, was sufficient to block p75NTR-ICD retrograde trafficking when added exclusively to distal axons (Figure 3.5). Furthermore, addition of MS275 to the distal axons, but not the cell bodies, prevented cell death induced by BDNF (data not shown). Similarly, knockdown of HDAC1, using a lentivirus that reduces HDAC1 expression but not any of the other HDACs (Kim et al., 2010), also blocked p75NTR-mediated apoptosis (data not shown).

To determine the molecular substrates that may be deacetylated upon NGF-withdrawal or BDNF treatment, sympathetic neurons were grown in the presence of NGF and MS275, to maximize acetylation. The axons were collected, lysates immunoprecipitated with anti-acetyl lysine and the precipitates analyzed by mass spectrometry. Of the approximately 100 putative substrates, one candidate that was of peak interest to us was dynactin1/p150^{Glued}. We hypothesized that HDAC deacetylation of p150^{Glued} increases retrograde trafficking of p75NTR and leads to apoptosis.

Retrograde signaling typically depends on active transport of the signaling components by the dynein-dynactin complex. To validate that activation of p75NTR could affect this interaction in neurons, we treated the neurons with BDNF, to stimulate deacetylation of $p150^{Glued}$, or NGF + MS275, to maximize acetylation. In the presence of BDNF there was a significant increase in the co-immunoprecipitation of $p150^{Glued}$ and DIC compared to NGF + MS275, supporting a role for HDAC1 in regulating the interaction and subsequent pro-apoptotic signaling (Figure 3.6).

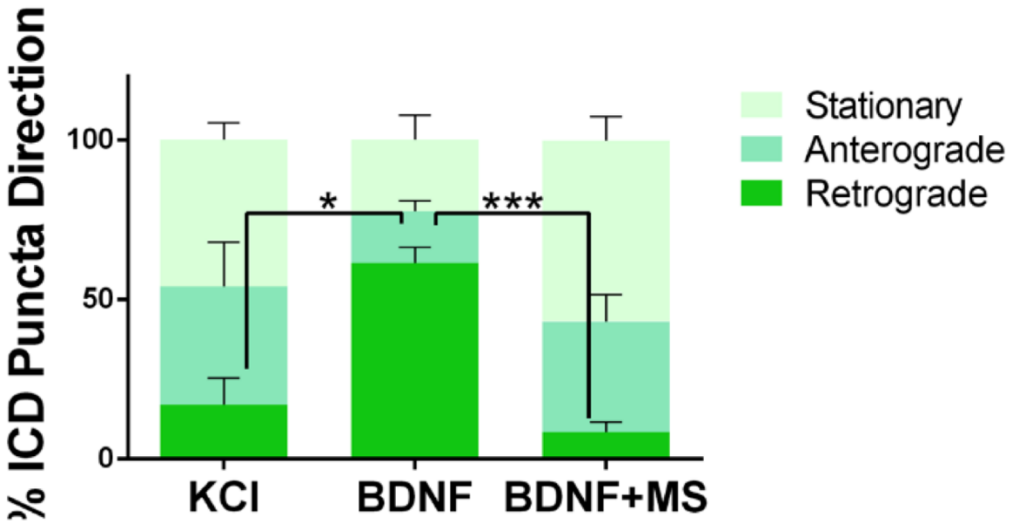

Figure 3.5. HDAC1 activity is required for retrograde transport of the intracellular domain of p75NTR. Quantification of moving and stationary p75NTR ICD and directionality of traffic in neurons cultured in KCl, BDNF (200ng/ml) or BDNF+MS275 (5 uM). KCl vs BNDF, $p<0.05$; BDNF vs BDNF+MS, $p<0.001$; NGF vs TFD, $p<0.05$; TFD vs TFD+MS, $p<0.05$. **Figure courtesy of Amrita Pathak**

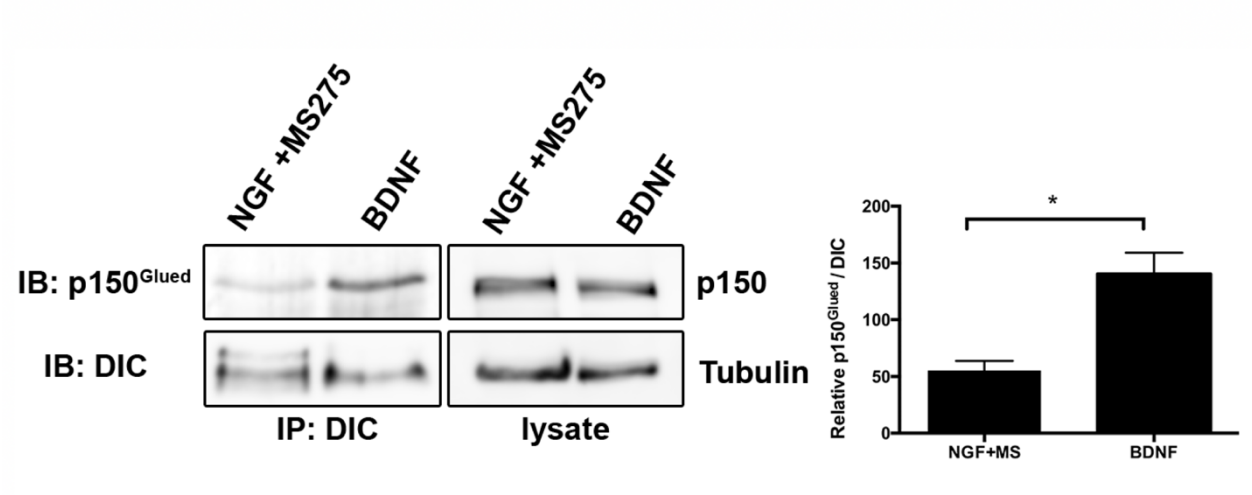

Figure 3.6. Activation of p75NTR enhances the interaction of $p150^{Glued}$ with dynein. (Left) Neurons were treated with 200 ng/ml BDNF or 20 ng/ml NGF + 5 □M MS275 for 48 hrs. The neurons were then lysed, and immunoprecipitated with an antibody to DIC and western blotted for $p150^{Glued}$ or DIC. (Right) The average ratio of p150Glued pulled down with DIC to total DIC in the lysates ± SEM for n=3; ∗, $p < 0.05$, student's t-test.

3.4 Discussion

In this study, we identified a novel role for HDAC1 in the formation of the p75NTR retrograde signaling complex, which is required for both BDNF-induced and NGF-withdrawal-induced apoptosis in sympathetic neurons. Additionally, we have shown that retrograde transport of the p75NTR-ICD is necessary to induce apoptosis. My specific goals for this project were 1) to create p75NTR mutant constructs that were unable to be cleaved by TACE and 2) to confirm mass spectrometry data that $p150^{Glued}$ deacetylation by HDAC increases its association with dynein to form the retrograde apoptotic signaling complex.

Retrograde signaling following deprivation of neurotrophic factors has only recently been recognized (Ghosh et al., 2011; Simon et al., 2016); however, the nature of this signal has yet to be studied. The involvement of p75NTR in retrograde pro-apoptotic signaling following NGF-withdrawal corroborates previous findings that apoptosis of sensory and sympathetic neurons in *trkA-/-* mice can be rescued by simultaneous deletion of *p75ntr* (Majdan et al., 1997; Nikoletopoulou et al., 2010). We hypothesize that the elimination of p75NTR would attenuate pro-apoptotic signaling.

The proteolysis of p75NTR by γ-secretase has been reported to occur in endosomes following internalization in PC12 cells (Urra et al., 2007). Therefore, we predicted that the p75ICD may be generated on endosomes within the axon that are then trafficked back to the cell soma. In a previous study in the chick visual system, retrograde p75NTR transport and cell death correlated with the accumulation of NGF in cell somas. Notably, the retrogradely transported NGF localized to multivesicular bodies (MVBs) (Butowt and Bartheld, 2009). Further,

p75NTR has also been detected in MVBs following endocytosis in PC12 cells (Escudero et al., 2014). These results suggest that retrograde transport of p75NTR and perhaps even receptor proteolysis may occur within MVBs. In results not shown here, we found that NGF withdrawal or BDNF treatment increased p75NTR ICD colocalization with the MVB marker CD63 in axons, suggesting that these vesicles transport p75NTR ICD back to the cell body (Pathak et al., submitted).

We show in this study through a combination of mass spectrometry and biochemical validation that HDAC1 acts in neuronal axons to promote p75NTR ICD retrograde transport and cell death. Specifically, HDAC1 can deacetylate $p150^{Glued}$ after BNDF treatment to increase its association with the retrograde transport motor complex. The nature of how this post-translational modification alters the association of $p150^{Glued}$ with other proteins has yet to be studied. Additionally, our mass spectrometry data on proteins that show enriched acetylation after HDAC inhibition revealed dozens more putative substrates for HDAC1. Future work should focus on elucidating whether any affect retrograde trafficking and p75NTR-mediated cell death. This may reveal additional novel insights into p75NTR retrograde trafficking and the regulation of neuron apoptotic signaling. Gaining a complete understanding of how p75NTR cleavage, in response to ligand and non-ligand mediated receptor activation, and how the retrograde signal is produced and transported may reveal novel molecular components in this process. Given the proposed role of p75NTR signaling and trophic factor deprivation in many neurodegenerative conditions, further elucidation of the mechanisms involved in these processes will have important implications for an understanding and treating a variety of neuropathologies.

4. Discussion

4.1 Limitations and future directions

The data provided in Chapters 2 and 3 provide novel and exciting insights into the regulation of p75NTR trafficking in sympathetic neurons. Now that we have a better understanding of this molecular process, we can address the limitations of the current study and how future experiments can strengthen our observations.

Our data indicate that sympathetic neurons respond to NGF by upregulating p75NTR surface expression through Arf6 activation in order to amply NGF signaling and reduce NT3-TrkA signaling. To complete this study, we utilized primary sympathetic neurons isolated from perinatal rodents. While they never received NGF *in vitro* prior to our experimental timepoints, postnatal neurons have already reached NGF-producing targets *in vivo*, prior to isolation. Unfortunately, collecting embryonic sympathetic neurons prior to their exposure to NGF is technically not feasible. This introduces a new level of complexity to the study as well as new questions that need to be addressed. We observed low surface expression of p75NTR in neurons kept in the absence of NGF under depolarizing conditions (Figure 2.1). The minimal levels of p75NTR at the plasma membrane suggest that in the time between isolation from the animal and our experimental timepoints, previously surface-residing p75NTR is internalized and stored in vesicles that are trafficked to the cell surface upon acute NGF treatment. Alternatively, p75NTR that was at the surface may be endocytosed and degraded

in the absence of NGF and newly synthesized p75NTR is stored until NGF activates its movement to the plasma membrane. While our findings represent a model for sympathetic neurons first reaching NGF-producing targets, investigating this *in vivo* would give us a better understanding of this process.

There are several ways this could be addressed experimentally. While *ngf*$^{-/-}$ sympathetic neurons do not survive target innervation and are completely absent by post-natal day 14 (Crowley et al., 1994), *ngf*$^{-/-}$*; bax*$^{-/-}$ mice*,* have larger than wildtype SCGs (Glebova and Ginty, 2004), due to the role Bax plays in normal developmental apoptosis. Culturing sympathetic neurons from *ngf*$^{-/-}$*; bax*$^{-/-}$ mice*,* would allow us to study truly NGF naïve neurons and investigate p75NTR trafficking, only when neurons are exposed to NGF for the first time in culture. Repeating our experiments using these neurons would illuminate how this process happens without prior exposure NGF. If our results differ, it would indicate that perhaps the previously surface-residing p75NTR was internalized and stored in GGA3+ vesicles when maintained in the absence of NGF, only to be shuttled to back to the surface upon NGF exposure. Alternatively, surface p75NTR may be degraded after dissection and the p75NTR that is trafficked to the cell surface comes from a separate pool.

To investigate this process *in vivo* would require closely investigating embryos at various early developmental time points around the time when sympathetic target innervation occurs. Using precise sectioning and immunofluorescence techniques, it is possible to capture a snapshot of growing sympathetic axons toward their target tissues and measure p75NTR levels at the surface of these growth cones as well as examining where p75NTR is localized

intracellularly before it is shuttled to the surface. Since live p75NTR immunostaining on unpermeablized growth cones *in vivo* would be technically challenging, super resolution microscopy may be necessary to dissect the precise subcellular localization of the full-length receptor. Simultaneous detection of various membrane-localized and intracellular markers would aide in measuring *in vivo* p75NTR trafficking. This would verify that this is a mechanism occurring during sympathetic nervous system development to ensure proper target innervation. Due to the complexity and density of the early rodent embryos, it may be necessary to use lineage tracing techniques to fluorescently label only a subset of growing axons in order to more easily visualize their location and morphology *in vivo.*

Another limitation of this study arises from our lack of understanding regarding the role that neuron depolarization plays in neurotrophin receptor trafficking. In a study by Ascano et al, it was reported that TrkA can be inserted into growth cones within minutes of NGF treatment (Ascaño et al., 2009). In the absence of NGF, neurons in this study were kept alive by inhibiting caspase activation. In contrast, our data suggest that maintaining neurons in mildly depolarizing conditions with KCl leads to considerable TrkA surface expression (Figure 2.4). It is possible that KCl, similar to its effect on TrkB surface expression (Meyer-Franke et al., 1998; Du et al., 2000), induces up regulation of TrkA in neurons, but this has yet to be studied. If indeed depolarization leads to up regulation of TrkA surface expression in our culture conditions, it would be interesting to define the mechanism by which this occurs. One hypothesis is that electrical activity in developing neurons upregulates TrkA surface expression in

order to ensure NT-3 mediated outgrowth signaling. Neurons that have not yet established proper dendritic arbors and presynaptic connections may be fated to undergo apoptosis and, therefore, would not require TrkA surface expression. Meyer-Franke et al (1998) showed that TrkB was rapidly trafficked to the surface of peripheral neuron subtypes after depolarization in a transcriptional independent manner through activation of type-1 adenylyl cyclase (Meyer-Franke et al., 1998). It has also been shown that depolarizing conditions can increase transcription of TrkA, but not p75NTR in a sympathoadrenal-derived cell line over the course of several days. Whether previously translated and stored TrkA is regulated in a similar fashion to p75NTR remains to be studied.

The neurotrophic “switch” from NT3 to NGF responsiveness is due to the formation of TrkA-p75NTR heterodimers that increase TrkA’s affinity for NGF (Hempstead et al., 1991). How these complexes form *in vivo* is unknown. It is unclear even whether the formation is an active process that requires intracellular signaling or if simply co-expressing the two receptors at the cell surface is sufficient. Recently, it has been determined that TrkA is recycled in neurons in response to its own activation, through activation of Arf6 (Li et al., 2015). This study showed that direct interaction between the cytoplasmic tail of TrkA with GGA3 promotes recycling over degradation of the receptor to prolong NGF signaling. Our data indicate that reserve pools of p75NTR are colocalized with GGA3+ vesicles in neurons prior to NGF-induced membrane trafficking (Figure 2.6). Therefore, it remains a possibility that these pathways converge and GGA3 may play a role in proper complex formation. We have shown p75NTR-GGA3 co-localization prior to NGF treatment, while Li, et al (2015) showed TrkA-GGA3

interaction after NGF treatment. Live imaging of neurons immediately after acute NGF treatment and precise localization of p75NTR, TrkA, and GGA3 over time may reveal novel insights into this rapid and dynamic process and provide insight as to how high affinity NGF receptors are formed.

