**The role of Coronin-1 in neurotrophin signaling during
sympathetic nervous system development**

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Abstract

Long-distance signaling is a property inherent to neurons and neural circuits. Communication between axonal targets and neuronal cell bodies is increasingly recognized as critical for developmental processes and for normal function in adulthood. How this retrograde long-distance signal maintains high fidelity as it traffics to the cell body remains unknown, but could be achieved by the enhanced signal durations observed in some growth factor signaling. I found that the retrograde Nerve growth factor (NGF)-TrkA signaling endosome recruits a novel effector protein known as Coronin-1, which protects the endosome from lysosomal degradation during development. Indeed, in the absence of Coronin-1, the NGF-TrkA signaling endosome fuses to lysosomes 6-10-fold faster than in wild-type neurons. Furthermore, loss of Coronin-1 affects several NGF-dependent processes including neuron survival. These phenotypes are consistent with the finding that Coronin-1 stabilizes the NGF-TrkA signaling endosome, providing a plausible mechanism for long-distance retrograde signaling. Further, I demonstrated that Coronin-1 protects the signaling endosome by facilitating NGF-dependent calcium release and subsequent calcineurin activation. This novel mechanism for NGF-dependent calcium release provides insight into the mechanistic details underlying NGF-dependent transcription, axon growth, and cell survival. Above all, my findings argue for a critical role for Coronin-1 in sympathetic nervous system development.

In addition, to investigate the process by which neurotrophins direct neuronal survival, I examined another key developmental process mediated by neurotrophic factors: the molecular mechanisms governing how axons traverse distinct axon growth environments/niches. The neurotrophins, NT3 and NGF are derived, respectively, from intermediate targets such as blood vessels and final targets such as heart. Both NT3 and NGF are critical for proper development of the sympathetic nervous system, and interestingly both signal through the TrkA receptor tyrosine kinase. Given that both NT3 and NGF promote axon growth, the question remains: how do sympathetic axons switch preference from intermediate to final target fields in order to form an integrated circuit? One might argue that since NGF-TrkA, but not NT3-TrkA, undergoes retrograde signaling to induce transcriptional programs, the mechanistic switch may be linked to NGF-dependent transcription. Coronin-1 represents an NGF-induced gene that may mediate this transition. Using *in vivo* and *in vitro* axon growth assays I demonstrate opposing roles of Coronin-1 in NGF versus NT3 mediated signaling and axon extension. This provides critical insight into the mechanisms underlying the transition of axons from intermediate to final targets during development.

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Abbreviations

BDNF: brain-derived neurotrophic factor

CCV: clathrin-coated vesicle

CNS: central nervous system

CREB: cAMP response element-binding protein

DRG: dorsal root ganglia

EGF: epidermal growth factor

NGF: nerve growth factor

NT3: neurotrophin-3

NT4/5: neurotrophin-4/5

PBS: Phosphate buffered saline

PNS: peripheral nervous system

PI3K: phosphatidylinositol 3-kinase

PLC- γ : phospholipase C-gamma

SCG: super cervical ganglia

Trk: tyrosine kinase

TrkA: high affinity tropomyosin kinase A

WT: wild type

Chapter I: Introduction

Proper neural circuit formation during development is critical for animal survival. In this thesis, I investigate two key events, cell death and target innervation, which are necessary for correct neural circuit formation. Using the sympathetic nervous system as a simple platform to research circuit formation, previous research revealed that target derived growth factors drive the formation of the peripheral nervous system. For instance, growth factors undergo long-distance retrograde transport from target organs (e.g. eye, skin, muscle) to neuron soma, in addition to influencing signaling locally at axon tips. As neurons innervate their final target, they compete for limiting amounts of growth factors in order to survive and form appropriate synapses with targets. However, the detailed mechanisms underlying these processes are still unknown. Therefore, the goal of my research is to answer two main questions: 1) How does target derived growth factor maintain high-fidelity transfer of information during long distance retrograde signaling? 2) How does target derived growth factor determine proper target innervation? Herein, I will introduce the relevant background on the nervous system and current growth factor-based models. In the end, I will explain how Coroinin-1 functions as a novel signaling endosome stabilizing factor required for proper final target innervation and neuron survival during development.

