p75-NGFR and TNFR1 Interact and Cooperate During the Sympathetic Nervous System Development

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A Dissertation presented to the Graduate Faculty
of the University of Virginia in Candidacy for the Degree of
Doctor of Philosophy

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University of Virginia
May, 2014

Abstract

During development, components of the nervous system are overproduced and then refined to achieve optimal connectivity and functionality. During this refinement process, excess neurons are weakened and eliminated, while others are strengthened and stabilized. The molecular basis for these refinement decisions remains unclear. This thesis examines how TNFR family members coordinate their activity to antagonize NGF-TrkA signaling in order to regulate neuronal cell death, axon growth, and cell size during development. First, the closely related TNFR family members, *p75-NGFR* and *TNFR1*, and their cognate ligands, *BDNF* and *TNF*, are expressed in sympathetic ganglia. *In vitro*, p75-NGFR and TNFR1 act cooperatively to countermand NGF-TrkA trophic signaling in order to cause ligand-initiated axon growth inhibition. However, such concurrent availability of the two receptors is required to cause cell death initiated by BDNF, but not by TNFα. Further, using co-immunoprecipitation and bimolecular fluorescence complementation (BiFC) assays, these receptors are shown to interact on the cellular surface. *In vivo,* this codependence does not appear to be necessary for developmental cell death, but is required for regulation of sympathetic soma size and proper axon patterning during target innervation. Taken together, these findings suggest cooperativity between p75-NGFR and TNFR1 in mediating a range of developmental processes including neuronal morphology regulation.

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Chapter 1

Background

Development of the Sympathetic Nervous System

The nervous system is wired through a series of developmental events coordinated by complex signaling pathways during development. In this developmental process, it is necessary for the organism to connect a proper amount and type of neurons with their correct partners, as well as to modify neuronal morphology. Toward this end, components of the nervous system including axons and neurons are overproduced and then subpopulations are either strengthened or eliminated. Such refinement is as important as construction of the components to achieve the optimal wiring of the nervous system.

As a part of the autonomic nervous system, the sympathetic nervous system (SNS) maintains homeostasis and enables an animal's "fight or flight" response by increasing heart rate and blood pressure, dilating pupils, promoting perspiration and delaying digestion (Cannon, 1932). The sympathetic nervous system comprises two types of neurons, preganglionic and postganglionic, connecting the central nervous system (CNS) and target organs such as eyes, salivary glands, heart, stomach, and kidneys via spinal cord and sympathetic ganglia (Fig. 1-1). Since Levi-Montalcini and Hamburger discovered the effects of mouse sarcoma promoting nerve growth using the sensory and

sympathetic nervous system of chick embryos in 1951, the postganglionic sympathetic neurons have been an archetypal model to study and dissect the complex signaling that takes place during nervous system development. There are two great merits of using postganglionic sympathetic neurons; 1) the neurons are nearly homogeneously noradrenergic, and 2) the ganglia receive inputs from preganglionic spinal cord neurons and send outputs to target organs (Cane and Anderson, 2009). These simple chemical and structural traits extremely simplify categorization of neuronal type and are beneficial in analyzing molecular influences of the inputs and outputs on development of postganglionic neurons.

In addition to the simpler molecular identity of neurons, the structure of the sympathetic nervous system is somewhat simpler than the central nervous system. Preganglionic neurons reside in the spinal cord and extend their axons towards postganglionic neurons clustered in ganglia along the spinal cord, which is known as the sympathetic chain. Postganglionic axons travel from the sympathetic ganglia toward the aforementioned target organs. Thus, development of the sympathetic nervous system requires both preganglionic and postganglionic neurons to form proper connections with the right targets. This requirement is a great challenge especially for postganglionic neurons because their axons have to find and innervate target organs, which are often located far from the sympathetic ganglia in a dynamically changing environment. To achieve this in a limited time during development, the organism coordinates developmental events in a specific order.

Extensive studies using mouse embryos have shown that postganglionic neurons undergo key milestones from embryonic day 10 (E10) (Rubin, 1985). Sympathetic precursors are generated from neural crest cells, which have ventrally migrated from dorsal neural tube to the adjacent vicinity of the dorsal aorta around E10 (Rubin, 1985). Signals secreted by the surrounding tissues, including the aorta, induce neuronal differentiation into sympathetic precursors and ganglia formation by activating a cascade of specific transcription factors. At E12-14, sympathetic cell bodies, which have exited from the mitotic cycle and differentiated as noradrenergic, start to extend axons and dendrites (Rubin, 1985). At E15, the axons begin to reach the target tissues and final target innervation commences (Rubin, 1985). Simultaneously, dendrites continue to be elaborated in the sympathetic ganglia. Then, following the final target innervation, the postganglionic dendrites and preganglionic axons form functional synapses in the ganglia. This process is regulated by a retrograde signal, which has traveled inside the axon from the final target to the dendrites (Sharma et al., 2010). The stabilization of sympathetic neurons during development depends on the target-derived neurotrophic signal, identified as nerve growth factor (NGF) (Cohen, 1960). Between E17 and postnatal day 0 (P0), as final targets receive sympathetic innervation, postganglionic neurons undergo NGF-dependent survival where only the neurons acquiring survival signaling are strengthened and fixed in the nervous system. In contrast, the neurons failing to gain access to NGF commit to die by activating signaling that encodes apoptosis (Oppenheim, 1991). In this way, the spinal cord and target organs are wired by postganglionic sympathetic neurons together with preganglionic neurons prior to birth. Elaboration of this network continues after birth.

During development, the survival/death decision of neurons, as well as final target innervation, play a crucial role because the functionality of the nervous system lies in the appropriate amount of connections in the nervous system. Here, two influencing parameters are the number and morphology of neurons, both of which are controlled by the amount of target-derived NGF in the sympathetic nervous system. Thus, developmental cell death and axonal patterning on target organs play an important role in removing errors and refining the nervous system.

Neurotrophins and Neurotrophin Receptors

Development of the sympathetic nervous system requires cell extrinsic trophic factors, which activate intrinsic signaling cascades to initiate a hierarchy of developmental events (Bibel and Barde, 2000). One class of trophic factors that is particularly important for nervous system development and function is the neurotrophin family. Neurotrophins are growth factors crucial for neuronal precursor proliferation, neuronal differentiation, and survival as well as axon growth and target innervation (Glebova and Ginty, 2005). Mammals utilize four neurotrophins: NGF, brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT3) and neurotrophin-4 (NT4). Each neurotrophin is generated from the precursor known as proneurotrophin, which must be cleaved in order to function as a mature form of neurotrophin (Teng et al, 2010). To transduce signaling from the neurotrophins, there are two different classes of neurotrophin receptors: 1) a family of receptor tyrosine kinases, termed the Trks; and 2) the p75 nerve growth factor receptor

(p75-NGFR), a tumor necrosis factor receptor (TNFR) family member. Each Trk family member binds to a specific neurotrophin with high affinity. These are as follows: NGF to TrkA, BDNF and NT4 to TrkB, and NT3 to TrkC, with p75-NGFR displaying low affinity promiscuous binding with all neurotrophins (Fig. 1-2A) (Bibel and Barde, 2000; Chao, 2003). One aspect that makes p75-NGFR a puzzling receptor is that its ligand partners include not only the neurotrophins, but also proneurotrophins and myelin-derived molecules. However, a classic view in the neurotrophin field is that distinct activation of Trk receptors vs p75-NGFR ultimately results in supporting vs inhibiting neuronal survival/growth, respectively.

Almost all developmental events of the postganglionic sympathetic neurons are supported by NGF-TrkA signaling (Glebova and Ginty, 2005; Ichim et al., 2012). When NGF binds to TrkA, the ligand dimerizes the receptor, leading to autophosphorylation of the intracellular kinase domain of TrkA. This event generates a signaling cascade downstream involving Ras activation, which further activates several pathways, such as the phosphophatidyl inositol-3 (PI3)-kinase pathway and/or the MAP kinase pathway, resulting in the expression of pro-survival/pro-differentiation genes, promoting neuronal survival, differentiation, neurite growth or synaptic plasticity (Fig. 1-2B) (Chao, 2003; Reichardt, 2006; Ichim et al., 2012). On the other hand, the pro-apoptotic functions of p75-NGFR is triggered as follows: when p75-NGFR is activated by neurotrophins, proneurotrophins or nonneurotrophic ligands, it leads to the activation of the Jun kinase (JNK) pathway, or RhoA, which results in either upregulating pro-apoptotic genes or the reduction of growth cone motility, thus promoting cell death and axon pruning (Fig.1-2B)

(Bibel and Barde, 2000; Chao, 2003; Glebova and Ginty, 2005; Reichardt, 2006). As such, the signal initiated by target-derived neurotrophins is transduced in a complicated manner by their receptors and downstream effectors. Neurons orchestrate neurotrophin signaling by regulating the spatial and temporal expression of neurotrophins as well as Trk receptors and p75-NGFR.

TNFR Family

Tumor Necrosis Factor Receptor (TNFR) family consists nearly 30 distinct members and regulates a wide variety of functions such as immune responses, cell proliferation, differentiation and death (Locksley et al., 2001; Twohig et al., 2012). Structurally, the members are characterized by multiple (usually one to four) cysteine-rich domains (CRDs) in the extracellular part of the receptor. Six highly conserved cysteines in these domains create three intrachain disulfide bridges, forming the binding groove for the corresponding ligand, which is typically a trimer (Locksley et al., 2001; Twohig et al., 2012). Of these TNFR family members, eight receptors with a certain intracellular interaction motif, known as the death domain (DD), are further categorized as death receptors including TNFR1 and p75-NGFR, both of which are reported to cause apoptosis and refine the nervous system (Twihog et al., 2012). The DD acts as a docking site and can recruit various adaptor proteins, such as Fas-associated DD protein (FADD), TNFR-associated DD protein (TRADD), to recruit even more pro-death signaling molecules and transduce death signaling downstream (Locksley et al., 2001).

Even though many TNFR family members do not possess the DD, 20 members of the TNFR family have another intracellular domain that can recruit an adaptor protein known as TNFR-associated factor (TRAF) (Arch et al., 1998; Silke and Brink, 2010). One reason signaling generated by TNFR family members is complex is because TRAFs interact with a variety of cytoplasmic proteins and regulate signaling for cell survival as well as apoptosis and stress responses (Arch et al., 1988).

Programmed Cell Death and the Neurotrophic Theory

Programmed cell death (PCD) occurs as an active and natural process whereby cells die so that the proper number and types of cells should efficiently arise during development and be maintained throughout life. Almost all tissues utilize PCD in vertebrates for component quality- and quantity control and error corrections by removing unneeded, nonfunctional or harmful cells (Glückman, 1951; Ellis et al., 1991; Oppenheim, 1991).

A classic and well-studied form of PCD is apoptosis, in which cells commit to die by activating an intracellular suicide mechanism. Development of the nervous system hugely relies on apoptosis to shape the circuit during the late stage. Apoptosis is linked to certain microscopic features such as cellular shrinkage, chromatin condensation, endonuclease degradation of DNA and nuclear fragmentation (Kerr et al., 1972; Wu et al., 2011). At the molecular level, apoptosis is characterized by the intracellular cascade activation of specific cysteine proteases known as caspases, which are executors in the death signaling pathway. This caspase cascade activation is a two-step process; first, the initiator

caspases (caspase-2, -8, -9, -10) are auto-activated, often following the formation of a multi-component complex, then the activated initiator caspases further activate the effector caspases (caspase-3, -7, -8) by cleavage (Riedl and Shi, 2004; Parrish et al., 2013). Following such cleavage, the effector caspases are committed to cleave a broad spectrum of cellular substrates to destroy the cell (Riedl and Shi 2004; Parrish et al, 2013). The morphological features and caspase-dependence of apoptosis is distinct from necrosis, which is another form of cell death characterized by cell swelling and caspase-independent properties (Wu et al., 2011). Recent accumulating studies report that programmed cell death is not limited to apoptosis, but also includes a form of necrosis. The process of programmed necrosis, also known as Necroptosis, has been reported to cause cell death by a distinct mechanism (Edinger and Thompson, 2004; Wu et al., 2011). Although this is an attractive topic, it is beyond the scope of this thesis, i.e., the refinement functions of apoptotic signaling during the sympathetic nervous system development. Thus, hereafter cell death will refer only to apoptosis in this thesis.

During development of the sympathetic nervous system, which is one of the best models for studying apoptosis, as much as 50% of developing neurons undergo apoptosis soon after the axons reach and innervate the target tissues (Oppenheim, 1991). This phenomenon is explained by the “neurotrophic theory” formulated following the discovery of NGF by Levi-Montalcini and Hamburger and Hamburger in the 1940s and 1950s. The theory addresses the survival of neurons as well as innervation depends on the limited amount of target-derived neurotrophic factor (Oppenheim, 1989; Davies, 1991). Further evidence, discovered in the 1980s, verified three aspects of the theory; 1) under

normal conditions, only a limited population of NGF-dependent ganglion neurons can survive, 2) the massive cell death observed under normal condition is rescued by the addition of exogenous NGF, and 3) the degree of innervation positively correlates with the levels of NGF secreted in the vicinity of targets (Oppenheim, 1989). The further understanding of the molecular mechanism for these phenomena has progressed with the discovery of the receptors to NGF, TrkA and p75-NGFR, which have already been described to play positive or negative roles in neuronal survival during development. Thus, the optimal number of neurons innervating a target tissue in the sympathetic nervous system is determined by competition between pro-death signaling from p75-NGFR and pro-survival signaling from TrkA (Deppmann and Janes, 2012). In this competition, either pro-survival signaling or pro-apoptotic signaling becomes dominant in the cell, resulting in its survival/death decision.

Interestingly, the competition between pro-survival and pro-death signaling has recently been found to be more complex. New discoveries demonstrated that TrkA causes neuronal death in a p75-dependent manner (Nikoletopoulou et al., 2010; Dekkers et al., 2013). This suggests that TrkA is involved in generating both pro-survival- and pro-death signaling, implying that TrkA function in cell survival/death depends on the NGF availability because neurons survive when NGF-TrkA signaling is dominant (Bamji et al., 1998). In other words, TrkA can detect the presence and absence of NGF, leading to distinct functions of the receptor. Thus, TrkA is now categorized as a “dependence receptor”. Since p75-NGFR can also be activated by both the presence and absence of NGF, sympathetic neurons utilize multiple dependence receptors. The molecular

mechanisms by which TrkA and p75-NGFR cooperate to cause cell death remains unknown. The merit of using dependence receptors during developmental cell death is probably enabling cells to control its sensitivity to NGF by upregulating or downregulating TrkA and/or p75-NGFR, which is a key mechanism for a neuron to become NGF-dependent during target innervation. Here, it is interesting to note that survival of developing sensory neurons is supported by either BDNF or NT3 prior to target innervation, while NGF-dependence is acquired during the early stages of target innervation (Paul and Davies, 1995). This suggests that neurotrophin sensitivity of developing neurons is fundamentally different before and during target innervation.

Developmental Cell Survival and Death in the SNS

In developing mouse sympathetic neurons, TrkA, TrkC and p75-NGFR, but not TrkB, are expressed (Fagan et al., 1996). Thus, as the neurotrophic theory states, neuronal survival during sympathetic nervous system development is supported by acquiring target-derived NGF via TrkA and activating survival signaling inside the neuron. When the distal axons reach the final target after traveling a long distance following the vasculature, NGF secreted from target organs binds to neuronal TrkA on the axon. This leads to endocytosis of the NGF-TrkA complex often referred to as the signaling endosome. Following endocytosis, the signaling endosome is trafficked retrogradely toward the cell body, where it engages transcriptional programs that support neuronal survival and axon growth, including upregulationg TrkA expression (Ibáñez et al., 1992; Kohn et al., 1999; Ye et al., 2003; Deppmann et al, 2008). Interestingly, the restrograde TrkA signaling is

attenuated by inhibition of PI3-kinase, suggesting that the retrograde trafficking of the signaling endosome is PI3-kinase-dependent (Kuruvilla et al., 2004).

The other important aspect that the neurotrophic theory indicates is that developmental cell death is initiated when the neuron is unable to gain access to NGF. This has been successfully modeled in an *in vitro* system where developing sympathetic neurons cultured in NGF-deprived media undergo apoptosis (Deckwerth and Johnson, 1993a and 1993b). After this *in vitro* manipulation became available, accumulating studies have described molecular mechanisms of sympathetic apoptosis induced by NGF-deprivation. According to elegant work from the laboratory of Eugene Johnson, Jr., neuronal degradation and death can be prevented by inhibiting mRNA, which suggests that de novo synthesized gene products participate to cause apoptosis (Martin et al., 1988; Deckwerth and Johnson, 1993b). Furthermore, the neuronal death is prevented by addition of NGF, cyclic AMP or KCL, which is blocked by inhibition of protein synthesis, suggesting that the cell possess a mechanism to halt the apoptotic progress at the post translational level (Edwards et al., 1991). These findings further led to investigations identifying gene components unregulated or required in the apoptotic pathway. It is now established that NGF deprivation activates the MLK-JNK-cJun pathway followed by the intrinsic mitochondrial pathway in a p75-NGFR-mediated manner to cause apoptosis in developing sympathetic neurons (Deshmukh and Johnson, 1997; Ham et al, 2000; Kole et al., 2013). The intrinsic pathway during the development of sympathetic neurons utilizes common machinery with common components also used for apoptosis of other cell types. The apoptotic upstream signals activate Bcl-2-associated X protein (Bax) and release

mitochondrial cytochrome c, which binds to Apaf-1 and recruits and forms the apoptosome complex with Apaf-1 in the cytoplasm. (Deshmuk and Johnson, 1997; Dekkers et al., 2013). This recruits and activates caspase-9, leading to activation of the effector caspases. Here, the X-linked inhibitor of apoptosis (XIAP) specifically polyubiquitylates active caspase-9 to tightly regulate the intrinsic pathway. Thus, in order to activate caspase cascade, the neuron is required to decrease the level of XIAP (Potts et al., 2003; Parrish et al., 2013). Recently, a global gene expression analysis discovered additional genes associated with the ER unfolded protein response are upregulated after NGF withdrawal, suggesting that ER stress response takes place during sympathetic apoptosis (Kristiansen et al., 2011). Compared to the downstream NGF-deprivation induced refinement, however, the upstream mechanism, i.e., how the cell detects the "absence" of NGF and transduces the signal to the inside, has been understudied. As a death receptor expressed on the sympathetic cell membrane, p75-NGFR is a good candidate to influence how this process is initiated.

p75-NGFR is a classic death receptor accelerating apoptosis during the sympathetic nervous system development. p75-NGFR knockout animals have been reported to increase neuronal numbers at the postnatal sympathetic ganglia (Bamji et al., 1998; Deppmann et al., 2008). However, the apoptotic effect of p75-NGFR in the sympathetic nervous system does not remain until adulthood. In adult *p75-NGFR*-deficient sympathetic ganglia the neuronal number is normal compared to wild type controls, while the neuronal number remains increased in the adult *Bax*-null sympathetic ganglia (Bamji et al., 1998; Deppmann et al., 2008; Li et al., 2011). These data suggest that p75-NGFR

causes rapid cell death, but that sympathetic apoptosis does not completely rely on p75-NGFR. One plausible explanation for this would be that other pro-apoptotic receptors participate in this apoptotic process.

Indeed, apoptotic signaling induced by other TNFR family members is implicated in the apoptotic signaling during nervous system development. One example is TNF-TNFR1 signaling. The sympathetic neurons express TNFα and its death receptor TNFR1 during development, and addition of TNFα significantly kills cultured developing sympathetic neurons in the presence of a suboptimal level of NGF that can support sufficient neuronal survival but can be overridden by activation of death signaling (Barker et al., 2001). In the same report, the *in vivo* neuronal number of the sympathetic ganglia from *TNF*-deficient mice is higher than that of wild type controls. Another example known to cause cell death is death receptor 6 (DR6) signaling. DR6 is a member of the TNFR family and has been demonstrated to cause apoptosis in sensory developing neurons, which are also NGF-dependent like the sympathetic neurons (Nikolaev et al., 2009). In this process, NGF deprivation releases N terminal β-amyloid precursor protein (APP), which acts as a DR6 ligand (Nikolaev et al., 2009). On the other hand, two other TNFR family members, Fas and glucocorticoid-induced tumor necrosis factor receptor (GITR), do not contribute to developmental cell death in sympathetic neurons. This is despite the coexpression of the receptors and their ligands, FasL and GITRL, respectively (Putcha et al., 2002; O'Keefe et al., 2008). Taken together, developing neurons utilize multiple sets of pro-death signaling to hasten the apoptotic process. However, whether (and how) these receptors cooperate during this process remains unknown. One possibility is that distinct

receptors form a complex on the cell membrane to generate apoptotic signaling. For example, recent studies show that p75-NGFR and DR6 form a complex allowing β-amyloid-induced death to occur in cortical neurons (Hu et al., 2013). Additionally, DR6 is reported to form a nonapoptotic complex with another TNFR member, TROY, during brain vascular development and promotes vascular endothelial growth factor (VEGF)-induced JNK phosphorylation (Tam et al., 2011). However, whether p75-NGFR has such other co-receptor partners among TNFR family members is completely unknown.

Target Innervation and Developmental Axon Pruning in the SNS

The neurotrophic theory argues that the axons of developing neurons compete for a limited amount of target-derived NGF during the final target innervation. To reach the distal target tissues, differentiated sympathetic neurons extend their axons from the sympathetic ganglia by following vasculature and intermediate targets that attract sympathetic axons by secreting trophic factors such as Artemin (ARTN) and NT3 which induce ret proto-oncogene (RET) signaling and TrkA signaling, respectively, locally at the extending axons (Enomoto et al., 2001, Honma et al., 2002; Kuruvilla et al., 2004; Glebova and Ginty, 2005). Once the target has been reached, sympathetic innervation commences. As shown in the initial discovery and following reports of the effects of NGF on the promotion of sympathetic axon extension, the development event is promoted by NGF (Levi-Montalcini, 1987; Glebova and Ginty, 2005; Davies, 2009). The relative relevance of NGF-TrkA signaling in target innervation *in vivo* was first discovered by Glebova and Ginty by generating a mouse line that lacks both *NGF* and

Bax. Since neuronal death caused by lack of *NGF* has been rescued by ablating *Bax*, the role of NGF in target innervation has been successfully determined (Glebova and Ginty, 2004). According to their report, the NGF-dependency in target innervation varies among sympathetic targets *in vivo*. For example, sympathetic innervation is absent in the heart ventricles whereas partially reduced in the kidneys in *Bax*-/-;*NGF*-/- mice, supporting that additional players are required in promoting the sympathetic innervation in majority of target organs (Glebova and Ginty, 2004).

Developmental axon pruning is necessary to correct neuronal misconnections and achieve nervous system development for proper functions. Toward this end, not only must the developing axons be guided properly to the target tissue, but also excess and nonfunctional axons must be eliminated.

Accumulating studies show that p75-NGFR promotes accurate sympathetic axon branching on the target by locally mediating repulsive, inhibitory or degenerative signaling in the axon (Wong et al, 2002; Yamashita et al., 1999; Kohn et al, 1999; Signh et al, 2008). The sympathetic target innervation in *p75-NGFR*-deficient mice is impaired (Lee et al., 1994; Jahed and Kawaja, 2005). In the repulsive- and inhibitory signaling, myelin-derived proteins such as Nogo, myelin associated glycoprotein (MAG) and oligodendrocyte glycoprotein (OMpg), activate RhoA and result in inhibition of axon growth (Yamashita and Tohyama, 2003). For such guidance cues, an increasing number of factors have been reported, such as semaphorin (Sema) 3A and 3F, ephrin B2 and proNGF (Naska et al., 2010; Deinhardt et al., 2011).

In axon-degenerative signaling, BDNF-activated p75-NGFR promotes axon growth inhibition in the developing sympathetic neurons (Kohn et al., 1999). Recent data showing an increased sympathetic innervation in mice carrying a mutation in the BDNF promoter region suggest that this axon pruning induced by BDNF via p75-NGFR takes place in an activity-dependent manner (Singh et al., 2008). Although both the local degenerative signaling used in axon pruning and the global degenerative signaling used in apoptosis are caspase-dependent events, recent reports indicate that a prominent difference lies in the type of caspases participating in the aforementioned processes. The downstream signaling during axon pruning specifically utilizes caspase-6, the absence of which protects sympathetic axons from pruning induced by NGF withdrawal (Nikolaev et al., 2009; Simon et al., 2012; Cusack et al., 2013). However, further detail of the axon pruning-specific signaling pathway, particularly how caspase-6 is activated, has been controversial and not fully elucidated.

Other TNFR family members, not only p75-NGFR, are also reported to regulate target innervation. DR6 is reported to promote sensory axon pruning via caspase-6 activation (Nikolaev et al., 2009; Cusack et al., 2013). Interestingly, GITR has been shown to promote sympathetic neurite growth *in vitro* via supporting NGF-induced ERK phosphorylation (O'Keefe et al., 2008). In *GITR*-null mice, sympathetic innervation density at iris and nasal mucosa is greatly reduced (O'Keefe et al., 2008). Recent studies reporting similar pro-neurite-growth effects of the reverse signaling of TNF, i.e., TNFR1-TNF signaling, add more complexity to the functions of the TNFR family members

during development. In this report, significant growth of developing sympathetic axons is shown after exogenous addition of extracellular domains of TNFR1 in culture, and reduction of sympathetic innervation density is observed in iris, nasal tissue and submandibular glands from both *TNF*-deficient- and *TNFR1*-deficient mice (Kisiswa et al., 2013). However, as seen in pro-death complexes formed by p75-NGFR and DR6, or non-apoptotic complexes regulating CNS vascular development formed by DR6 and TROY, whether multiple TNFR family members cooperate for either axon growth or pruning during the target innervation or denervation still remains a mystery.

Neuronal Soma Size Regulation during Development

Compared to developmental cell survival/death and axon growth/pruning, the physiological impact of neuronal soma size during development has been understudied.