TrkA activation by NGF causes a transcriptional up regulation of p75NTR in sympathetic neurons and neuroblasts (Miller et al., 1994; Verdi and Anderson, 1994; Wyatt and Davies, 1995). However, we showed that acute NGF treatment caused an upregulation of p75NTR at the cell surface even when translation was blocked with cycloheximide, suggesting that newly synthesized p75NTR does not account for the observed increase in surface expression (Figure 2.1). We show in Chapter 2, that intracellular stores of p75NTR exist in neurons, contained in GGA3+ vesicles, and that acute NGF treatment induces rapid trafficking to the cell surface (Figure 2.6). Since adjacent neurons are competing for limited trophic support at the sympathetic target tissues and since NGF binds to TrkA-p75NTR dimers with 100 times more affinity, it is likely that *in vivo,* a neuron that fails to upregulate p75NTR surface expression would be outcompeted. Future studies could be designed to explore the survival of sympathetic neurons when p75NTR upregulation is blocked in just a few neurons. I hypothesize that the neurons lacking p75NTR upregulation would die due to being outcompeted for the limiting amounts of NGF. Of course, this analysis would be complicated by the fact that p75NTR also plays a role in sympathetic development by actively inducing apoptosis when activated in the absence of TrkA, for example, by BDNF.

It would also be interesting to investigate the route of p75NTR trafficking in the presence to NGF. Once TrkA retrograde signaling endosomes reach the soma

to initiate p75NTR transcription, is new protein shuttled directly to the membrane or does all p75NTR get trafficked through GGA3+ vesicles and surface expression regulated through Arf6 activation?

The data presented in Chapter 2 indicate that while TrkA activation can upregulate p75NTR surface expression through activation of Arf6, but activation of Arf6 alone is sufficient to increase p75NTR trafficking (Figure 2.4). This represents a novel role for Arf6 in mediating p75NTR membrane expression. It remains unknown whether Arf6 activation can shuttle p75NTR to the cell surface in TrkA-negative cell types. TrkA can activate Arf6 through PI3K (Figure 2.5), as well as through Src (Li et al., 2015). Both can be activated by a variety of other kinases and kinase receptors. I hypothesize that activation of Arf6 represents a novel mechanism for promoting p75NTR surface expression, even in cells not expressing TrkA.

A subset of ventricular-subventricular zone (V-SVZ) stem cells in adult rats express p75NTR. p75NTR positive neural stem cells persist into adulthood and respond to both BDNF and NGF to promote generation of daughter neuroblasts and mature neurons both *in vitro* and *in vivo* (Young et al., 2007b). The p75NTR positive neural precursor cells (NPC) were highly proliferative as compared to p75NTR negative cells, which never formed neurospheres in culture. p75NTR-expressing cells responded to BNDF to increase NPC proliferation and generate an increased number of neurons. The authors propose this as a mechanism by which BDNF, which increases in the brain after exercise, can promote neurogenesis by activating p75NTR-expressing cells to divide (Young et al., 2007b). The study explicitly showed that TrkA mRNA was not present in any

population of neural stem cells, and in p75NTR-expressing cells neither TrkB or TrkC mRNA was detected. This suggests that BDNF's effect on NSC activation is not to do its action on TrkB-p75NTR heterodimers, but on p75NTR directly (Young et al., 2007b). The study did not investigate whether p75NTR surface availability had any effect on BDNF or NGF responsiveness. Thus, this system may provide an interesting model to study whether p75NTR surface expression and therefore neurogenic capacity can be altered through Arf6 activation, in the absence of TrkA. Young et al. (2007) used FACS on live, unpermeablized cells to isolate populations of p75NTR-expressing cells, therefore it is more accurate to say p75NTR *surface-expressing* cells. They found a gradient of expression levels, but a positive correlation between p75NTR *surface* expression and neurogenic capacity and sphere formation after exposure to NGF and BDNF. If our current study identified a novel molecular player involved in surface trafficking of p75NTR in all cells type, it would be worth pursuing whether Arf6 activation in neural stem cells can increase V-SVZ neurogenesis. This could be assessed by ectopically expressing the Arf6Q67L constitutively active construct in p75NTR positive NPCs and measuring changes in p75NTR surface expression as well as sphere forming and neurogenic capacity.

4.2 Significance

A full understanding of p75NTR trafficking in sympathetic nervous system development and beyond has broad clinical implications for various disease states. Recently, a functional polymorphism of p75NTR in humans was reported to increase risk of major depressive disorders and cardiac disease through vagal

autonomic dysfunction (Chang et al., 2015). Human subjects homozygous for this polymorphism showed significantly reduced vagal activity compared to control subjects. Reduction in vagal output is correlated with coronary heart disease, myocardial infarction, and congestive heart failure (Chang et al., 2015). Understanding the mechanism by which this polymorphism alters p75NTR function and expression in the human autonomic nervous system would be a useful area for future studies.

Another example of how the regulation of p75NTR expression, trafficking and activation may have an impact on human health is related to the recently reported role of p75NTR in the regulation of glucose uptake and insulin resistance (Li et al., 2012). $p75NTR^{-/-}$ mice showed an increase in insulin sensitivity and specifically depleting p75NTR in adipocytes or myoblasts caused an increase in glucose uptake induced by insulin stimulation. Additionally, glucose transporters GLUT4 in $p75NTR^{-/-}$ cells were aberrantly trafficked to the cell membrane after glucose exposure. Of particular relevance to our study, p75NTR appears to interact directly with GTPases Rab5 and Rab31 to regulate GLUT4 trafficking and glucose sensitivity (Li et al., 2012). Understanding how p75NTR mediates this trafficking and whether p75NTR trafficking itself is involved in this process could reveal novel therapeutic targets for treating Type 2 diabetes and insulin resistance.

Finally, it is important to point out that our studies in Chapter 3, on p75NTR retrograde apoptotic signaling, have significant implications for understanding a variety of neurodegenerative conditions. A role for p75NTR has been suggested in a wide variety of neuropathologies, including Alzheimer's disease (Chao, 2016), amyotrophic lateral sclerosis (ALS) (Chao et al., 2006), stroke (Irmady et al., 2014)

and degeneration after spinal cord injury (Beattie et al., 2002). It is likely that active retrograde degenerative signaling involving p75NTR contributes to many of these conditions. For example, a number of recent studies have suggested that there is apoptotic retrograde signaling in some forms of ALS (Siu et al., 2018). Moreover, the p75NTR ICD was detected in a retrograde dynein complex in the SOD1 (G93A) model of ALS (Perlson et al., 2009). I suggest that our study on p75NTR ICD retrograde trafficking could serve as a model system for understanding the mechanisms involved in such neurodegeneration. Future studies should be aimed at exploring the function of p75NTR trafficking and the role of HDAC1 in such pathologies.

Appendix I.

Loss of Jedi-1 results in increased proliferation of neural progenitor cells

Abstract

We previously identified Jedi-1 as an engulfment receptor necessary for clearance of apoptotic sensory neurons in the dorsal root ganglia during normal development (Wu et al., 2009b). Its role in the central nervous system, however, has not been investigated. We found Jedi-1 mRNA expressed in neural progenitor cells (NPCs) derived from the ventricular-subventricular zone (V-SVZ), one of the two niches where neurogenesis continues during adult life. Interestingly, NPCs derived from perinatal *jedi-1*$^{-/-}$ mice exhibited a significant increase in proliferation relative to wild type, as measured by short term BrdU incorporation. Most proliferating cells were GFAP^{+} and Nestin^{+}, common markers of early NPCs. Additionally, NPCs derived from *jedi-1*$^{-/-}$ mice produced significantly more oligodendrocytes after 14 days of mitogen withdrawal. As previously reported (Lu et al., 2011), we found that NPCs have the ability to engulf carboxylated microspheres. However, preliminary data indicate that *jedi-1*$^{-/-}$ NPCs do not have a deficit in engulfment capability, suggesting that Jedi-1 regulates proliferation and differentiation of these cells through an alternate mechanism. Current experiments are focused on determining the mechanistic basis for Jedi-1's effects on proliferation and differentiation *in vitro* and *in vivo.*

Introduction

Programmed cell death (PCD) is a normal part of mammalian nervous system development. It is used as a way to establish proper cell numbers and connectivity as well as eliminating damaged or defective cells (Buss et al., 2006). In the central nervous system, PCD is observed throughout proliferation, migration, axon guidance, and synaptogenesis. It is estimated that up to 50% of all neurons born will undergo apoptosis (Burek, 1996); these cells must be cleared efficiently to prevent a potentially damaging, inflammatory response. While much of development occurs embryonically, cell death continues in the postnatal brain and therefore mechanisms of cell clearance must persist into adulthood. One role of cell elimination is to reduce excess neural progenitors. Apoptosis of progenitor cells has been shown to occur in the postnatal cerebellum and in the subventricular zone in order to limit the numbers of proliferating cells and influence brain size (Blaschke et al., 1996). A second wave of cell death occurs in post-mitotic neurons during the process of synaptogenesis to eliminate superfluous innervation (Gohlke et al., 2004).

It is now well established that neural stem cells (NSCs) are present in the adult mammalian brain and have a role in regulating cognition. In the adult, new neurons can arise from neural stem cells found in the ventricular-subventricular zone (V-SVZ) lining the ventricles and in the subgranular zone (SGZ) of the dentate gyrus of the hippocampus (Bond et al., 2015). By definition, these stem cells are self-renewing and can generate multiple lineages, including neurons, astrocytes and oligodendrocytes. In rodents, the NSCs in the V-SVZ generate neural precursor cells (NPCs) that give rise to olfactory bulb interneurons (Lim and

Alvarez-Buylla, 2016), which are important for olfactory learning and memory (Lepousez et al., 2013; Sakamoto et al., 2014). In humans, the degree of newly generated neurons in the V-SVZ is highly controversial but appears to decrease significantly after birth (Sorrells et al., 2018). However, there is evidence for migration of adult neuroblasts to the olfactory bulb, prefrontal cortex or striatum (Sanai et al., 2011; Ernst et al., 2014). Within the mammalian V-SVZ, there are subdomains of NSCs that generate specific progeny. For example, dorsal, Pax6-positive NSCs generate more superficial granule cells in the olfactory bulb, while the ventral Nkx2.1-expressing NSCs become deep granule cells (Toresson et al., 2000; Merkle et al., 2007; Young et al., 2007a; Xu et al., 2008; Merkle et al., 2014). There are 4 types of cells in the V-SVZ neurogenic niche, the ependymal cells, lining the ventricle, as well as type A, B and C cells. The B cells are the stem cells, which are self-renewing, divide very slowly and asymmetrically and give rise to transiently-amplifying type C cells. The type C cells generate type A cells, which are neuroblasts (Doetsch et al., 1999).

A variety of secreted factors have been identified that modulate proliferation of NSCs, including morphogens, growth factors, neurotransmitters and cytokines (Gonçalves et al., 2016). Many factors that promote neuron generation have also been identified *in vivo*, including glutamate and BDNF (Mu et al., 2010). In contrast, there is little known about what promotes oligodendrogenesis from NSCs. Sonic hedgehog (Shh) signaling has been shown to modulate the formation of oligodendrocytes (OLs) from NSCs following demyelination in a mouse model of MS (Samanta et al., 2015). Shh is required for the positional identity of NSCs in the ventral V-SVZ and their maintenance (Lai et al., 2003; Machold et al., 2003;

Ahn and Joyner, 2005; Ihrie et al., 2011). However, at early postnatal ages, there is a transient expression of Gli1, a downstream effector of Shh, in the dorsal V-SVZ and these NSCs generate large numbers of OLs (Tong et al., 2015). In the adult, few OLs are normally produced from NSCs; however, following a demyelinating lesion, the Gli1 expressing ventral NSCs migrate into the lesioned area, down regulate Gli1 and differentiate into OLs (Samanta et al., 2015). The down regulation of Gli1 is necessary for new myelin to form. Thus, NSCs responding to Shh can generate OLs, but this signal must be blocked for mature OLs to form. The receptor Notch also regulates NSC proliferation by inhibiting differentiation of the NSCs, thereby keeping them dividing. In the absence of Notch signaling, there is an initial increase in NSC proliferation, due to differentiation into transient amplifying cells, but then a decrease due to depletion of the type B NSCs (Ehm et al., 2010).

Similar to what is observed during neurodevelopment in both central and peripheral tissues, many of the newborn NSCs in these germinal zones die before incorporation into the circuitry (Ma et al., 2009). Therefore, efficient and robust mechanisms for apoptotic cell clearance must exist to ensure that dying neural precursors don't become necrotic and trigger a detrimental inflammatory or autoimmune response. Unchallenged microglia present in the SGZ of mice rapidly and efficiently clear dying neural progenitors via phagocytosis. This clearance mechanism persists into adulthood, even when the levels of neurogenesis have drastically declined (Sierra et al., 2010). Surprisingly, DCX+ neuroblasts in the SGZ and V-SVZ were recently identified as an additional cell type capable of phagocytosis during adult neurogenesis. The engulfment receptor adaptor protein

ELMO was required for efficient DCX+ cell-mediated engulfment and when blocked, either pharmacologically or genetically, apoptotic precursors accumulated. Along with this decrease in phagocytosis, an increase in TUNEL+ apoptotic cells as well as a decrease in newly born neuroblasts was observed, suggesting a link between efficient apoptotic cell clearance and the generation of new neurons (Lu et al., 2011).

Our lab has identified Jedi-1 expression in V-SVZ derived NPCs. Jedi-1 was initially identified as an engulfment receptor expressed by satellite glial cells in the dorsal root ganglia (DRG) and was shown to be necessary for the clearance of apoptotic sensory neurons during neurodevelopment (Wu et al., 2009b). Jedi-1 is an engulfment receptor characterized by an extracellular domain containing multiple EGF repeats, a single transmembrane domain, and an intracellular domain with several signaling motifs such as ITAMs and NPXY (Scheib et al., 2012). Jedi-1 is homologous to engulfment receptors Ced-1 and Draper, found in *Caenorhabditis elegans* and *Drosophila,* respectively (Figure A1). Jedi-1's ability to mediate engulfment of apoptotic targets is dependent on several intracellular signaling cascades. While an extracellular ligand for Jedi-1 has not been identified, receptor internalization and signal propagation are consistently induced by exposure to apoptotic targets. Previous work done in our lab has shown that several members of the Src family of kinases phosphorylate conserved immunoreceptor tyrosine-based activation motif (ITAM) motifs on Jedi-1 and recruit Syk to act as a signal transducer required for engulfment (Scheib et al., 2012). Further analysis indicated that Jedi-1 interacts with adaptor protein GULP through a conserved NPXY motif. GULP acts as an adaptor between Jedi-1 and

clathrin heavy chain (CHC) to promote internalization through actin cytoskeletal rearrangement. Phosphorylation of the clathrin heavy chain is required for engulfment and actin rearrangement. It has been previously shown that CHC can be phosphorylated by Src family kinases during internalization. Syk recruitment to activated Jedi-1 after apoptotic cell binding could directly phosphorylate CHC and promote its association with actin, which directly facilitates engulfment (Scheib et al., 2012; Sullivan et al., 2014) (Figure A1). While the role of Jedi-1 in PNS development is still being fully elucidated, its role in the central nervous system has not been studied.