Development and divisions of the nervous system

Despite the existence of species differences in the final format of the mammalian nervous system, the early developmental stages across species are remarkably conserved and therefore similar. All start with a single fertilized egg cell, which divides and differentiates into three distinct layers: the endoderm, mesoderm and ectoderm. On day 14, after these three layers have formed, the ectoderm further differentiates to form the neural plate, a disc-like structure that serves as the starting material for the nervous system. The ends of the neural plate begin to fold toward each other forming the neural groove, which by day 14 has deepened and at around day 27, has completely folded over and merged together at the ends to form a tubular structure, the neural tube (Figure 1). As this structure continues to grow and differentiate, the frontal portion develops into what will eventually become the brain and the rostral portion, into what will become the spinal cord. Once fully developed the brain will have differentiated into three parts: the forebrain, midbrain and hindbrain (Sanes DH, Reh TH, 2011).

Figure 1 Formation of neural tube from neural plate

During formation, neural plate folds to form neural groove. The dorsal part closes to form the neural tube

The mammalian nervous system is segregated into the central (CNS) and peripheral (PNS) nervous systems. The CNS is comprised of neurons in the brain and spinal cord, with the brain receiving and interpreting enormous quantities of external (sight, sound, taste and smell), and internal neural inputs (chemosensors, stretch, balance and homeostasis sensors). The brain is composed of four main parts: the cerebrum, cerebellum, brain stem, and meninges, and the cerebrum can be further divided into frontal, temporal,

occipital, and parietal lobes. The spinal cord acts as a bridge between the brain and other parts of the body. Its role is to transport sensory signals from the PNS to the brain, and motor signals from the brain to muscles and glands. Additionally, the spinal cord provides a center for simple reflexes, which do not necessarily require central integration and involvement of higher regions in the brain (Kandel, 2000) (Figure 2).

The nerves and ganglia that exist outside of the brain and spinal cord comprise the PNS, and play an important role in connecting the CNS with peripheral organs, such as muscle, blood vessels and glands (Figure 2). Unlike the CNS, which is separated from the peripheral circulation by the blood-brain barrier, the PNS is readily accessed by blood born elements, both intrinsic (immune cells) and extrinsic (pathogens). Two main subsystems of the PNS include the somatic nervous system and autonomic nervous system. The somatic nervous system contains nerve fibers conducting impulses from the CNS, which controls body movements with conscious control. On the other hand, the autonomic nervous system innervates and controls organs (such as smooth muscle, cardiac muscle and glands) without conscious control, using the simple reflex arc mentioned above. Within the autonomic nervous system, there are the sympathetic and parasympathetic systems and the enteric nervous system (Burnstock, 1981).

As a subdivision of the autonomic nervous system, the sympathetic nervous system contains two main neuron types - preganglionic neurons are primarily located in the grey matter of the thoracolumbar region of spinal cord, while postganglionic neuron synapse on to effector organs to control endocrine and exocrine function. The superior cervical ganglion (SCG) provides sympathetic innervation to targets in the head and neck. Its target organs include eye, pineal gland, carotid body, blood vessels of the skin, submaxillary gland, heart, trachea, to name a few. It is critical for maintaining homeostasis and is responsible for the fight-or-flight response, the physiological reaction to the environmental threats (Jansen et al., 1995). These response include: pupil dilation in the eye, increased renin release in the kidney, increased rate and force of cardiac contraction, blood vessel dilation in skeletal muscle and so on (Dee Unglaub Silverthorn, 2009). As one of the three largest ganglion (middle, inferior, super) in the sympathetic chain, SCG is frequently used as a simple platform for cell survival research, which is useful because it contains a relatively homogeneous population of sympathetic neurons (Li and Horn, 2006).

Figure 2 Diagram showing the subdivisions of the nervous system

Competition for neuronal survival

During the development of nervous system, neurons have to compete with each other for survival and to form a proper nervous circuit. How does the system decide which neuron population to eliminate? Early experiments by Levi-Montalcini demonstrated that the survival of motor neurons innervating a particular target could be influenced by manipulating that target (muscle in their particular study). For instance, if extra tissue was explanted, motor neuron number increased, whereas a decreased motor neuron number was observed if original tissue was removed. However, Levi-Montalcini's interpretation was that the limb bud released a diffusible factor promoting axon growth (Hamburger and Levi-Montalcini, 1949; Levi-Montalcini and Hamburger, 1951). Next, Stanley Cohen, working with Levi-Montalcini to purify a cell free solution containing a