Neuronal hypertrophy induced by NGF has been observed as one of the nerve-growth promoting effects NGF exhibits during development. Levi-Montalcini and Bookers found that an increase in size of chick sympathetic neuronal cell bodies is evoked by addition of NGF isolated from mouse salivary glands (Levi-Montalcini and Bookers, 1960). The following studies demonstrated that the cell body diameter of developing neurons is positively correlated with NGF levels in the media *in vitro* and that neuronal size at mouse sympathetic ganglia is decreased by approximately half *in vivo* after NGF antiserum injection (Chun and Patterson,1977; Ruit and Snyder, 1991). Unlike sympathetic neuronal death, this NGF deprivation-induced neuronal atrophy does not

seem to require Bax since the *Bax*-deficient neurons after NGF deprivation still show a diameter size reduction by about half compared to *Bax*-deficient neurons cultured in the presence of NGF (Deckwerth et al., 1996). Further research using *BDNF*-deficient- and *p75-NGFR*-deficient mice revealed that neuronal size in the two mutant sympathetic ganglia decreased compared to wild type (Bamji et al., 1998). However, since those experiments were not carried out in the *Bax*-null background, ablating *BDNF* or *p75-NGFR* rescued a certain neuronal population that otherwise would have undergone cell death. Thus, the neuronal number at the sympathetic ganglia from such mice differ from that of wild type, in which cell death occurs and 50% of the neuronal population is lost. Thus, such difference in neuronal number among genotpes may have resulted in misinterpretation of the effects of p75-NGFR and BDNF. In order to accurately understand their effects in regulating the developing sympathetic neuronal size, *Bax*-dificient neurons need to be used.

Molecular mechanisms of p75-NGFR signaling

p75-NGFR is TNFR superfamily (TNFRSF) member 16 and it is required for proper nervous system development. Expression of p75-NGFR has been widely observed in the CNS and PNS during development, although its postnatal expression level decreases in most cells and is restricted to subpopulations of neurons, including sympathetic and sensory neurons (Roux and Barker, 2002; Bartkowska et al., 2010). Interestingly, the p75-NGFR expression is elevated in several types of adult neurons and glial cells after neuronal injury and cellular stress (Ibanes and Simi, 2012). As previously mentioned, by

interacting with a large number of molecules p75-NGFR possesses diverse functions including apoptosis, neurite growth inhibition, myelination, and axon pruning (Fig.1-2B) (Roux and Barker, 2002; Dekkers et al., 2013).

p75-NGFR has a variety of ligands and co-receptors, thus possessing a wide range of subsequent signaling pathways. p75-NGFR is also capable of binding all neurotrophins and proneurotrophins (Bibel and Barde, 2000; Hempstead, 2006). The well-known pathways activated by neurotrophin binding of p75-NGFR are the JNK pathway and caspase cascade, which both result in apoptosis, as well as the NFkB, which is known to promote cell proliferation and survival (Yoon et al., 1998; Linggi et al., 2005; Carter et al., 1996). Neurotrophins bind to a subpopulation of p75-NGFR that pre-exists as a homodimer on the cell surface via disulfide-linkage of the specific cysteine in the transmembrane region (Vilar et al., 2009a). This pre-ligand assembly of p75-NGFR is regarded to be responsible for activation of the neurotrophin-induced pathways including NFkB, JNK and caspase-3 (Vilar et al., 2009a, 2009b). On the other hand, binding of the precursors of mature neurotrophins, known as proneurotrophins, requires p75-NGFR, as well as another transmembrane protein known as Sortilin, and causes apoptosis in cultured sympathetic neurons and axonal growth cone retraction in hippocampal neurons (Nykjaer et al., 2004; Jensen et al., 2007; Deinhardt et al., 2011). In proNGF binding, p75-NGFR/Sortilin has higher affinity than TrkA, which indicates that proNGF acts as a death ligand whereas NGF acts as a survival ligand in cells expressing p75-NGFR, Sortilin and TrkA, such as sympathetic neurons. However, the neuronal number at sympathetic ganglia from *Sortilin*-deficient mice does not show a significant change

compared to controls during naturally occurring cell death (Jensen et al., 2007). This suggests that apoptosis during sympathetic nervous system development does not rely on the proneurotrophin signaling pathway.

Although roles of unprocessed vs mature neurotrophins seem to be distinctive during development, one common feature of proneurotrophin and neurotrophin is that both can induce sequential cleavage of p75-NGFR by secretases and release of the intracellular domain (ICD) of p75-NGFR into the cytoplasm (Kanchappa et al., 2006; Bronfman, 2007). In cultured sympathetic neurons it is shown that the level of p75-NGFR-ICD is elevated after addition of proBDNF and BDNF, but not NGF. (Kanchappa et al., 2006). This can explain the great neuronal loss in the sympathetic ganglia from the mice constitutively expressing p75-NGFR-ICD (Majdan et al., 1997). The proteolytic processing of p75-NGFR is a two-step shedding event; first, the extracellular domain (ECD) of p75-NGFR is cleaved by metalloproteases including "a disintegrin, metalloprotease" 17 (ADAM17) and ADAM10, then the membrane bound C-terminal fragment (CTF) of p75-NGFR is cleaved by the γ-secretase complex (Bronfman, 2007). In PC12 cells, which are a neuron-like cell line expressing both p75-NGFR and TrkA, NGF induces ICD production (Urra et al., 2007). Interestingly, the NGF-induced cleavage of p75-NGFR is blocked by a Trk kinase inhibitor, suggesting that the cleavage process is TrkA-dependent in PC12 cells. However, NGF-inducibility of p75-NGFR cleavage is context-dependent. In sympathetic neurons, ICD production is not induced by addition of NGF (Kanchappa et al., 2006). The mechanism that induces p75-NGFR proteolysis in the sympathetic neurons remains unknown.

Additionally, p75-NGFR can bind to nonneurotrophic, myelin-derived molecules such as MAG, Nogo, and OMpg (Wong et al., 2002). It is shown that this binding does not require the disulfide linkage of p75-NGFR through the transmembrane cysteine, which is seen in neurotrohin binding of p75-NGFR (Ibanez and Simi, 2012; Vilar et al., 2009). Upon binding of nonneurotrohic molecules, p75-NGFR requires the Nogo receptor (NgR) and Lingo1 as coreceptors, and this complex facilitates the interaction of p75-NGFR and Rho dissociation inhibitor (Rho-GDI), which leads to release of active RhoA, ultimately leading to axon growth inhibition and axon degeneration (Yamashita and Tohyama, 2003). Interestingly, RhoA activity is restricted under neurotrophin-induced p75-NGFR signaling (Yamashita and Tohyama, 2003).

Furthermore, p75-NGFR is a dependence receptor and can be activated by the absence of ligands to cause apoptosis. A dependence receptor is defined as an apoptotic mediator detecting the withdrawal of a trophic factor and transducing death signaling downstream (Rabizadeh and Bredesen, 2003). R2 cells in serum-free medium expressing p75-NGFR experienced significantly decreased cell death with the presence of the p75-NGFR antibody compared to a control culture without the antibody (Rabizadeh et al., 1993). How does a receptor detect a signal without a ligand? Even though how p75-NGFR functions as a dependent receptor remains unknown, it is possible that it functions together with TrkA, which is also a dependence receptor. Interestingly, p75-NGFR and TrkA have been shown to interact through their intracellular domains in a ligand-independent fashion in PC12 cells and hippocampal neurons using FRET technique

(Iacaruso et al., 2011). This may suggest that when TrkA is not occupied with NGF, it interacts with p75-NGFR and that the TrkA/p75-NGFR complex might generate death signaling. How p75-NGFR and TrkA cooperate to cause cell death remains to be fully elucidated.

Molecular mechanisms of TNF-TNFR1 signaling

TNFR1, also known as TNFRSF1A, is a classic death receptor initially identified as a mediator for immunological responses. TNFR1 and its ligand TNFα have also been implicated in non-immunological functions during development of the nervous system (Twohig et al., 2012). TNFR1 and p75-NGFR share similar functions and downstream pathways, such as cell proliferation via NFkB activation and apoptosis via activation of JNK and caspase cascade (Fig. 1-2C) (Chen and Goeddel, 2002; Naude et al., 2011).

As members of the TNF family, TNF and lymphotoxin (LT) can bind to both TNFR1 and TNFR2. TNFR2 is structurally similar to TNFR1, but does not possess the DD. While expression of LT and TNFR2 is restricted to hematopoietic and endothelial cells, TNF and TNFR1 are broadly expressed, including in the nervous system. Thus, TNF-TNFR1 signaling has been implicated in regulating nervous system development (Remouchamps et al., 2011; Tracey et al., 2008).

Distinctions between TNF-TNFR1 signaling compared to p75-NGFR signaling include a fewer number of ligands that TNFR1 has and the formation of death-inducing signaling

complexes (DISC). As a TNFR1 ligand, TNF is biologically active in two forms; a soluble form known as TNFα and a membrane-bound form called tmTNF. tmTNF is cleaved by ADAM17 and its extracellular part is released as TNFα (Naude et al., 2011). Both forms can bind to TNFR1 and TNFR2, but TNFα binds toTNFR1 with higher affinity whereas tmTNF preferentially binds to TNFR2 (Tracey et al., 2008). Prior to ligand binding, TNFR1 exists as a homotrimer via the first cysteine-rich domain in the extracellular part. This is called pre-ligand assembly and this ligand-independent self-association is required for TNF binding (Chan et al., 2000). In this pre-ligand stage, a protein called the silencer of death domain (SODD) is bound to the cytoplasmic tail of TNFR1, in order to avoid inadvertent activation of TNFR1 (Chan, 2007). Upon binding, a TNF trimer interacts with the extracellular domain of TNFR1. This conformational change recruits several adapter proteins to the DD of TNFR1 including TRADD, FADD, TRAF2, receptor interacting protein (RIP) and even procaspase-8. Aggregation of these proteins at TNFR1-DD is called the death-inducing signaling complex (DISC) and acts to further transduce the death signaling to the cytoplasm (Chen and Goeddel, 2002; Schutze et al., 2008). In p75-NGFR signaling, on the other hand, no complex equivalent to DISC has been found and activation of caspase-8 is not involved in p75-NGFR-mediated cell death. Thus, the p75-NGFR apoptotic signaling pathway is regarded to be distinct from that of TNFR family members (Roux and Barker, 2002).

Aside from the canonical TNF-TNFR1 pathway, there is an emerging, non-canonical pathway called reverse signaling in TNF biology. In this signaling, binding of TNFRs or TNF antagonists to tmTNF triggers signaling inside the tmTNF-bearing cell, leading to

suppression of cell proliferation in human T cells (Tracey et al., 2008; Eissner et al., 2004). A recent study has discovered an important role of this TNF reverse signaling during sympathetic nervous system development. A soluble form of TNFR1 was shown to act via neuronal tmTNF to facilitate axon growth in sympathetic neurons and promote sympathetic target innervation in iris and submandibular glands in an ERK1/ERK2-dependent manner (Kisiswa et al., 2013). This suggests that target-derived soluble TNFR1 influences the target innervation and implies that soluble TNFR1 plays a role as an attraction guidance cue in axon branching on target organs.

Figure 1-1. Schematic representation of the sympathetic nervous system. Preganglionic sympathetic neurons: *orange*, Postganglionic sympathetic neurons: *green*. Postganglionic neurons receive inputs from preganglionic neurons and extend axons to target organs such as eyes, salivary glands, heart, stomach, and kidneys.

Figure 1-1

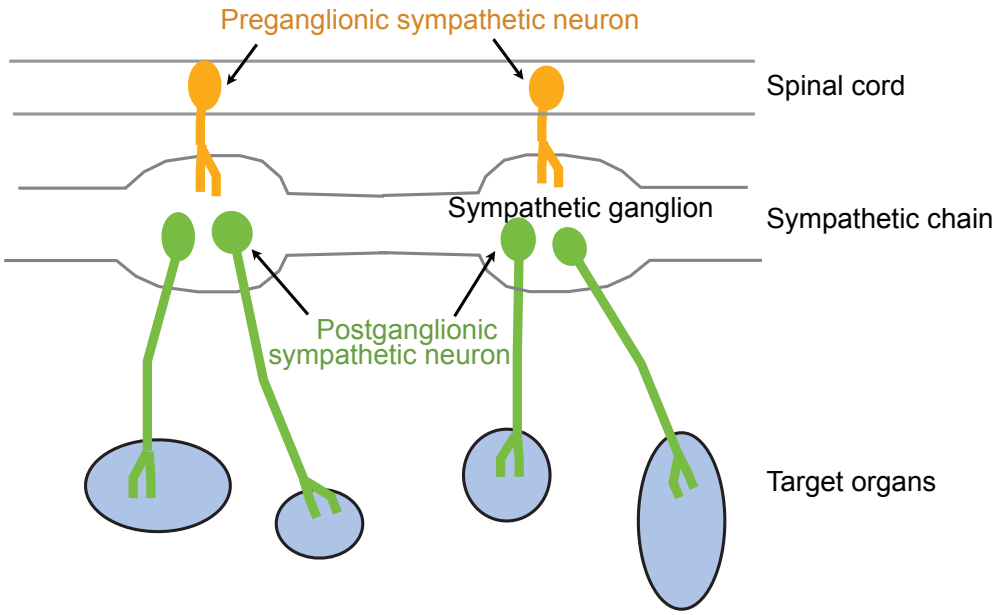
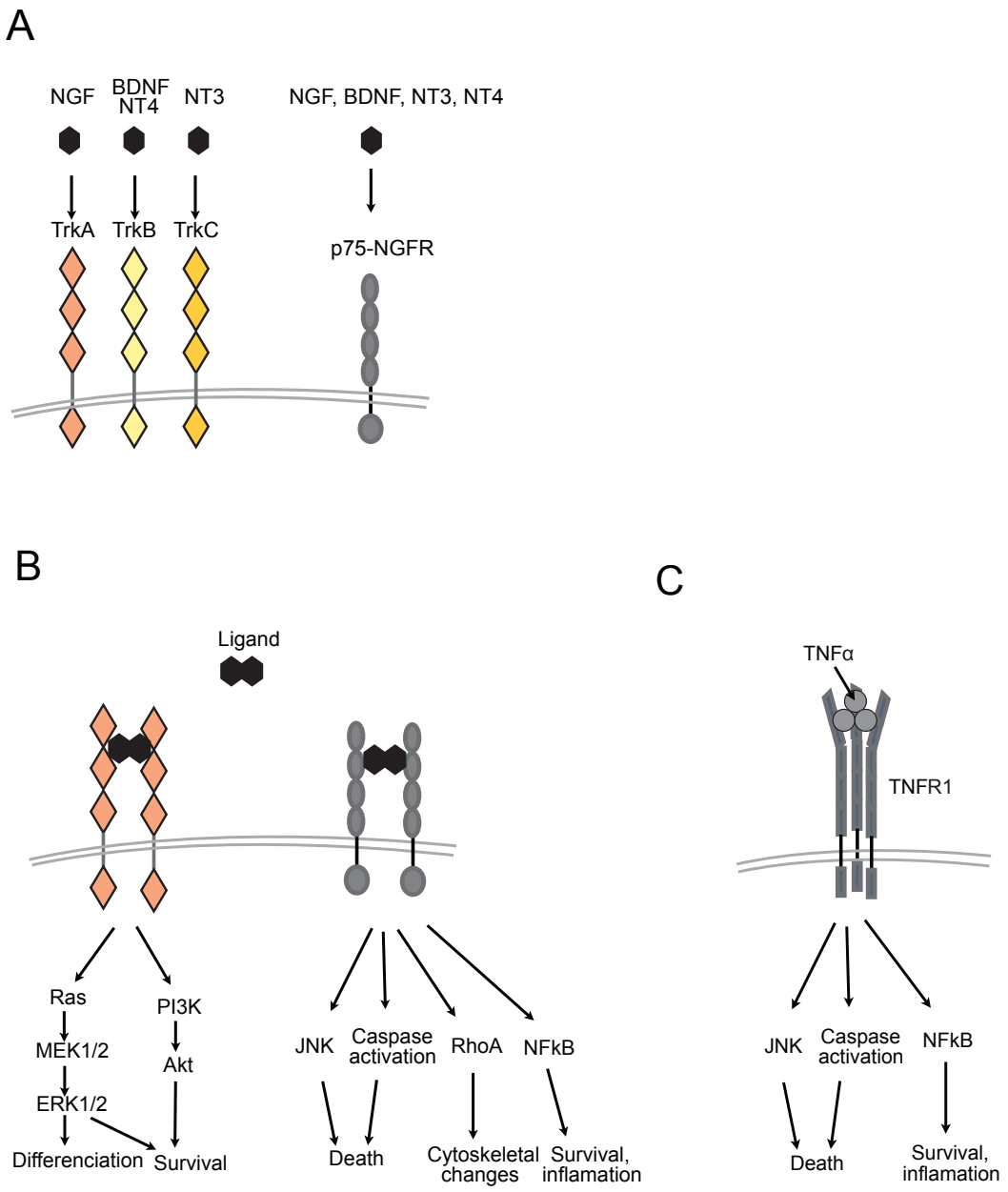

Preganglionic sympathetic neuron
Spinal cord
Sympathetic ganglion
Sympathetic chain
Postganglionic
sympathetic neuron
Target organs

Figure 1-2. Neurotrophin signaling via TrkA and p75-NGFR, and TNF-TNFR1 signaling. (A) Neurotrophins and neurotrophin receptors. (B) TrkA signaling and p75-NGFR signaling. (C) TNF-TNFR1 signaling.

Figure 1-2


A
NGF
BDNF
NT4
NT3
NGF, BDNF, NT3, NT4
TrkA
TrkB
TrkC
p75-NGFR
B
Ligand
Ras
PI3K
MEK1/2
Akt
ERK1/2
Differenciation
Survival
JNK
Caspase activation
RhoA
NFkB
Death
Cytoskeletal changes
Survival, inflamation
C
TNFα
TNFR1
JNK
Caspase activation
NFkB
Death
Survival, inflamation

Chapter 2

p75-NGFR and TNFR1 cooperate during sympathetic nervous system development

This chapter is based on work submitted to eLife journal.

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Introduction

Nervous system development in complex organisms employs a strategy of "trial and error", which relies on a dynamic and tightly controlled cycle of construction and destruction. The vertebrate nervous system takes advantage of this strategy by overproducing circuit components, such as axons, synapses and even neurons and then refining them to achieve appropriate connectivity and function (Hamburger and Levi-Montalcini, 1949; reviewed in Chao, 2003; Fitzsimonds and Poo, 1998). An emergent principal in developmental neuroscience is that pro-growth and pro-refinement signaling pathways compete with one another to promote or inhibit connectivity in order to define the architecture of the nervous system (Deppmann *et al.,* 2008; Singh *et al.,* 2008;

Sharma *et al.*, 2010). How cross-talk between construction and destruction cues occurs to mediate nervous system wiring remains an open question.

In the sympathetic nervous system, TrkA and p75-nerve growth factor receptor (p75-NGFR) promote pro-growth and pro-refinement events, respectively (reviewed in Majdan and Miller, 1999). Target organ-derived nerve growth factor (NGF) binds axonal TrkA thereby transducing signaling cascades, which promote target innervation, neuronal survival, synapse initiation, and a variety of other important developmental processes (McMahon *et al.* 1994; Glebova and Ginty, 2004; Sharma *et al.,* 2010). p75-NGFR signaling, on the other hand, represents a counterbalance to these TrkA-mediated pro-growth signals, as it mediates neuronal death, axon degeneration, and even synapse restriction (Bamji *et al.,* 1998; Singh *et al.,* 2008; Sharma *et al.,* 2010). p75-NGFR refines the peripheral nervous system (PNS) by hastening developmental competition between neurons through the promotion of apoptosis and axon pruning (Deppmann *et al.,* 2008; Singh *et al.,* 2008).

Competition for survival during sympathetic neuron target innervation is perhaps the best characterized developmental event in which both TrkA and p75-NGFR participate. This process was first described in the neurotrophic factor hypothesis, which posits that neuronal death during development is initiated via competition for limiting amounts of target-derived trophic factor, such as NGF (Hamburger and Montalcini, 1949). In sympathetic neurons, two candidate signaling pathways have been proposed to facilitate apoptosis during competition for trophic factor: (1) NGF withdrawal (Deckwerth and

Johnson, 1993b) and (2) ligand-induced death signaling which over-rides low levels of NGF-dependent survival signaling (termed suboptimal by Miller and colleagues, Majdan *et al.,* 2001). Death signaling in sympathetic neurons can occur through brain-derived neurotrophic factor (BDNF) or neurotrophin 4 (NT4) signaling through p75-NGFR (Majdan *et al.,* 2001; Deppmann *et al.,* 2008). Importantly, BDNF-induced apoptosis is blocked at saturating (optimal) levels of NGF (Majdan *et al.,* 2001). While there is evidence that these are two fundamentally different pathways for neuron death, several studies have suggested that p75-NGFR may be involved in both processes, however, whether p75-NGFR requires a ligand in the context of trophic withdrawal remains in question (Nikoletopoulou *et al.,* 2010; Bamji *et al.*, 1998).

We have observed that loss of *p75-NGFR* slows, but does not prevent, developmental sympathetic neuron death *in vivo* (Deppmann *et al.,* 2008). Therefore, we speculate that other death receptors may be able to compensate for loss of *p75-NGFR*. p75-NGFR is a member of tumor necrosis factor receptor (TNFR) family and signaling related to several of these receptors has been reported to build or refine the sympathetic nervous system (O'Keeffe *et al.*, 2008; Kisiswa *et al.*, 2013; Barker *et al.,* 2001; Nikolaev *et al.*, 2009). TNFR1 and death receptor 6 (DR6) are expressed in developing sympathetic neurons and play similar pro-destruction roles to p75-NGFR in sympathetic nervous system refinement. What is the purpose of having several death receptors expressed at the same place and time, performing similar functions? Perhaps these receptors are purely redundant and this is a way for a cell to diversify the ligands that it can respond to. Alternatively, different combinations of death receptors may reflect unique functionality.

To distinguish between these possibilities, it is critical to understand the relative contribution and functional relationship of TNFR family members in developmental circuit refinement.

Here we focus on the relationship between two pro-refinement receptors expressed in the sympathetic nervous system, p75-NGFR and TNFR1. These family members share several properties, for example, both receptors are capable of triggering common signaling pathways including caspase cascades, Jun N-terminal kinase (JNK), NFkB (reviewed in Hempstead, 2002) and RhoA (Yamashita *et al.,* 1999; Neumann *et al.,* 2002). There are also several key differences between these family members, including the variation in the number of ligands that each cognate receptor can bind to: TNFR1 is more limited in the ligands it prefers (e.g., TNFα and LTα), whereas p75-NGFR promiscuously binds to all immature and mature neurotrophins (NGF, BDNF, NT4/5, NT3), as well as non-neurotrophic factors secreted by glial cells such as Nogo, myelin-associated glycoprotein (MAG), and oligodendrocyte myelin glycoprotein (OMgp) (Bibel and Barde, 2000; Jansen *et al.,* 2007; Wang *et al.,* 2002). Whether these two distinct TNFR family members cooperate during refinement signaling to affect an individual neuron's life/death or morphology is unknown.

In this study, we examine how TNFR family members coordinate their activity to mediate neuronal cell death, axon growth inhibition and neuronal soma size during development. We first found that the closely related TNFR family members, *p75-NGFR*, *TNFR1*, and *DR6* are highly expressed in sympathetic ganglia. We also report that p75-

NGFR requires TNFR1 to transduce BDNF-dependent cell death (unidirectional codependence) and p75NTR and TNFR1 require each other to transduce ligand dependent inhibition of axon growth (bidirectional codependence). The mechanism for this apparent TNFR family codependence appears to be via physical interaction between p75NTR and TNFR1 as measured by co-immunoprecipitation and bimolecular fluorescence complementation (BiFC). Our *in vivo* studies suggest that this functional codependence between p75-NGFR and TNFR1 is also required for proper regulation of sympathetic soma size and target innervation, but not for expediting sympathetic neuron death during development.

Results

Closely related TNFR family members and their ligands are expressed in sympathetic neurons

To determine which death receptors are most similar to p75-NGFR, we performed phylogenetic analysis of all TNFR family members using their rat amino acid sequences as previously described (Dereeper *et al.,* 2008, Fig. 2-1A). We found that p75-NGFR, DR6 and TNFR1 cluster in the same phylogenetic clade, which may explain the similarities in previously reported functions including cell death and axon degeneration (Nikolaev *et al.,* 2009; Barker *et al.,* 2001; Neumann *et al.,* 2002). We next sought to examine the expression pattern of TNFR family members using cDNA from the superior cervical ganglia (SCG) and brain of P0 rats, via RT-PCR (Fig. 2-1B.). Interestingly p75-NGFR, TNFR1 and DR6 display robust expression in SCG. TNFR1 and DR6 also show

high expression in the brain, whereas p75-NGFR does not. This observation is consistent with a number of previous studies demonstrating that developmental expression of p75-NGFR is restricted to the PNS and a few subsets of neurons in the CNS (Bibel and Barde, 2000).

The above expression analysis was performed on acutely dissected SCGs or brain. To determine which of these cell types express *p75-NGFR*, *TNFR1* and *DR6* and their ligands, we performed RT-PCR using cDNA from dissociated cultures of neurons or glia from sympathetic ganglia (Fig. 2-1D). Developing sympathetic neurons are NGF-dependent and glial populations can be eliminated by addition of aphidicolin (Oorschot and Jones, 1989). Maintaining cultures in the presence of NGF and aphidicolin enriched neurons, whereas glia can be enriched by maintaining cultures in the absence of both. We demonstrated that all three of the receptors are expressed in neurons, but only *TNFR1* appears to be enriched in glia (Fig. 2-1E). We also found cell-type specific expression of some of the ligands: *TNFα* and *BDNF* were expressed in both neurons and glia, *MAG* was specifically expressed in glia and *amyloid precursor protein (APP*) expression was specific to neurons. Because *APP* and *DR6* appear to be the only pair expressed specifically in neurons, this might suggest an autocrine/paracrine mechanism of amyloid products signaling through DR6 during PNS development, which is consistent with previous reports (Nikolaev *et al.,* 2009; Hu *et al.,* 2013).

TNFα or BDNF induce neuronal apoptosis and inhibit axon growth in sympathetic neurons grown in suboptimal NGF

The simultaneous expression of BDNF and TNFα along with their cognate receptors in the SCG led us to ask whether these two death ligands independently antagonize NGF-TrkA survival signaling in sympathetic neurons? Indeed, previous studies have demonstrated that BDNF and TNFα are involved in sympathetic neuron death both *in vitro* and *in vivo* (Bamji *et al.,* 1998; Barker *et al.*, 2001). We sought to examine the relative ability of p75-NGFR or TNFR1 to override NGF-TrkA survival signaling. We examined sympathetic neuronal cell death in response to saturating BDNF (250ng/ml) or TNFα (2ng/ml) using sympathetic neurons from postnatal day 0-3 (P0-P3) rats (Fig. 2-2A,B). Neurons were cultured for 36 hours in suboptimal NGF (2ng/ml) or an anti-NGF function-blocking antibody, which display robust survival or death, respectively. Neurons cultured in NGF were also exposed to BDNF or TNFα for 36 hours prior to assessment of survival/death. Consistent with previous reports, we found that both ligands were capable of overcoming suboptimal NGF-TrkA survival signaling to induce apoptosis (Bamji *et al.*, 1998; Barker *et al.*, 2001).