The goal of this project was to determine if Jedi-1, required for the engulfment of apoptotic sensory neurons, is involved in cell clearance in the adult V-SVZ and if its loss can alter the proper development and progression of NPCs. We initially hypothesized that Jedi-1 regulates the engulfment of apoptotic cells by NPCs in the CNS during development and in adult neurogenesis, thereby promoting proliferation of NPCs.

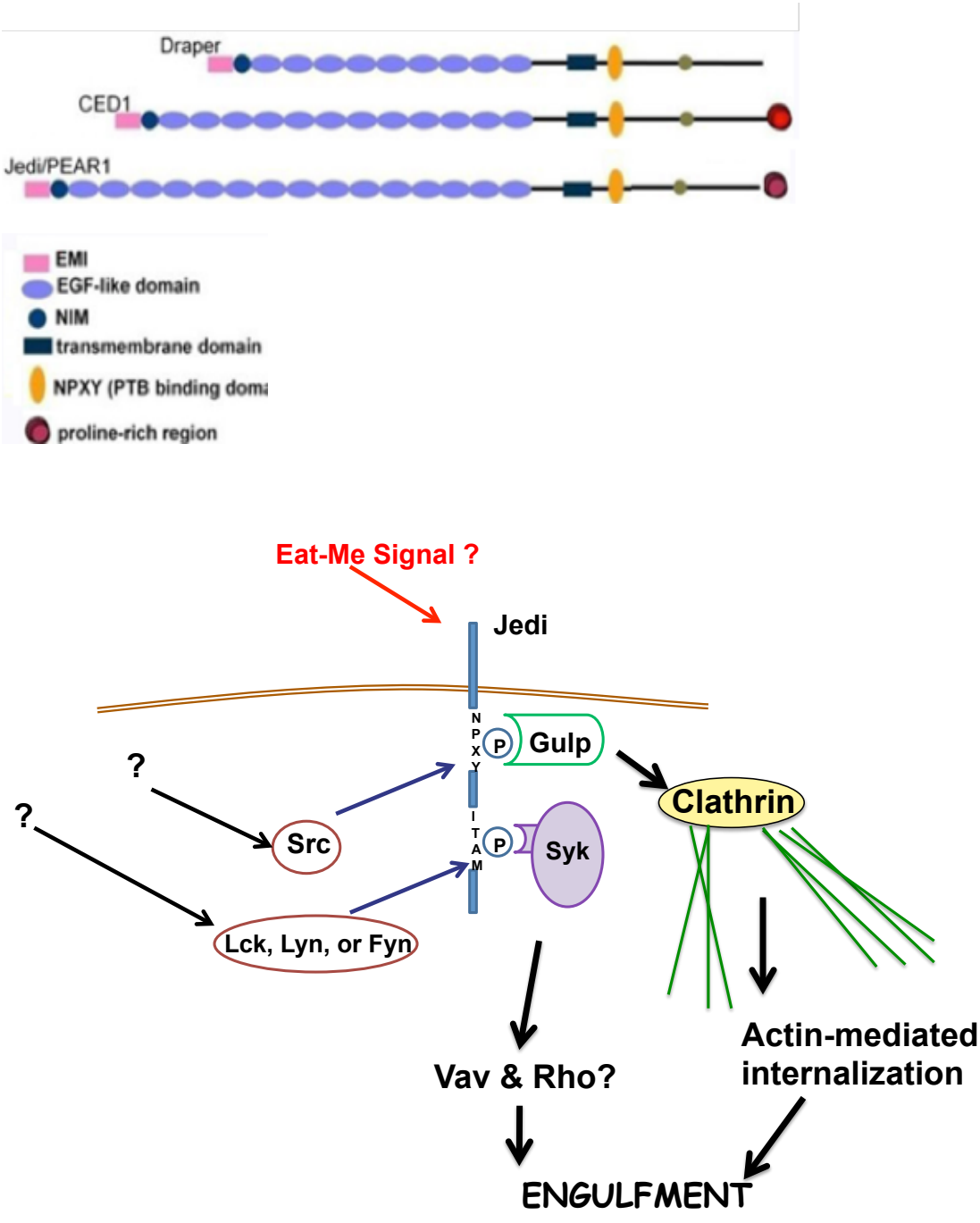

Figure A1. Jedi-1 is homologous to CED1 and Draper (upper panel). Jedi-1 activation by a still unknown “eat-me” signal induces phosphorylation of intracellular NPXY and ITAM motifs, which leads to apoptotic cell engulfment (lower panel).

Methods

Animals - All experiments with animals were approved by the Animal Care and Use Committee at Vanderbilt University. C57BL/6J mice were used as wildtype controls and Jedi-1 null embryonic stem cells were obtained from the KOMP Repository (Pear1$^{tm1a(KOMP)Wtsi}$) (Figure A2). The stem cells were injected into C57BL/6 blastocyst by the Vanderbilt Transgenic Mouse Shared Resource and chimeras bred to wild type and progeny carrying the Jedi-1 null allele were crossed to generate mice homozygous for the null allele (referred to from here on as *jedi-1*$^{-/-}$). Breeders were maintained on a normal fat diet and the genetic background of progeny was confirmed by PCR of genomic DNA.

Figure A2. Diagram illustrating the construct used to create the *jedi-1* null allele. Image from www.Komp.org, adapted by Alexandra Trevisan.

RT-PCR – Total RNA was obtained from tissue or cell samples using Trizol. RNA was purified using RNeasy columns (Qiagen # 74104). Random hexamers were used to generate cDNA and PCR was completed using the following primers:

JediFoward: CCTGCAGCTGCCCACCGGGCTGGA

JediReverse: CCTGGCAGCCCGGGCCATGCGTGT

PCR settings were: 94°C for 5 min, then 30 cycles of 94°C for 30 seconds, 69°C for 30 seconds, 72°C for 40 seconds, followed by 72°C for 10 minutes. Product

size (509bp) was confirmed by running the samples on a 2% agarose TAE gel.

Neurosphere culture- To generate perinatal V-SVZ neurospheres, at least 4 pups aged p4-6 were pooled from either WT or *Jedi-1*$^{-/-}$ mice. After total brain isolation, the V-SVZ region was microdissected under a dissection microscope to isolate the tissue proximal to the lateral ventricles. The pooled tissue was dissociated using Neurocult Enzymatic Dissociate Kit (need product) and then plated in stem cell media containing 20 ug/ml EGF, 10ug/ml bFGF, and 0.02% heparin to promote proliferation. Cells were grown in sterile 10cm^2 non-coated Petri dishes to discourage cells from adhering and kept in a tissue culture incubator at 37°C and 5% CO_2. Cells and/or spheres in suspension were spun down at 700rpm every other day to complete a half media change.

Neural precursor cell differentiation – To differentiate NSCs into mature progeny, neurospheres were gently dissociated using the Neurocult Chemical Dissociate Kit until a single cell suspension was achieved. 50,000 cells were seeded onto coverslips placed into 24-well plates coated overnight with poly-d-lysine and for 2 hours with laminin. To differentiate the cells, they were plated into neurobasal media containing 1% FBS, N2 supplement, and 2 mM L-glutamine. For long term experiments, half the media was changed every other day. Plates were kept at 37°C and 5% CO_2. To quantify the number of differentiated progeny, cells were immunostained with anti-GFAP, anti-DCX (Cell signaling, 1:1000), and anti-04 (Millipore MAB345, 1:100). Nuclei were visualized with DAPI.

Phagocytosis assay – For engulfment assays with NPCs, cells that had been maintained in differentiation media for 1 day, were placed into differentiation media containing fluorescently-labelled, carboxylated 2 um microspheres at 1:100 dilution and incubated at 37°C for 3 hours. Cells were washed with PBS containing 1% BSA before fixation in 4% PFA or 10% neutral buffered formalin (NBF) for 15 minutes. NPC coverslips were stained with anti-GFAP (Milipore, 1:500) or anti-DCX (Santa Cruz, 1:50) to visualize putative B cells and A cells, respectively. Z-stacks of cells were imaged using Leica SP5 Confocal microscope to determine if fluorescent beads were completely internalized. Phagocytic index was determined using the following equation: Phagocytic index, PI = (number of beads engulfed ÷ total cells counted) × (number of engulfing cells/total cell counted) × 100).

In vitro BrdU assay - For the BrdU pulse of NPCs, cells that had been maintained in differentiation media for various timepoints, were placed into differentiation media containing 10uM BrdU for 2 hours before fixation with 4% PFA. Fixed cells were treated with 4M HCl for 10 minutes to denature DNA and neurtralized 0.1M sodium borate buffer for 10 minutes before immunostaining with anti-BrdU (BDBioscience, 1:50) at 37°C . Nuclei were counterstained using DAPI (Prolong Gold + DAPI) to assess total cell number.

In vivo EdU pulse – For *in vivo* analysis of NPC proliferation 6 week old C57BL/6 wild type or *jedi-1-/-* mice were injected i.p. with 25mg/kg EdU and sacrificed after 1 hour. After transcardial perfusion with 10% NBF, the brain was isolated and post fixed for 24 hours in NBF for paraffin embedding. Brains were oriented for coronal

sections. 10-micron sections from Paraffin-embedded brains were taken starting from the anterior opening of the lateral ventricles and through the entire V-SVZ niche. After deparaffinization, rehydration, and permeabilization, brain sections were stained using the ClickIt reaction (a copper catalyzed reaction to chemically link the alkyne-linked nucleotide with the azide conjugated fluorophore(Cappella et al., 2008)), and Cy5-azide (Lumiprobe, A3330) to visualize EdU+ cells. DAPI was used to visualize total cells and overall brain morphology. Each EdU+ nucleus from both left and right ventricles was counted from the twenty most anterior V-SVZ sections and normalized to the V-SVZ area.

Microscopy – Bead engulfment assays were visualized using Leica SP5 confocal microscope. Z-stacks were obtained to confirm bead internalization. BrdU incorporation was visualized using a Zeiss Axioskop 2 epifluorescent microscope. For visualization of differentiated NPCs and *in vivo* EdU incorporation, a Nikon Eclipse Ti inverted fluorescent microscope was utilized.

Results

Jedi-1 is expressed in brain and cultured V-SVZ neurospheres

Jedi-1 was initially identified as a putative engulfment receptor expressed by satellite glial cells in the dorsal root ganglia (DRG) necessary for the clearance of apoptotic sensory neurons during development (Wu et al., 2009b). We first sought to determine Jedi-1 expression in mouse brain. Using both RT-PCR and Western blot analysis on total brain tissue, we confirmed expression of Jedi-1 in the brain. Primer and antibody specificity were confirmed using *jedi-1*$^{-/-}$ tissues (Figure A3). Additionally, analysis of lacZ reporter mice, showed expression in brain endothelial cells (data not shown), as previously reported (Nanda, 2005).

To determine whether Jedi-1 is expressed in neural stem cells, neurospheres derived from mouse V-SVZ region were cultured from both wild type and *jedi-1*$^{-/-}$ P4-6 mice and harvested for RT-PCR analysis. Spheres cultured in EGF and FGF-containing media expressed Jedi-1 transcript, as well as NPCs that have been differentiated for 1 day in vitro (1 DIV) in the absence of mitogens (Figure A3).

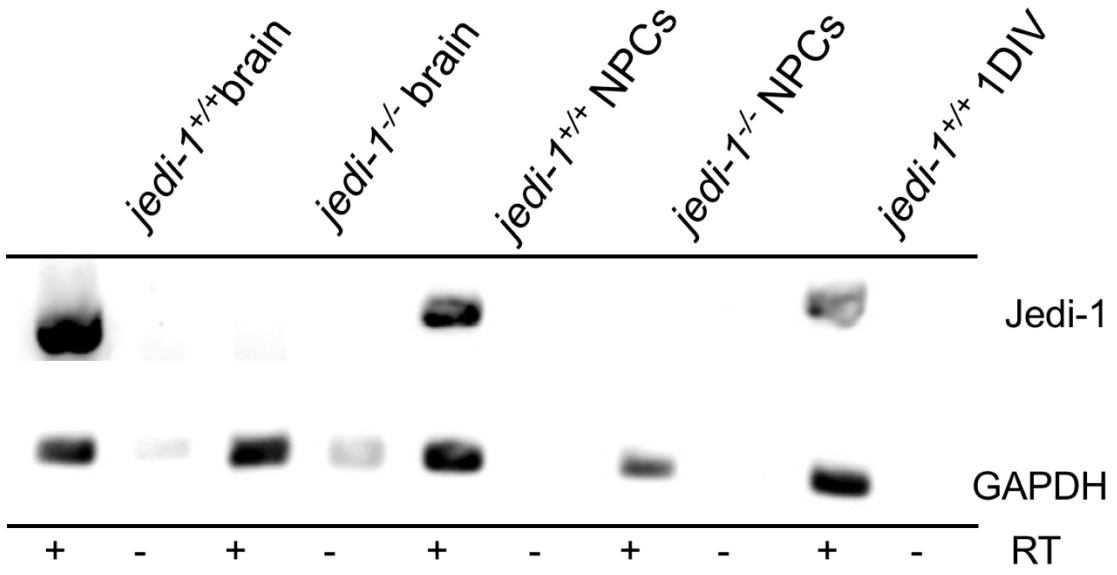

Figure A3. Expression analysis using cDNA obtained from total RNA shows *jedi-1* expression in both total brain, in primary neurospheres and in spheres differentiated for 1 day (1DIV NPCs). *Jedi-1*$^{-/-}$ cells do not express any transcript.

Loss of Jedi-1 does not alter phagocytic capacity of NPCs

Recently, Lu et al. (2011) reported that doublecortin positive (DCX+) cells are capable of engulfing dying neural progenitor cells in both cultured NPCs and within the V-SVZ neurogenic niche, although the phagocytic receptor involved was not identified (Lu et al., 2011). We speculated that due to its expression in total brain and in neural stem cell-enriched neurospheres that Jedi-1, similar to its function in the PNS, may play a role in apoptotic neural precursor cell engulfment. To test this, we cultured V-SVZ neurospheres from wildtype and *jedi-1*$^{-/-}$mice followed by mitogen withdrawal for 1 day to induce differentiation and to force non-adherent cells to settle onto coverslips. Carboxylated fluorescent microspheres, 2 microns in diameter are commonly used as an apoptotic target for *in vitro* engulfment assays (Lu et al., 2011; Sullivan et al., 2014). We added the spheres to

our 1DIV neural precursor cells for 3 hours and assessed engulfment by imaging confocal z-stacks through individual cells. While subtypes of NPCs from both genotypes possessed a moderate ability to engulf these beads, there was no significant difference in engulfment ability between wild type and *jedi-1*$^{-/-}$ cells. We assessed two measurements to quantify engulfment: total percentage of all cells engulfing at least one bead, and phagocytic index, which weights the percentage based on how efficiently cells engulf multiple beads (Figure A4). While Lu, et al (2011) reported that DCX+ neuroblasts were the primary engulfing cell type, we found that GFAP+ neural stem cells engulfed beads more efficiently, and in our culture conditions, DCX+ cells rarely engulfed beads (Figure A4).