diffusible factor from mouse sarcomas. Application of this purified diffusible tumor factor to sympathetic and sensory neurons resulted in enhanced neurite growth *in vitro* (Levi-Montalcini and Booker, 1960). Therefore, this important fate determining factor, nerve growth factor (NGF), was identified as being synthesized by target organs to initiate cell death related to local and cell body mechanisms (Levi-Montalcini, 1987) and has subsequently been shown to be one of a family of structurally related proteins known as neurotrophins. NGF seems to provide the dominant control in early developmental stages, because all neurons of the developing sympathetic nervous system depend on NGF for survival. Interestingly, function blocking antibodies toward NGF dramatically decreased sympathetic and sensory neuron survival (Levi-Montalcini and Booker, 1960) and deprivation of neurotrophin results in generation of a stereotypic apoptotic signaling pathway that causes cell death, via mechanisms that include translocation of Bax and activation of cytochrome C and caspase signaling (Klein, 1994). Moreover, in the absence of NGF *in vivo* sympathetic neurons were completely lost (Crowley et al., 1994). These works culminated in the neurotrophic hypothesis which suggested that the target tissue or organ secretes limited amounts of neurotrophic factors that bind receptors on the axon terminals and influence neuronal survival (Levi-Montalcini, 1987). An overabundance of neurons is initially produced in both the CNS and PNS, but approximately half of the neurons generated are ultimately eliminated by programmed cell death (Oppenheim, 1991).

Why evolve a mechanism for developing the nervous system that is so inefficient?

One possible explanation is that this serves as an adaptation to complex body plans in which some neurons are used as pioneers. Another possible explanation is that this is a quality control process. For example the neuron axon innervation number is defined by the target organ size. Although the neurotrophic hypothesis, in which neurons getting more retrograde neurotrophin are able to survive, explains the process of competition survival, how neurotrophin maintains high signal fidelity during a long distance retrograde transport still needs to be explored.

Target innervation

As the nervous system develops, neurons extend their axons for long distances to reach their targets to eventually establish complex neuronal circuits. As nascent axons emerge, they first encounter intermediate targets on their journey toward their peripheral final targets. In the PNS these intermediate targets are often blood vessels. For example, the SCG is the most cranial of the sympathetic ganglia, and sits between the internal and external carotid arteries. Axons projecting along the external carotid arteries innervate the sublingual and parotid glands while projections growing along the internal carotid arteries innervate the eye, blood vessels, lacrimal gland, pineal gland, skin of head, oral mucosa and nasal cavity mucosa (Makita et al., 2008). Neural crest cells aggregate into the sympathetic chain around embryonic day 10.5, in mice. NGF plays an important role in sympathetic final target innervation, and the amount of NGF received by

rat sympathetic neurons has been shown to correlate with the levels of target innervation in organs like submandibular gland, heart atrium and iris (Korsching and Thoenen, 1983).

Axonal path finding relies on an interplay between attractive and inhibitory guidance cues (Tessier-Lavigne and Goodman, 1996) which are detected by the active growing structure located in the tip of the axon (Cajal, 1890), known as the growth cone. The growth cone is made of dynamic cytoskeletal machinery receiving input from internal and external sources to guide the extension of the cytoskeleton and lead to persistent protrusion and growth of the axon. There are three distinct zones in the growth cone, which are referred to as the central core, lamellipodia and filopodia. In response to guidance cues such as calcium, cAMP and netrins, the growth cone can turn, retract or move forward, and these cues in turn interact with other cytoskeletal regulators such as the RhoGTPases which are critically important in the regulation of growth cone actin dynamics (Huber et al., 2003). Target innervation involves not only axon outgrowth, but also branching, defasciculation, and penetration into the final target (Tessier-Lavigne and Goodman, 1996). Despite a significant amount of valuable research in this field, it remains unclear how axons make the transition from intermediate to final target organs.