A similar antagonism between p75-NGFR and NGF-TrkA has been observed in the context of axon degeneration and stabilization/growth (Kohn *et al.*, 1999; Singh *et al.*, 2008; Park *et al.*, 2010). We next examined the relative contribution of p75-NGFR and TNFR1 in inhibiting axon growth in microfluidic devices, which separates axons from cell bodies (Park *et al.*, 2006). Once axons extend through the microfluidic device, they were imaged, and either maintained in the presence or absence of suboptimal NGF (10ng/ml for axon growth assays) and/or supplemented with BDNF (250ng/ml) or TNFα (2ng/ml) for an additional 24 hours. Notably the suboptimal level of NGF in this context

is five-fold higher than for cell survival suggesting that the threshold concentrations of NGF that are required to suppress p75-NGFR signaling (this demarcates optimal versus suboptimal) depends on functional context (Park *et al.*, 2010). The difference in positive or negative growth between days 0 and 1 was assessed and is represented as an average growth in length over 24 hours (Fig. 2-2C,D). As expected, those maintained in the presence of NGF displayed a positive growth in length, while those maintained without NGF retracted and/or degenerated. Moreover, axon growth was inhibited to similar extents by saturating BDNF or TNFα, suggesting that ligand-induced p75-NGFR and TNFR1 are both capable of actively antagonizing NGF-TrkA dependent axon growth signaling.

The similar abilities of p75-NGFR and TNFR1 to antagonize NGF-TrkA-dependent cell survival and axon growth led us to ask whether these receptors operate in a cooperative or independent manner. We propose 2 models: 1) 'codependent'; where two death receptors require each other to antagonize NGF-TrkA signaling to promote refinement (Fig. 2-3A) or 2) 'Independent': the two receptors function 'independently' to initiate cell death or axon degeneration (Fig. 2-3B). In this model cell death or axon growth inhibition would occur when either receptor is activated independent of the presence of the other. We next seek to examine these models using *in vitro* and *in vivo* loss of function experiments.

p75-NGFR requires TNFR1 but not *vice versa* for ligand dependent refinement.

If p75-NGFR and TNFR1 work independently rather than co-dependently, then ablating one should not affect the function of the other. However, if they require one another, ablating one receptor should weaken or eliminate the refinement function of the other. Sympathetic neurons from wild type, *p75-NGFR*$^{-/-}$ and *TNFR1*$^{-/-}$ mice, were cultured in the presence of NGF and BDNF or TNFα for 36 hours and cell survival was assessed as described in Figure 2-2. BDNF and TNFα efficiently promoted apoptosis of neurons isolated from P0 wild-type mice grown in suboptimal NGF (Fig. 2-4A,B). Interestingly, BDNF does not induce death in neurons isolated from *p75-NGFR*$^{-/-}$ or *TNFR1*$^{-/-}$ suggesting that the pro-death function of BDNF requires the concurrent availability of p75-NGFR and TNFR1. On the other hand, addition of TNFα to *p75-NGFR*$^{-/-}$ neurons displays increased death, suggesting that TNFR1 does not require p75-NGFR to promote ligand-induced apoptosis (Fig. 2-4A,B). These data suggest that in a suboptimal NGF survival paradigm, these receptors display a unidirectional co-dependence where p75-NGFR is dependent on TNFR1 but not *vice versa* (i.e., TNFR1 induces death independent of p75-NGFR) (Fig. 2-4C). It is also important to note that both *p75-NGFR*$^{-/-}$ and *TNFR1*$^{-/-}$ neurons were capable of undergoing apoptosis as a result of NGF withdrawal. Rather, these neurons are more susceptible to NGF-withdrawal. These data suggest that NGF deprivation is capable of inducing cell death via mechanisms independent of BDNF- or TNFα-induced cell death.

We next examined whether there is a unidirectional codependence between p75-NGFR and TNFR1 in a different function context: inhibition of axon growth. We returned to the paradigm used in Figure 2-2C, where sympathetic axons from *p75-NGFR*$^{-/-}$ and *TNFR1*$^{-/-}$

mice are measured after 24 hours in the presence of suboptimal NGF and saturating BDNF or TNFα. Interestingly, the results indicate codependence of the two receptors: adding BDNF to *TNFR1*$^{-/-}$ neurons or adding TNFα to *p75-NGFR*$^{-/-}$ neurons did not suppress axon growth suggesting bidirectional codependence between these two receptors (Fig. 2-4D,E). Interestingly, *p75-NGFR*$^{-/-}$ neurons exhibited greater axon growth with or without exogenous BDNF or TNFα, which may suggest that p75-NGFR plays an additional role in repressing NGF-TrkA signaling in axon growth.

p75-NGFR and TNFR1 physically associate resulting in altered subcellular localization

To understand the nature of codependence between p75-NGFR and TNFR1 toward NGF-TrkA-dependent processes, we asked whether these receptors are physically associated. To this end, we generated HA-tagged p75-NGFR and FLAG-tagged TNFR1 expression constructs (Fig. 2-6A). These constructs were expressed in HEK293 cells followed by analysis of interaction by co-immunoprecipitation (co-IP) and immunoblot analysis. In the immunoprecipitated lanes, we detected FLAG-TNFR1 only when HA-p75-NGFR and TNFR1 were expressed, suggesting association of p75-NGFR and TNFR1 (Fig. 2-5A).

We next sought to determine the subcellular localization of p75-NGFR/TNFR1 heteromeric complexes compared to their respective homomeric interactions. For this, we used an experimental paradigm where the association of the two receptors can be observed in a single cell assay (Fig. 2-5B). To visualize the association of p75-NGFR and TNFR1, we used bimolecular fluorescence complementation (BiFC), where p75-NGFR

and TNFR1 are cloned in frame with split-portions of the Venus fluorescent protein (N-terminus or C-terminus) (Fig. 2-6B) (Hu and Kerppola, 2003). To avoid overexpression, which could lead to inappropriate interaction, these fusion constructs were expressed in the pTRE2hyg vector backbone, a tetracycline-regulated expression system. We expressed the constructs in HEK293 cells cultured in tetracycline-free media in order to maintain minimal "leaky" heterologous expression. As positive- and negative controls, cells expressing TrkA-VN and TrkA-VC were exposed to media with or without 50 ng/ml NGF prior to imaging, respectively. Cells expressing the homomeric pair of p75-NGFR exhibited uniform fluorescence in the cytoplasm and plasma membrane as well as a few intracellular puncta; whereas cells expressing homomeric TNFR1 displayed fluorescent patches suggesting that these receptors have distinct subcellular distributions in the cell (Fig. 2-5C). Each homomeric pair - p75-NGFR or TNFR1- exhibited fluorescence in the absence of its cognate ligand, which is consistent with previous reports (Vilar *et al.*, 2009; Chan *et al.*, 2000). Interestingly, in cells expressing the heteromeric pair of p75-NGFR and TNFR1, punctate fluorescence was similar to homomeric TNFR1. This indicates that the subcellular localization of the p75-NGFR:TNFR1 complex is principally influenced by TNFR1. Localizing p75-NGFR to a particular microenvironment may provide an explanation for the unidirectional codependence observed above where p75NGFR requires TNFR1 (but not *vice versa*) for ligand-induced death.

p75-NGFR and TNFR1 are bidirectionally codependent *in vivo* for sympathetic soma size regulation

In vitro, p75-NGFR and TNFR1 display unidirectional co-dependence with respect to inhibiting NGF-TrkA dependent survival but bidirectional co-dependence for inhibition of axon growth. We next sought to determine which, if any, of these scenarios also applies *in vivo* using several assays reflecting different aspects of neuronal trophic state: soma size, final target innervation and survival (Deckwerth *et al.*, 1996; Glebova and Ginty, 2004; Deppmann *et al.*, 2008). Observing similar phenotypes between mice null for *p75-NGFR* or *TNFR1* would be consistent with a codependent relationship between these receptors. However, if these receptor knockout animals do not phenocopy each other or if double knockout animals have additive phenotypes we would conclude that these receptors function uniquely or independently, respectively.

We began by examining neuronal size. It has previously been demonstrated that NGF regulates soma size in sympathetic neurons (Deckwerth and Johnson, 1993b). The most extreme example of this is when naturally occurring cell death is blocked in SCGs, as is the case in *Bax*$^{-/-}$ mice (Deckwerth *et al.*, 1996). These neurons still compete for target-derived NGF but rather than competition resulting in a life or death decision, all the neurons survive. This manipulation has allowed us to study developmental competition programs other than survival/death. To examine soma size, SCGs from P5 mice (a time after the majority of apoptosis would have happened) were cryosectioned and neurons were visualized with Nissl staining (Coggeshall *et al.*, 1990). The areas of neurons displaying nucleolus, which is a characteristic landmark for the center of the cell, was measured and are represented in 3 bins: small (less than 175μm²), medium (175~350μm²), large (more than 350μm^2). Comparing wild-type and *Bax*$^{-/-}$ SCGs reveals a shift in soma

size related to competition for target derived NGF: large cells presumably won the competition for NGF and the small neurons presumably lost the competition and would have died if *Bax* was present (Fig. 2-7A*)*. We next used this paradigm to examine how loss of *p75-NGFR* or *TNFR1* influences trophic withdrawal induced sympathetic soma atrophy using *Bax*$^{-/-}$*; p75-NGFR*$^{-/-}$ and *Bax*$^{-/-}$*;TNFR1*$^{-/-}$, mice. If the receptors promote cell atrophy, the small soma population will decline compared to the *Bax*$^{-/-}$ phenotype. Remarkably, somas of SCG neurons in *TNFR1*$^{-/-}$; *Bax*$^{-/-}$ and *p75-NGFR*$^{-/-}$; *Bax*$^{-/-}$ mice display size distributions similar to wild-type animals, suggesting that the receptors rescue the neuron size distribution phenotype observed in *Bax*$^{-/-}$ mice (Fig. 2-7A,B) and that TNFR1 and p75-NGFR are both required for promoting cell atrophy during developmental competition for target derived trophic factor (Fig. 2-7C).

p75-NGFR and TNFR1 promote final target innervation both independently and codependently in a context dependent manner

We examined whether p75-NGFR and TNFR1 are co-dependent in the context of target innervation (Glebova and Ginty, 2004). We harvested heart and kidney from P0 mice and examined sympathetic axonal innervation via whole mount tyrosine hydroxylase staining (Glebova and Ginty, 2004). We first examined innervation in the kidney by assessing the degree of axonal tiling from WT, *TNFR1*$^{-/-}$, *p75-NGFR*$^{-/-}$, and *TNFR1*$^{-/-}$*;p75-NGFR*$^{-/-}$ mice (Fig. 2-8). Kidneys from wild type mice exhibit axon bundles spread evenly over the surface of the tissue while kidneys from *TNFR1*$^{-/-}$ or *p75-NGFR*$^{-/-}$ mice display axon bundles that remain thick and fasciculated leading to decreased tiling (Fig. 2-9A,B). Interestingly tiling was not further reduced in kidneys from *TNFR1*$^{-/-}$*;p75-NGFR*$^{-/-}$ mice

indicating that these receptors operate co-dependently rather than independently to promote defasciculation and tiling (Fig. 2-9C).

We next assessed sympathetic innervation of hearts from the same animals used for the above kidney analysis. Sympathetic axons entering the heart spread out in part by avoiding one another through an axonal competition process that parallels competition for survival as described previously (Park *et al.*, 2010; Deppmann *et al.*, 2008). We assessed axonal self-avoidance by quantifying the number of axonal crossovers in the heart (Fig. 2-8). Sympathetic axons from *p75-NGFR*$^{-/-}$ and *TNFR1*$^{-/-}$ mice display increase axon crossovers relative to hearts from wild-type mice (Fig. 2-9A,D). Interestingly, hearts from *p75-NGFR*$^{-/-}$;*TNFR1*$^{-/-}$ mice displayed a further elevation of crossover which appears to be additive suggesting that these receptors behave independently to promote axonal self-avoidance (Fig. 2-9E). Taken together, we conclude that the manner in which p75-NGFR and TNFR1 influence final target innervation is context dependent. This context may be specific to the final target being innervated or the process being promoted (i.e., defasciculation or axonal self-avoidance).

p75-NGFR but not TNFR1 promotes developmental death *in vivo*

We next examined whether p75-NGFR and TNFR1 function codependently in expediting death of sympathetic neurons *in vivo*. To test this idea, we examined neuronal number within SCGs from P0 wild type mice, p*75-NGFR*$^{-/-}$, *TNFR1*$^{-/-}$ and *p75-NGFR*$^{-/-}$*;TNFR1*$^{-/-}$ mice as previously described (Deppmann *et al.*, 2008). Surprisingly, we observed no difference in the number of neurons within the SCG between wild type and *TNFR1*$^{-/-}$

animals, in contrast to the increase observed in *p75-NGFR*-deficienct animals (Fig. 2-10A). Interestingly, double knockout mice exhibited similar neuronal numbers as *p75-NGFR*$^{-/-}$ mice, suggesting that TNFR1 does not support p75-NGFR in expediting naturally occurring neuron death *in vivo* (Fig. 2-10A). This finding suggests that p75-NGFR and TNFR1 are not codependent, or independent but rather function uniquely in the context of developmental cell death *in vivo* (Fig. 2-10B). This is an outcome that is different than what we observed *in vitro* (Fig. 2-4B,C), which we suggest reflects a fundamental difference in the mechanisms governing apoptosis in these paradigms: (1) Antagonizing survival signals through ligand regulated death receptors or (2) Trophic deprivation, which may still involve death receptors but perhaps not their ligands (Deckwerth & Johnson, 1993b; Nikoletopoulou *et al.*, 2010).

Discussion

Death receptors have been reported to initiate several developmental refinement events during nervous system assembly including: cell death, axon pruning, and inhibition of dendrite formation (Bamji *et al.*, 1998; Barker *et al.*, 2001; Singh *et al.*, 2008; Nikolaev *et al.*, 2009; Neumann *et al.*, 2002). However, it has been unclear whether, or how, those death receptors cooperate. In our study, we have focused on how two death receptors, p75-NGFR and TNFR1, antagonize NGF-TrkA dependent signaling and elucidated three novel characteristics of this combination of death receptors (Fig. 2-11). First, BDNF and TNFα require each other's cognate receptor, p75-NGFR and TNFR1, in order to inhibit axon growth whereas BDNF, but not TNFα, requires both of p75-NGFR and TNFR1 to

cause apoptosis in neurons grown in culture in the presence of suboptimal concentrations of NGF. Second, these receptors associate with TNFR1 moving p75-NGFR to a unique domain within the cell. Third, p75-NGFR and TNFR1 require each other for the regulation of neuronal soma size and axon patterning during target innervation, but not for developmental cell death *in vivo*.

The distinction between independent and codependent TNFR family signaling is provocative because it provides a framework by which refinement signaling can be diversified. We found that different refinement events utilize this diversity in different ways. For example, p75-NGFR and TNFR1 seem to be equally codependent in the context of regulating cell atrophy, ligand-induced axon growth inhibition *in vitro*, and axon patterning in kidney innervation, whereas they appear to be partially codependent in the context of ligand-induced cell death *in vitro* and completely unique in naturally occurring cell death *in vivo* (Fig. 2-11). Interestingly, *in vitro* and *in vivo* death paradigms reveal codependent and independent roles for p75-NGFR and TNFR1, respectively *(*Fig. 2-4B,C and 2-10A,B). We suggest that this difference between *in vitro* and *in vivo* paradigms may reflect two distinct triggers for neuronal apoptosis: death receptor suppression of survival signaling versus trophic withdrawal, respectively. What is the purpose of such a combinatorial code of TNFR family signaling mediating a diversity of refinement events? It's possible that this is a way in which a single trophic cue like NGF-TrkA might influence several different building events on the same cell by virtue of inhibiting p75-NGFR that is participating in unique, independent and codependent signaling. In this way, a relatively complex combinatorial code of TNFR family pro-

destruction interactions may act as a rosetta stone to interpret a relatively simple yet highly mobile trophic, pro-construction cue.

How might TNFR family members work together to change one another's signaling properties? In general, these receptors are thought to initiate similar pathways such as JNK, NFKB, Rho, and Caspase8/10 cascades (Locksley *et al.*, 2001; Hempstead, 2002). However, it is possible that individual TNFR family members induce subtle variations in distinct pathways, the combination of which may be sufficient to create diversified outcomes. Alternatively, interaction between family members may be permissive for a particular signaling functionality perhaps through a shift in subcellular locale. A portion of p75-NGFR dependent signaling is thought to require hierarchal cleavage by BACE followed by gamma secretase in order to liberate the intracellular domain (Kenchappa *et al.*, 2010; Kanning *et al.*, 2003). Importantly, BACE and gamma secretase activity are thought to be most active in endosomes and lipid rafts, respectively (Osenkowski *et al.*, 2008; Das *et al.*, 2013). It is known that TNFR1 is enriched in lipid rafts whereas p75NGFR is detected in both raft and non-raft compartments (Cottin *et al.*, 2002; Zhang *et al.*, 2013). Our finding that homomeric TNFR1 as well as p75-NGFR:TNFR1 heteromers exist in punctate microdomains suggests that TNFR1 may deliver p75NGFR to microenvironment(s) necessary for p75-NGFR cleavage and signaling.

Given the size of the TNFR family, as well as the number of co-receptors reported to play a role in their function, we suggest that interaction between p75-NGFR and TNFR1 merely scratches the surface of how many heteromeric receptor combinations are

possible. For example, p75-NGFR has been reported to interact with Sortilin which mediates pro-neurotrophin signaling (Nykjaer *et al.*, 2004), Nogo Receptor (NgR) which mediates Nogo, MAG, and OMGp signaling (Wang *et al.*, 2002), and DR6 which mediates N-APP and beta amyloid signaling (Nikolaev *et al.*, 2009; Hu *et al.*, 2013). Future studies will examine the role of codependent signaling in mediating processes that were previously described as Sortilin, NgR, or DR6 dependent.

Figure 2-1. TNFR super family expression analysis.

(A) Phylogenetic tree of TNFR super family members generated using their amino acid sequences (Dereeper *et al.*, 2008).

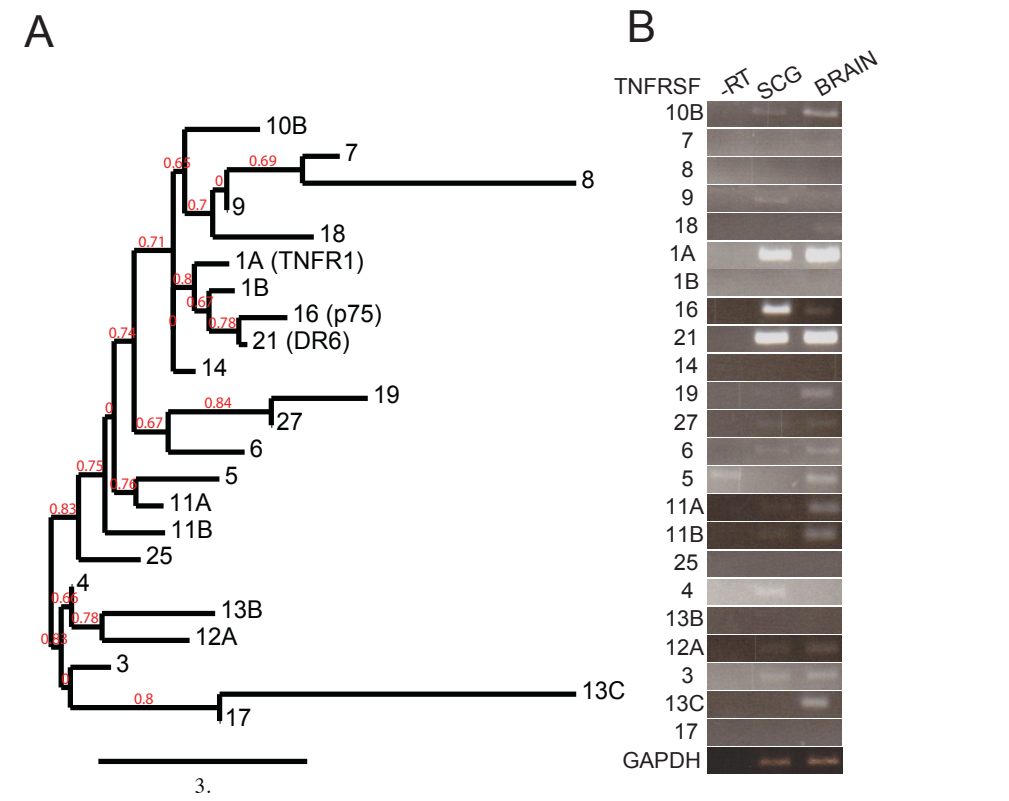
(B) RT-PCR of TNFR super family members. The expression of TNFR super family members was analyzed using mRNA isolated from the superior cervical ganglia (SCG) of P0 rats, via reverse transcriptase polymerase chain reaction (RT-PCR). Each experiment was performed at least 3 times (n=3).

(C) TNFR superfamily member aliases.

(D) RT-PCR of ligands of TNFR1, p75NGFR and DR6 using mRNA isolated from SCG neurons (n) and glia (g). Neuronal and glial mRNAs were obtained after 4 DIV in the presence or absence of NGF and AraC, respectively. Each experiment was performed at least 3 times (n=3).

(E) Schematic representation of ligand and receptor expression.

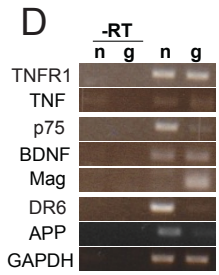
Figure 2-1



3.

C

TNFRSF	Alias
TNFRSF10B	DR5, TRAILR2, KILLER/DR5, TRICK2
TNFRSF7	CD27, S152
TNFRSF8	CD30, Lymphoid activation antigen
TNFRSF9	CD137, ILA, Ly63, 4-1BB
TNFRSF18	AITR, GITR
TNFRSF1A	TNFR1, TNFAR, CD120A
TNFRSF1B	TNFR2, TNFBR, CD120B
TNFRSF16	p75, NGFR, CD271
TNFRSF21	DR6
TNFRSF14	HEVM, HVEA, TR2
TNFRSF19	TAJ, TROY
TNFRSF27	EDA2R, XEDAR, EDA-A2R
TNFRSF6	FAS antigen, CD90, APO1, APT1
TNFRSF5	CD40
TNFRSF11A	RANK, ODFR, PDbeta2GENE, TRANCER
TNFRSF11B	OPG, OCIF
TNFRSF25	DR3, APO3, LARD
TNFRSF4	CD134, TXGP1L, OX40 antigen, ACT35
TNFRSF13B	TACI
TNFRSF12A	FN14, TWEAKR
TNFRSF3	LTBR, TNFCR
TNFRSF13C	BAFFR
TNFRSF17	BCM, BCMA



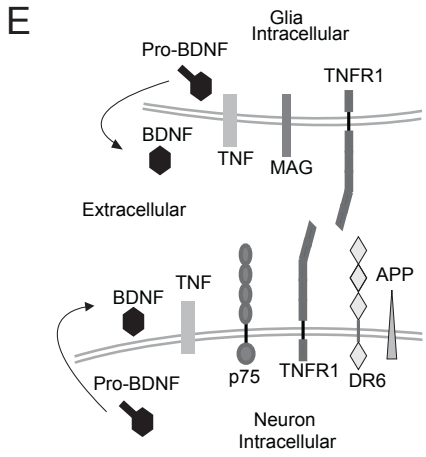


Figure 2-2. BDNF and TNFα promote apoptosis and inhibit axon growth *in vitro*.

(A) Ligand-induced apoptosis. Dissociated sympathetic neurons were isolated from P0 rats and plated in mass culture on poly-lysine and laminin. Neurons were established in 50ng/ml NGF and Ara-C for 2 days. Cultures were then changed to the indicated conditions: anti-NGF (Millipore catalog#AB927, 1:1000), NGF (2ng/ml), BDNF (250ng/ml), TNFα (2ng/ml) for 36 hours followed by assessment of cell survival via Hoechst staining. Survival in each condition was relative to the survival of neurons grown in NGF (2ng/ml). Each experiment was performed 4 times (n=4) with >100 cells counted for each.

(B) Quantification of (A). p values were determined with one-way ANOVA.

(C) Ligand-induced axon growth inhibition. Dissociated sympathetic neurons from P0 mouse SCG were established in 50ng/ml of NGF for 2 days, then cellbodies (CB) and distal axons (DA) are exposed to 10ng/ml NGF and either 250ng/ml BDNF or 2ng/ml TNFα. An NGF-free medium with anti-NGF (Millipore catalog#AB927, 1:1000) was also used as control. A pancaspase inhibitor, BAF **(**boc-aspartate fluoromethylketone), was used at 20nM in all conditions to prevent cell death. Individual axons were measured after 24 hours in response to various ligands (anti-NGF: n=12, NGF: n=28, BDNF:n=27, TNF:n=13). Yellow heads: tracked individual axons before and after the media change.

(D) Quantification of (C). p values were determined with one-way ANOVA.

Contributions. A: Original pictures taken by Kelvin Chan. B: Cells counted by Irene Cheng. C, D: Original pictures taken and axons measured by Danielle Heffner.