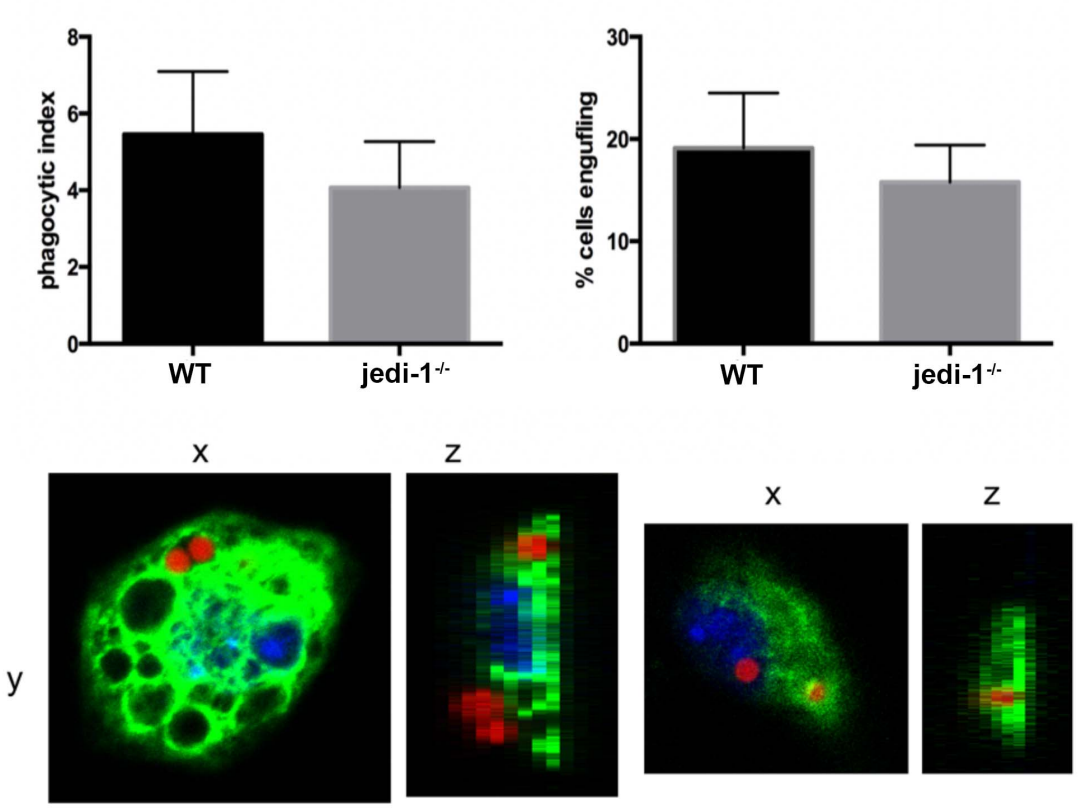

Figure A4. No change in phagocytic index in *jedi-1-/-* NPC when carboxylated microspheres are the target. Primary neurospheres from WT and *jedi-1*$^{-/-}$ P4-6 mice were grown for 7-9 days *in* vitro and dissociated into single cells, plated into differentiation media. After 1 day *in vitro* (1DIV), carboxylated fluorescent microspheres were added for 3 hours. Cells were stained with GFAP and engulfment was scored using confocal microscopy. There was no significant difference between wild type and *jedi-1-/-* in terms of the number of engulfing cells or in the phagocytic index (PI) (n=3 independent experiments). Phagocytic index, PI = (number of beads engulfed ÷ total cells counted) × (number of engulfing cells/total cell counted) ×100). Representative images are depicted below each graph showing GFAP+ cells(green) containing beads in the x,y,z plane.

Loss of Jedi-1causes an increase in NPC proliferation

In addition to the novel observation that neural precursor cells had phagocytic capacity and were able to engulf other dying NPCs both *in vitro* and *in vivo*, Lu et al (2011) also reported that this engulfment was required for proper neurogenesis and an accumulation of dying cells negatively impacted the number of dividing cells in the adult neurogenic niches. We tested whether *jedi-1*$^{-/-}$ NPCs had any differences in proliferative capacity by completing an acute, 2-hour BrdU pulse on NPCs in mitogen free media. Surprisingly, NPCs generated from *jedi-1*$^{-/-}$ mice incorporated BrdU at twice the rate of wildtype cells (Figure A5, p=0.002, n=6 replicates). While all of the cells in culture were counted for the analysis, we noted that the vast majority of the cells at this stage were GFAP+ and Nestin+ neural stem cells (Figure A6), suggesting that loss of Jedi-1 increased the proliferation of neural stem cells.

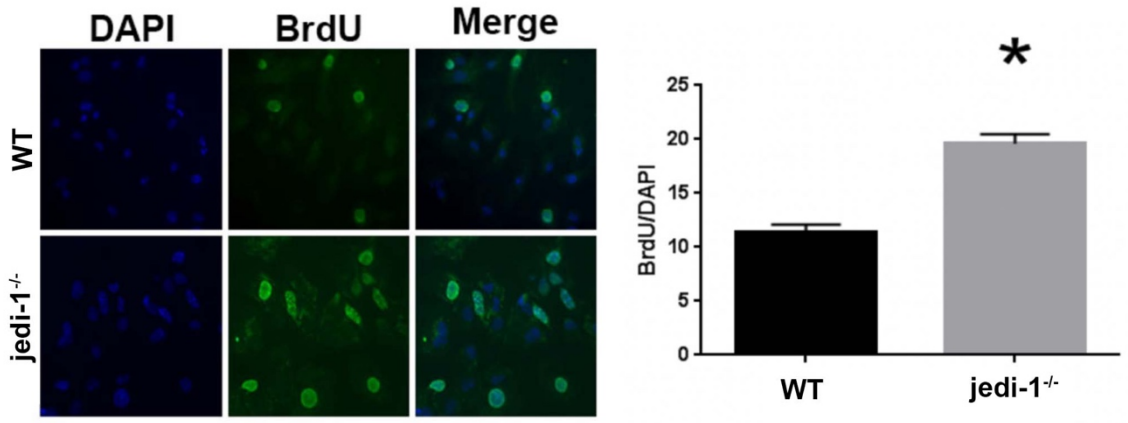

Figure A5. Percentage of proliferating cells compared to total cell population in wild type vs. *jedi-1*$^{-/-}$ NPCs. NPCs from *Jedi-1*$^{+/+}$ and *jedi-1*$^{-/-}$ mice were grown as neurospheres, then dissociated and plated in differentiation media. After 2 days, the cells were given a BrdU pulse 2 hours before fixation and quantification of the number of BrdU+ nuclei. A student's t-test was carried out for n=6 samples. Error bars indicate SEM. Representative fluorescence microscopy images of +/+ and Jedi-1-/- NPCs are shown above the graph. Cells were stained with anti-BrdU (green) to identify proliferating cells and DAPI (blue) for nuclear visualization.

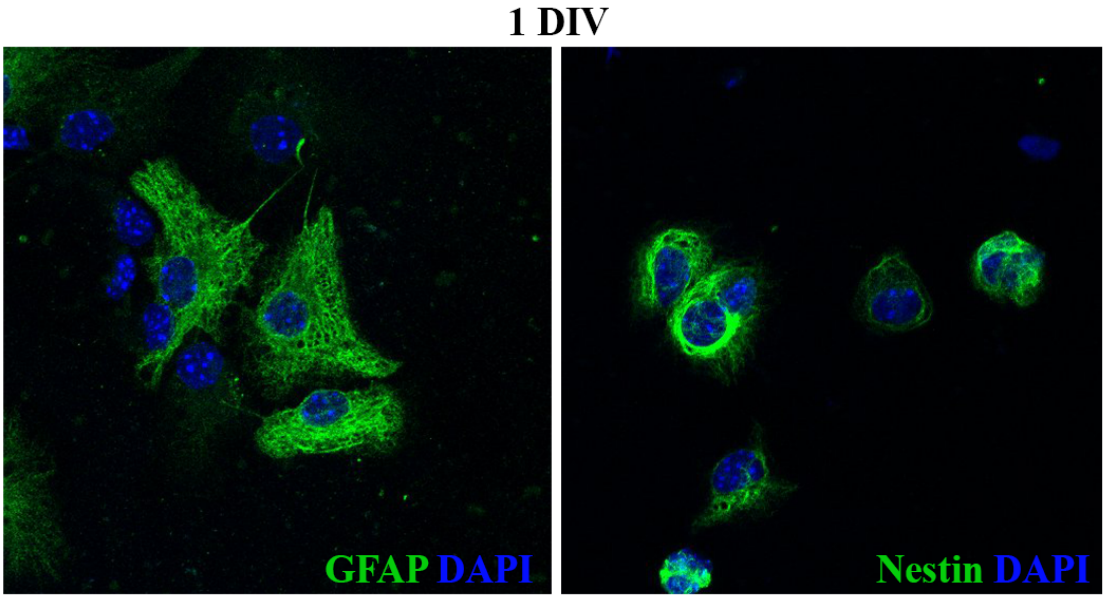

Figure A6. NPCs cultured for 1 day in differentiation media are primarily GFAP+ and Nestin+. NPCs from *WT* mice were grown as neurospheres, then dissociated and plated in differentiation media. After 1 day in culture, cells were fixed and stained for GFAP and Nestin.

Loss of Jedi-1 leads to increase in oligodendrogenesis in vitro

Given the increase in proliferation of *jedi-1*$^{-/-}$ NPCs, we wondered if they maintained their pluripotency. NPC cells from wildtype and *jedi-1*$^{-/-}$ mice were cultured first in EGF and FGF as neurospheres and then dissociated and seeded equally on coated coverslips and allowed to differentiate in mitogen free media for 14 days. The *jedi-1*$^{-/-}$ cells retained their ability to differentiate into neurons and glia (Figure A7); however, we observed an approximately 150% increase in the fraction of cells that were early stage oligodendrocytes, based on staining for the marker O4, in *jedi-1*$^{-/-}$ cultures compared to wildtype ($p < 0.05$, $n = 3$) (Figure A8). These data indicate that the increase in proliferation of *jedi-1-/-* NPCs may be specific to oligodendrocyte-producing precursors or that loss of *jedi-1* increases oligodendrogenesis at the expense of other cell types.

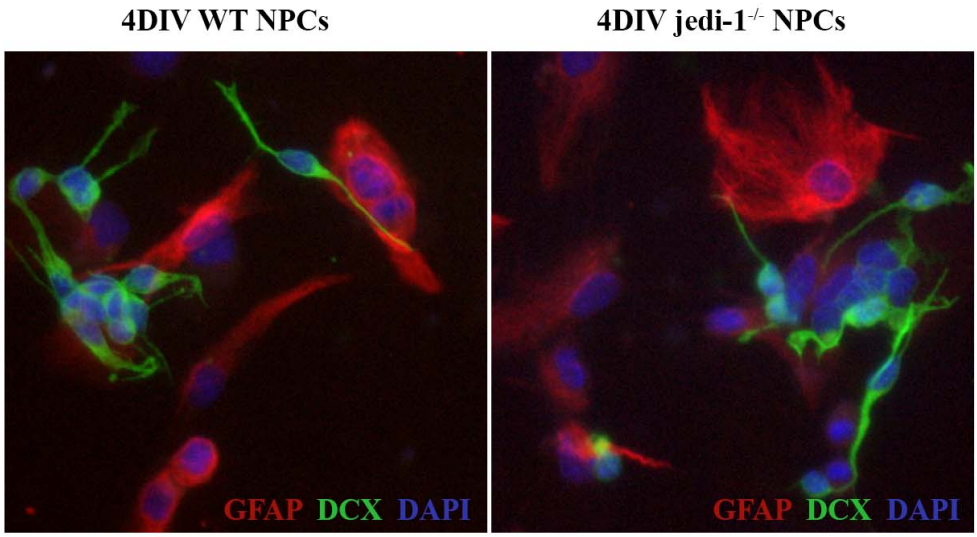

Figure A7. WT and *jedi-1*$^{-/-}$ derived neurospheres from the V-SVZ were differentiated for 4 days in mitogen free media before fixation. Immunostaining with GFAP (to label astrocytes) and DCX (to label neuroblasts) reveals that both wildtype and *jedi-1*$^{-/-}$ NSCs can form both neurons and glia in culture.

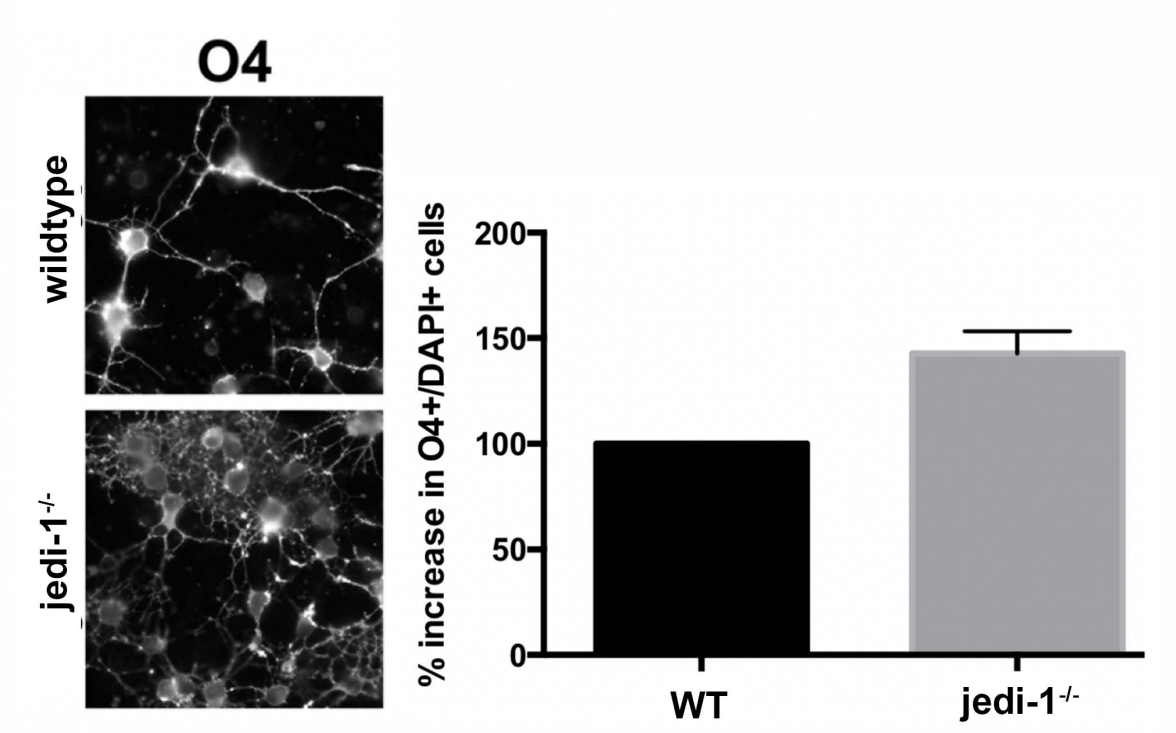

Figure A8. NPCs cultured for 14 days *in vitro* following mitogen withdrawal formed significantly more O4+ oligodendrocytes. Primary neurospheres from wild type (WT) and *jedi-1*$^{-/-}$ P4-6 mice were dissociated and allowed to differentiate for 14 days without EGF or FGF before fixation. The cells were then immunostained for the early oligodendrocyte marker O4. Results are shown as % of wildtype NPCs. (O4+/DAPI+) n=3; *, $p<0.05$.