Neurotrophins

Neurotrophins and their receptor family members

Neurotrophic factors are a family of proteins involved widely in neural development and survival. Most neurotrophic factors can be classified into three main groups: neurotrophins, glial cell-line derived neurotrophic factor family ligands (GFLs), and neuropoietic cytokines. Neurotrophins are well known as a family of low molecular weight, target-secreted proteins that are structurally related to NGF and are critical for neuron differentiation, function and survival (Deister and Schmidt, 2006). Interestingly, animals lacking neurotrophins or their receptor display marked loss of neurons in the PNS, but not in the CNS (Lewin and Barde, 1996). This indicates that neurotrophins play a more important role in the PNS for neuron survival. The four neurotrophin family members share 50% sequence similarity and are known as NGF, Brain derived growth factor (BDNF), Neutrophin-3(NT3) and Neutrophin-4(NT4) (Lindsay et al., 1994). Neurotrophins have a variety of functions, including regulating cell death, differentiation, synapse, neurotransmitter release and long-term potentiation (Chao, 2003), but they are perhaps best known for their role in cell death and target innervation. A hallmark of vertebrate nervous system development is that excess neurons are generated during embryogenesis, and pruned and refined as the system develops. In the PNS, a proportion of the neurons die after target innervation. Moreover, neurotrophins such as NGF and NT3 are indispensable for neuronal target matching in the sympathetic system (Francis et al., 1999; Glebova and Ginty, 2004a). In addition, neurotrophins have a high affinity receptor group called

neurotrophic tyrosine kinase family, including TrkA, TrkB and TrkC (Figure 3). Although much research has been done, we are still defining the breadth of a process, which these factors control, and how they work together to coordinate nervous system development and function.

Figure 3 Neurotrophins and their high affinity receptor

Top level lists ligand including NGF, BDNF, NT4/5 and NT3. Bottom level lists receptor including TrkA, TrkB and TrkC. Solid arrow means high affinity binding. Dash arrow means low affinity binding.

NGF

Nerve growth factor (NGF) was found by Rita Levi-Montalcini and Stanley Cohen in 1950s. By transplanting tumor tissue to a chicken embryo, they observed a hyper-innervation of axons, suggesting that there was a diffusible factor

produced by the tumor that stimulated nerve growth (Levi-Montalcini and Hamburger, 1951). An extract of the tumor tissue stimulated axon growth in *in vitro* assays and Levi-Montalcini and Cohen subsequently purified the factor and named it NGF. NGF is particularly important for cell survival, axon branching and extension in sympathetic and sensory neurons. NGF is mainly secreted by target tissue, but after nerve injury Schwann cells and fibroblasts can locally synthesize NGF. NGF was found in limb or organs to support sympathetic neurons and some sensory neurons (Lindsay et al., 1994). NGF has also been found in hippocampus and neocortex where it supports basal forebrain cholinergic neurons (Large et al., 1986), the principal neuron type affected in Alzheimer's Disease. NGF-TrkA signaling is crucial for the survival and growth of sympathetic neurons as well as small-diameter nociceptive sensory neurons (Levi-Montalcini and Booker, 1960; Smeyne et al., 1994). Mice lacking NGF (NGF^{-/-}) showed marked depletion of all sympathetic and nociceptive sensory neurons. However NGF was not essential for survival of all PNS sensory neurons, mainly for nociceptive neurons (e.g. 40% in DRG need NGF) (Lewin and Barde, 1996a) . Differential affinity between ligands is observed at the high affinity tropomyosin kinase A (TrkA) receptor. TrkA is a member of the Receptor protein tyrosine kinases (RPTK) family, and NGF binds exclusively to TrkA, which is expressed primarily in the PNS and defined regions of the CNS, as evidenced by the loss of trigeminal and dorsal root ganglia neurons in TrkA^{-/-} mice (Barbacid, 1994). Because all nociceptive neurons in DRG and trigeminal ganglia express TrkA at some point in their development, these neurons are lost in TrkA or NGF knockout

mice (Crowley et al., 1994; Smeyne et al., 1994). In addition, cholinergic neuronal projections, not neural survival, decreased in the forebrain of these knockouts (forebrain is a known final target secreting NGF to the hippocampus and cortex (CNS)) (Smeyne et al., 1994).

BDNF

Brain-derived neurotrophic factor (BDNF) primarily regulates neuron development in the CNS, such as hippocampus, cortex and basal forebrain, having a critical role in synaptic plasticity and axon guidance (Cellerino et al., 1997; Cohen-Cory et al., 2010). Unlike NGF, BDNF is not absolutely necessary for competitive survival, but may act as an autocrine survival factor during target independent survival, given the fact that BDNF and its receptors were found in some population of neurons themselves (Barde et al., 1982; Davies and Wright, 1995). However, BDNF could promote growth, differentiation, polarization, long-term memory and is involved in depression (Cohen-Cory et al., 2010; Martinowich et al., 2007). For instance, *in vitro* experiments in hippocampal neurons showed that autocrine secreted BDNF resulted in local cAMP/protein kinase A activity promoting axon differentiation and growth (Cheng et al., 2011). In BDNF^{-/-} mice, neurons innervating the pineal gland failed to stop at the target (Kohn et al., 1999). BDNF binds exclusively and with high affinity to TrkB, which is expressed in both CNS and PNS. TrkB^{-/-} mice exhibit severe neuron loss in trigeminal, nodose and petrosal sensory ganglia (Klein et al., 1994). In some instances BDNF is required for TrkB⁺ neuron survival in the PNS. This is in