Figure 2-2

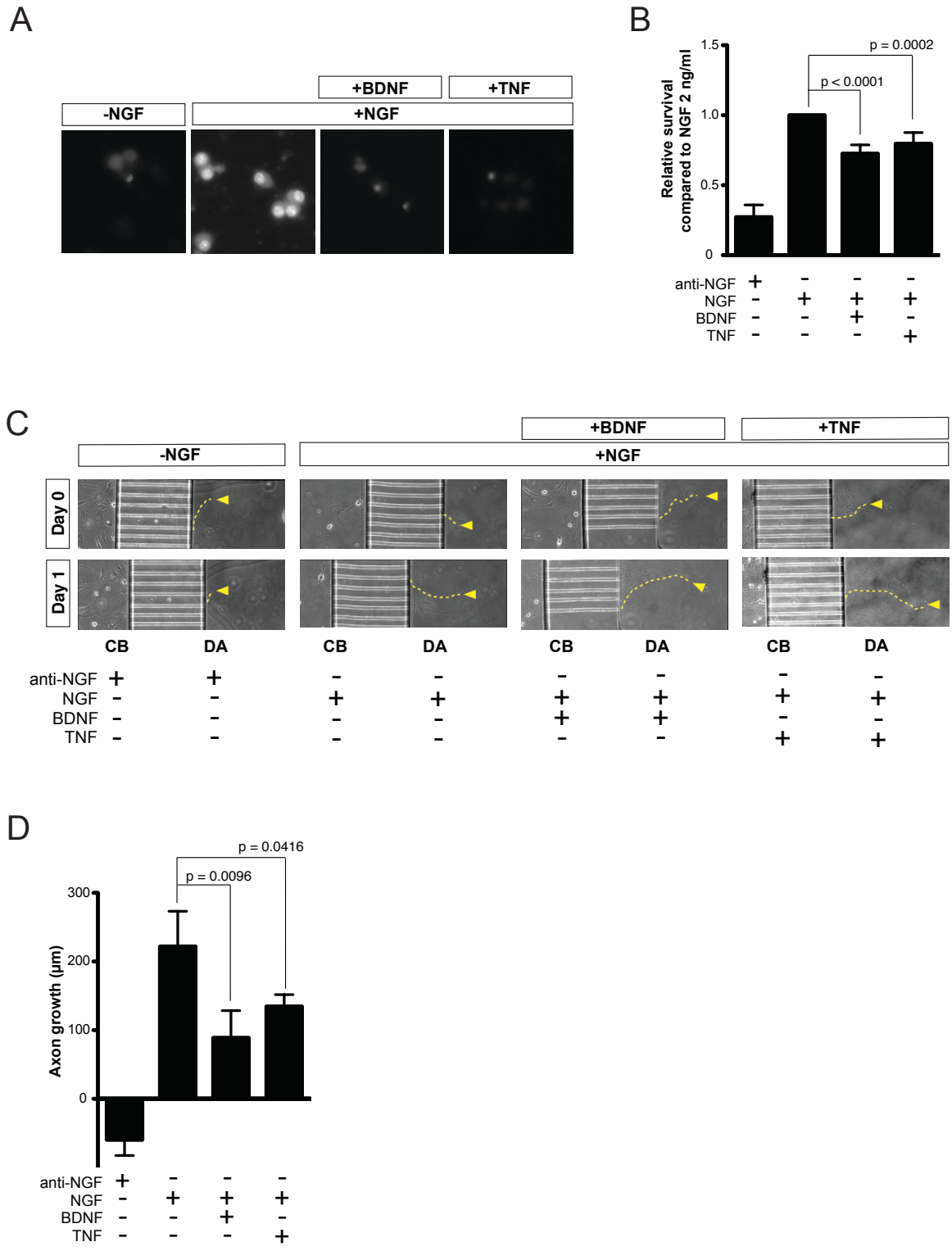

A
+BDNF
+TNF
-NGF
+NGF
B
Relative survival compared to NGF 2 ng/ml
1.5
1.0
0.5
0
p = 0.0002
p < 0.0001
anti-NGF + - - -
NGF - + + +
BDNF - - + -
TNF - - - +
C
+BDNF
+TNF
-NGF
+NGF
Day 0
Day 1
CB DA CB DA CB DA CB DA
anti-NGF + + - - - - - -
NGF - - + + + + + +
BDNF - - - - + + - -
TNF - - - - - - + +
D
Axon growth (µm)
300
200
100
0
p = 0.0416
p = 0.0096
anti-NGF + - - -
NGF - + + +
BDNF - - + -
TNF - - - +

Figure 2-3. Models for multiple death receptors to antagonize NGF-TrkA signaling. (A) Codependent model. p75-NGFR and TNFR1 cooperate to generate inhibitory signaling against NGF-TrkA signaling. Activation of both receptors is required to suppress cell survival and axon growth.

(B) Independent model. p75-NGFR and TNFR1 independently generate inhibitory signaling against NGF-TrkA signaling. Activation of either receptor is required.

Figure 2-3

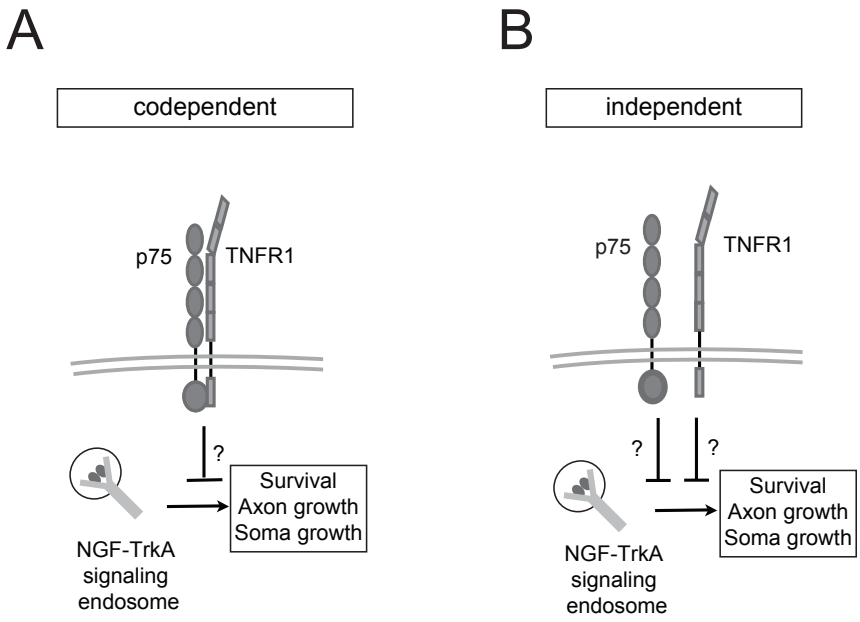

A
codependent
p75
TNFR1
?
Survival
Axon growth
Soma growth
NGF-TrkA
signaling
endosome
B
independent
p75
TNFR1
?
?
Survival
Axon growth
Soma growth
NGF-TrkA
signaling
endosome

Figure 2-4. Functional codependence between p75-NGFR and TNFR1 in ligand-induced cell death and axon growth inhibition *in vitro*.

(A) p75-NGFR and TNFR1 require one another in cell death initiated by BDNF but not TNF *in vitro*.

(B) Quantification of (A). Each experiment was performed 4 times (n=4) with >100 cells counted for each. p values were determined with two-way ANOVA.

(C) Schematic of TNFR1-dependent p75NGFR death signaling representing unidirectional codependence.

(D) p75-NGFR and TNFR1 require each other in ligand-induced axon growth inhibition. p values were determined with one-way ANOVA. The counted number of individual axons for each condition was as below: in WT, anti-NGF:n=11, NGF:n=28, BDNF:n=26, TNF:n=12. In *p75-NGFR*$^{-/-}$, anti-NGF:n=5, NGF:n=23, BDNF:n=21, TNF:n=25. In *TNFR1*$^{-/-}$, anti-NGF:n=21, NGF:n=29, BDNF:n=36, TNF:n=35.

(E) Schematic of codependence between p75-NGFR and TNFR1 in ligand-induced axon growth inhibition *in vitro*.

Contributions. A: Original pictures taken by Kelvin Chan and Irene Cheng. B: Cells counted by Irene Cheng. D: Axons measured by Danielle Heffner.

Figure 2-4

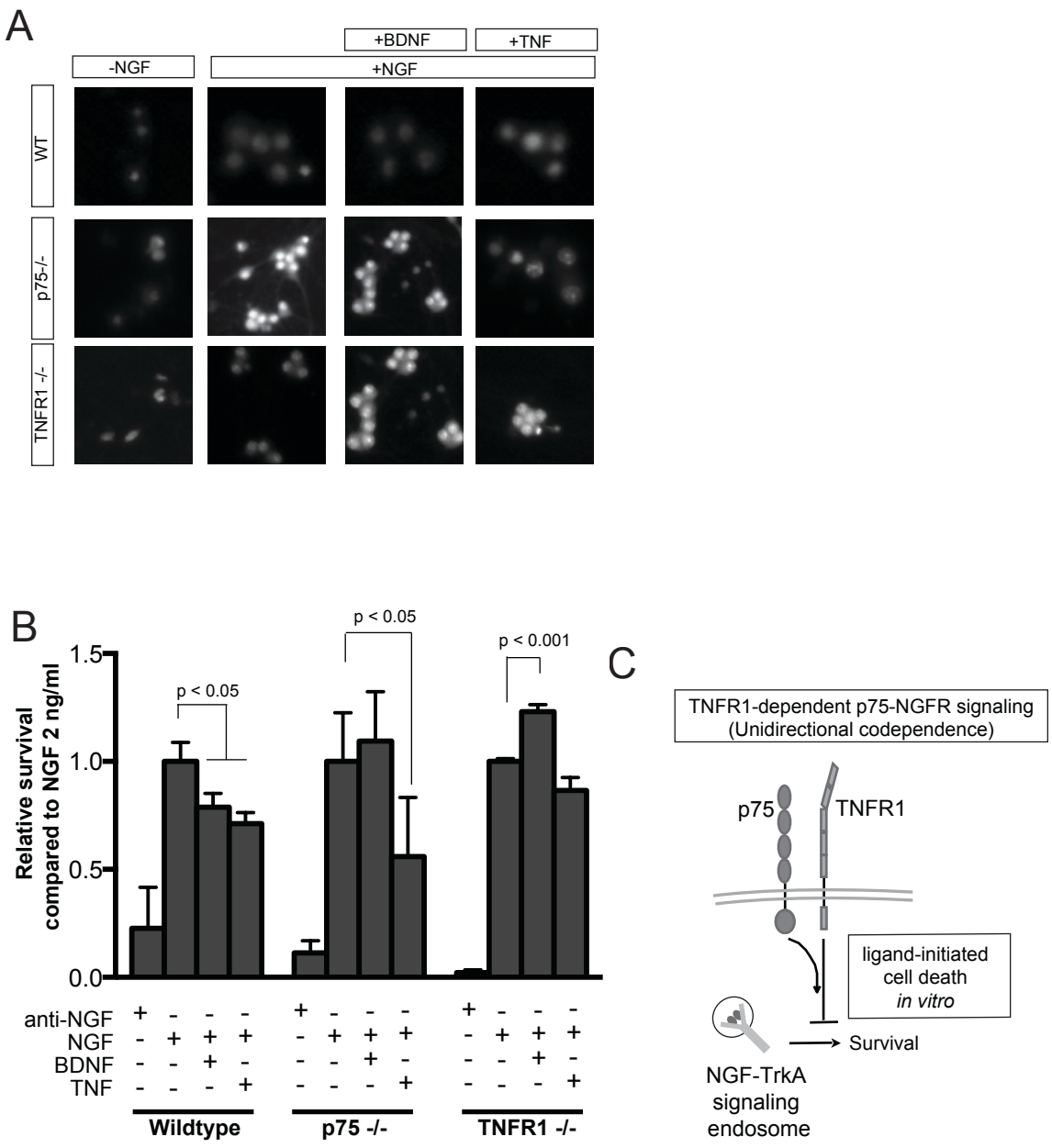

A
-NGF
+NGF
+BDNF
+TNF
WT
p75-/-
TNFR1 -/-
B
Relative survival compared to NGF 2 ng/ml
1.5
1.0
0.5
0.0
p < 0.05
p < 0.05
p < 0.001
anti-NGF
NGF
BDNF
TNF
Wildtype
p75 -/-
TNFR1 -/-
C
TNFR1-dependent p75-NGFR signaling
(Unidirectional codependence)
p75
TNFR1
ligand-initiated
cell death
in vitro
Survival
NGF-TrkA
signaling
endosome

Figure 2-4 (continued)

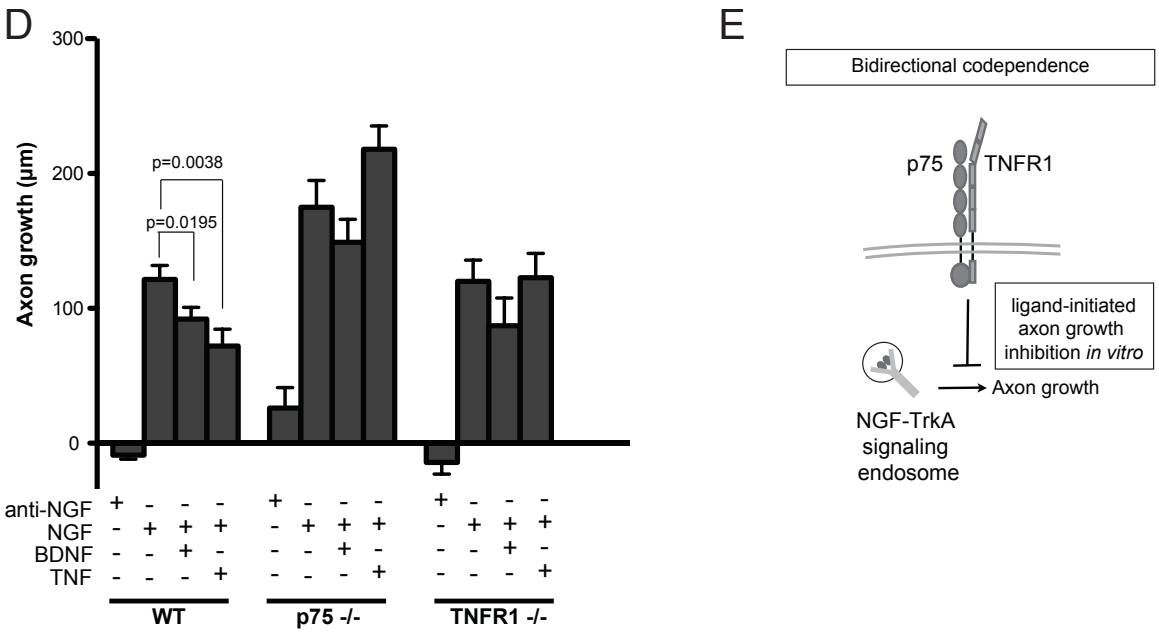


Figure 2-5. Interaction of p75-NGFR and TNFR1.

(A) Co-immunoprecipitation (co-IP) of p75-NGFR and TNFR1 constructs using HEK293 cells. The experiment was performed at least 4 times (n=4).

(B) Schematic of bimolecular fluorescence complementation (BiFC).

(C) Bimolecular fluorescence complementation (BiFC) of p75-NGFR and TNFR1 constructs using HEK293 cells. As a positive control, cells expressing TrkA-VN and TrkA-VC were exposed to media containing NGF 50 ng/ml for 15 minutes prior to imaging. In comparison, as a negative control, cells expressing TrkA-VN and TrkA-VC were maintained in media without NGF prior to imaging. Transfected cells were imaged using fluorescence excitation at 514 nm. Scale bar: 5 μm. The experiment was performed at least 3 times (n=3).

Contributions. C: Pictures taken by Irene Cheng and Kazusa Edamura.

Figure 2-5

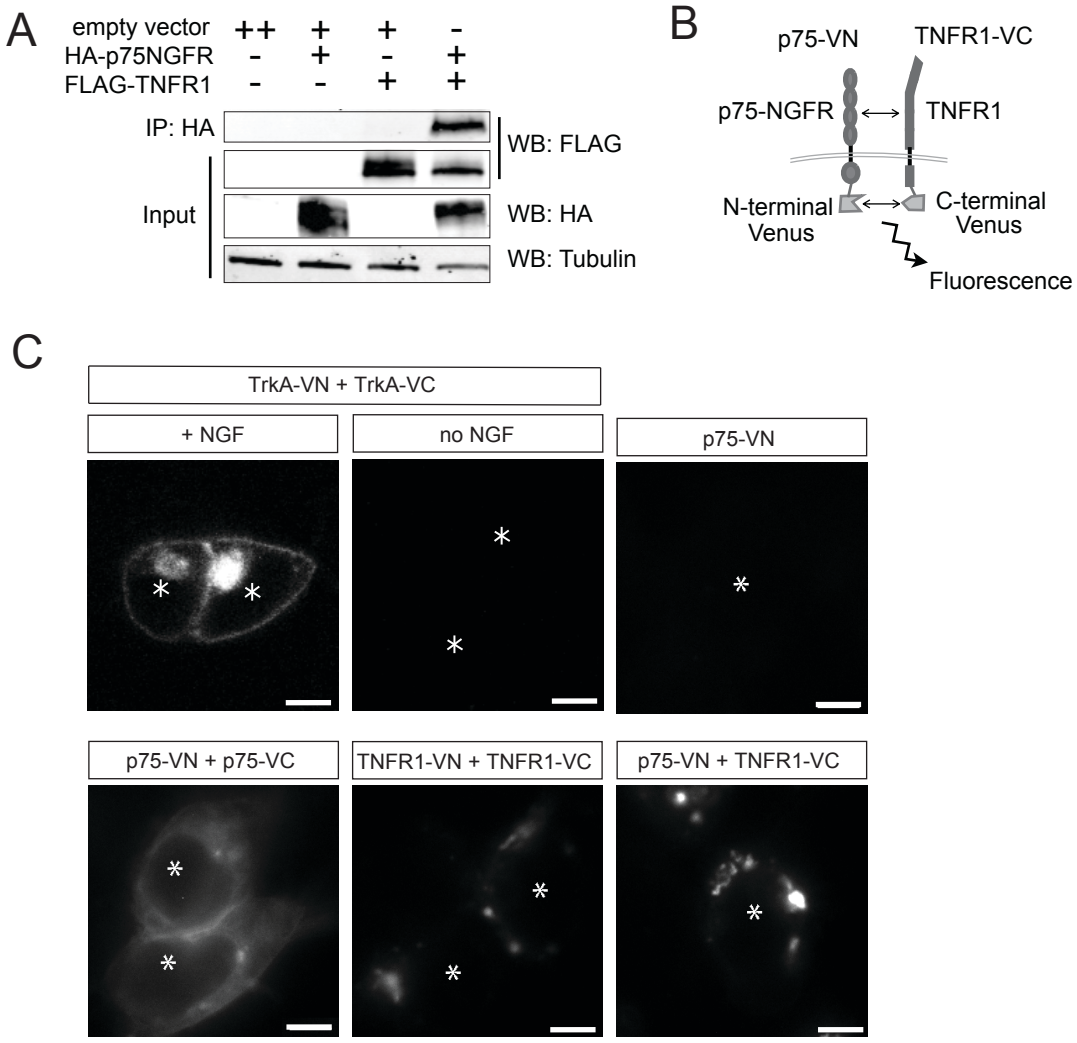

A
empty vector ++ + + -
HA-p75NGFR - + - +
FLAG-TNFR1 - - + +
IP: HA
WB: FLAG
Input
WB: HA
WB: Tubulin
B
p75-VN
TNFR1-VC
p75-NGFR
TNFR1
N-terminal Venus
C-terminal Venus
Fluorescence
C
TrkA-VN + TrkA-VC
+ NGF
no NGF
p75-VN
p75-VN + p75-VC
TNFR1-VN + TNFR1-VC
p75-VN + TNFR1-VC

Figure 2-6. Schematic representation of DNA constructs and BiFC of additional combination.

(A) HA-p75-NGFR and FLAG-TNFR1. HA and FLAG are inserted between signal sequence and mature peptide sequence of p75-NGFR and TNFR1, respectively.

(B) p75-VN, p75-VC, TNFR1-VN and TNFR1-VC.

(C) Bimolecular fluorescence complementation (BiFC) of additional combination of p75-NGFR and/or TNFR1 constructs using HEK293 cells. Transfected cells were imaged using fluorescence excitation at 514 nm. Scale bar: 5 μm. The experiment was performed at least 3 times (n=3).

Contributions. C: Pictures taken by Irene Cheng.

Figure 2-6

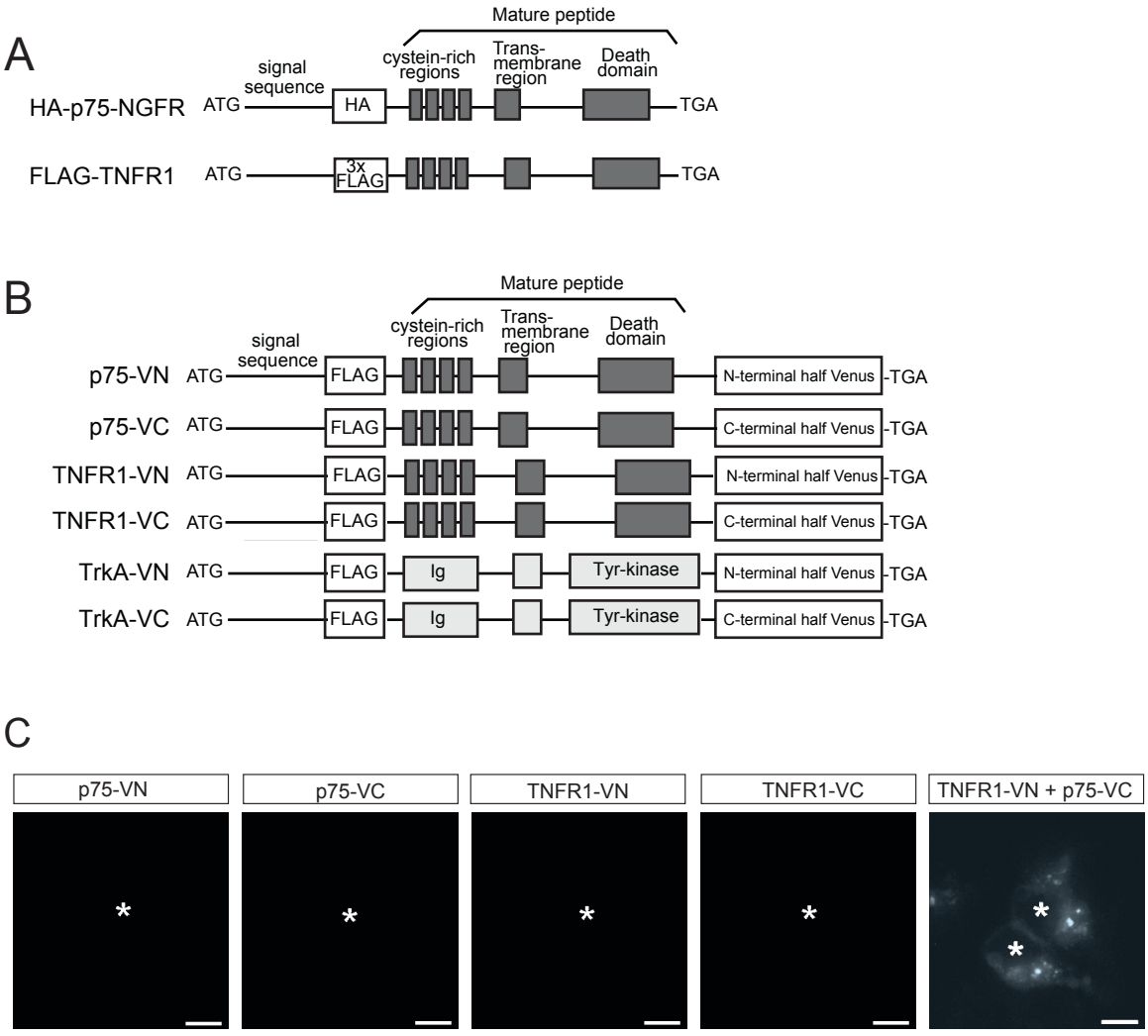

A
Mature peptide
signal sequence
cystein-rich regions
Trans-membrane region
Death domain
HA-p75-NGFR
ATG
HA
TGA
FLAG-TNFR1
ATG
3x FLAG
TGA
B
Mature peptide
signal sequence
cystein-rich regions
Trans-membrane region
Death domain
p75-VN
ATG
FLAG
N-terminal half Venus
TGA
p75-VC
ATG
FLAG
C-terminal half Venus
TGA
TNFR1-VN
ATG
FLAG
N-terminal half Venus
TGA
TNFR1-VC
ATG
FLAG
C-terminal half Venus
TGA
TrkA-VN
ATG
FLAG
Ig
Tyr-kinase
N-terminal half Venus
TGA
TrkA-VC
ATG
FLAG
Ig
Tyr-kinase
C-terminal half Venus
TGA
C
p75-VN
p75-VC
TNFR1-VN
TNFR1-VC
TNFR1-VN + p75-VC

Figure 2-7. Cooperativity of p75-NGFR and TNFR1 in sympathetic soma size distribution *in vivo*.

(A) Loss of *p75-NGFR* or *TNFR1* rescue altered cell body size distribution observed in $Bax^{-/-}$ SCGs. p values were determined with one-way ANOVA. n=3 at least for all genotypes with ~200 cells counted for each. Bottom panels shows higher magnification of area encompassed in the white rectangle in the upper panel. Blue arrow: large soma (>350μm²), Red arrow: medium soma (175-350μm²), Green arrow: small soma (<175μm²).

(B) quantification of (A).

(C) Schematic for codependence between p75-NGFR and TNFR1 in cell size regulation *in vivo*.

Contributions: A, B: Original pictures taken and cell size measured by Catherine Jansch.