Preliminary in vivo analysis

Neural stem cell dynamics *in vivo* are different from cultured cells. To determine if the observed *in vitro* increase in short-term BrdU incorporation also occurs in the adult V-SVZ, we measured cell proliferation using EdU. 6 week-old mice from both genotypes were injected i.p. with 25mg/kg EdU and sacrificed after 1 hour. Preliminary analysis of sections taken through the V-SVZ niche reveal an approximately 2-fold increase in the number of the EdU+ cells per V-SVZ area

(Figure A9). A more comprehensive analysis of EdU counts in the various V-SVZ subdomains as well as immunostaining for various markers to determine if one particular subtype of cell is more susceptible to loss of Jedi-1 is needed to clarify Jedi-1's role in NSC proliferation.

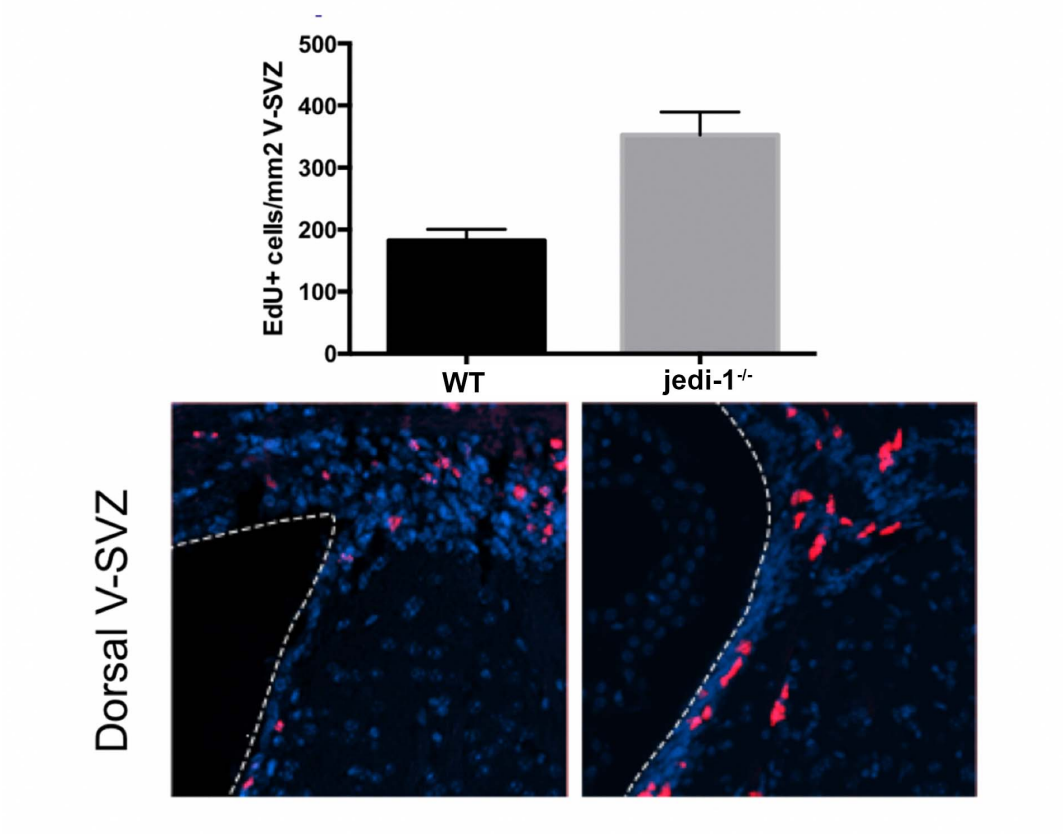

Figure A9. Preliminary data reveals an increase in EdU incorporation in adult mouse V-SVZ. 6-week old WT or *jedi-1*$^{-/-}$ mice were injected with 25mg/kg EdU i.p. 1 hour before sacrifice. Fixed brains were serial-sectioned at 10um and EdU incorporation was detected using the Click-IT™ system. EdU+ cells were counted in every 6th section in the V-SVZ region and are displayed as EdU+ cells/mm^2 V-SVZ. (n=2 brains, 20 sections each). Representative images of EdU labeling (red) in the V-SVZ are depicted below the graph (blue staining is DAPI and the dotted white line indicates the edge of the dorsal ventricle).

Discussion

During development of the nervous system and continuing throughout adulthood in several neurogenic regions in the brain, many newly born neurons undergo programmed cell death. Removal of these apoptotic corpses is required for proper neurodevelopment and brain homeostasis. Previous work has identified microglia as at least one cell type responsible for this clearance in the adult brain (Sierra et al., 2010). Surprisingly, it has also been reported that neural precursor cells themselves may possess phagocytic ability and regulate their own clearance in the adult neurogenic niche in order to ensure efficient neurogenesis (Lu et al., 2011). We previously identified the engulfment receptor, Jedi-1 responsible for the clearance of apoptotic sensory neurons during peripheral nervous development (Wu et al., 2009b) but determining its role in the central nervous system and whether it regulates neurogenesis was the focus of this project.

Since we observed Jedi-1expression in V-SVZ neurospheres, we sought to determine if it has a role in neural precursors clearance in this niche as well. Lu et al. (2011) previously reported that DCX+ neuroblasts generated from V-SVZ derived neurospheres as well as neuroblasts *in vivo* were able to engulf apoptotic cells. In our study, DCX+ cells rarely engulfed carboxylated microspheres, while the majority of engulfing cells appeared to be GFAP+/Nestin+ B cells. These differences could be attributed to subtle differences in culture conditions. The DCX+ neuroblasts in the Lu (2011) study appeared ameboid in shape with a slight polarization. In contrast, the DCX+ cells we observed in culture had a significantly different morphology, with extensive neurite outgrowth and small cell somas.

These obvious morphological differences suggest that the DCX+ cells in our cultures were more differentiated along the neural lineage, while those in the Lu et al. study were likely more immature and still retained their phagocytic ability, like the GFAP+/Nestin+ cells in our cultures.

Unsurprisingly, efficient neurogenesis requires proper clearance of apoptotic cells in order to avoid a detrimental inflammatory response that may arise from secondary necrosis (Sierra et al., 2010). While we did not observe any phagocytic deficits in NPCs from *jedi-1*$^{-/-}$ knockout mice *in vitro*, we did observe a baseline increase in proliferation. Jedi-1, therefore, may play a novel role in suppressing neurogenesis, independent of phagocytic capacity. Additionally, preliminary studies show that the observed increase in NPC proliferation *in vitro* appears to also be evident in the V-SVZ of adult *jedi-1*$^{-/-}$ mice, suggesting that this is not specific to cultured NPCs (Figure A9). This leads us to speculate how, mechanistically, Jedi-1 may regulate neural stem cell dynamics.

Jedi-1, also known as PEAR1, has been reported to play a role in regulating hematopoiesis. Overexpression of Jedi-1 in bone marrow cells decreased the proliferation of myeloid progenitors *in vitro* and may regulate this proliferation through interaction with Notch, due to structural similarities to Notch ligands Jagged and Delta (Krivtsov et al., 2007). Jedi-1/PEAR1 knockdown in megakaryocytes (platelet precursors) induced a two-fold increase in colony formation in clonogenic assays. This increase is thought to be related to the reduced expression of phosphatase and TENsin homolog (PTEN) and several PI3K-Akt and Notch-related genes. Knockdown of PEAR1 reduces PTEN

expression, which decreases Akt dephosphorylation leading to increased megakaryocyte precursor proliferation (Kauskot et al., 2013). Future work on the role of Jedi-1 in NPC proliferation should focus on determining if loss of Jedi-1 in these cells alters proliferation through a PTEN/p-Akt-dependent mechanism.

At present, it is unclear whether Jedi-1 is affecting NPC proliferation through cell autonomous mechanisms or through cell-cell signaling. Although Jedi-1 mRNA has been detected in NPCs in culture, we do not know if the protein is expressed in these cells. We have detected Jedi-1 expressed at high levels in brain endothelial cells and in the choroid plexus (not shown). Notably, there is evidence that soluble factors released from these cell populations in the V-SVZ can regulate the proliferation of neural stem cells. For example, neurotrophin 3 (NT3) released by brain endothelial cells and choroid plexus supports B1 cell quiescence through nitrous oxide (NO) production via TrkC. Loss of NT3 resulted in increased cycling of B1 cells (Delgado et al., 2014). B1 cells make direct cellular contact to the cerebral spinal fluid via cilia protrusion between ependymal cells into the ventricular space and they also make deep projections to niche blood vessels. It remains possible that the increase in NPC proliferation we detected is due to Jedi-1 expression on endothelial cells and/or on the NSCs themselves. To investigate this possibility, one could use immunofluorescence techniques; first, to immunostain the V-SVZ of the brain for Jedi-1 and co-stain for markers of NSCs, such as GFAP. In addition, one could measure the number of contacts that B1 cells make with blood vessels and the ventricular space in *jedi-1*$^{-/-}$ animals at various ages in development to determine if Jedi-1 expression is required for B1

cell process extension or proper positioning.

The role of endothelial cells in regulating NPC proliferation could also be investigated *in vitro* by culturing endothelial cells from wildtype or *jedi-1*$^{-/-}$ brains and seeding wildtype or knockout NPCs over them and measuring proliferation and morphology of the stem cells. To test whether direct contact is necessary, mesh trans well plates could be utilized to allow for soluble molecules released by endothelial cells to pass through but prevent direct interaction between the cell types. This experiment would dissect out how endothelial cells regulate the proliferation and differentiation. In our study, neural precursors are isolated and cultured independent from endothelial cells, but we can't rule out that lack of Jedi-1 expression in endothelial cells prior to the isolation of NPCs reprogrammed the NPCs to be intrinsically more proliferative.

NSCs in culture can differentiate into mature astrocytes, oligodendrocytes, and neurons in culture after mitogen withdrawal. We report that while *jedi-1*$^{-/-}$ NSCs maintain their pluripotency, they generate significantly more O4+ oligodendrocytes after 14 days of differentiation *in vitro (*Figure A8). Whether Jedi-1 has a direct role in regulating the proliferation of oligodendrocyte precursor cells or shifting the fate of NSCs to oligodendrogenesis remains to be determined. Current studies are aimed at fully dissecting the cell type(s) affected by loss of Jedi-1 *in vitro*, by determining the fraction of mature oligodendrocytes, astrocytes and neurons that arise from knockout NSCs. These studies will be done with secondary spheres, derived from primary spheres, dissociated and plated at clonal density to confirm that they self-renew and that individual cells maintain

pluripotency. In addition, future work will focus on whether this increase in oligodendrocyte generation is phenocopied *in vivo.* Little is known about the generation of oligodendrocytes during adult neurogenesis, but sonic hedgehog (Shh) appears to modulate the formation of OLs from NSCs after a demyelinating lesion in a mouse Multiple Sclerosis (MS) model (Samanta et al., 2015). Determining whether loss of Jedi-1 affects remyelination after injury in MS models is a goal of future work in the lab. If the loss of Jedi-1 affects the production of neurons *in vitro*, for example, by shifting NSC fate toward oligodendrocytes, then it will be important to determine if the production of new neurons is affected in *jedi-1*$^{-/-}$ mice *in vivo*. In particular, the number of V-SVZ- generated neurons in the rostral migratory steam and olfactory bulb of adult mice should be evaluated.

If our preliminary results showing an increase in Edu+ cell *in vivo* hold true (Figure A9), it would be informative to investigate this effect at older ages. Excess cell cycling of B cells in early adulthood can lead to an exhaustion of the neural stem cell niche (Ehm et al., 2010). By pulsing older mice with EdU, we may observe the opposite effect: fewer proliferating cells in the V-SVZ. This would indicate that perhaps loss of Jedi-1 early in development increases cycling of stem cells, which depletes the pool of B1 cells in mature mice. Additionally, future studies should determine if this phenotype is specific to V-SVZ derived stem cells or if NPCs isolated from the SGZ, the other main germinal zone in the adult brain also show an increase in proliferation. If so, older mice may perform poorly on learning and memory-focused behavioral tasks compared to wildtype littermates.

Conclusion

While still in progress, this project has provided insights in to the complex process of neural stem cell maintenance and differentiation as well as providing a novel role for the engulfment receptor Jedi-1 in this process. Future work in the lab will determine if Jedi-1 possesses multiple distinct roles in various cell types or if these roles converge on a central mechanism that explains the observed phenotypes.

BIBLIOGRAPHY

Agerman K, Baudet C, Fundin B, Willson C, Ernfors P (2000) Attenuation of a caspase-3 dependent cell death in NT4-and p75-deficient embryonic sensory neurons. Molecular and Cellular Neuroscience 16:258–268.

Ahn S, Joyner AL (2005) In vivo analysis of quiescent adult neural stem cells responding to Sonic hedgehog. Nature 437:894–897.

Andres R, Herraez-Baranda LA, Thompson J, Wyatt S, Davies AM (2008) Regulation of sympathetic neuron differentiation by endogenous nerve growth factor and neurotrophin-3. Neuroscience Letters 431:241–246.

Arimura N, Kimura T, Nakamuta S, Taya S, Funahashi Y, Hattori A, Shimada A, Ménager C, Kawabata S, Fujii K, Iwamatsu A, Segal RA, Fukuda M, Kaibuchi K (2009) Anterograde transport of TrkB in axons is mediated by direct interaction with Slp1 and Rab27. Dev Cell 16:675–686.

Ascaño M, Richmond A, Borden P, Kuruvilla R (2009) Axonal targeting of Trk receptors via transcytosis regulates sensitivity to neurotrophin responses. J Neurosci 29:11674–11685.

Baldwin AN, Bitler CM, Welcher AA, Shooter EM (1992) Studies on the structure and binding properties of the cysteine-rich domain of rat low affinity nerve growth factor receptor (p75NGFR). Journal of Biological Chemistry 267:8352–8359.

Baloh RH, Tansey MG, Lampe PA, Fahrner TJ, Enomoto H, Simburger KS, Leitner ML, Araki T, Johnson EM, Milbrandt J (1998) Artemin, a novel member of the GDNF ligand family, supports peripheral and central neurons and signals through the GFRalpha3-RET receptor complex. Neuron 21:1291–1302.

Bamji SX, Majdan M, Pozniak CD, Belliveau DJ, Aloyz R, Kohn J, Causing CG, Miller FD (1998) The p75 neurotrophin receptor mediates neuronal apoptosis and is essential for naturally occurring sympathetic neuron death. J Cell Biol 140:911–923.

Baum JS, St George JP, McCall K (2005) Programmed cell death in the germline. Semin Cell Dev Biol 16:245–259.

Beattie MS, Harrington AW, Lee R, Kim JY, Boyce SL, Longo FM, Bresnahan JC, Hempstead BL, Yoon SO (2002) ProNGF induces p75-mediated death of oligodendrocytes following spinal cord injury. Neuron 36:375–386.

Belliveau DJ, Krivko I, Kohn J, Lachance C, Pozniak C, Rusakov D, Kaplan D, Miller FD (1997) NGF and Neurotrophin-3 Both Activate TrkA on Sympathetic Neurons but Differentially Regulate Survival and Neuritogenesis. J Cell Biol 136:375–388.