contrast to the absolute requirement of NGF and NT3 for TrkA⁺ and TrkC⁺ neurons in the PNS, respectively.

NT3

Like NGF, Neurotrophin-3 (NT3) influences the PNS (Ernfors et al., 1990), supporting axon growth, branching, synapse formation, as well as sensory and sympathetic neuron survival and differentiation. In the sympathetic system, NT3 appears to be important for intermediate target innervation, whereas NGF is important for specifying final target innervation. NT3 is secreted by intermediate targets, such as the vasculature (Figure 4). Half of sympathetic neurons were lost in NT3^{-/-} animals, which occur because the neurons could not reach NGF from the final target. Moreover, peripheral sensory neurons, such as proprioceptive neuron afferents from subpopulation of the DRG, were also lost, but motor neuron populations appear unaffected (Ernfors et al., 1994). In sensory neurons, the survival of cochlear neurons is dependent on NT3/TrkC; however, vestibular neuron survival requires BDNF/TrkB-dependent mechanisms (Fritzsche et al., 1997). An *in vitro* experiment has shown that NT3 can activate TrkA to regulate survival and axon growth in sympathetic neurons (Belliveau et al., 1997). Although NT3 preferentially binds to TrkC, in absence of TrkC, NT3 binds other receptors like TrkA. TrkC is expressed in the PNS and CNS, as well as in tissues outside of nervous system, and TrkC^{-/-} mice experienced a loss of all spinal muscle afferents (Klein et al., 1994; Matsuo et al., 2000). The subpopulation in

the DRG, which innervates muscle and conveys proprioceptive signals to the spinal cord, were lost in NT3^{-/-} mice (Ernfors et al., 1994).

Figure 4 NT3 and NGF expression source

NT3 is mainly secreted by the intermediate targets, such as blood vessel, while NGF is from final targets, such as heart.

NT4

Neurotrophin-4 (NT4), first identified in *Xenopus laevis* and sometimes referred to as NT5 (Berkemeier et al., 1991; Hallböök et al., 1991), supports neuron or glial survival. In mammals, NT4 was the last neurotrophin member to be discovered; it is expressed in various regions of the brain and supports rat retinal ganglion cell survival and neurite outgrowth *in vitro* in rats (Huang and Reichardt, 2003). NT4^{-/-} mice are the only viable neurotrophin knockout mice unlike other neurotrophin knockout mice (Liu et al., 1995). Neither sympathetic neurons of the

SCG nor facial motor neurons are affected by loss of NT4, but there is a significant loss of sensory neurons in nodose-petrosal ganglion (inferior ganglion of vagus nerve, which are responsible for visceral sensory innervation) and geniculate ganglion (ganglion of facial nerve). Therefore, like the other neurotrophins, NT4 is required for the development of a subpopulation of peripheral sensory neurons (Liu et al., 1995). Although no overt behavioral phenotypes were observed, defects in long-term memory were observed in NT4-/- mice (Xie et al., 2000). Similar to BDNF, NT4 binds exclusively to TrkB. Much more work is required to fully characterize NT4 in nervous system development and function.

Low affinity receptor, P75

Beside the high affinity receptors TrkA, TrkB and TrkC, all neurotrophins bind with similar affinity to a low-affinity neurotrophin receptor (p75NTR), a single trans-membrane glycoprotein in the tumor necrosis factor receptor superfamily (Rodríguez-Tébar et al., 1991). It is well-established that target-secreted neurotrophins support neuron survival. In contrast early genetic experiments suggest that p75 promotes neuron death (Chao and Hempstead, 1995). p75 serves as a candidate as death signaling factors that play a role in neuronal development. Interestingly, if the neurotrophin binds to the p75 receptor, apoptosis will occur through Jun kinase-mediated signaling pathway (Aloyz et al., 1998; Barrett and Bartlett, 1994). In the sensory and sympathetic system, neurotrophin has been shown to activate NF- κ B dependent neuron survival in a p75-dependent