Figure 2-7

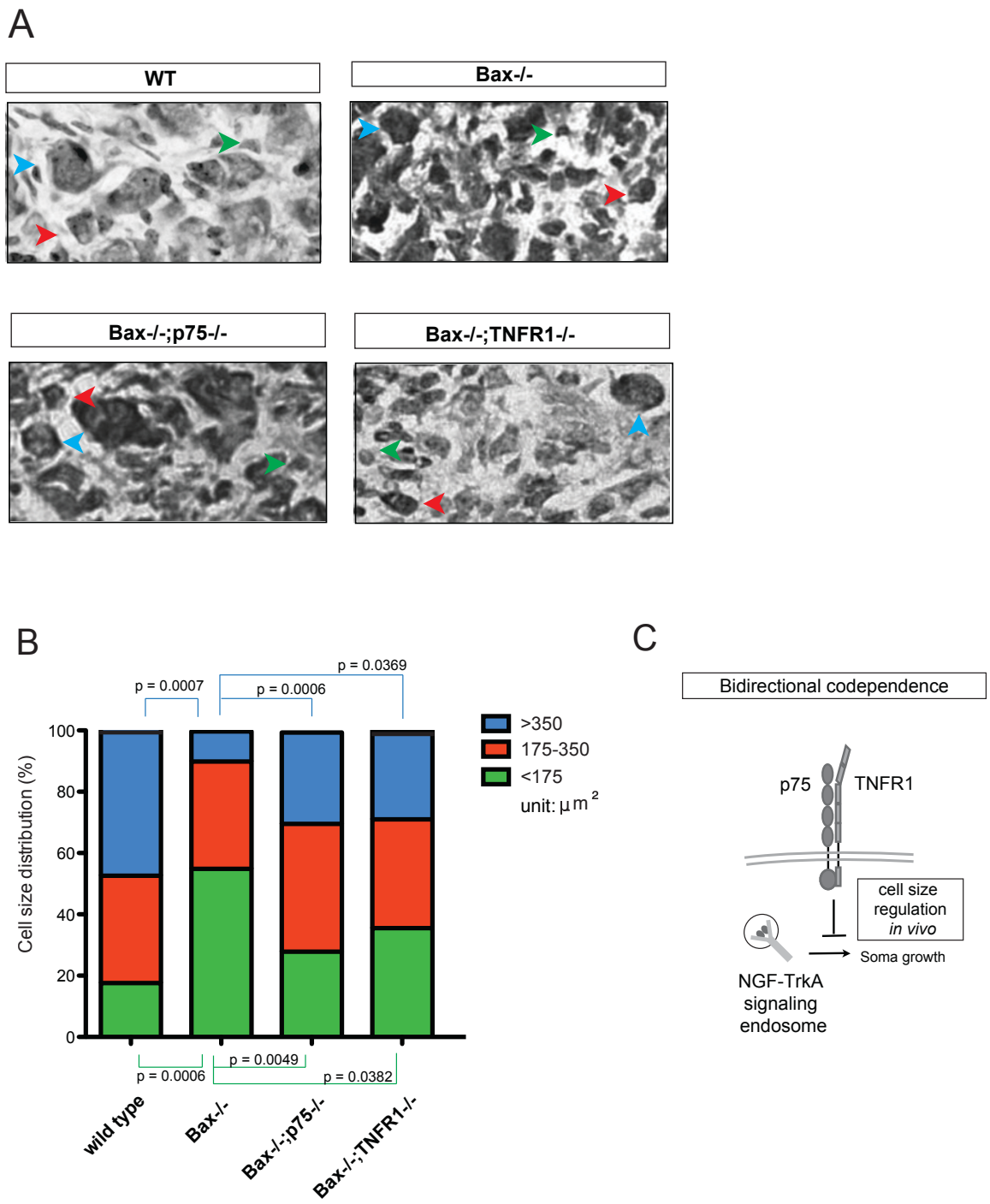

A
WT
Bax-/-
Bax-/-;p75-/-
Bax-/-;TNFR1-/-
B
p = 0.0007
p = 0.0006
p = 0.0369
Cell size distribution (%)
100
80
60
40
20
0
>350
175-350
<175
unit: μm 2
p = 0.0006
p = 0.0049
p = 0.0382
wild type
Bax-/-
Bax-/-;p75-/-
Bax-/-;TNFR1-/-
C
Bidirectional codependence
p75
TNFR1
cell size
regulation
in vivo
Soma growth
NGF-TrkA
signaling
endosome

Figure 2-8. Quantification of axonal patterning in kidney and heart *in vivo.*

To quantify axonal tiling of sympathetic kidney innervation, 3 parallel lines were placed so that the middle line (Line 2) overlies the major axis of the oval shape of a kidney. The number of axons crossing with Line 1, 2 and 3 was quantified in each kidney. Arrows on the enlarged image denote actual counting of axons crossing Line 2 in wild type kidney. At least 3 kidneys were examined per genotype. To assess the degree of axonal crossovers of sympathetic innervation on heart, the number of crossovers along the length of an individual axon was examined. At least 5 axonal regions were examined per heart. Arrows on the enlarged image shows actual counting of axonal crossovers in Axon 4. At least 3 hearts were analyzed per genotype.

Contributions: Original pictures taken by Danielle Heffner.

Figure 2-8

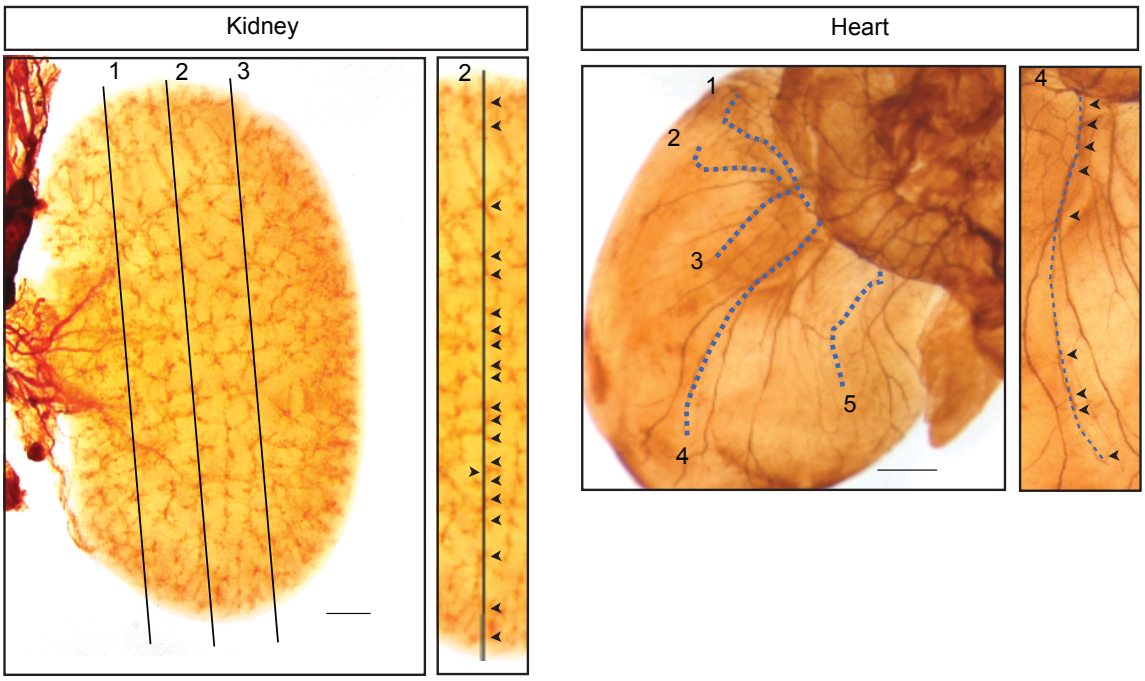


Figure 2-9. Cooperativity of p75-NGFR and TNFR1 in axon patterning during sympathetic target innervation *in vivo*.

(A) Ligand dependent p75-NGFR and TNFR1 are required for proper sympathetic innervation of kidney and heart. Whole-mount tyrosine hydroxylase immunostaining of P0 mouse kidneys and hearts were taken from Wild-type (wt), *TNFR1*$^{-/-}$, *p75*$^{-/-}$, and *TNFR1*$^{-/-}$;*p75*$^{-/-}$ (DKO) mice. Blue arrow heads indicate fasciculated axon bundles and black arrow heads indicate abnormally crossed-over axons. Each experiment was performed at least 3 times (n=3).

(B) Quantification of axon bundle tiling on kidneys shown in (A). At least 6 lines were analyzed per condition. p values were determined with one-way ANOVA.

(C) Schematic for codependence between p75-NGFR and TNFR1 in sympathetic axon patterning during kidney innervation *in vivo*.

(D) Quantification of axonal crossovers on hearts shown in (A). At least 10 individual axons were analyzed per condition. At least 3 hearts per genotype were analyzed. p values were determined with one-way ANOVA.

(E) Schematic for independent roles of p75-NGFR and TNFR1 in sympathetic axon patterning during heart innervation *in vivo*.

Contributions: A: Original pictures taken by Danielle Heffner.

Figure 2-9

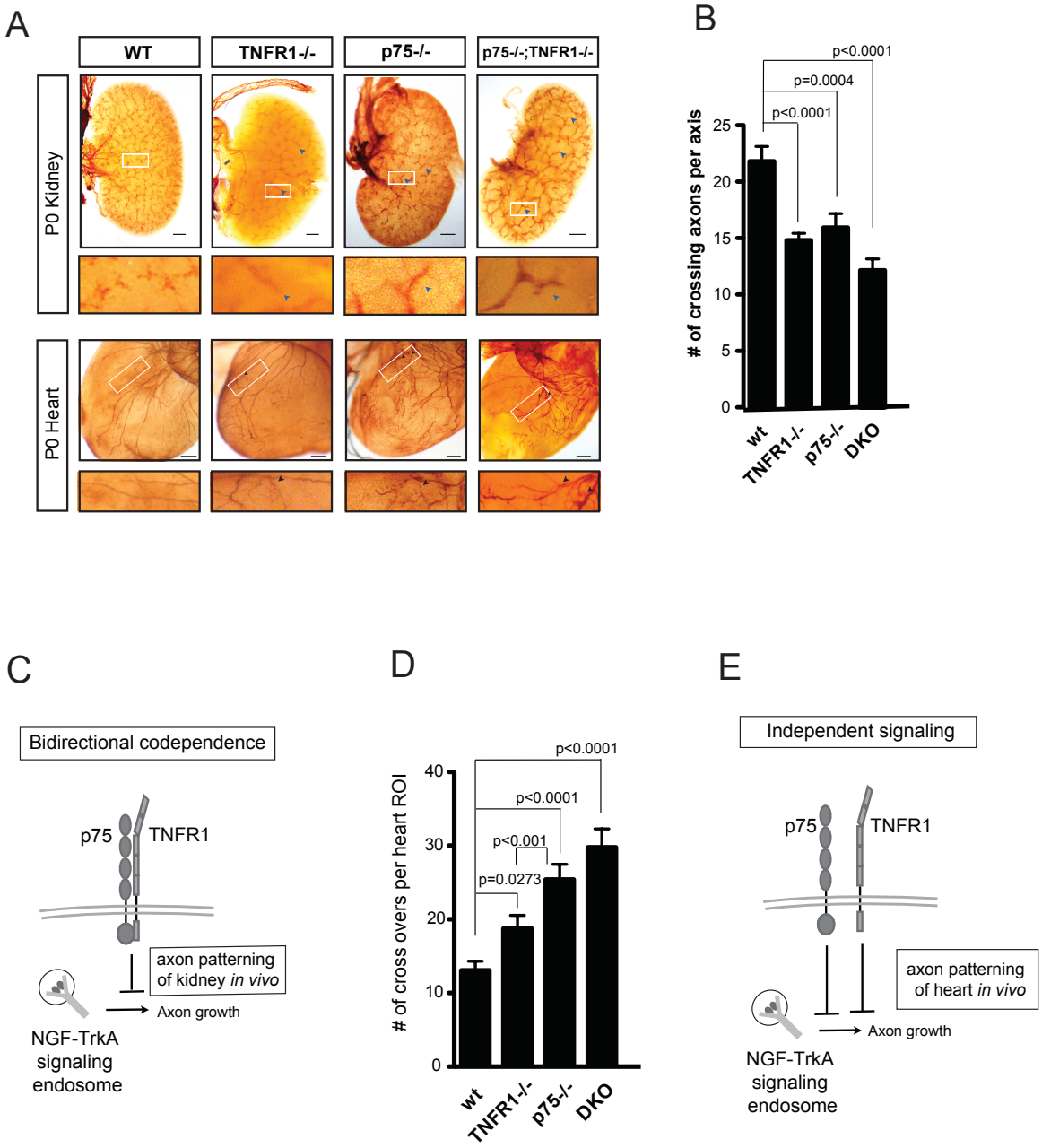

A
WT
TNFR1-/-
p75-/-
p75-/-;TNFR1-/-
P0 Kidney
P0 Heart
B
p<0.0001
p=0.0004
p<0.0001
# of crossing axons per axis
25
20
15
10
5
0
wt
TNFR1-/-
p75-/-
DKO
C
Bidirectional codependence
p75
TNFR1
axon patterning
of kidney in vivo
Axon growth
NGF-TrkA
signaling
endosome
D
p<0.0001
p<0.0001
p<0.001
p=0.0273
# of cross overs per heart ROI
40
30
20
10
0
wt
TNFR1-/-
p75-/-
DKO
E
Independent signaling
p75
TNFR1
axon patterning
of heart in vivo
Axon growth
NGF-TrkA
signaling
endosome

Figure 2-10. p75-NGFR, but not TNFR1, is required in developmental neuron death *in vivo*.

(A) The neuronal number was counted using central profiling on 10μm mouse SCG sections after Nissl-stained. p values were determined with one-way ANOVA. WT:n=6, *TNFR1*$^{-/-}$:n=6, *p75*$^{-/-}$:n=7, DKO:n=5.

(B) Schematic for unique functions in promoting developmental apoptosis p75-NGFR and TNFR1 *in vivo*.

Contributions. A: Cells counted by Catherine Jansch.

Figure 2-10

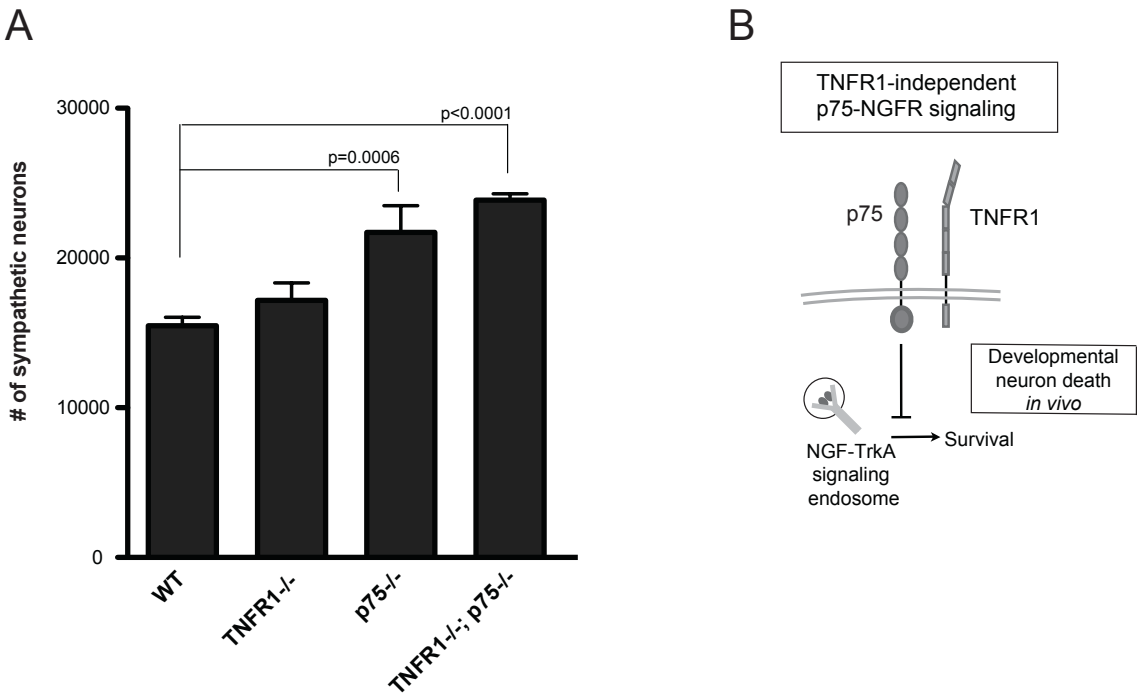
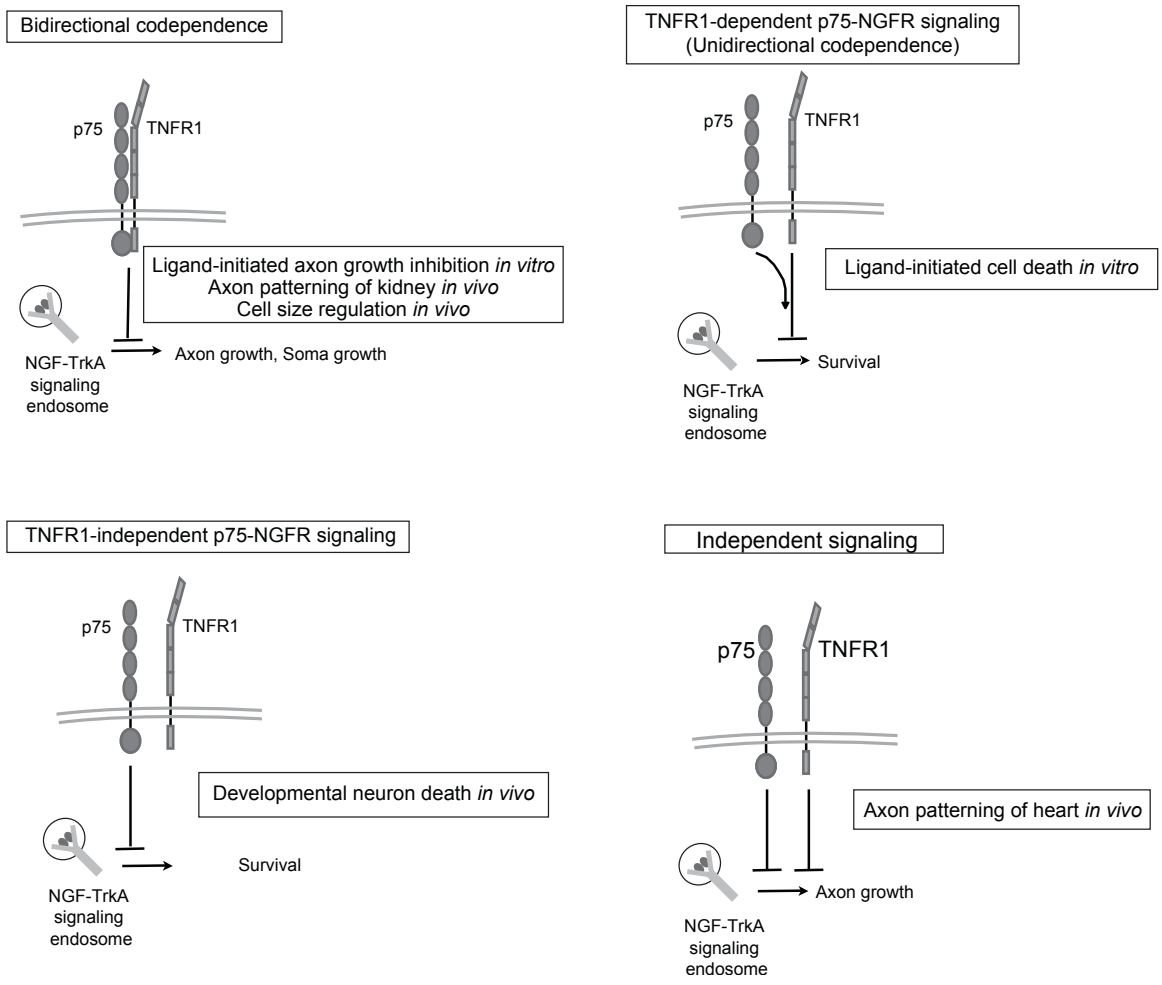

A
30000
20000
10000
0
# of sympathetic neurons
p<0.0001
p=0.0006
WT
TNFR1-/-
p75-/-
TNFR1-/-; p75-/-
B
TNFR1-independent
p75-NGFR signaling
p75
TNFR1
Developmental
neuron death
in vivo
Survival
NGF-TrkA
signaling
endosome

Figure 2-11. Model for p75-NGFR and TNFR1 cooperation during sympathetic nervous system development.

Four characteristics of p75-NGFR and TNFR1 cooperation. *Bidirectional codependence*: BDNF and TNFα require each other's cognate receptor - p75-NGFR and TNFR1 - in order to inhibit axon growth. *Unidirectional codependence (TNFR1-dependent p75-NGFR signaling)*: BDNF, but not TNFα, requires both p75-NGFR and TNFR1 to cause apoptosis in neurons grown in culture in the presence of suboptimal concentrations of NGF. *Independent*: p75-NGFR and TNFR1 do not require one another to influence a particular function as is the case in axon restriction during heart innervation. *Unique (TNFR1-independent p75-NGFR signaling)*: p75-NGFR and TNFR1 do not require one another and do not influence the same processes function as is the case in expediting neuron death *in vivo*.

Figure 2-11


Bidirectional codependence
p75
TNFR1
Ligand-initiated axon growth inhibition *in vitro*
Axon patterning of kidney *in vivo*
Cell size regulation *in vivo*
NGF-TrkA signaling endosome
Axon growth, Soma growth
TNFR1-dependent p75-NGFR signaling (Unidirectional codependence)
p75
TNFR1
Ligand-initiated cell death *in vitro*
Survival
NGF-TrkA signaling endosome
TNFR1-independent p75-NGFR signaling
p75
TNFR1
Developmental neuron death *in vivo*
Survival
NGF-TrkA signaling endosome
Independent signaling
p75
TNFR1
Axon patterning of heart *in vivo*
Axon growth
NGF-TrkA signaling endosome

Chapter 3

Preliminary Results on the Interaction of p75-NGFR and TNFR1

Association of p75-NGFR and TNFR1 is possibly ligand-independent

In Chapter 2, p75-NGFR and TNFR1 have been found to interact with each other. However, the molecular basis of this complex is completely unknown. To examine whether addition of ligands enhances or diminishes the association of p75-NGFR and TNFR1, HA-tagged p75-NGFR and FLAG-tagged TNFR1 were expressed in HEK293 cells and then the cells were cultured with 200 ng/ml BDNF or 40 ng/ml TNFα in order to perform co-immunoprecipitation (co-IP). HA-p75-NGFR was immunoprecipitated and FLAG-TNFR1 as well as HA-p75-NGFR were detected by immunoblot (Fig. 3-1). The immunoprecipitated lanes show that a similar level of association of p75-NGFR and TNFR1 in the absence or presence of BDNF or TNFα. This implies that the association of p75-NGFR and TNFR1 may be ligand-independent just like pre-ligand assembly.

Localization of p75-NGFR on neurites is upregulated after NGF deprivation

If adding their ligands does not impact formation of the p75-NGFR/TNFR1 complex, perhaps localization of the two receptors is regulated in response to changes in the trophic environment. NGF deprivation is a great candidate for regulating localization of dependence receptors including p75-NGFR. Thus, I hypothesized that localization of p75-NGFR on cell surface is responsive to NGF deprivation. To test this idea, primary

culture of sympathetic neurons from P0 wild type rats was established and maintained in 50 ng/ml of NGF for 14 days until the neurons form a typical neuronal morphology with fully extended neurites. Neurons were then deprived of NGF for 90 minutes and immunostained using p75-NGFR antibody. The results show that localization of p75-NGFR on neurites is upregulated after NGF deprivation (Fig 3-2). This suggests that the absence of NGF can induce the localization of p75-NGFR on cell membrane.

Transmembrane domain similarity analysis of p75-NGFR, TNFR1 and DR6.

It is now established that pre-ligand binding assembly is a common feature of TNFR family members including TNFR1, TNFR2, p75-NGFR and DR5. Of these, TNFR1, TNFR2 and DR5 form homooligomers via their first cysteine-rich domains whereas p75-NGFR does so via its transmembrane cysteine (Chan et al., 2001; Mukai et al., 2010; Vilar et al., 2009; Valley et al., 2012). Interestingly, Valley and colleagues show that DR5 trimers dimerize and form a highly organized receptor network on the cell membrane. The dimerization of receptor-trimers is through a specific cysteine and two glycines in the DR5 transmembrane domain (Valley et al., 2012). This led me to hypothesize that TNFR1 and p75-NGFR may interact with each other via their cysteines in their transmembrane regions. To assess this idea, I first analyzed the similarity of the amino acid sequences of the transmembrane region of mouse p75-NGFR, TNFR1 and DR6, using an automated program called PRALINE Multiple Sequence Alignment (http://www.ibi.vu.nl/programs/pralinewww/). The results show that both TNFR1 and DR6 possess a cysteine (mouse TNFR1-C224, mouse DR6-C368) near C257 of p75-

NGFR, through which p75-NGFR is known to form a homodimer. This suggests that p75-NGFR, TNFR1 and DR6 have a possibility of forming a heterodimer through these cysteines. Next I asked whether C224 of TNFR1 is conserved among different species. Now the amino acid sequences of TNFR1 transmembrane domains from mouse, Xenopus and zebrafish were analyzed. The results indicate that the C224 of mouse TNFR1 is not conserved among the species. This suggests that perhaps TNFR1-C224 does not play the same "hinge" role as p75-NGFR-C257, which is, in contrast, conserved among difference species including mouse, Xenopus and zebrafish (Vilar et al., 2009). Thus, possible binding site of p75-NGFR and TNFR1 would be either in extracellular or intracellular domain.

Figure 3-1. Association of p75-NGFR and TNFR1 is possibly ligand-independent. HEK293 cells expressing HA-tagged p75-NGFR and FLAG-tagged TNFR1 were cultured with 200 ng/ml BDNF and/or 40 ng/ml TNFα in order to perform co-immunoprecipitation (co-IP). HA-p75-NGFR was immunoprecipitated and FLAG-TNFR1 as well as HA-p75-NGFR was detected by immunoblot.

Figure 3-2. Localization of p75-NGFR on neurites is upregulated after NGF deprivation. Sympathetic neurons from P0 wild type rats were established and maintained in 50 ng/ml of NGF for 14 days. Neurons were then deprived of NGF for 90 minutes and immunostained for p75-NGFR.

Figure 3-1.

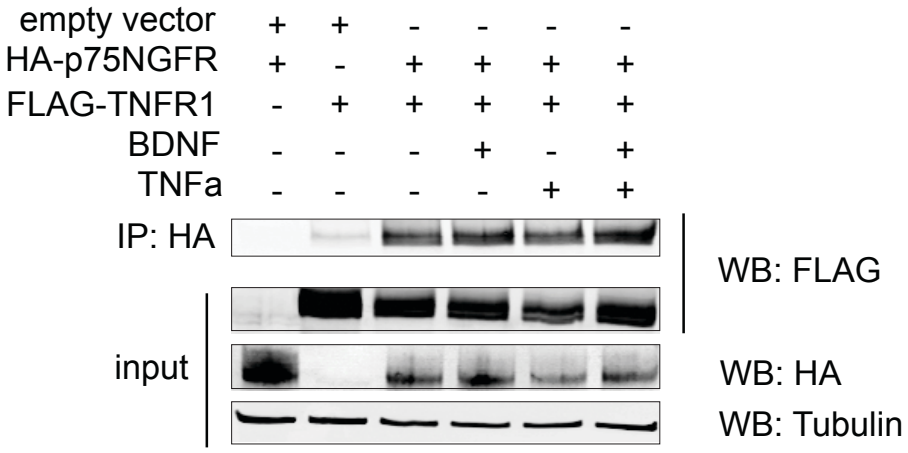


Figure 3-2.