Benedetti M, Levi A, Chao MV (1993) Differential expression of nerve growth factor receptors leads to altered binding affinity and neurotrophin responsiveness. Proceedings of the National Academy of Sciences 90:7859–7863.

Bertrand MJM, Kenchappa RS, Andrieu D, Leclercq-Smekens M, Nguyen HNT, Carter BD, Muscatelli F, BARKER PA, De Backer O (2008) NRAGE, a p75NTR adaptor protein, is required for developmental apoptosis in vivo. Cell Death Differ 15:1921–1929.

Birren SJ, Lo L, Anderson DJ (1993) Sympathetic neuroblasts undergo a developmental switch in trophic dependence. Development 119:597–610.

Blaschke AJ, Staley K, Chun J (1996) Widespread programmed cell death in proliferative and postmitotic regions of the fetal cerebral cortex. Development 122:1165–1174.

Bodmer D, Ascaño M, Kuruvilla R (2011) Isoform-Specific Dephosphorylation of Dynamin1 by Calcineurin Couples Neurotrophin Receptor Endocytosis to Axonal Growth. Neuron 70:1085–1099.

Bond AM, Ming G-L, Song H (2015) Adult Mammalian Neural Stem Cells and Neurogenesis: Five Decades Later. Cell Stem Cell 17:385–395.

Bonifacino JS (2004) The GGA proteins: adaptors on the move. Nat Rev Mol Cell Biol 5:23–32.

Bononi J, Cole A, Tewson P, Schumacher A, Bradley R (2008) Chicken protocadherin-1 functions to localize neural crest cells to the dorsal root ganglia during PNS formation. Mech Dev 125:1033–1047.

Brennan C, Rivas-Plata K, Landis SC (1999) The p75 neurotrophin receptor influences NT-3 responsiveness of sympathetic neurons in vivo. Nature Publishing Group 2:699–705.

Bronfman FC, Tcherpakov M, Jovin TM, Fainzilber M (2003) Ligand-induced internalization of the p75 neurotrophin receptor: a slow route to the signaling endosome. J Neurosci 23:3209–3220.

Bronner-Fraser M (1986) Analysis of the early stages of trunk neural crest migration in avian embryos using monoclonal antibody HNK-1. Dev Biol 115:44–55.

Burek MJ, Oppenheim RW (1996) Programmed cell death in the developing nervous system. Brain Pathol 6:427–446.

Buss RR, Sun W, Oppenheim RW (2006) Adaptive roles of programmed cell death during nervous system development. Annu Rev Neurosci 29:1–35.

Butowt R, Bartheld von CS (2009) Fates of neurotrophins after retrograde axonal transport: phosphorylation of p75NTR is a sorting signal for delayed degradation. J Neurosci 29:10715–10729.

Cappella P, Gasparri F, Pulici M, Moll J (2008) A novel method based on click chemistry, which overcomes limitations of cell cycle analysis by classical determination of BrdU incorporation, allowing multiplex antibody staining. Cytometry A 73:626–636.

Carney TJ, Dutton KA, Greenhill E, Delfino-Machín M, Dufourcq P, Blader P, Kelsh RN (2006) A direct role for Sox10 in specification of neural crest-derived sensory neurons. Development 133:4619–4630.

Casaccia-Bonnefil P, Carter BD, Dobrowsky RT, Chao MV (1996) Death of oligodendrocytes mediated by the interaction of nerve growth factor with its receptor p75. Nature 383:716–719.

Casademunt E, Carter BD, Benzel I, Frade JM, Dechant G, Barde YA (1999) The zinc finger protein NRIF interacts with the neurotrophin receptor p75(NTR) and participates in programmed cell death. The EMBO Journal 18:6050–6061.

Ceni C, Unsain N, Zeinieh MP, Barker PA (2014) Neurotrophins in the regulation of cellular survival and death. Handb Exp Pharmacol 220:193–221.

Chang C-C, Fang W-H, Chang H-A, Huang S-Y (2015) Functional Ser205Leu polymorphism of the nerve growth factor receptor (NGFR) gene is associated with vagal autonomic dysregulation in humans. Sci Rep 5:13136.

Chao MV (2003) Neurotrophins and their receptors: a convergence point for many signalling pathways. Nat Rev Neurosci 4:299–309.

Chao MV (2016) Cleavage of p75 neurotrophin receptor is linked to Alzheimer's disease. Mol Psychiatry 21:300–301.

Chao MV, Rajagopal R, Lee FS (2006) Neurotrophin signalling in health and disease. Clin Sci 110:167–173.

Cho Y, Cavalli V (2014) HDAC signaling in neuronal development and axon regeneration. Current Opinion in Neurobiology 27:118–126.

Clay MR, Halloran MC (2014) Cadherin 6 promotes neural crest cell detachment via F-actin regulation and influences active Rho distribution during epithelial-to-mesenchymal transition. Development 141:2506–2515.

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

Crowley C, Spencer SD, Nishimura MC, Chen KS, Pitts-Meek S, Armanini MP, Ling LH, McMahon SB, Shelton DL, Levinson AD (1994) Mice lacking nerve growth factor display perinatal loss of sensory and sympathetic neurons yet develop basal forebrain cholinergic neurons. Cell 76:1001–1011.

Culmsee C, Gerling N, Lehmann M, Nikolova-Karakashian M, Prehn JHM, Mattson MP, Krieglstein J (2002) Nerve growth factor survival signaling in cultured hippocampal neurons is mediated through TrkA and requires the common neurotrophin receptor P75. Neuroscience 115:1089–1108.

D'Souza-Schorey C, Chavrier P (2006) ARF proteins: roles in membrane traffic and beyond. Nat Rev Mol Cell Biol 7:347–358.

Davies AM (2009) Extracellular signals regulating sympathetic neuron survival and target innervation during development. Auton Neurosci 151:39–45.

Davies AM, Minichiello L, Klein R (1995) Developmental changes in NT3 signalling via TrkA and TrkB in embryonic neurons. The EMBO Journal 14:4482–4489.

Debby-Brafman A, Burstyn-Cohen T, Klar A, Kalcheim C (1999) F-Spondin, expressed in somite regions avoided by neural crest cells, mediates inhibition of distinct somite domains to neural crest migration. Neuron 22:475–488.

Dechant G, Rodriguez-Tebar A, Barde YA (1994) Neurotrophin receptors. Progress in Neurobiology 42:347–352.

Deinhardt K, Chao MV (2014) Trk receptors. Handb Exp Pharmacol 220:103–119.

Deinhardt K, Reversi A, Berninghausen O, Hopkins CR, Schiavo G (2007) Neurotrophins Redirect p75NTR from a clathrin-independent to a clathrin-dependent endocytic pathway coupled to axonal transport. Traffic 8:1736–1749.

Deinhardt K, Salinas S, Verastegui C, Watson R, Worth D, Hanrahan S, Bucci C, Schiavo G (2006) Rab5 and Rab7 control endocytic sorting along the axonal retrograde transport pathway. Neuron 52:293–305.

Delgado AC, Ferrón SR, Vicente D, Porlan E, Perez-Villalba A, Trujillo CM, D'Ocón P, Fariñas I (2014) Endothelial NT-3 delivered by vasculature and CSF promotes quiescence of subependymal neural stem cells through nitric oxide induction. Neuron 83:572–585.

Deppmann CD, Mihalas S, Sharma N, Lonze BE, Niebur E, Ginty DD (2008) A model for neuronal competition during development. Science 320:369–373.

Dhanasekaran DN, Reddy EP (2008) JNK signaling in apoptosis. Oncogene 27:6245–6251.

Dobrowsky RT, Werner MH, Castellino AM, Chao MV, Hannun YA (1994) Activation of the sphingomyelin cycle through the low-affinity neurotrophin receptor. Science 265:1596–1599.

Doetsch F, Caillé I, Lim DA, García-Verdugo JM, Alvarez-Buylla A (1999) Subventricular zone astrocytes are neural stem cells in the adult mammalian brain. Cell 97:703–716.

Dong Z, Sinanan A, Parkinson D, Parmantier E, Mirsky R, Jessen KR (1999) Schwann cell development in embryonic mouse nerves. J Neurosci Res 56:334–348.

Du J, Feng L, Yang F, Lu B (2000) Activity- and Ca2+-Dependent Modulation of Surface Expression of Brain-Derived Neurotrophic Factor Receptors in Hippocampal Neurons. J Cell Biol 150:1423–1434.

Durbec PL, Larsson-Blomberg LB, Schuchardt A, Costantini F, Pachnis V (1996) Common origin and developmental dependence on c-ret of subsets of enteric and sympathetic neuroblasts. Development 122:349–358.

Ehm O, Göritz C, Covic M, Schäffner I, Schwarz TJ, Karaca E, Kempkes B, Kremmer E, Pfrieger FW, Espinosa L, Bigas A, Giachino C, Taylor V, Frisén J, Lie DC (2010) RBPJkappa-dependent signaling is essential for long-term maintenance of neural stem cells in the adult hippocampus. J Neurosci 30:13794–13807.

Eilers A, Whitfield J, Shah B, Spadoni C, Desmond H, Ham J (2001) Direct inhibition of c-Jun N-terminal kinase in sympathetic neurones prevents c-jun promoter activation and NGF withdrawal-induced death. Journal of Neurochemistry 76:1439–1454.

ElShamy WM, Ernfors P (1996) A local action of neurotrophin-3 prevents the death of proliferating sensory neuron precursor cells. Neuron 16:963–972.

Erickson CA (1985) Control of neural crest cell dispersion in the trunk of the avian embryo. Dev Biol 111:138–157.

Ernst A, Alkass K, Bernard S, Salehpour M, Perl S, Tisdale J, Possnert G, Druid H, Frisén J (2014) Neurogenesis in the striatum of the adult human brain. Cell 156:1072–1083.

Escudero CA, Lazo OM, Galleguillos C, Parraguez JI, Lopez-Verrilli MA, Cabeza C, Leon L, Saeed U, Retamal C, Gonzalez A, Marzolo M-P, Carter BD, Court FA, Bronfman FC (2014) The p75 neurotrophin receptor evades the endolysosomal route in neuronal cells, favouring multivesicular bodies specialised for exosomal release. J Cell Sci 127:1966–1979.

Esposito D, Patel P, Stephens RM, Perez P, Chao MV, Kaplan DR, Hempstead BL (2001) The cytoplasmic and transmembrane domains of the p75 and Trk A receptors regulate high affinity binding to nerve growth factor. Journal of Biological Chemistry 276:32687–32695.

Frade JM, Barde YA (1999) Genetic evidence for cell death mediated by nerve growth factor and the neurotrophin receptor p75 in the developing mouse retina and spinal cord. Development 126:683–690.

Frade JM, RodriguezTebar A, Barde YA (1996) Induction of cell death by endogenous nerve growth factor through its p75 receptor. Nature 383:166–168.

Francis N, Farinas I, Brennan C, Rivas-Plata K, Backus C, Reichardt L, Landis S (1999) NT-3, like NGF, is required for survival of sympathetic neurons, but not their precursors. Dev Biol 210:411–427.

Geetha T, Kenchappa RS, Wooten MW, Carter BD (2005) TRAF6-mediated ubiquitination regulates nuclear translocation of NRIF, the p75 receptor interactor. The EMBO Journal 24:3859–3868.

George L, Kasemeier-Kulesa J, Nelson BR, Koyano-Nakagawa N, Lefcort F (2010) Patterned Assembly and Neurogenesis in the Chick Dorsal Root Ganglion. J Comp Neurol 518:405–422.

Ghosh AS, Wang B, Pozniak CD, Chen M, Watts RJ, Lewcock JW (2011) DLK induces developmental neuronal degeneration via selective regulation of proapoptotic JNK activity. J Cell Biol 194:751–764.

Glebova NO, Ginty DD (2004) Heterogeneous requirement of NGF for sympathetic target innervation in vivo. J Neurosci 24:743–751.

Glebova NO, Ginty DD (2005) Growth and survival signals controlling sympathetic nervous system development. Annu Rev Neurosci 28:191–222.

Gohlke JM, Griffith WC, Faustman EM (2004) The role of cell death during neocortical neurogenesis and synaptogenesis: implications from a computational model for the rat and mouse. Developmental Brain Research 151:43–54.

Gonçalves JT, Schafer ST, Gage FH (2016) Adult Neurogenesis in the Hippocampus: From Stem Cells to Behavior. Cell 167:897–914.

Guillory G, Bronner-Fraser M (1986) An in vitro assay for neural crest cell migration through the somites. J Embryol Exp Morphol 98:85–97.

Harrington AW, Ginty DD (2013) Long-distance retrograde neurotrophic factor signalling in neurons. Nat Rev Neurosci 14:177–187.

Harrington AW, St Hillaire C, Zweifel LS, Glebova NO, Philippidou P, Halegoua S, Ginty DD (2011) Recruitment of actin modifiers to TrkA endosomes governs retrograde NGF signaling and survival. Cell 146:421–434.

Harrison SM, Jones ME, Uecker S, Albers KM, Kudrycki KE, Davis BM (2000) Levels of nerve growth factor and neurotrophin-3 are affected differentially by the presence of p75 in sympathetic neurons in vivo. J Comp Neurol 424:99–110.

Hayden MS, Ghosh S (2012) NF-κB, the first quarter-century: remarkable progress and outstanding questions. Genes Dev 26:203–234.

Heerssen HM, Pazyra MF, Segal RA (2004) Dynein motors transport activated Trks to promote survival of target-dependent neurons. Nat Neurosci 7:596–604.

Hempstead BL (2014) Deciphering proneurotrophin actions. Handb Exp Pharmacol 220:17–32.

Hempstead BL, Martin-Zanca D, Kaplan DR, Parada LF, Chao MV (1991) High-affinity NGF binding requires coexpression of the trk proto-oncogene and the low-affinity NGF receptor. Nature 350:678–683.

Hirata H, Hibasami H, Yoshida T, Ogawa M, Matsumoto M, Morita A, Uchida A (2001) Nerve growth factor signaling of p75 induces differentiation and ceramide-mediated apoptosis in Schwann cells cultured from degenerating nerves. Glia 36:245–258.

Hongu T, Funakoshi Y, Fukuhara S, Suzuki T, Sakimoto S, Takakura N, Ema M, Takahashi S, Itoh S, Kato M, Hasegawa H, Mochizuki N, Kanaho Y (2015) Arf6 regulates tumour angiogenesis and growth through HGF-induced endothelial β1 integrin recycling. Nat Commun 6:7925.

Hongu T, Kanaho Y (2014) Activation machinery of the small GTPase Arf6. Adv Biol Regul 54:59–66.

Honma Y, Araki T, Gianino S, Bruce A, Heuckeroth RO, Johnson EM Jr., Milbrandt J (2002) Artemin Is a Vascular-Derived Neurotropic Factor for Developing Sympathetic Neurons. Neuron 35:267–282.

Howe CL, Mobley WC (2005) Long-distance retrograde neurotrophic signaling. Current Opinion in Neurobiology 15:40–48.

Howe CL, Valletta JS, Rusnak AS, Mobley WC (2001) NGF signaling from clathrin-coated vesicles: evidence that signaling endosomes serve as a platform for the Ras-MAPK pathway. Neuron 32:801–814.