manner (Hamanoue et al., 1999; Maggirwar et al., 1998). Sympathetic neurons could receive dynamic proapoptotic signaling through p75 signaling to cause cell death. There is evidence *in vivo* suggesting that the total number of sympathetic neurons increased significantly in P0 $p75^{-/-}$ mice (Bamji et al., 1998), while the neuron numbers in $p75^{-/-}$ adults showed no difference from WT mice (Lee et al., 1992). This suggests that the p75 receptor is critical for inducing rapid cell death during neuronal development, but has no effect on cell survival rate in adults.

Downstream signaling

There are 10 conserved tyrosines in the cytoplasmic domains of Trk receptors, with the auto-regulatory loop of the kinase domain containing Y670, Y674 and Y675 (Stephens et al., 1994). Y785 has been shown to interact with PLC- γ producing inositol tris-phosphate and diacylglycerol (Vetter et al., 1991). Phosphorylation of Y490 recruits and phosphorylates the adapter protein, Shc, via binding to Shc PTB domain (Atwal et al., 2000) (Figure 5). Common downstream signaling for neurotrophin includes the extracellular-signal-regulated kinases (ErK/MAPK), mitogen-activated protein kinase kinases (MAPKK/MEK) and phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K) signaling pathways. TrkA activates PI3K to increase protein kinase AKT activity, which can induce expression of Bcl-2, NF κ B and human caspase-9 (Huang and Reichardt, 2003).

Figure 5 Trk receptor common downstream signaling

The Trk receptor recruits PLC, Shc and PI3K via different phosphorylation sites to activate different signaling pathways, such as AKT, Raf/Mek/Erk and calcium. “P” stands for the phosphorylation site.

Signaling endosome

Long-distance coordination of neural development was illustrated by Viktor Hamburger and Rita Levi-Montalcini over 50 years ago in their work that led to the neurotrophic factor hypothesis, in which initially neurons compete for limited neurotrophic factor in order to construct an optimal target innervation pattern and

neural circuits during early development (Hamburger and Levi-Montalcini, 1949). Their studies showed that the survival of spinal ganglia is dictated by a limiting amount of target-derived trophic factor. Survival is thought to be mediated through transcriptional responses. Transcriptional controls, such as via c-jun, increase cell death after removal of NGF (Estus et al., 1994). How can a signal initiated at distal axons in the final target influence a transcriptional response? We now know that long-distance retrograde communication between sympathetic target organs and neuronal cell bodies occurs via the so called “NGF-TrkA signaling endosome” (Grimes et al., 1996). In this scenario NGF binds to a receptor tyrosine kinase, TrkA, causing receptor trans-phosphorylation and internalization at the distal portion of axons. NGF-TrkA is internalized to form signaling endosomes via various mechanisms. The most common and widely known way is via clathrin-mediated endocytosis (Howe et al., 2001; Wiley and Burke, 2001). In addition evidence supports other mechanisms such as caveolin-mediated and Pincher-(a novel protein that enhances NGF-TrkA pinocytosis) mediated endocytosis (Shao et al., 2002; Zweifel et al., 2005).

The retrograde signaling endosome model describes a process, in which the whole signaling endosome itself, not just neurotrophin ligand or Trk receptor alone, is transported a long distance from the distal axon to the cell body, where it induces downstream P-AKT and CREB signaling, for example, to support cell survival related activities. This model has been controversial for many years, but is now widely considered to be the major mechanism for long-distance Trk