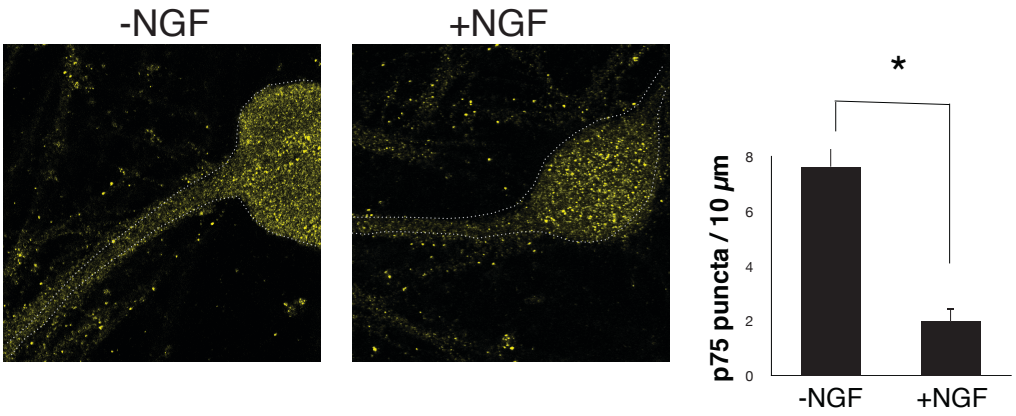


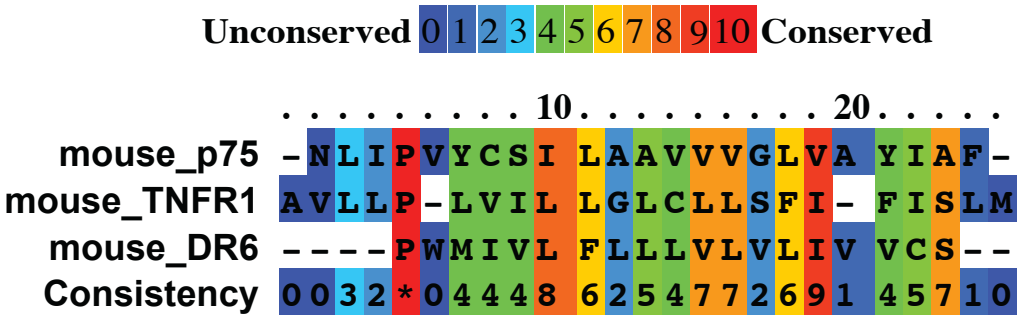
Figure 3-3. Transmembrane domain similarity analysis of p75-NGFR, TNFR1, and DR6. (A) Sequences of the transmembrane region of mouse p75-NGFR, TNFR1, and DR6 as well as sequences of the transmembrane region of TNFR1 from Xenopus TNFR1 and zebrafish. (B) Sequence similarity analysis of the transmembrane region of mouse p75-NGFR, TNFR1, and DR6. Sequences were analyzed using PRALINE Multiple Sequence Alignment (http://www.ibi.vu.nl/programs/pralinewww/). (C) Sequence similarity analysis of the transmembrane region of TNFR1 from mouse, Xenopus and zebrafish.

Figure 3-3

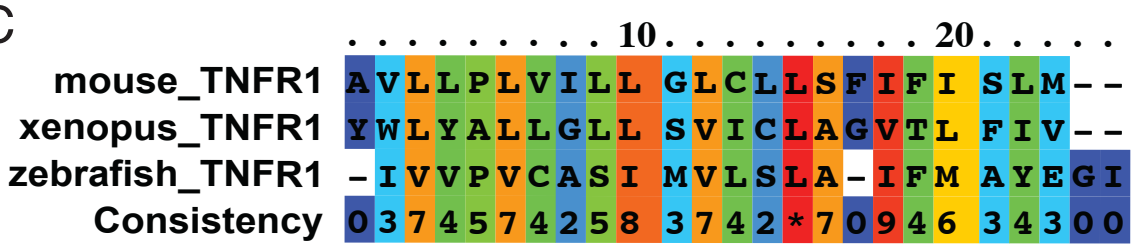
A

mouse p75 TM (253-275 aa)	NLIPVYCSILAAVVVGLVAYIAF
mouse TNFR1 TM (212-234 aa)	AVLLPLVILLGLCLLSFIFISLM
mouse DR6 TM (351-370 aa)	PWMIVLFLLLVLVLIVVCS
Xenopus TNFR1TM	YWLYALLGLLSVICLAGVTLFIV
zebrafish TNFR1 TM	IVVPVCASIMVLSLAIFMAYEGI

B



C



Chapter 4:

Preliminary results on the molecular mechanisms of p75-dependent synapse restriction.

Introduction

The functional nervous system is encoded by the specificity of its synaptic connections. Errors in the number, location, timing and type of synaptic connection during development lead to abnormal nervous system function. Understanding not only the mechanisms that trigger neuronal synaptogenesis, but also the mechanisms that inhibit improper synapse formation is important.

Peripheral nervous system development requires cell extrinsic trophic factors, which activate intrinsic signaling cascades to initiate a hierarchy of developmental events (Bibel and Barde, 2000). One class of trophic factors that is particularly important for nervous system development and function is the neurotrophin family. Mammals utilize four neurotrophins: nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT3) and neurotrophin-4 (NT-4). There are 2 different classes of neurotrophin receptors: (i) a family of receptor tyrosine kinases, termed the Trks; and (ii) the p75 neurotrophin receptor. Each Trk family member binds to a specific neurotrophin as follows: NGF-TrkA, BDNF-TrkB, NT3-TrkC, whereas p75 displays promiscuous binding with all neurotrophins (Bibel and Barde, 2000).

The spatial and temporal pattern of signaling by neurotrophins and their receptors regulates the development of all populations of peripheral neurons. In a simple model system, the sympathetic nervous system, NGF secreted from target organs binds to neuronal TrkA on the distal axon. This leads to endocytosis of the NGF-TrkA complex herein referred to as the signaling endosome. It is trafficked retrogradely toward the cell body, where it engages transcriptional programs that support neuronal survival and axon growth (Ibanez et al., 1992; Kohn et al., 1999). Conversely, p75 is reported to function antagonistically to TrkA; it causes neuronal death and axon degeneration (Bamji et al., 1998; Kohn et al., 1999). As a mechanism of cross talk, NGF-TrkA silencing of p75 is implicated (Majdan et al., 2001). Importantly, high levels of TrkA activation is observed to dominantly suppress p75 signaling. This suggests that there exists a threshold of TrkA signaling that is the key determinant for the choice between neuron survival/death (Bamji et al., 1998) and axon growth/degeneration (Singh et al., 2008). Recently, Deppmann and colleagues discovered that this antagonistic framework governed by NGF-TrkA and p75 also holds true for synapse formation in sympathetic ganglia (Sharma et al., 2010). This antagonistic framework, more specifically the inhibitory function of p75, plays an important role to regulate the location and timing of sympathetic synaptogenesis.

Sympathetic synaptic connections are made between preganglionic neurons from the spinal cord and postganglionic dendrites from neurons in sympathetic ganglia (Glebova and Ginty, 2005). It has long been debated which synaptic component (pre- versus post-) determines when and where a synapse is formed. The finding from Deppmann and colleagues indicates that the postsynaptic specialization can drive differentiation of the

presynaptic specialization. This suggests that components of the postsynaptic density (PSD) signals to the presynaptic axon. The PSD is made up of a highly dense and complex structure consisting of neurotransmitter receptors and proteins associated with scaffolding, cell adhesion, signaling, and cytoskeletal tethering (Sheng and Hoogenraad, 2007).

The molecular basis by which p75 restricts synapse formation remains completely unknown, mainly due to the complicated postsynaptic architecture of the synapse. Not only are the targets of p75 signaling within the PSD unknown, the specific p75-dependent signal is also unknown. Part of the challenge in defining these signals lies in the complex nature of p75 activation. It displays promiscuous interaction with many different ligands yielding different modes of activation and engagement of various downstream signaling pathways (Fig. 4-1). The three modes of p75 activation include: (i) liberation of the p75 intracellular domain (ICD) via proteolytic cleavage by several secretases such as β-, γ-secretases, and tumor necrosis factor α converting enzyme (TACE); (ii) conformational change of p75 dimers upon engagement by a subset of ligands; and (iii) heterodimerization between p75 and a co-receptor upon engagement of a different subset of ligands (Gentry et al., 2004). Beyond the mode of activation, the pathways downstream of p75 that are critical for synapse restriction remain unknown. Potential downstream p75 signaling includes activation of NF-kB, c-Jun N-terminal Kinase (JNK), caspases, and RhoA (Chao, 2003).

It is possible that other p75 family member(s) also participate in synapse restriction. p75 is member 16 of the tumor necrosis factor receptor super family. Interestingly, two other members of this family: (i) tumor necrosis factor receptor (TNFR1, also known as TNFR super family member 1A) and (ii) death receptor 6 (DR6, also known as TNFR super family member 21) have been reported to mediate cell death and axon degeneration, respectively, in developing sympathetic neurons (Barker et al., 2001; Nikolaev et al., 2009). Although these family members have been widely studied in several systems beyond neurodevelopment, there have been no reports describing whether they are functionally redundant or working together in a single pathway. Thus, whether (and how) p75, TNFR1 and DR6 crosstalk during neuronal development is completely unknown. Moreover, whether TNFR1 and DR6 can induce synapse restriction is also unknown.

Results and Discussion

β-, γ-secretases are required for p75-dependent synapse restriction.

As described in Background, different modes of p75 oligomerization can lead to activation of diverse downstream pathways. One mechanism by which downstream pathways are activated is through a series of secretase-mediated cleavage events eventually leading to ICD liberation, which activates further signaling and transcriptional programs. To preliminarily examine the requirement of the secretases in restricting synapse formation, pharmacological inhibitors against β- and γ-secretases (compound E and BACEI, respectively) were added to sympathetic mature neurons cultured until they

fully extend dendrites. Following immunostaining the neurons for a PSD marker using a Maguk (Membrane-associated guanylate kinase) antibody, PSD clustering was measured by examining the number of fluorescent puncta per unit length of dendrites. The PSD levels were elevated by addition of secretase inhibitors in the absence of NGF (Fig. 4-2). This is suggestive of a requirement of the p75 ICD in p75-dependent synapse restriction. However, addition of the inhibitors did not increase the number of PSDs in the presence of NGF relative to the control (Fig. 4-2). This suggests that β- and/or γ-secretases cannot reduce PSD clustering when NGF-TrkA signaling is high, implying that sufficient levels of NGF-TrkA signaling may inhibit β- and/or γ-secretases. The results regarding ICD liberation demand further molecular interrogation.

p75 signals through Rho, but not JNK, to restrict synapse formation.

Among downstream players of p75, JNK and RhoA are particularly good candidates for synapse restriction due to their reported involvement related to synapse functions in the CNS (Zhu et al., 2005; Margolis et al., 2010). To test if these downstream players restrict synapse formation in sympathetic neurons, pharmacological inhibitors were added to primary cultures. In the absence of NGF, more PSD clustering was observed in the presence of RhoA inhibitor (CT04) while no difference was observed in presence of JNK inhibitor (SP600125) (Fig. 4-3). These results suggest that RhoA, but not JNK, is involved in synapse restriction. Interestingly, in the presence of NGF the RhoA- and JNK inhibitors could not reduce the number of PSD clustering compared to control, suggesting that RhoA requires low NGF-TrkA signaling to restrict synapse formation. These data

imply that NGF-TrkA signaling inhibits activation of RhoA. Whether RhoA is sufficient to suppress PSD clustering and the signaling pathways downstream of RhoA, which are relevant to synapse restriction would be worthwhile topics for future investigations.

Activation of TNFR1 phenocopies p75-dependent synapse restriction.

p75 and TNFR1 have both been shown to induce sympathetic neuron death. Why have 2 receptors performing the same function(s)? I hypothesized that TNF receptor family members activate either independent pathways or may work together in an interdependent pathway. To test whether TNFR1 is able to restrict synapses, TNFα, the cognate ligand for TNFR1 was added to cultured wild type rat neurons. BDNF was also added as a control to activate p75. TNFα was indeed able to suppress PSD clustering similar to what is observed with p75 activation (Fig. 4-4). Interestingly, concurrent addition of BDNF and TNFα led to a similar reduction of PSD clustering compared to the PSD level affected by the addition of BDNF or TNFα. This implies that p75 and TNFR1 may cooperate to suppress synapse formation. Assuming TNFα specifically activates TNFR1, these data indicate that there is overlapping function between p75 and TNFR1 in synapse restriction, as well as in axon growth inhibition, axon patterning on target and cell size regulation as previously discussed in Chapter 2. The further molecular basis for their redundant functions should be examined.

Ligand-activation of TNFR1 restricts PSD clustering in a p75-dependent manner.

This functional overlap could arise from these receptors initiating separate parallel pathways, or participating in a single pathway to suppress synapse formation. Therefore, I next sought to examine PSD clustering in the absence of p75. Using *p75*-/- mouse neurons, I examined the ability of TNFα or BDNF to restrict synapse formation. As expected, BDNF was no longer able to suppress PSD clustering due to a lack of its cognate receptor. Surprisingly, TNFα was also unable to suppress PSD clustering even though the TNFR1 locus remained intact (Fig. 4-5). This preliminary observation supports the idea that they participate in a single pathway to restrict synapses, rather than independent parallel pathways. This codependent relationship of p75 and TNFR1 is consistent with what was observed in axon growth inhibition *in vitro* as well as axon patterning and cell size regulation *in vivo* as previously described. As a molecular mechanism by which p75 and TNFR1 cooperate during sympathetic nervous system, interaction of the two receptors on cell surface is suggested in Chapter 2. One way to pursue the role of p75 and TNFR1 in synapse restriction would be to examine whether or not association of p75 and TNFR1 inhibits PSD clustering in the vicinity of the p75/TNFR1 complex. Furthermore, whether β- and γ- secretase activity induces formation of the p75/TNFR1 complex could be an interesting potential future endeavor.

Figure 4-1. Modes of activation and downstream signaling effectors of p75. Different ligands yield different modes of activation and engage various downstream signaling effectors. The identified ligands are: the neurotrophins (NGF, BDNF, NT-4, NT-3) as well as non-neurotrophins such as Nogo, myelin-associated glycoprotein (MAG) and oligodendrocyte myelin glycoprotein (OMG). However, which ligand causes which mode of activation or downstream effectors is not clearly known. Abbreviations: extracellular domain (ECD), intracellular domain (ICD), β-secretase (β), γ-secretases (γ).

Figure 4-1

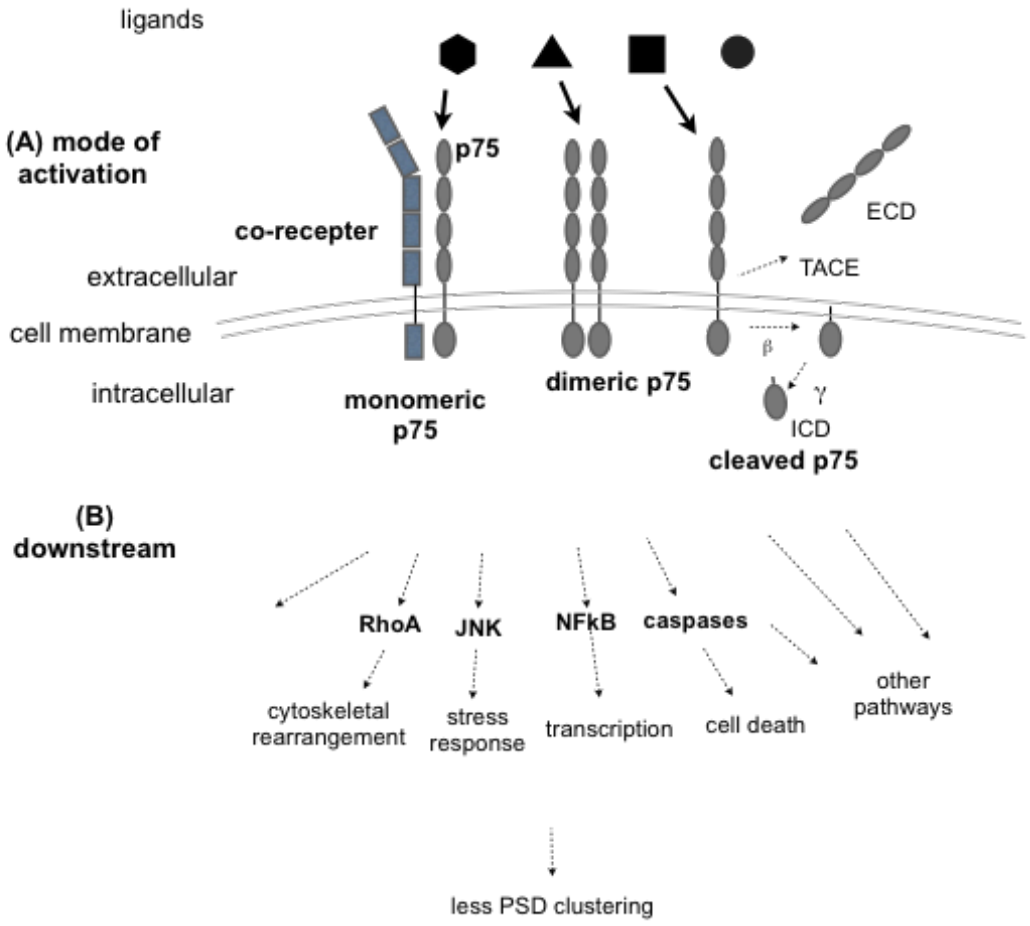

ligands
(A) mode of activation
p75
co-recepter
ECD
extracellular
TACE
cell membrane
β
intracellular
γ
monomeric p75
dimeric p75
ICD
cleaved p75
(B) downstream
RhoA
JNK
NFkB
caspases
other pathways
cytoskeletal rearrangement
stress response
transcription
cell death
less PSD clustering

Figure. 4-2. β-, γ-secretases are required for p75-dependent synapse restriction. Wild type rat neurons were treated in culture medium containing 100 nM Compound E (γ-secretase inhibitor), or 10 μM BACEI (β-secretase inhibitor) for 3 hours. In the minus NGF condition, the culturing medium contained 1 μg/ml anti-NGF. p values were determined with Student's T-test.

Figure. 4-3. p75 signals through RhoA, but not JNK, to restrict synapse formation. Wild type rat neurons were treated in culture medium containing 4 ng/ml CT04 (Rho inhibitor), or 25 nM SP600125 (JNK inhibitor) for 3 hours. In the minus NGF condition, the culturing medium contained 1 μg/ml anti-NGF. p values were determined with Student's T-test.

Figure 4-2

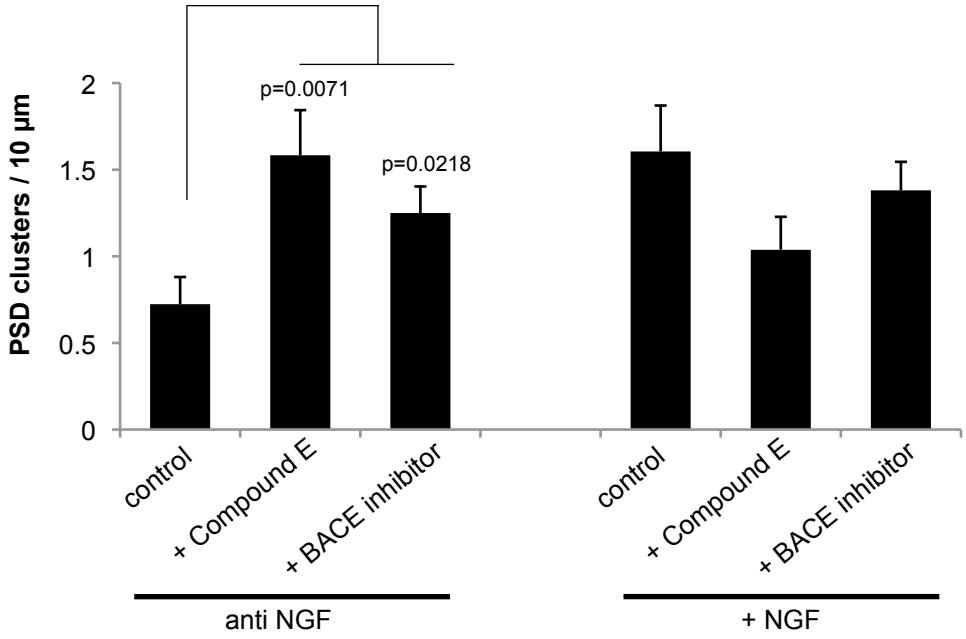


Figure 4-3

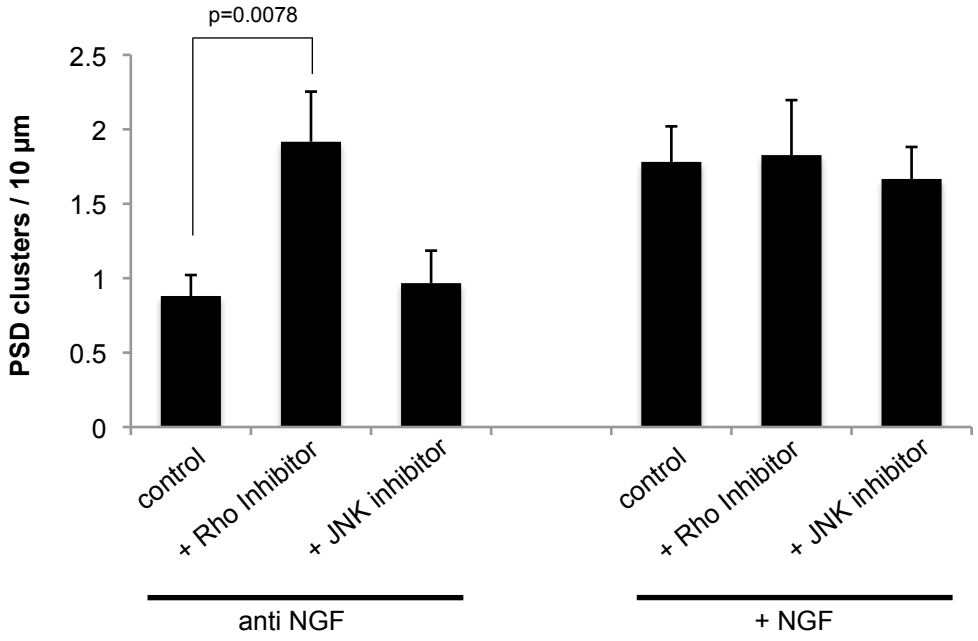


Figure 4-4. Activation of TNFR1 phenocopies p75-dependent synapse restriction.
Wild type rat neurons were treated in culture medium containing i) 1 μg/ml anti-NGF and ii) BDNF 250 ng/ml, TNFα 2 ng/ml, or nothing as control overnight. Anti-NGF was added to lower NGF-TrkA signaling. p values were determined with Student's T-test.

Figure 4-5. TNFα restricts PSD clustering in a p75-dependent manner.
p75-/- mouse neurons were treated in culture medium containing i) 1 μg/ml anti-NGF and ii) BDNF 250 ng/ml, TNFα 2 ng/ml, or nothing as control overnight. p values were determined with Student's T-test.

Figure 4-4

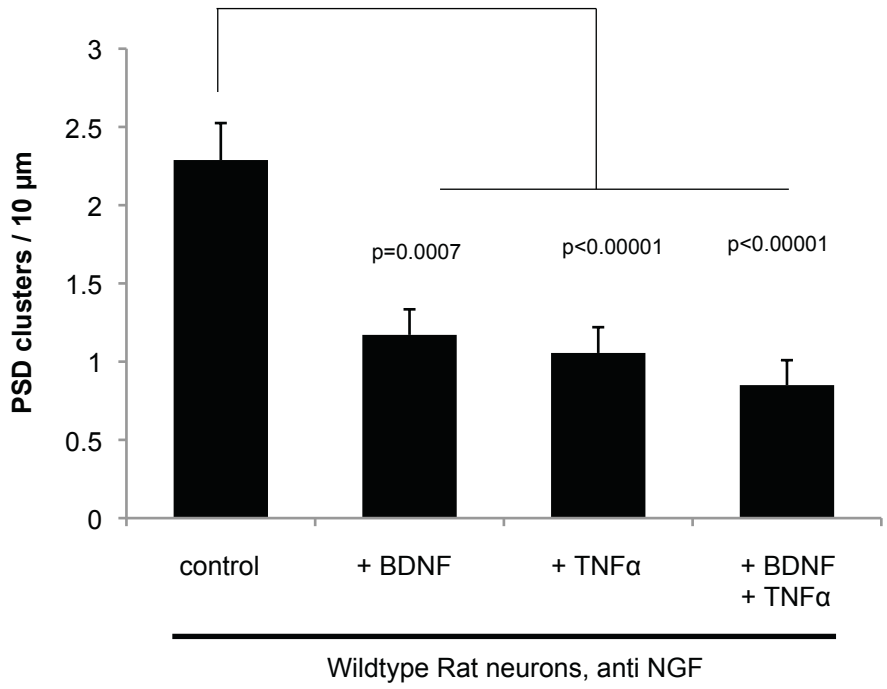
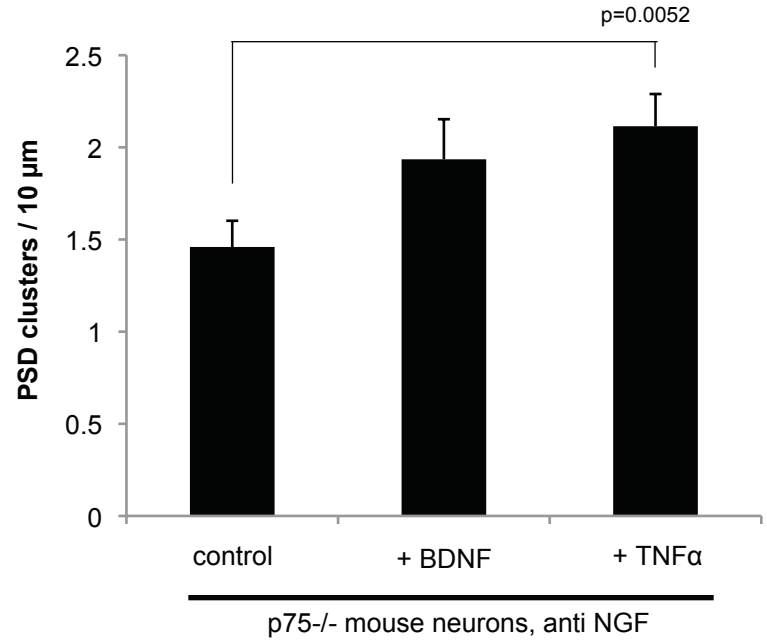


Figure 4-5



Chapter 5

Discussion and Future Directions

Crosstalk between NGF-TrkA signaling and p75-NGFR/TNFR1 signaling

It is a prevailing view that NGF-TrkA signaling promotes neuronal survival and axon maintenance whereas activation of p75-NGFR by BDNF suppresses those functions by overriding NGF-TrkA signaling (Bamji et al., 1998; Kohn et al., 1999; Singh et al., 2008). In Chapter 2, this was modeled *in vitro* by culturing neurons in suboptimal levels NGF with BDNF or TNFα.