Huang EJ, Reichardt LF (2001) Neurotrophins: roles in neuronal development and function. Annu Rev Neurosci 24:677–736.

Hutagalung AH, Novick PJ (2011) Role of Rab GTPases in membrane traffic and cell physiology. Physiol Rev 91:119–149.

Ihrie RA, Shah JK, Harwell CC, Levine JH, Guinto CD, Lezameta M, Kriegstein AR, Alvarez-Buylla A (2011) Persistent Sonic Hedgehog Signaling in Adult Brain Determines Neural Stem Cell Positional Identity. Neuron 71:250–262.

Irmady K, Jackman KA, Padow VA, Shahani N, Martin LA, Cerchietti L, Unsicker K, Iadecola C, Hempstead BL (2014) Mir-592 regulates the induction and cell death-promoting activity of p75NTR in neuronal ischemic injury. J Neurosci 34:3419–3428.

Jackson TR, Brown FD, Nie Z, Miura K, Foroni L, Sun J, Hsu VW, Donaldson JG, Randazzo PA (2000) ACAPs are arf6 GTPase-activating proteins that function in the cell periphery. J Cell Biol 151:627–638.

Jansen P, Giehl K, Nyengaard JR, Teng K, Lioubinski O, Sjoegaard SS, Breiderhoff T, Gotthardt M, Lin F, Eilers A, Petersen CM, Lewin GR, Hempstead BL, Willnow TE, Nykjaer A (2007) Roles for the pro-neurotrophin receptor sortilin in neuronal development, aging and brain injury. Nat Neurosci 10:1449–1457.

Jaworski J (2007) ARF6 in the nervous system. Eur J Cell Biol 86:513–524.

Johnson EM Jr,, Deckwerth TL (1993) Molecular Mechanisms of Developmental Neuronal Death. Annu Rev Neurosci 16:31–46.

Jung K-M, Tan S, Landman N, Petrova K, Murray S, Lewis R, Kim PK, Kim DS, Ryu SH, Chao MV, Kim T-W (2003) Regulated intramembrane proteolysis of the p75 neurotrophin receptor modulates its association with the TrkA receptor. Journal of Biological Chemistry 278:42161–42169.

Kanning KC, Hudson M, Amieux PS, Wiley JC, Bothwell M, Schecterson LC (2003) Proteolytic Processing of the p75 Neurotrophin Receptor and Two Homologs Generates C-Terminal Fragments with Signaling Capability. Journal of Neuroscience 23:5425–5436.

Karki S, Holzbaur EL (1995) Affinity chromatography demonstrates a direct binding between cytoplasmic dynein and the dynactin complex. Journal of Biological Chemistry 270:28806–28811.

Kasemeier-Kulesa JC, Kulesa PM, Lefcort F (2005) Imaging neural crest cell dynamics during formation of dorsal root ganglia and sympathetic ganglia. Development 132:235–245.

Kauskot A, Vandenbriele C, Louwette S, Gijsbers R, Tousseyn T, Freson K, Verhamme P, Hoylaerts MF (2013) PEAR1 attenuates megakaryopoiesis via control of the PI3K/PTEN pathway. Blood 121:5208–5217.

Kenchappa RS, Tep C, Korade Z, Urra S, Bronfman FC, Yoon SO, Carter BD (2010) p75 neurotrophin receptor-mediated apoptosis in sympathetic neurons involves a biphasic activation of JNK and up-regulation of tumor necrosis factor-alpha-converting enzyme/ADAM17. J Biol Chem 285:20358–20368.

Kenchappa RS, Zampieri N, Chao MV, Barker PA, Teng HK, Hempstead BL, Carter BD (2006) Ligand-dependent cleavage of the P75 neurotrophin receptor is necessary for NRIF nuclear translocation and apoptosis in sympathetic neurons. Neuron 50:219–232.

Khursigara G, Orlinick JR, Chao MV (1999) Association of the p75 neurotrophin receptor with TRAF6. Journal of Biological Chemistry 274:2597–2600.

Kim JY, Shen S, Dietz K, He Y, Howell O, Reynolds R, Casaccia P (2010) HDAC1 nuclear export induced by pathological conditions is essential for the onset of axonal damage. Nature Publishing Group 13:180–189.

King SJ, Schroer TA (2000) Dynactin increases the processivity of the cytoplasmic dynein motor. Nat Cell Biol 2:20–24.

Klarlund JK, Guilherme A, Holik JJ, Virbasius JV, Chawla A, Czech MP (1997) Signaling by phosphoinositide-3,4,5-trisphosphate through proteins containing pleckstrin and Sec7 homology domains. Science 275:1927–1930.

Korade Z, Kenchappa RS, Mirnics K, Carter BD (2009) NRIF is a regulator of neuronal cholesterol biosynthesis genes. J Mol Neurosci 38:152–158.

Korsching S (1993) The neurotrophic factor concept: a reexamination. Journal of Neuroscience 13:2739–2748.

Kraemer BR, Snow JP, Vollbrecht P, Pathak A, Valentine WM, Deutch AY, Carter BD (2014) A role for the p75 neurotrophin receptor in axonal degeneration and apoptosis induced by oxidative stress. J Biol Chem 289:21205–21216.

Krammer PH (2000) CD95's deadly mission in the immune system. Nature 407:789–795.

Kristiansen M, Ham J (2014) Programmed cell death during neuronal development: the sympathetic neuron model. Cell Death Differ 21:1025–1035.

Krivtsov AV, Rozov FN, Zinovyeva MV, Hendrikx PJ, Jiang Y, Visser JWM, Belyavsky AV (2007) Jedi--a novel transmembrane protein expressed in early hematopoietic cells. J Cell Biochem 101:767–784.

Krull CE (2001) Segmental organization of neural crest migration. Mech Dev 105:37–45.

Kuruvilla R, Zweifel LS, Glebova NO, Lonze BE, Valdez G, Ye H, Ginty DD (2004) A neurotrophin signaling cascade coordinates sympathetic neuron development through differential control of TrkA trafficking and retrograde signaling. Cell 118:243–255.

Lai K, Kaspar BK, Gage FH, Schaffer DV (2003) Sonic hedgehog regulates adult neural progenitor proliferation in vitro and in vivo. Nat Neurosci 6:21–27.

Lee KF, Davies AM, Jaenisch R (1994) p75-deficient embryonic dorsal root sensory and neonatal sympathetic neurons display a decreased sensitivity to NGF. Development 120:1027–1033.

Lepousez G, Valley MT, Lledo P-M (2013) The impact of adult neurogenesis on olfactory bulb circuits and computations. Annu Rev Physiol 75:339–363.

Levi-Montalcini R (1966) The nerve growth factor: its mode of action on sensory and sympathetic nerve cells. Harvey Lect 60:217–259.

Levi-Montalcini R (1987) The nerve growth factor 35 years later. Science 237:1154–1162.

Li P, Le Moan N, Sachs BD, Schachtrup C, Vagena E, Olefsky JM, Akassoglou K (2012) p75 neurotrophin receptor regulates glucose homeostasis and insulin sensitivity. Proc Natl Acad Sci USA 109:5838–5843.

Li X, Lavigne P, Lavoie C (2015) GGA3 mediates TrkA endocytic recycling to promote sustained Akt phosphorylation and cell survival. 26:4412–4426.

Lim DA, Alvarez-Buylla A (2016) The Adult Ventricular-Subventricular Zone (V-SVZ) and Olfactory Bulb (OB) Neurogenesis. Cold Spring Harbor Perspectives in Biology 8:a018820.

Linggi MS, Burke TL, Williams BB, Harrington A, Kraemer R, Hempstead BL, Yoon SO, Carter BD (2005) Neurotrophin receptor interacting factor (NRIF) is an essential mediator of apoptotic signaling by the p75 neurotrophin receptor. Journal of Biological Chemistry 280:13801–13808.

Lu Z, Elliott MR, Chen Y, Walsh JT, Klibanov AL, Ravichandran KS, Kipnis J (2011) Phagocytic activity of neuronal progenitors regulates adult neurogenesis. 13:1076–1083.

Ma DK, Kim WR, Ming G-L, Song H (2009) Activity-dependent Extrinsic Regulation of Adult Olfactory Bulb and Hippocampal Neurogenesis. Annals of the New York Academy of Sciences 1170:664–673.

Machold R, Hayashi S, Rutlin M, Muzumdar MD, Nery S, Corbin JG, Gritli-Linde A, Dellovade T, Porter JA, Rubin LL, Dudek H, McMahon AP, Fishell G (2003) Sonic hedgehog is required for progenitor cell maintenance in telencephalic stem cell niches. Neuron 39:937–950.

Maggirwar SB, Sarmiere PD, Dewhurst S, Freeman RS (1998) Nerve growth factor-dependent activation of NF-kappaB contributes to survival of sympathetic neurons. Journal of Neuroscience 18:10356–10365.

Mahadeo D, Kaplan L, Chao MV, Hempstead BL (1994) High affinity nerve growth factor binding displays a faster rate of association than p140trk binding. Implications for multi-subunit polypeptide receptors. Journal of Biological Chemistry 269:6884–6891.

Maina F, Hilton MC, Andres R, Wyatt S, Klein R, Davies AM (1998) Multiple roles for hepatocyte growth factor in sympathetic neuron development. Neuron 20:835–846.

Maina F, Klein R (1999) Hepatocyte growth factor, a versatile signal for developing neurons. Nat Neurosci 2:213–217.

Majdan M, Lachance C, Gloster A, Aloyz R, Zeindler C, Bamji S, Bhakar A, Belliveau D, Fawcett J, Miller FD, BARKER PA (1997) Transgenic mice expressing the intracellular domain of the p75 neurotrophin receptor undergo neuronal apoptosis. Journal of Neuroscience 17:6988–6998.

Makita T, Sucov HM, Gariepy CE, Yanagisawa M, Ginty DD (2008) Endothelins are vascular-derived axonal guidance cues for developing sympathetic neurons. Nature 452:759–763.

Marmigere F, Ernfors P (2007) Specification and connectivity of neuronal subtypes in the sensory lineage. Nat Rev Neurosci 8:114–127.

Mayo LD, Donner DB (2002) The PTEN, Mdm2, p53 tumor suppressor-oncoprotein network. Trends Biochem Sci 27:462–467.

McCorry LK (2007) Physiology of the autonomic nervous system. American Journal of Pharmaceutical Education 71.

McIlwain DR, Berger T, Mak TW (2013) Caspase functions in cell death and disease. Cold Spring Harbor Perspectives in Biology 5:a008656–a008656.

McKenney RJ, Huynh W, Tanenbaum ME, Bhabha G, Vale RD (2014) Activation of cytoplasmic dynein motility by dynactin-cargo adapter complexes. Science 345:337–341.

Merino R, Gañan Y, Macias D, Economides AN, Sampath KT, Hurle JM (1998) Morphogenesis of Digits in the Avian Limb Is Controlled by FGFs, TGFβs, and Noggin through BMP Signaling. Dev Biol 200:35–45.

Merkle FT, Fuentealba LC, Sanders TA, Magno L, Kessaris N, Alvarez-Buylla A (2014) Adult neural stem cells in distinct microdomains generate previously unknown interneuron types. Nature Publishing Group 17:207–214.

Merkle FT, Mirzadeh Z, Alvarez-Buylla A (2007) Mosaic organization of neural stem cells in the adult brain. Science 317:381–384.

Meyer-Franke A, Wilkinson GA, Kruttgen A, Hu M, Munro E, Hanson MG, Reichardt LF, Barres BA (1998) Depolarization and cAMP elevation rapidly recruit TrkB to the plasma membrane of CNS neurons. Neuron 21:681–693.

Miller FD, Speelman A, Mathew TC, Fabian J, Chang E, Pozniak C, Toma JG (1994) Nerve Growth Factor Derived from Terminals Selectively Increases the Ratio of p75 to trkA NGF Receptors on Mature Sympathetic Neurons. Dev Biol 161:206–217.

Mitchell DJ, Blasier KR, Jeffery ED, Ross MW, Pullikuth AK, Suo D, Park J, Smiley WR, Lo KW-H, Shabanowitz J, Deppmann CD, Trinidad JC, Hunt DF, Catling AD, Pfister KK (2012) Trk activation of the ERK1/2 kinase pathway stimulates intermediate chain phosphorylation and recruits cytoplasmic dynein to signaling endosomes for retrograde axonal transport. J Neurosci 32:15495–15510.

Mok S-A, Lund K, Campenot RB (2009) A retrograde apoptotic signal originating in NGF-deprived distal axons of rat sympathetic neurons in compartmented cultures. Cell Res 19:546–560.

Mu Y, Lee SW, Gage FH (2010) Signaling in adult neurogenesis. Current Opinion in Neurobiology 20:416–423.

Nanda N (2005) Platelet Endothelial Aggregation Receptor 1 (PEAR1), a Novel Epidermal Growth Factor Repeat-containing Transmembrane Receptor, Participates in Platelet Contact-induced Activation. Journal of Biological Chemistry 280:24680–24689.

Naumann T, Casademunt E, Hollerbach E, Hofmann J, Dechant G, Frotscher M, Barde YA (2002) Complete deletion of the neurotrophin receptor p75(NTR) leads to long-lasting increases in the number of basal forebrain cholinergic neurons. Journal of Neuroscience 22:2409–2418.

Newbern JM (2015) Molecular control of the neural crest and peripheral nervous system development. Curr Top Dev Biol 111:201–231.

Nikoletopoulou V, Lickert H, Frade JM, Rencurel C, Giallonardo P, Zhang L, Bibel M, Barde Y-A (2010) Neurotrophin receptors TrkA and TrkC cause neuronal death whereas TrkB does not. Nature 467:59–63.

Nishino J, Mochida K, Ohfuji Y, Shimazaki T, Meno C, Ohishi S, Matsuda Y, Fujii H, Saijoh Y, Hamada H (1999) GFR alpha3, a component of the artemin receptor, is required for migration and survival of the superior cervical ganglion. Neuron 23:725–736.

Nykjaer A, Lee R, Teng KK, Jansen P, Madsen P, Nielsen MS, Jacobsen C, Kliemannel M, Schwarz E, Willnow TE, Hempstead BL, Petersen CM (2004) Sortilin is essential for proNGF-induced neuronal cell death. Nature 427:843–848.

Palmada M, Kanwal S, Rutkoski NJ, Gustafson-Brown C, Johnson RS, Wisdom R, Carter BD (2002) c-jun is essential for sympathetic neuronal death induced by NGF withdrawal but not by p75 activation. J Cell Biol 158:453–461.

Perlson E, Jeong G-B, Ross JL, Dixit R, Wallace KE, Kalb RG, Holzbaur ELF (2009) A switch in retrograde signaling from survival to stress in rapid-onset neurodegeneration. J Neurosci 29:9903–9917.

Potts PR, Singh S, Knezek M, Thompson CB, Deshmukh M (2003) Critical function of endogenous XIAP in regulating caspase activation during sympathetic neuronal apoptosis. J Cell Biol 163:789–799.

Powelka AM, Sun J, Li J, Gao M, Shaw LM, Sonnenberg A, Hsu VW (2004) Stimulation-Dependent Recycling of Integrin β1 Regulated by ARF6 and Rab11. Traffic 5:20–36.

Prigent M, Dubois T, Raposo G, Derrien V, Tenza D, Rossé C, Camonis J, Chavrier P (2003) ARF6 controls post-endocytic recycling through its downstream exocyst complex effector. J Cell Biol 163:1111–1121.