signaling. ^{125}I -radiolabeled nerve growth factor (^{125}I -NGF) was used to demonstrate for the first time that NGF transported retrogradely in the noradrenergic neurons of the sympathetic nervous system of rats and mice (Hendry et al., 1974a). Additionally, NGF stimulated retrograde transport of phosphorylated TrkA (P-TrkA) receptor (Ehlers 1995). Moreover, TrkA endocytosis was not only enhanced by NGF but also required NGF for long-distance signaling (Grimes et al., 1996; Riccio, 1997; Tsui-Pierchala and Ginty, 1999). The earliest model of the retrograde signaling endosome was proposed by Halegoua and colleagues (1991), who demonstrated that CCVs in NGF-treated PC12 cells contained NGF, TrkA, and elements of the Ras-MAPK pathway. This was suggested as a platform for retrograde signaling (Mobley 1996). In DRG neurons, the signaling endosome was shown to contain NGF, activated TrkA and signaling proteins of the Rap1/Erk1/2, p38MAPK, and PI3K/AKT pathway. An *in vitro* experiment, in which the endocytic compartment traveled retrogradely in isolated sciatic nerve, provided strong evidence for the existence of retrograde signaling endosomes (Figure 6) (Delcroix et al., 2003). In compartmentalized cultures of sympathetic neurons, which separate the neuron cell body from the axon, the (NGF-P-TrkA)-signaling complex was shown to be transported retrogradely (Kuruvilla et al., 2000; Tsui-Pierchala and Ginty, 1999). Although several other models exist, including Trk trafficking without NGF and secondary messenger trafficking, Ye and others provided significant evidence supporting the retrograde signaling endosome hypothesis. For instance when NGF was applied only on the distal axon, activated TrkA was required both in the

cell body and in the distal axon in order for sympathetic neurons to survive. Ligand-receptor internalization was mandatory for distal NGF to support survival. Furthermore, lowering proximal axon TrkA activity had no impact on cell survival and retrograde signaling (Ye et al., 2003). All neurotrophins bound to their high affinity receptors are thought to traffic retrogradely. For example NGF-TrkA and BDNF-TrkB form signaling endosomes in compartmentalized cultures of DRG neurons (Watson *et al.*, 1999). Interestingly, not all growth factors can initiate long-distance signaling. Although both epidermal growth factor (EGF) and NGF cause transient activation of Ras, only NGF induced the activation of Rap1 and the formation of the C3G/CrkL/Shp2/Gab2/TrkA complex, which might serve as mediator of NGF long distance signaling. Moreover NGF induced prolonged Rap1 and Ras-MAPK signaling (Wu *et al.*, 2001). Thus, different signaling endosomes may elicit different signaling outcomes.

Figure 6 Select components of Signaling endosome relative to this research

The main adapter signaling proteins associated with the NGF-TrkA signaling endosome are Shc, PLC, PI3K, Rab5, Rap and EEA (Delcroix et al., 2003a; Grimes et al., 1996a; Harrington et al., 2011a; Valdez et al., 2005a; Wu et al., 2001a).

An internalized signaling endosome is transported back to the neuronal cell body via a dynein motor (Yano et al., 2001). A feature of the signaling endosome is

the ability to deliver signals to a variety of subcellular locales, providing exquisite specificity toward a given task. For example, transportation of the NGF-TrkA signaling endosome from distal axons into the dendrite was necessary and sufficient to initiate synapse formation between the spinal cord and sympathetic ganglia (Sharma et al., 2010). Transport of NGF-TrkA to the cell body has also been implicated in complex processes such as developmental competition for survival (Deppmann et al., 2008). In adults, it was suggested that general disruption of NGF-TrkA transport in cholinergic forebrain neurons may contribute to Alzheimer's phenotypes, given the fact that cholinergic forebrain neuron survival is NGF dependent (Chen et al., 1997; Wu et al., 2001) .

Consistent with the neurotrophic hypothesis, a popular model for sympathetic neuron survival (Figure 7) suggests that target-derived NGF binds axonal TrkA to form a signaling endosome which is retrogradely transported to the cell body to induce gene expression (Lonze et al., 2002; Riccio, 1997; Ye et al., 2003a). Once an axon reaches its target and is exposed to NGF, the expression of TrkA is increased to support and reinforce cell survival signaling. At the same time, proapoptotic p75 can also be induced by NGF, however, it is suppressed by active TrkA expression (Majdan et al., 2001). On the other hand, if the neuron does not have sufficient NGF or innervates the wrong target, TrkA activity decreases and correspondingly p75 signaling would not be inhibited. Ligands such as BDNF or proNGF will bind p75 resulting in cell death (Deppmann et al., 2008). In the end neurons with high TrkA activity survive, while neurons with low

TrkA activity die. Thus this signaling endosome mediated model is often also referred to as the developmental competition model.

Figure 7 Model of sympathetic neuron competition for survival (modified from Glebova and Ginty 2005)

NGF secreted from the final target binds TrkA and after internalization becomes part of signaling endosome. Signaling endosomes (blue) are retrogradely transported to cell body where they support survival and regulated the expression of genes, such as TrkA, p75, BDNF and NT4. Although p75 causes cell apoptosis, NGF-induced TrkA can inhibit this outcome. Neurons, which do not receive sufficient NGF from the final target, die due to the dominating p75 signaling.