How do neurons respond when there is no NGF-TrkA signaling? As previously shown in Figure 2-4, wild type, *p75-NGFR*-null and *TNFR1*-null neurons showed low survival and axon growth when NGF was deprived (-NGF) whereas high survival and axon growth was observed in the suboptimal level of NGF (+NGF) (Fig. 2-4B,D). These data suggest that neuronal death and axon growth inhibition induced by NGF deprivation do not require p75-NGFR or TNFR1. This implies that inhibitory functions of p75-NGFR and TNFR1 take place by suppressing NGF-TrkA signaling, which is consistent with the previous report showing antagonizing effects of BDNF-activated p75-NGFR against NGF-TrkA signaling in neuron survival and neurite growth (Bamji et al., 1998; Kohn et al., 1999), as well as our codependence model where p75-NGFR and TNFR1 cause cell death and axon growth inhibition by antagonizing NGF-TrkA signaling (Fig. 2-3A).

In order for p75-NGFR to override NGF-TrkA signaling, suboptimal levels of NGF are required. The level of NGF that can be overridden by p75-NGFR depends on the developmental context (Kohn et al., 1999; Singh et al, 2008). In Chapter 2, the suboptimal level of NGF used in the *in vitro* cell death assay was 2 ng/ml. The same cell death assay using wild type neurons at higher levels of NGF will exhibit less death in the presence of NGF and BDNF because BDNF-p75-NGFR signaling is shown to be unable to override NGF-TrkA signaling when NGF levels are high (10 ng/ml) (Bamji et al., 1998). Likewise, TNFα addition to higher levels of NGF should not cause cell death in wild type neurons as previously shown by Barker and colleagues (Barker et al, 2001). Thus, the apoptotic effect of TNFα in *p75-NGFR*-null neurons observed in Figure 2-4B may be blocked at higher levels of NGF. Then, do ligand-activated p75-NGFR and TNFR1 enhance their signaling to cause cell death in lower levels of NGF? The *in vitro* cell death assay in lower levels of NGF will probably display similar promoting effects of BDNF and TNFα relative to what was observed at 2 ng/ml NGF, as addition of TNFα has been demonstrated to promote cell death at <1 ng/ml NGF (Barker et al., 2001). Taken together, ligand-activated p75-NGFR and TNFR1 will override NGF-TrkA signaling to cause cell death when NGF levels are not high (<2 ng/ml), probably leading to TNFR1-dependent p75-NGFR signaling and p75-NGFR-independent TNFR1 signaling as previously observed at 2 ng/ml NGF. However, when NGF levels are high (>10 ng/ml), death signaling generated by BDNF-p75-NGFR and TNFα-TNFR1 will not be capable of antagonizing NGF-TrkA signaling to cause cell death. In the context of axon degeneration or axon growth inhibition, on the other hand, crosstalk of NGF-TrkA and

p75-NGFR/TNFR1 signaling has been understudied. In Chapter 2, the suboptimal level of NGF used in the *in vitro* axon growth measurement assay was 10 ng/ml. If NGF-TrkA signaling can be similarly overridden at low levels of NGF (<10 ng/ml), but not at high levels (>10 ng/ml), the same axon growth assay using wild type neurons at higher levels of NGF would show rescue of axon growth inhibition when BDNF or TNFα is added, whereas the same assay at lower levels of NGF would display similar inhibitory effects by BDNF and TNFα in promoting further inhibition of axon growth in the presence of BDNF or TNFα, compared to that of low NGF only. Further investigation is required to identify how crosstalk between NGF-TrkA signaling and p75-NGFR/TNFR1 signaling takes place in axon growth.

One attractive molecular mechanism by which ligand-activated p75-NGFR and TNFR1 antagonize NGF-TrkA signaling is attenuation of ERK phosphorylation. When BDNF-activated p75-NGFR overrides NGF-TrkA signaling, phosphorylation of ERK, a downstream pathway of NGF-TrkA signaling, is attenuated (Singh et al., 2008). Since activation of ERK is a function of TrkA activation, low level of ERK phosphorylation attenuated by addition of BDNF to suboptimal NGF might be regarded as a similar result caused by low TrkA activation by low levels of NGF. Examination of ERK phosphorylation in the presence of TNFα and suboptimal NGF should reveal whether ERK is also a target attenuated by TNFα-TNFR1 signaling.

Distinct requirements of p75-NGFR and TNFR1 for NGF-deprivation-induced neuron death *in vitro* and developmental neuron death *in vivo*

NGF deprivation is a classic *in vitro* model of developmental neuron death. However, it does not perfectly mimic molecular properties of neurons during developmental cell death *in vivo*. As previously mentioned above, NGF-deprivation-induced cell death *in vitro* was observed in all of wild type, *p75-NGFR*-null and *TNFR1*-null neurons in the anti-NGF condition, suggesting p75-NGFR and TNFR1 are not required to cause NGF-deprivation death (Fig. 2-4B). In comparison, developmental cell death *in vivo* requires p75-NGFR, but not TNFR1 (Fig. 2-10A). This discrepancy may be due to fundamental difference between the two experimental paradigms. Whereas the sympathetic neurons used *in vitro* assays were separated from target organs and glial cells, cell-cell communication with them was retained in neurons analyzed *in vivo*. This may be an explanation for the heterogeneous requirement of p75-NGFR and TNFR1. Perhaps target organs or glial cells secrete an additional trophic factor *in vivo*, which can be overridden by p75-NGFR, but not by TNFR1. Lack of such a trophic factor *in vitro* may relatively strengthen TNFR1's capability of killing neurons. This may be the reason why TNFR1 could not cause apoptosis in *p75-NGFR*-null mice *in vivo* whereas TNFα-activated TNFR1 could kill *p75-NGFR*-null neurons *in vitro*.

Developmental neuron death in the sympathetic neurons requires TNF and p75-NGFR, but not TNFR1, BDNF or NT4 *in vivo*

As stated in the neurotrophic theory, developing sympathetic neurons compete for a limited amount of target-derived NGF and superfluous neurons undergo apoptosis due to

the failure to acquire NGF. p75-NGFR facilitates this NGF-deprivation-induced apoptotic process. However, whether NGF-deprivation induces a death ligand to promote cell death has remained unknown and controversial. One great candidate is BDNF, which has been shown to cause cell death in developing sympathetic neurons via p75-NGFR both *in vitro* and *in vivo* (Bamji et al., 1998). However, a different report indicates that BDNF and NT4 do not affect the neuronal number at SCG (Liu et al., 1995). As shown in Appendix 3, the data in this thesis support the latter, as the sympathetic neuronal number of SCG from *BDNF*$^{-/-}$; *NT4*$^{-/-}$ mice is not significantly different from that of wild type controls (Fig. A-3F). This eliminates neurotrophins as possible ligands activating p75-NGFR during the sympathetic developmental neuron death *in vivo*. Then what is activating p75-NGFR to facilitate the developmental cell death *in vivo*? Jensen and colleagues have eliminated sortilin as a possibility by showing that neonatal *Sortilin*-deficient mice have a similar number of neurons in the SCG compared to controls (Jensen et al., 2007). Myelin-derived molecules including MAG, Nogo, and Ompg, activate RhoA, however this signaling does not overlap with activation of JNK or caspase cascade, so these ligands are not capable of directly causing apoptosis either (Yamashita and Tohyama, 2003). Taken together, these data imply that p75-NGFR is activated either without ligands or by an unidentified ligand. Here it is interesting to note that TrkA is required to cause cell death in a p75-dependent manner and that p75-NGFR and TrkA interact through their intracellular domain (Nikoletopoulou et al., 2010; Iacaruso et al., 2011). It may be possible that interaction of TrkA and p75-NGFR generate death signaling to cause developmental neuron death *in vivo*. One potential future direction to test this idea is to ask whether TrkA and p75-NGFR form a complex after NGF withdrawal as well as

whether inhibiting the association of these two receptors can rescue cell death from NGF deprivation in sympathetic neurons.

Furthermore, we found that TNF is required in the apoptotic process *in vivo* (Appendix 3, Fig. A-3F). This makes the scenario more puzzling, because it has been established that the apoptotic function of TNFα is exerted via its death receptor, TNFR1 (Barker et al., 2001). This raises an additional question of how TNF transduces the apoptotic signaling. Does TNF act through p75-NGFR as its novel ligand? This possibility is eliminated by the *in vitro* experiment in Chapter 2. If TNFα acts through p75-NGFR to cause apoptosis, adding TNFα to *TNFR1*-null neurons, where p75-NGFR is intact, would kill the neurons. However, the data show that the TNFα addition does not cause apoptosis in *TNFR1*-null neurons *in vitro* (Fig. 2-4B). This suggests that TNFα is not capable of activating p75-NGFR to cause apoptosis. Additionally, the ligand-receptor binding screen performed by Bossen and colleagues does not support the TNF-p75-NGFR binding (Bossen et al., 2006). In their report, all possible ligand-receptor combinations were comprehensively examined using 20 human TNF family members, 20 mouse TNF family members, and 28 human TNFR family members as well as 28 mouse TNFR family members. In their results, no interaction was observed between TNF and p75-NGFR. How TNF transduces death signaling to cause cell death requires further investigation.

p75-NGFR and TNFR1 are required to inhibit axon growth *in vitro* and promote proper axon patterning on target *in vivo*

Aside from apoptosis, axon-refining functions of p75-NGFR and TNFR1 were examined. The *in vitro* study shows that 1) TNFα-induced axon growth inhibition does not occur in *p75-NGFR*-deficient neurons, and 2) BDNF-induced axon growth inhibition is also blocked in *TNFR1*-deficient neurons (Fig. 2-4D). Since both ligands can activate their cognate death receptors and inhibit axon growth in wild type neurons, this study is the first evidence that p75-NGFR and TNFR1 require one another to inhibit axon growth upon stimulus of death ligands. This implies that TNFR1 is actively involved in the p75-NGFR signaling induced by myelin-derived molecules. MAG, Nogo, and OMgp activate RhoA in downstream via the receptor complex of p75-NGFR, NgR and Lingo1, and this results in axon growth inhibition (Yamashita et al., 1999). Perhaps TNFR1 may be also required in the receptor complex of p75-NGFR, NgR and Lingo1. The biochemical and microscopic study showing association of p75-NGFR and TNFR1 supports this idea. One possible future endeavor would be to test whether TNFR1 co-localizes with NgR and Lingo1. Understanding the precise formation of the receptor complex may provide an easier therapeutic target for axonal injury.

Furthermore, the *in vivo* study using the kidney and heart shows that axon patterning is regulated by p75-NGFR and TNFR1 on both targets but slightly differently. In kidney innervation, sympathetic axons of single receptor KO mice as well as DKO mice remain in thick bundles and less evenly innervate the target (Fig. 2-9). This suggests that p75-NGFR and TNFR1 are equally important and required to properly distribute axons on target via repulsive signaling. However, the axon patterning in the DKO kidney shows slightly more severe impairment than *p75-NGFR*-null- or *TNFR1*-null neurons. This

suggests that the mechanism for cooperation of p75-NGFR and TNFR1 in axon patterning on the kidney might not be quite the same as what was seen in the axon growth inhibition *in vitro*. One possibility is that p75-NGFR in this situation is capable of influencing several Rho GTPases, including RhoA and Rac1, and that cooperation with TNFR1 is required in only RhoA activation. Indeed, proNGF decreases Rac1 activity via p75-NGFR/Sortilin and causes growth cone collapse in hippocampal neurons *in vitro* (Deinhardt et al., 2011). An examination of whether p75-NGFR influences both activities of RhoA and Rac1 in the sympathetic axons might provide a clearer understanding on the mechanism of axon patterning on the kidney.

In heart innervation, the single receptor KO mice and DKO mice show more axonal crossovers on the target compared to control, suggesting p75-NGFR and TNFR1 are involved in proper axon patterning on the heart. However, among the receptor KO mice, *p75-NGFR*-null mice show significantly more axonal crossovers on the heart than *TNFR1*-null mice. This suggest that axon patterning on the heart is more dependent on p75-NGFR and that p75-NGFR and TNFR1 independently function in axon patterning of heart. How the two receptors differently work in kidney and heart may be due to the distinct cellular contexts among target organs. For example, Glebova and Ginty discovered that the sympathetic target organs exhibit different requirement of NGF for target innervation. In their study, the absence of NGF greatly reduces the heart innervation, but only partially impairs the kidney innervation (Glebova and Ginty, 2004). This suggests that an unidentified signaling may be supporting the kidney innervation. Furthermore, the heterogeneous requirements of NGF suggests that cooperation of p75-

NGFR and TNFR1, i.e., the p75-NGFR/TNFR1 complex, may be available only when target innervation has lower requirement of NGF. It could be that the p75-NGFR/TNFR1 complex may function when the local NGF-TrkA signaling is low, but be disrupted by high levels of NGF-TrkA signaling. The preliminary data that p75-NGFR localization is induced after NGF-TrkA support this idea. Thus, one great future possibility is to test whether the p75-NGFR/TNFR1 complex is disrupted in high levels of NGF.

p75-NGFR and TNFR1 are required to regulate neuronal cell size *in vivo*

As discussed in Chapter 1, previous studies on the regulatory role of p75-NGFR in neuronal soma size distribution were based on comparison among different neuronal populations did not exclude the apoptotic function of p75-NGFR (Bamji et al., 1998). In this thesis, the neuronal populations were compared among different genotypes using the *Bax*-null background. This allows us to accurately examine the effects of p75-NGFR and TNFR1 in regulation of neuronal soma size. The results demonstrated that compared to *Bax*-null control neurons, further ablation of p75-NGFR, TNFR1 or TNF shifts the entire population to a larger size, suggesting that they function to shift the population to a smaller size in wild type (Fig. 2-7B, A-3B). Since a large soma is capable of maintaining many neurites, soma size control might be one active developmental process to indirectly reduce the number of dendrites in order to achieve a proper circuitry. In this sense, p75-NGFR and TNFR1 codependently function to shape the soma size during development. Taken together with the roles in axon inhibition and patterning, this study revealed that

p75-NGFR and TNFR1 modify and shape neuronal morphology during development of the sympathetic nervous system.

Detecting interaction of p75-NGFR and TNFR1

In Chapter 2, p75-NGFR and TNFR1 have been shown to interact each other using biochemical and microscopy techniques. Although both techniques have been applied to study a wide variety of protein-protein interactions including receptor-receptor complexes, several caveats arise.

i) Coimmunoprecipitation (coIP)

Coimmunoprecipitation (coIP) was performed to detect interaction between p75-NGFR and TNFR1 in Chapter 2 (Fig. 2-5A). One caveat of coIP is that results of the assay are sometimes susceptible to position of the epitope tag in the protein. In this biochemical assay, protein samples were prepared by transiently transfecting HEK293 cells with two DNA constructs coding for p75-NGFR and TNFR1 extracellularly tagged with HA and FLAG, respectively. Each epitope tag is located at the N-terminus of the receptor, inserted between signal sequence and mature peptide sequence. This manipulation prevents disruption of the signal sequence, thus helps appropriate localization of the receptor in a cell. Interestingly, when p75-NGFR and TNFR1 were internally epitope-tagged at the C-termini, coIP was not successful. Although C-terminal fusions are beneficial for p75-NGFR and TNFR1 to avoid the risk of interfering ligand binding, it may influence intracellular structure of the receptors. Moreover, if interaction of p75-

NGFR and TNFR1 occurs in their intracellular domains, internal tags may be buried in the association, which may result in false negatives in coIP.

Another caveat of coIP is mislocalization of the proteins induced by the epitope tags or their ectopic overexpression. Although the constructs used in this thesis were designed such that the signal sequences would remain intact, it is possible that localization of HA-p75-NGFR and FLAG-TNFR1 in HEK293 cells is not the same as that of sympathetic neurons. To exclude this possibility, coIP of endogenous p75-NGFR and TNFR1 using sympathetic neurons will be needed. In addition, performing westernblot using protein samples from sympathetic neurons as well as to HEK293 cells expressing HA-p75-NGFR and TNFR1 will also allow us to compare levels of expression of p75-NGFR and TNFR1 in HEK293 cells to that of endogenous p75-NGFR and TNFR1 in sympathetic neurons. An additional caveat regarding coIP approach is that solubilizing membrane-associated proteins by detergent could cause unnatural conformations of p75-NGFR and TNFR1. Also, coIP does not exclude the possibility that p75-NGFR and TNFR1 are associated through a different protein, thus whether or not p75-NGFR and TNFR1directly interact cannot be determined only by coIP.

In order to identify which domains of p75-NGFR and TNFR1 are required for their interaction, it is necessary to generate deletion mutants of p75-NGFR and TNFR1 that lose the binding ability. CoIP using constructs of p75-NGFR and TNFR1 mutants carrying a deletion of their extracellular, transmembrane or intracellular domain should provide a clue on where the association domains of p75-NGFR and TNFR1 reside in the

proteins. If interaction of p75-NGFR and TNFR1 utilizes disulfide linkage through a conserved cysteine as previously observed in homodimer of p75-NGFR utilizing a specific transmembrane cysteine (C257), it may be interesting to find if extracellular or intracellular domains of p75-NGFR and TNFR1 possess conserved cysteines at similar locations. As shown in Chapter 3, probably p75-NGFR and TNFR1 do not interact through their transmembrane cysteines, since the potential cysteine in the transmembrane of TNFR1 is not conserved among species (Fig. 3-3).

To improve the experimental design of coIP used in this thesis, there are a few future directions. One future direction is to perform reciprocal immunoprecipitation (IP) where FLAG is immunoprecipitated and then HA is immunoblotted. This IP has been attempted, however, anti-FLAG IP protocol needs rigorous optimization by choosing an optimal antibody and incubation procedure for immunoprecipitation. If successfully performed, it will provide more validation on the interaction of p75-NGFR and TNFR1. Another direction to improve the current coIP is to reprobe the membrane with proteins anti-HA-immunoprecipitated. This will allow us to assess stoichiometry of the interaction between p75-NGFR and TNFR1. This was not pursued in this thesis to avoid detecting a robust 70 kDa signal of heavy chain of chicken IgY located near the 75kDa band of p75-NGFR. The anti-HA used in IP is a chicken IgY, and if reprobed using the same anti-HA, the heavy chain (70 kDa) will be detected too closely to p75-NGFR (75 kDa). Choosing a different anti-HA that has been produced in mouse or rabbit will solve this technical issue. An additional improvement for the coIP can be applied to transfection procedure. In the transfection performed in this thesis, the incubation time before media change was set to

2-3 hours and cells were incubated for 6-9 hours during transfection, both of which are shorter than that of the commercial protocol. Although these shorter time windows were intentionally selected to avoid cell death caused by transfecting two death receptors, they resulted in lower transfection efficiency than reported standards. To increase the transfection efficiency, it may be useful to add a caspase inhibitor during transfection, which will prevent cell death and allow a longer transfection period.

ii) Bimolecular fluorescence complementation (BiFC)

Bimolecular fluorescence complementation (BiFC) technique was utilized to visualize interaction of p75-NGFR and TNFR1 in Chapter 2 (Fig 2-5C). In this assay, distinct subcellular localization of homomeric oligomers of p75-NGFR and TNFR1 has been detected and the subcellular localization of p75-NGFR/TNFR1 is phenotypically similar to that of TNFR1. Although this microscopic approach has advantages in enabling detection of protein-protein interaction under a standard microscope setup without rigorous analysis procedure unlike Förster resonance energy transfer (FRET), a limitation arises. Complementation of N-terminal- and C-terminal fragments of a fluorophore, Venus in this thesis, is an irreversible step, resulting in accumulation of protein-protein association over time. Thus, fluorescent signal detected in BiFC technique does not reflect real-time dynamics of the interaction, which results in detecting disassociation that might have occurred after association as positive association. This may potentially affect our understanding of the interaction of p75-NGFR and TNFR1. The actual interaction between the two receptors might be less stable. One future possibility to study kinetics of the association of p75-NGFR and TNFR1 would be to utilize FRET technique.

Chapter 6

Materials and Methods

Animals

Sprague Dawley rats were purchased from Harlan. Sympathetic rat neurons were isolated from P0-P2 rat pups as previously described (Zareen and Green, 2009). All animals were maintained in a c57bl6/C3H hybrid background and littermate controls were used wherever possible. *Bax*$^{-/-}$, *TNFR1*$^{-/-}$, and *p75NGFR*$^{-/-}$ were bred and genotyped as previously described (Knudson *et al*., 1995; Peschon *et al*., 1998; Lee *et al*., 1992). All experiments were carried out in compliance with the Association for assessment of laboratory animal care policies and approved by the University of Virginia animal care and use committee.

Primary neuronal culture

Superior Cervical Ganglia (SCG) dissected from postnatal day 0-3 (P0-3) rats or mice as previously described (Suo *et al*., 2014) underwent enzymatic dissociation. After the ganglia were completely dissociated and grown in DMEM supplemented with 10% fetal bovine serum (FBS), 1U/ml penicillin/streptomycin (penstrep), and 50ng/ml NGF purified from mouse salivary glands. Neurons were plated on cover glass coated with poly-D-lysine (PDK) and laminin and hereafter incubated at 37˚C in 10% CO_2. After 24-36 hours, the neuronal culture was treated with 5μM cytosine β-D-arabinofuranoside (Ara-C, Sigma, Cat#C6645) to avoid glial proliferation. Media was changed every 48-60 hours.

HEK293 cell culture

Cells were kept in 37˚C in 5% CO_2. HEK293FT cells were cultured in DMEM containing 20% FBS and 1 U/ml penstrep (HEK media) and split at 1:20 to 1:10 dilution every 72-96 hours following a PBS wash and 0.05% trypsin-EDTA treatment. Cells were transfected using lipofectamine 2000 according to manufacturer's instructions.

Phylogenetic analysis and PCR primers

Amino acid sequences of all rat TNFR family members were analyzed on Phylogeny.fr. The platform performed sequence alignment by MUSCLE (v3.7), and reconstituted the phylogenetic tree using the maximam likelihood method on the PhyML (v3.0 aLRT). Then the platform performed the graphical representation and editing of the phylogenetic tree using TreeDyn (198.3) (Dereeper *et al*., 2008). PCR was performed using standard reactions with cDNA materials synthesized from mRNA isolated from P0-3 rat SCG. The following sequences are used as primers:

TNFRSF1A- F	ACCAAGTGCCACAAAGGAAC
TNFRSF1A- R	CTGGAAATGCGTCTCACTCA
TNFRSF1B- F	AAATGCAAGCACAGATGCAG
TNFRSF1B- R	CAGCAGACCCAGAGTTGTCA
LTBR- F	GAGCCCTAAACATGGCAGAG
LTBR- R	CTGCCCTTCTCACTGTCCTC

TNFRSF4- F	CTTGTACCTGCTCCGAAAGG
TNFRSF4- R	AGGATATGGGCTGTCTGTGC
CD40- R	TCTGAGCCCTGGAACTGTTT
CD40- F	TATTACTGCGGACCCCTGAC
FAS- F	ACCTGGTGACCCTGAATCTG
FAS- R	TGATACCAGCACTGGAGCAG
CD27- F	TGTGCAGCTCCGACTGTATC
CD27- R	GGCAGCTGTAAGGACAAAGC
TNFRSF8- F	TGCAGAGAAGTGGGTCAGTG
TNFRSF8- R	GTGGCTCTGGAGGTTCTCTG
TNFRSF9- F	CTGGTTCTCTGTGCCCAAAT
TNFRSF9- R	AGTGCTTCTCGGTTTCCTGA
TNFRSF10B- F	AAACCAGGCAGCTTTGAAGA
TNFRSF10B- R	AGCTGGGTTGTTTCCATTTG
TNFRSF11A- F	GCCAGCAAGAAGTGTGTGAA
TNFRSF11A- R	CCGGTCCGTGTACTCATCTT
TNFRSF11B- F	TGGGAATGAAGATCCTCCAG
TNFRSF11B- R	GAGGAAGGAAAGGGCCTATG
TNFRSF12A- F	CACTGATCCAGTGAGGAGCA
TNFRSF12A- R	CTCTCTGTCTGCCCCAGAAC
TNFRSF13B- F	GGCCGGATAACTTAGGAAGG

TNFRSF13B- R	TGGGAAGTGGCTCTCCTCTA
TNFRSF13C- F	GTGGGTCTGGTGAGTCTGGT
TNFRSF13C- R	TTGAATGGAGGCCAGTTAGG
TNFRSF14- F	CAGCTAGATCGGCCTACCAC
TNFRSF14- R	GCTGTTCCACAGCATGAGAA
NGFR-RT F	TTGCTTGCTGTTGGAATGAG
NGFR-RT R	AGCTCCTGGGGAGGAAAATA
TNFRSF17- F	ACTAAGAGCAGGGCTGGTGA
TNFRSF17- R	CTTGCCATAGTCACCCGTTT
TNFRSF18- F	CTGTGCCATGGGTACCTTCT
TNFRSF18- R	AAGCAGCCACACTAGGAGGA
TNFRSF19- R	TCAATCCCGAAAATGAAAGC
TNFRSF19- F	GTCCTTTGAGCATCCTGAGC
TNFRSF21- F	CTCGCGGTACCTTCTCTGAC
TNFRSF21- R	CGTGTGCTCAGGATGAGAAA
TNFRSF25- F	GTGCTGAGGACCTTCGTAGC
TNFRSF25- R	GCCCCTTCTGGTATTTCTCC
EDA2R- F	GGCCAACTGCACAAATACCT
EDA2R- R	TCCTACCAGTGCGACAAGTG

in vitro neuron death

Dissociated sympathetic neurons were isolated from P0-3 rats or mice and plated in mass

culture on poly-lysine and laminin. These neurons are established in 50g/ml NGF and Ara-C for 2 days. Cultures were then changed to the indicated conditions: anti-NGF (Millipore catalog#AB927, 1:1000), NGF (2ng/ml), BDNF (250ng/ml), TNFα (2ng/ml) for 36 hours followed by assessment of cell survival via Hoechst staining (Invitrogen, cat#H3569) as previously described (Suo *et al*., 2014).

in vivo SCG neuron count/soma size

For cell number and soma size assays trunks of P0 mice or P5 mice, respectively, were flash-frozen in OCT and then cryosectioned into 10μm thick sections. The slides were stained using the Nissl protocol and the number or size of cell bodies including distinct nucleoli was determined on every fifth section. Neuronal numbers were then determined by multiplying the counts by five. Cell size distribution was determined by categorizing the area of soma into small (less than 175μm²), medium (175~350μm²), large (more than 350μm²).

in vitro axon growth measurement

Sympathetic neurons were cultured in microfluidic device for 3 to 5 days in 50ng/ml NGF. Once the axons were long enough for measurement, then cellbodies and distal axons were switched to media containing 10ng/ml NGF and either 250ng/ml BDNF or 2ng/ml TNFα. An NGF-free medium with anti-NGF (Millipore catalog#AB927, 1:1000) was also used as control. A caspase inhibitor, BAF (boc-aspartate fluoromethylketone), was used at 20nM in all conditions to prevent cell death. The axons were then imaged

using phase-contrast microscopy, and reimaged after 24 hours. The axons were then measured using ImageJ, with at least 12 axons measured per condition.