Reichardt LF (2006) Neuratrophin-regulated signalling pathways. Philosophical Transactions of the Royal Society B: Biological Sciences 361:1545–1564.

Reissmann E, Ernsberger U, Francis-West PH, Rueger D, Brickell PM, Rohrer H (1996) Involvement of bone morphogenetic protein-4 and bone morphogenetic protein-7 in the differentiation of the adrenergic phenotype in developing sympathetic neurons. Development 122:2079–2088.

Rickmann M, Fawcett JW, Keynes RJ (1985) The migration of neural crest cells and the growth of motor axons through the rostral half of the chick somite. J Embryol Exp Morphol 90:437–455.

Rubin E (1985) Development of the rat superior cervical ganglion: ganglion cell maturation. Journal of Neuroscience 5:673–684.

Saito D, Takase Y, Murai H, Takahashi Y (2012) The Dorsal Aorta Initiates a Molecular Cascade That Instructs Sympatho-Adrenal Specification. Science 336:1578–1581.

Sakamoto M, Ieki N, Miyoshi G, Mochimaru D, Miyachi H, Imura T, Yamaguchi M, Fishell G, Mori K, Kageyama R, Imayoshi I (2014) Continuous postnatal neurogenesis contributes to formation of the olfactory bulb neural circuits and flexible olfactory associative learning. J Neurosci 34:5788–5799.

Salehi AH, Roux PP, Kubu CJ, Zeindler C, Bhakar A, Tannis LL, Verdi JM, BARKER PA (2000) NRAGE, a novel MAGE protein, interacts with the p75 neurotrophin receptor and facilitates nerve growth factor-dependent apoptosis. Neuron 27:279–288.

Samanta J, Grund EM, Silva HM, Lafaille JJ, Fishell G, Salzer JL (2015) Inhibition of Gli1 mobilizes endogenous neural stem cells for remyelination. Nature 526:448–452.

Sanai N, Nguyen T, Ihrie RA, Mirzadeh Z, Tsai H-H, Wong M, Gupta N, Berger MS, Huang E, Garcia-Verdugo JM, Rowitch DH, Alvarez-Buylla A (2011) Corridors of migrating neurons in the human brain and their decline during infancy. Nature 478:382–386.

Santiago A, Erickson CA (2002) Ephrin-B ligands play a dual role in the control of neural crest cell migration. Development 129:3621–3632.

Santy LC, Casanova JE (2001) Activation of ARF6 by ARNO stimulates epithelial cell migration through downstream activation of both Rac1 and phospholipase D. J Cell Biol 154:599–610.

Sasselli V, Pachnis V, Burns AJ (2012) The enteric nervous system. Dev Biol 366:64–73.

Saxena S, Howe CL, Cosgaya JM, Hu M, Weis J, Krüttgen A (2004) Differences in the surface binding and endocytosis of neurotrophins by p75NTR. Molecular and Cellular Neuroscience 26:292–307.

Scheib JL, Sullivan CS, Carter BD (2012) Jedi-1 and MEGF10 signal engulfment of apoptotic neurons through the tyrosine kinase Syk. J Neurosci 32:13022–13031.

Schneider C, Wicht H, Enderich J, Wegner M, Rohrer H (1999) Bone morphogenetic proteins are required in vivo for the generation of sympathetic neurons. Neuron 24:861–870.

Schwarz Q, Maden CH, Vieira JM, Ruhrberg C (2009) Neuropilin 1 signaling guides neural crest cells to coordinate pathway choice with cell specification. Proc Natl Acad Sci USA 106:6164–6169.

Scott-Solomon E, Kuruvilla R (2018) Mechanisms of neurotrophin trafficking via Trk receptors. Molecular and Cellular Neuroscience:1–0.

Shao Y, Akmentin W, Toledo-Aral JJ, Rosenbaum J, Valdez G, Cabot JB, Hilbush BS, Halegoua S (2002) Pincher, a pinocytic chaperone for nerve growth factor/TrkA signaling endosomes. J Cell Biol 157:679–691.

Sierra A, Encinas JM, Deudero JJP, Chancey JH, Enikolopov G, Overstreet-Wadiche LS, Tsirka SE, Maletic-Savatic M (2010) Microglia shape adult hippocampal neurogenesis through apoptosis-coupled phagocytosis. Cell Stem Cell 7:483–495.

Simon DJ, Pitts J, Hertz NT, Yang J, Yamagishi Y, Olsen O, Tešić Mark M, Molina H, Tessier-Lavigne M (2016) Axon Degeneration Gated by Retrograde Activation of Somatic Pro-apoptotic Signaling. Cell 164:1031–1045.

Siu M, Sengupta-Ghosh A, Lewcock JW (2018) Dual Leucine Zipper Kinase Inhibitors for the Treatment of Neurodegeneration. J Med Chem:acs.jmedchem.8b00370.

Sorrells SF, Paredes MF, Cebrian-Silla A, Sandoval K, Qi D, Kelley KW, James D, Mayer S, Chang J, Auguste KI, Chang EF, Gutierrez AJ, Kriegstein AR, Mathern GW, Oldham MC, Huang EJ, Garcia-Verdugo JM, Yang Z, Alvarez-Buylla A (2018) Human hippocampal neurogenesis drops sharply in children to undetectable levels in adults. Nature 555:377–381.

Sullivan CS, Scheib JL, Ma Z, Dang RP, Schafer JM, Hickman FE, Brodsky FM, Ravichandran KS, Carter BD (2014) The adaptor protein GULP promotes Jedi-1-mediated phagocytosis through a clathrin-dependent mechanism. Holzbaur E, ed. Mol Biol Cell 25:1925–1936.

Syroid DE, Maycox PJ, Soilu-Hänninen M, Petratos S, Bucci T, Burrola P, Murray S, Cheema S, Lee KF, Lemke G, Kilpatrick TJ (2000) Induction of postnatal schwann cell death by the low-affinity neurotrophin receptor in vitro and after axotomy. Journal of Neuroscience 20:5741–5747.

Tanaka Y, Niwa S, Dong M, Farkhondeh A, Wang L, Zhou R, Hirokawa N (2016) The Molecular Motor KIF1A Transports the TrkA Neurotrophin Receptor and Is Essential for Sensory Neuron Survival and Function. Neuron 90:1215–1229.

Teng HK, Teng KK, Lee R, Wright S, Tevar S, Almeida RD, Kermani P, Torkin R, Chen Z-Y, Lee FS, Kraemer RT, Nykjaer A, Hempstead BL (2005) ProBDNF Induces Neuronal Apoptosis via Activation of a Receptor Complex of p75NTR and Sortilin. Journal of Neuroscience 25:5455–5463.

Thapa N, Anderson RA (2012) PIP2 signaling, an integrator of cell polarity and vesicle trafficking in directionally migrating cells. Cell Adhesion & Migration 6:409–412.

Thiery JP, Duband JL, Delouvée A (1982) Pathways and mechanisms of avian trunk neural crest cell migration and localization. Dev Biol 93:324–343.

Tong CK, Fuentealba LC, Shah JK, Lindquist RA, Ihrie RA, Guinto CD, Rodas-Rodriguez JL, Alvarez-Buylla A (2015) A Dorsal SHH-Dependent Domain in the V-SVZ Produces Large Numbers of Oligodendroglial Lineage Cells in the Postnatal Brain. Stem Cell Reports 5:461–470.

Toresson H, Potter SS, Campbell K (2000) Genetic control of dorsal-ventral identity in the telencephalon: opposing roles for Pax6 and Gsh2. Development 127:4361–4371.

Tucker RP, Hagios C, Chiquet-Ehrismann R, Lawler J, Hall RJ, Erickson CA (1999) Thrombospondin-1 and neural crest cell migration. Dev Dyn 214:312–322.

Underwood CK, Reid K, May LM, Bartlett PF, Coulson EJ (2008) Palmitoylation of the C-terminal fragment of p75(NTR) regulates death signaling and is required for subsequent cleavage by gamma-secretase. Molecular and Cellular Neuroscience 37:346–358.

Urra S, Escudero CA, Ramos P, Lisbona F, Allende E, Covarrubias P, Parraguez JI, Zampieri N, Chao MV, Annaert W, Bronfman FC (2007) TrkA receptor activation by nerve growth factor induces shedding of the p75 neurotrophin receptor followed by endosomal gamma-secretase-mediated release of the p75 intracellular domain. Journal of Biological Chemistry 282:7606–7615.

Vaegter CB, Jansen P, Fjorback AW, Glerup S, Skeldal S, Kjolby M, Richner M, Erdmann B, Nyengaard JR, Tessarollo L, Lewin GR, Willnow TE, Chao MV, Nykjaer A (2011) Sortilin associates with Trk receptors to enhance anterograde transport and neurotrophin signaling. Nature Publishing Group 14:54–61.

Valdez G, Akmentin W, Philippidou P, Kuruvilla R, Ginty DD, Halegoua S (2005) Pincher-mediated macroendocytosis underlies retrograde signaling by neurotrophin receptors. J Neurosci 25:5236–5247.

Venkateswarlu K, Gunn-Moore F, Tavare JM, Cullen PJ (1999) EGF-and NGF-stimulated translocation of cytohesin-1 to the plasma membrane of PC12 cells requires PI 3-kinase activation and a functional cytohesin-1 PH domain. J Cell Sci 112 (Pt 12):1957–1965.

Verdi JM, Anderson DJ (1994) Neurotrophins regulate sequential changes in neurotrophin receptor expression by sympathetic neuroblasts. Neuron 13:1359–1372.

Vicario A, Kisiswa L, Tann JY, Kelly CE, Ibáñez CF (2015) Neuron-type-specific signaling by the p75NTR death receptor is regulated by differential proteolytic cleavage. J Cell Sci 128:1507–1517.

Vilar M, Charalampopoulos I, Kenchappa RS, Simi A, Karaca E, Reversi A, Choi S, Bothwell M, Mingarro I, Friedman WJ, Schiavo G, Bastiaens PIH, Verveer PJ, Carter BD, Ibáñez CF (2009) Activation of the p75 neurotrophin receptor through conformational rearrangement of disulphide-linked receptor dimers. Neuron 62:72–83.

Volosin M, Trotter C, Cragnolini A, Kenchappa RS, Light M, Hempstead BL, Carter BD, Friedman WJ (2008) Induction of proneurotrophins and activation of p75NTR-mediated apoptosis via neurotrophin receptor-interacting factor in hippocampal neurons after seizures. J Neurosci 28:9870–9879.

Volpicelli-Daley LA, Li Y, Zhang C-J, Kahn RA (2005) Isoform-selective effects of the depletion of ADP-ribosylation factors 1-5 on membrane traffic. Mol Biol Cell 16:4495–4508.

Wakamatsu Y, Endo Y, Osumi N, Weston JA (2004) Multiple roles of Sox2, an HMG-box transcription factor in avian neural crest development. Dev Dyn 229:74–86.

Wang RN et al. (2014) Bone Morphogenetic Protein (BMP) signaling in development and human diseases. Genes & Diseases 1:87–105.

Weskamp G, Schlöndorff J, Lum L, Becherer JD, Kim T-W, Saftig P, Hartmann D, Murphy G, Blobel CP (2004) Evidence for a critical role of the tumor necrosis factor alpha convertase (TACE) in ectodomain shedding of the p75 neurotrophin receptor (p75NTR). Journal of Biological Chemistry 279:4241–4249.

Wieland F, Hartert C (1999) Mechanisms of vesicle formation: Insights from the COP system. Current Opinion in Cell Biology 11:440–446.

Wiesmann C, Ultsch MH, Bass SH, de Vos AM (1999) Crystal structure of nerve growth factor in complex with the ligand-binding domain of the TrkA receptor. Nature 401:184–188.

Wu C, Cui B, He L, Chen L, Mobley WC (2009a) The coming of age of axonal neurotrophin signaling endosomes. J Proteomics 72:46–55.

Wu H-H, Bellmunt E, Scheib JL, Venegas V, Burkert C, Reichardt LF, Zhou Z, Fariñas I, Carter BD (2009b) Glial precursors clear sensory neuron corpses during development via Jedi-1, an engulfment receptor. Nature Publishing Group 12:1534–1541 Available at: http://www.nature.com/doifinder/10.1038/nn.2446.

Wyatt S, Davies AM (1995) Regulation of nerve growth factor receptor gene expression in sympathetic neurons during development. J Cell Biol 130:1435–1446.

Wyatt S, Piñon LG, Ernfors P, Davies AM (1997) Sympathetic neuron survival and TrkA expression in NT3-deficient mouse embryos. The EMBO Journal 16:3115–3123.

Xu Q, Tam M, Anderson SA (2008) Fate mapping Nkx2.1-lineage cells in the mouse telencephalon. J Comp Neurol 506:16–29.

Yamashita N, Kuruvilla R (2016) ScienceDirect Neurotrophin signaling endosomes: biogenesis, regulation, and functions. Current Opinion in Neurobiology 39:139–145.

Yamauchi J, Miyamoto Y, Torii T, Takashima S, Kondo K, Kawahara K, Nemoto N, Chan JR, Tsujimoto G, Tanoue A (2012) Phosphorylation of cytohesin-1 by Fyn is required for initiation of myelination and the extent of myelination during development. Sci Signal 5:ra69–ra69.

Yang XM, Toma JG, Bamji SX, Belliveau DJ, Kohn J, Park M, Miller FD (1998) Autocrine hepatocyte growth factor provides a local mechanism for promoting axonal growth. Journal of Neuroscience 18:8369–8381.

Ye M, Lehigh KM, Ginty DD (2018) Multivesicular bodies mediate long-range retrograde NGF-TrkA signaling. Elife 7:295.

Yeiser EC, Rutkoski NJ, Naito A, Inoue J-I, Carter BD (2004) Neurotrophin signaling through the p75 receptor is deficient in traf6-/- mice. J Neurosci 24:10521–10529.

Yip JW (1986) Migratory patterns of sympathetic ganglioblasts and other neural crest derivatives in chick embryos. Journal of Neuroscience 6:3465–3473.

Young HM, Cane KN, Anderson CR (2011) Development of the autonomic nervous system: a comparative view. Auton Neurosci 165:10–27.

Young KM, Fogarty M, Kessaris N, Richardson WD (2007a) Subventricular zone stem cells are heterogeneous with respect to their embryonic origins and neurogenic fates in the adult olfactory bulb. J Neurosci 27:8286–8296.

Young KM, Merson TD, Sotthibundhu A, Coulson EJ, Bartlett PF (2007b) p75 neurotrophin receptor expression defines a population of BDNF-responsive neurogenic precursor cells. J Neurosci 27:5146–5155.

Zerial M, McBride H (2001) Rab proteins as membrane organizers. Nat Rev Mol Cell Biol 2:107–117.

Zheng N, Jeyifous O, Munro C, Montgomery JM, Green WN (2015) Synaptic activity regulates AMPA receptor trafficking through different recycling pathways. Elife 4:260.

Zhou B, Cai Q, Xie Y, Sheng Z-H (2012) Snapin Recruits Dynein to BDNF-TrkB Signaling Endosomes for Retrograde Axonal Transport and Is Essential for Dendrite Growth of Cortical Neurons. Cell Reports 2:42–51.