Wholemount tyrosine hydroxylase staining

Wholemount organ staining was performed as previously described (Glebova and Ginty, 2004). Kidneys and hearts were harvested from P0 mice and fixed in 4% paraformaldehyde overnight. They were then dehydrated in methanol. Endogenous peroxidase activity was quenched using a solution of 20% DMSO/ 77% methanol/ 3% hydrogen peroxide. The organs were rehydrated and blocked in 4% BSA overnight, then incubated in sheep anti-TH (Chemicon cat #AB1542) at 1:500 for 3 days. The organs were washed with PBST and transferred to a 1:200 solution of anti-sheep HRP overnight. The organs were washed again and their color was developed in a solution of DAB with hydrogen peroxide. They were then fixed with 4% paraformaldehyde, dehydrated in methanol, and cleared in a 2:1 solution of benzyl benzoate and benzyl alcohol.

Quantification of axonal pattering in kidney and heart

To examine the degree of axonal tiling/defasciculation in kidney 3 parallel lines were placed on an imaged kidney such that the middle line overlies the major axis of the oval kidney shape (Fig. 2-8). The number of axons crossing each line was assessed such that the more cross-overs observed represents the extent of tiling. For heart innervation we assessed the number of crossovers an individual axon encounters. These results were normalized according to the individual length of the axon. Hearts and kidneys from at least 5 animals per genotype were examined and 3 organs per condition were used for

quantification.

Construction of plasmids

HA-p75-NGFR and FLAG-TNFR1: The full-length coding sequence of wild type rat p75-NGFR (Gift from J. Tuttle) and TNFR1 (Obtained from the mammalian gene collection) were expressed from the pcDNA 3.1 backbone and pExpress1 backbone, respectively. N-terminal hemagglutinin (HA) epitope and triple repeats of FLAG epitope was inserted between signal sequence and mature peptide sequence of p75-NGFR and TNFR1, respectively. p75-VN, p75-VC, TNFR1-VN, TNFR-VC, TrkA-VN, TrkA-VC: The full length coding sequence of wild type rat p75-NGFR and TNFR1 were expressed from the pTREhyg backbone. Triple repeats of the FLAG epitope were inserted between the signal sequence and mature peptide sequence of p75-NGFR, TNFR1 and TrkA. In the C-terminus of p75-NGFR, TNFR1 and TrkA, Venus N - or C-terminal fragments were inserted.

Immunoblot analysis

HEK293 cells were transfected with the constructs of HA-p75-NGFR and/or FLAG-TNFR1 using Lipofectamine2000 (Invitrogen) overnight and then lysed in ice-cold lysis buffer containing 1% Triton X-100 (Sigma, cat#X100), 60mM Octyl β-D-glucopyranoside (Sigma, cat#O8001) and protease inhibitors (Sigma, cat#P8340). For immunoprecipitation, 100μg of lysed sample was incubated with agarose-conjugated chicken anti-HA (Aves Lab, cat#PHA-1010) overnight at 4˚C. After electrophoresis, gels were blotted to PVDF membranes. The blots were immunostained with anti-FLAG

(Sigma, cat#F1804), anti-HA (Aves Lab, cat#ET-HA100), and anti-Tubulin overnight at 4˚C, and then imaged using Odyssey Infrared system (Licor). For input controls, 10μg of lysed sample was loaded in each lane.

BiFC visualization

BiFC was performed as previously described (Hu and Kerppola, 2003). HEK293 cells were transfected with the BiFC constructs using Lipofectamine2000 (Invitrogen) for 8-10 hours and then imaged using fluorescence microscopy (514 nm excitation).

PSD immunocytochemistry, p75-NGFR immunocytochemistry

The different steps needed for PSD- and p75-NGFR immunohistochemistry are shown in a) and b) respectively. DIV 12~16 rat- or DIV 8-12 mouse neurons are fixed in a) 100% ethanol at -20 ˚C, or b) 4% PFA at room temperature for 15 minutes, followed by 2 washes in PBS for 5 minutes/wash. Then the sample is blocked in a) 0.2 % gelatin, 0.075 % Triton-X in PBS on ice, or b) 5 % goat serum, 0.05 % Triton-X in PBS at room temperature for 30-120 minutes, and incubated over night at 4˚C in blocking solution with primary antibodies of a) Maguk (Membrane-associated guanylate kinase) and MAP2 (Microtuble-associated protein 2), or b) p75, TNFR1 and MAP2. Following 2 washes in PBS, the sample is incubated in blocking solution with proper secondary Alexa Fluor fluorescents for 60 minutes at room temperature. Then after 2 PBS washes, the sample is mounted on a slide glass for confocal microscopy.

Appendix 1:

Validating gene expression in *p75-NGFR*-null and *TNFR1*-null neurons

The observations from Chapter 2 that axon growth in *p75-NGFR*-null neurons is not inhibited in the presence of TNFα *in vitro* led me to ask whether *TNFR1* is actually expressed in the *p75-NGFR*-null neurons. Likewise, the observation that axon growth in *TNFR1*-null neurons is not inhibited in the presence of BDNF *in vitro* led me to ask whether *p75-NGFR* is expressed in the *TNFR1*-null neurons. Toward this end, I examined gene expression of *p75-NGFR*, *TNFR1*, and other pro-death genes of interest using the SCG neurons collected from *p75-NGFR*-null- and *TNFR1*-null mice at P0 via RT-PCR. The data display *p75-NGFR* expression in *TNFR1*-null neurons (Fig. A-1A). *p75-NGFR*-null neurons also showed *p75-NGFR* expression due to the retained nucleotide sequence *p75-NGFR* in our mutant mice, which was originally generated by inserting NEO to exon 3, leading to a frame shift but not deletion (Lee et al., 1992). Thus, the transcript seen in this lane should be nonfunctional. On the other hand, *TNFR1* expression is present only in wild type and *p75-NGFR*-neurons. This suggests that *p75-NGFR* expression is not eliminated by ablation of TNFR1.

To confirm the *p75-NGFR* transcript in *p75-NGFR*-null neurons is nonfunctional, I examined expression of p75-NGFR at protein level. The results show that the protein expression of p75-NGFR in NGF-deprived sympathetic neurons is absent in *p75-NGFR*-/-*;TNFR1*-/- (DKO) neurons (Fig. A-1B). p75-NGFR shows smear in lanes for wild type and *TNFR1*-null, probably due to the p75-NGFR cleavage induced by NGF withdrawal.

Since DKO animal line was generated by crossing *p75-NGFR*-/- line and *TNFR1*-/- line, the *p75-NGFR*-/- mouse line is now proven to lack functional p75-NGFR protein. This suggests that the abnormal transcript observed in the RTPCR is nonfunctional in the *p75-NGFR*-null mice. Taken together, TNFR1 is expressed in the *p75-NGFR*-null neurons and p75-NGFR is expressed in the *TNFR1*-null neurons.

Interestingly, mRNA expression levels of death receptors and their ligands are altered in *p75-NGFR*-null- and *TNFR1*-null neurons (Fig. A-1A). Genes including *TNFR1*, *TNF* and *ADAM17* are upregulated in *p75-NGFR*-null neurons whereas genes including *p75-NGFR* and *BDNF* are upregulated in *TNFR1*-null neurons. These data suggest that TNF-TNFR1 signaling is enhanced in *p75-NGFR*-null neurons while BDNF-p75-NGFR signaling is enhanced in *TNFR1*-null neurons. Moreover, TNF-sensitivity is probably increased in *p75-NGFR*-null neurons whereas BDNF-sensitivity is increased in *TNFR1*-null neurons. Thus, the two distinct mutant neurons have strengthened the other receptor's signaling and ligand sensitivity. This might be another explanation for the significant cell death observed in *p75-NGFR*-null neurons when cultured with NGF and TNFα (Fig. 2-4B). Perhaps, the death may have been caused due to the enhanced TNF-TNFR1 signaling and TNF-sensitivity in these neurons, rather than the nature of TNFR1 which was concluded to be p75-NGFR-independent to cause cell death. Likewise, the enhanced signaling may be the reason for more significant cell death of both *p75-NGFR*-null- and *TNFR1*-neurons in the anti-NGF condition, compared to that of wild type neurons. In comparison, enhanced BDNF-p75-NGFR signaling and BDNF-sensitivity in *TNFR1*-null neurons do not seem to influence the codependence model, as p75-NGFR

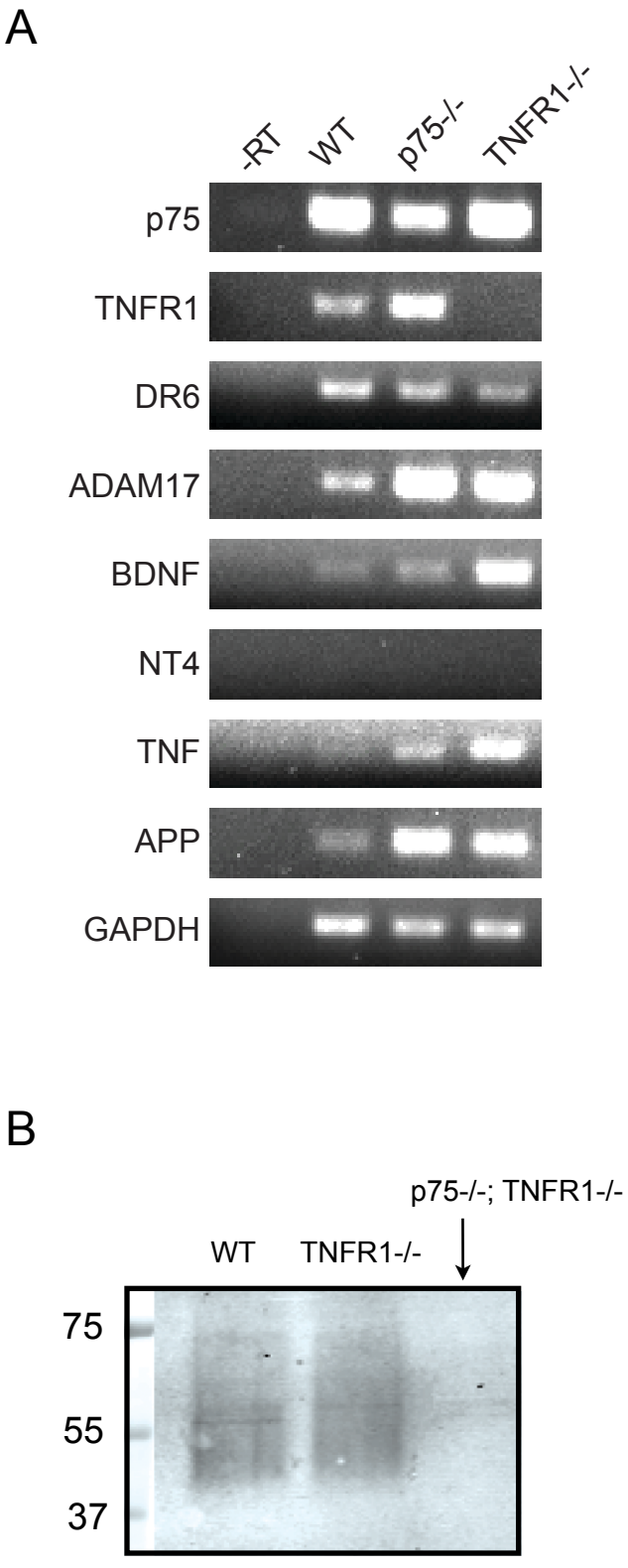
has been still incapable of cause significant cell death or axon growth inhibition in *TNFR1*-null neurons. In order to accurately understand the endogenously enhanced signaling in these mutant neurons, a future experiment will be to repeat the same *in vitro* assays with anti-TNFα or anti-BDNF added in the suboptimal NGF condition to remove TNFα or BDNF in media. If the increased expression of *TNF* and *BDNF* leads to an excess and toxic level of secretion of TNFα or BDNF in *p75-NGFR*-null- and *TNFR1*-neurons, respectively, neurons cultured in the suboptimal NGF with anti-TNFα or anti-BDNF will show higher survival and axon growth relative to that of the suboptimal NGF only.

Figure A-1. RT-PCR *of p75-NGFR*, *TNFR1*, and other selected genes using *p75-NGFR*-null- and *TNFR1*-null neurons.

(A) RT-PCR of p75-NGFR, TNFR1 and other selected genes using *p75-NGFR*-null- and *TNFR1*-null neurons.

(B) p75-NGFR Westernblotting of NGF-deprived SCG neurons from *TNFR1*-/- and *p75-NGFR*-/-;*TNFR1*-/- animals.

Figure A-1


A
-RT
WT
p75-/-
TNFR1-/-
p75
TNFR1
DR6
ADAM17
BDNF
NT4
TNF
APP
GAPDH
B
p75-/-; TNFR1-/-
WT
TNFR1-/-
75
55
37

Appendix 2:

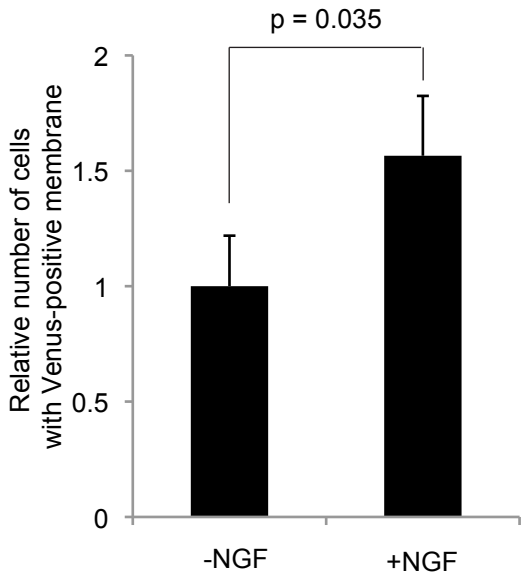
Analysis on BiFC controls using homomeric pair of TrkA

In order to provide more validation on the BiFC assay used in Chapter 2, quantification of controls using homomeric pair of TrkA was performed. HEK cells expressing homomeric pair of TrkA (TrkA-VN, TrkA-VC) were exposed to media containing 0 or 50 ng/ml of NGF prior to imaging as negative and positive controls, respectively. 25 randomly selected fields were imaged and the number of Venus-positive plasma membranes was examined. The cells exposed to 50 ng/ml NGF showed higher number of Venus-positive plasma membranes (Fig. A-2). Since the BiFC constructs of TrkA, p75-NGFR and TNFR1 have been designed similarly, this results provide more validation for the BiFC technique used in this thesis. To aid complete validation, a negative control that is a membrane protein and does not bind to p75-NGFR or TNFR1 will be required.

Figure A-2. Quantification of BiFC controls using homomeric pair of TrkA.

HEK cells expressing TrkA-VN and TrkA-VC were exposed to media containing 0 or 50 ng/ml of NGF for 15 minutes prior to imaging. 25 fields were randomly selected and imaged. The number of Venus-positive plasma membranes was examined and the results were normalized to the negative control where cells were exposed to media containing no NGF.

Figure A-2


p = 0.035
Relative number of cells with Venus-positive membrane
2
1.5
1
0.5
0
-NGF
+NGF

Appendix 3: Roles of p75-NGFR- and TNFR1 ligands *in vivo*

We examined roles of p75-NGFR and TNFR1 ligands *in vivo*. We again used the *Bax*$^{-/-}$ paradigm to assess competition induced soma atrophy without the byproduct of cell death. *TNF*$^{-/-}$; *Bax*$^{-/-}$ animals were generated and analyzed as previously in Figure 2-7 (Fig. A-3A). Interestingly *TNF*$^{-/-}$; *Bax*$^{-/-}$ mice exhibits size distribution similar to wild-type, suggesting that like the receptor knockouts, loss of TNF is also capable of rescuing the *Bax*$^{-/-}$ neuron size distribution phenotype (Fig. A-3B). We did not examine cell size using p75-NGFR ligand knockout animals, i.e., *BDNF*$^{-/-}$; *NT4*$^{-/-}$; *Bax*$^{-/-}$ mice, since they must be bred as triple heterozygous yielding low triple null frequency (1/64). Taken together with data from receptor knockout experiments, these findings are consistent with a model whereby p75-NGFR and TNFR1 are codependent in the context of regulating soma size and that this codependent function may also require ligand engagement.

Next, we examined the role of p75-NGFR and TNFR1 ligands in sympathetic target innervation. In contrast to TNFR1, p75-NGFR is highly promiscuous in its ligand binding preferences. We chose to focus on the mature neurotrophins, BDNF and NT4 rather than NGF and NT3 since the later two preferentially participate in TrkA-dependent trophic signaling. We examined sympathetic axon innervation of kidney and heart from P0 *BDNF*$^{-/-}$; *NT4*$^{-/-}$mice as well as *TNF*$^{-/-}$ mice as previously in Figure 2-9 (Fig. A-3C). Kidneys from both *BDNF*$^{-/-}$; *NT4*$^{-/-}$ mice and *TNF*$^{-/-}$ mice display normal axon tiling (Fig. A-3D). These data suggest that the receptor co-dependence of p75-NGFR and TNFR1

that we observe for tiling in the kidney in Figure 2-9 occurs either in a ligand-independent manner or in the presence of other ligand partners. As is the case for kidney innervation, *BDNF*$^{-/-}$; *NT4*$^{-/-}$ and *TNF*$^{-/-}$ hearts appear to be patterned normally suggesting a different set of ligands or a ligand-independent process (Fig. A-3E).

We next sought to address the apparent discrepancy between our *in vivo* versus *in vitro* cell death paradigms by asking whether ligands for p75-NGFR are used to expedite neuronal death as we and others have previously suggested (Deppmann et al., 2008; Bamji et al., 1998**)**. Toward this end, the neuron number in SCGs from P0 *BDNF*$^{-/-}$; *NT4*$^{-/-}$ as well as *TNF*$^{-/-}$ mice was examined. Interestingly, we did not observe any difference in neuron number between wild type and *BDNF*$^{-/-}$; *NT4*$^{-/-}$ mice (Fig. A-3F), indicating that the neurotrophin p75-NGFR ligands are not necessary to expedite developmental death. Surprisingly, *TNF*$^{-/-}$ mice phenocopy the *p75-NGFR*$^{-/-}$ phenotype, in which neuron number is increased by approximately 25% at P0, suggesting that TNF is required to cause developmental cell death *in vivo*. Taken together with our finding that *TNFR1*$^{-/-}$ mice do not phenocopy the *p75-NGFR*$^{-/-}$ cell death phenotype, these data suggest that TNF does not act through TNFR1 in promoting developmental sympathetic neuron death. Furthermore, our observation that TNFα promotes cell death in *p75-NGFR*$^{-/-}$ neurons *in vitro* eliminates the possibility that TNFα may work directly through p75-NGFR (Fig. 2-4B). How TNFα transduces its signaling remains unclear and will be attractive for ongoing inquiry.

Figure A-3. Influence by BDNF, NT4 and TNF in sympathetic soma size distribution, target innervation, and neuronal number *in vivo*.

(A) TNF is required to normal cell body size distribution *in vivo*. After Nissl-stained, the size of cell bodies of neurons on 10 μm mouse SCG sections were analyzed using central profiling. Blue arrow: large soma (>350 μm²), Red arrow: medium soma (175-350 μm²), Green arrow: small soma (<175 μm²). The control ($Bax^{-/-}$) was adapted from Figure 2-7A.

(B) Quantification of (A). The control ($Bax^{-/-}$) was adapted from Figure 2-7B.

(C) BDNF, NT4 and TNF are required for sympathetic innervation at kidneys and hearts at P0. Whole-mount tyrosine hydroxylase immunostaining of p0 mouse kidneys taken from Wild-type (WT), $BDNF^{-/-}$;$NT4^{-/-}$, and $TNF^{-/-}$. Each experiment was performed at least 3 times (n=3). The control (WT) was adapted from Figure 2-9A.

(D) Quantification of axon bundle tiling on kidneys shown in (C). At least 3 kidneys per genotype were analyzed. p values were determined with one-way ANOVA. The control (WT) was adapted from Figure 2-9B.

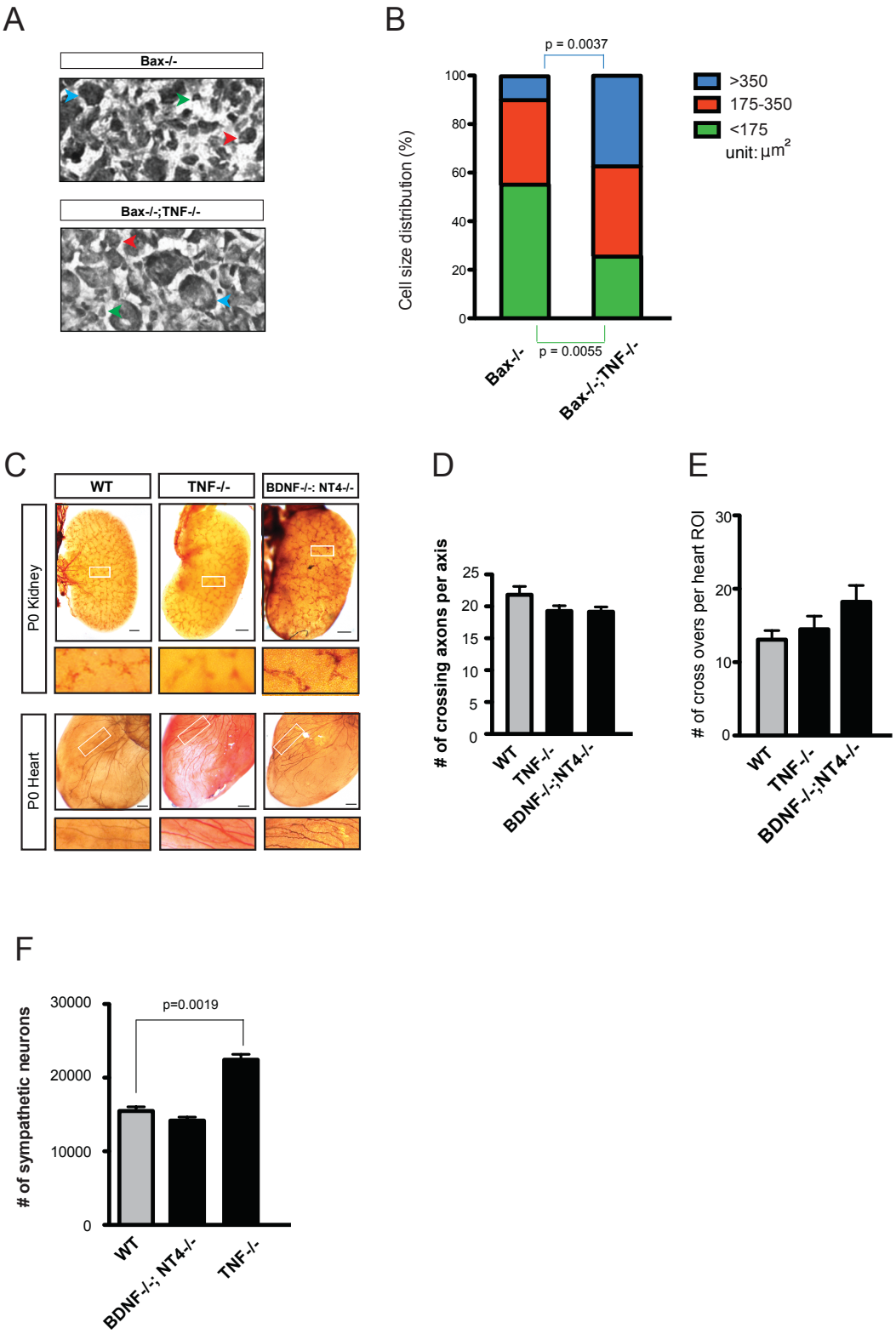
(E) Quantification of axonal crossovers on hearts shown in (C). At least 3 hearts per genotype were analyzed. p values were determined with one-way ANOVA. The control (WT) was adapted from Figure 2-9D.

(F) TNF is required in developmental cell death *in vivo*. The neuronal number was counted using central profiling on 10 μm mouse SCG sections after Nissl-stained. WT:n=6, $BDNF^{-/-}$;$NT4^{-/-}$:n=3, $TNF^{-/-}$:n=3. The control (WT) was adapted from Figure 2-10A.

Contributions. A, B: Original pictures taken and cell size measured by Catherine Jansch.

C: Original pictures taken by Danielle Heffner. F: Cells counted by Catherine Jansch.

Figure A-3


A
Bax-/-
Bax-/-;TNF-/-
B
p = 0.0037
Cell size distribution (%)
100
80
60
40
20
0
>350
175-350
<175
unit: μm2
p = 0.0055
Bax-/-
Bax-/-;TNF-/-
C
WT
TNF-/-
BDNF-/-: NT4-/-
P0 Kidney
P0 Heart
D
# of crossing axons per axis
25
20
15
10
5
0
WT
TNF-/-
BDNF-/-;NT4-/-
E
# of cross overs per heart ROI
30
20
10
0
WT
TNF-/-
BDNF-/-;NT4-/-
F
# of sympathetic neurons
30000
20000
10000
0
p=0.0019
WT
BDNF-/-; NT4-/-
TNF-/-

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