Taken together, these findings suggest that the retrograde signaling endosome is used as a means to tune neuron development and target innervation. One key characteristic of the retrograde signaling endosome is its high signal fidelity. For example EGF signaling half-life is short compared to NGF signaling because there are distinct signaling endosome stabilities. One question, that remains is how neurotrophin signaling at the axon terminls is retrogradely transported with high fidelity to influence survival of the neuron. This question will be addressed in Chapter 2.

Hierarchal signaling

In superior cervical ganglia, NT3 and NGF both signal through TrkA, however, only NGF induces the internalization and long-distance transport mechanisms associated with retrograde signaling endosomes, suggesting that NT3 only supports local axon signaling, having no influence on long-distance survival signaling, while NGF supports both. In addition NGF is required for final target innervation, but not for growth along intermediate targets (Kuruvilla et al., 2004). What is the mechanism for this difference?

NT3 can indirectly support neuron survival, differentiation and growth. Ginty and colleagues demonstrated that NT3 supported survival of mass cultures of sympathetic neurons, but unlike NGF, when applied exclusively to distal axons in compartmentalized chambers, it did not support survival (Harrington et al., 2011). In addition NT3 is required for intermediate target innervation in TrkA⁺ neurons of

the PNS. In P0 SCG 50% of neurons were lost in the absence of NT3 (Francis et al., 1999; Kuruvilla et al., 2004). In P12-P16 mice SCG, NT3^{-/-} lost 50% of neurons (Ernfors et al., 1994). Taken together with *in vitro* data it can be concluded that NT3 is required for intermediate target innervation, which is a prerequisite for final target innervation and exposure to NGF (Harrington et al., 2011). Thus NT3 and NGF support sympathetic neural development in a hierarchal manner (Belliveau et al., 1997; Kuruvilla et al., 2004). How do NGF and NT3 work together to facilitate neuron find its proper target? This question will be addressed in Chapters 3 and 4.

Coronin

Coronin was first identified as an actin/microtubule binding protein necessary for motility in the slime mold, *Dictyostelium discoideum*, where it was found associated crown structures on the dorsal surface of cells (de Hostos et al., 1991). In the absence of coronin, cell movement slowed (de Hostos et al., 1993). Coronin was also found to be enriched at the cell leading edge and in phagocytic cups, further supporting a role in regulating the cytoskeletal machinery of the cell (Maniak et al., 1995). In yeast, coronin is called Crn1, and interacts with both F-actin and microtubules. However loss of Crn1 alone had no effect on the actin cytoskeleton or endocytosis (Goode et al., 1999). Crn1 blocked cofilin from severing newly assembled actin filaments, while on the other hand, promoted cofilin mediated disassembly of aged filaments (Gandhi et al., 2009). Other evidence showed that Crn1 had dual effects on Arp2/3 binding to actin filament;

high concentrations of Crn1 inhibited binding but a low concentration of Crn1 activated binding (Liu et al., 2011). In *Drosophila* a mutation in gene Dpod1, a homolog of mammalian coronin, caused abnormalities in the actin cytoskeleton of the wing imaginal disc (Bharathi et al., 2004) where Dpod1 played a critical role in motility and phagocytosis. In *C. elegans*, Coronin was reported to regulate actin organization and cell morphology during neuroblast migration and neuritogenesis (Shen et al., 2014).

Mammals express up to 7 coronin paralogs, across a range of different tissues; the functions of these paralogs remain ill defined. Coronin-1A is mainly expressed in hematopoietic and neuronal tissues (Ferrari et al., 1999a; Nal et al., 2004; Suzuki et al., 1995a). Coronin-1B is the most broadly studied vertebrate coronin paralog and it is expressed in most tissues (Cai et al., 2005). Coronin-1B has been shown to decrease actin turnover by inhibiting ARP2/3-mediated nucleation of actin and cooperating with ADF/cofilin to disassemble cellular actin (Cai et al., 2005). Vertebrate coronin has similar and additional functions compared to invertebrate coronin. In vertebrate these roles seem to be found in individual coronin paralogs (de Hostos, 1999). Coronin function has likely diverged through evolution.

All paralogs share some structural similarities: an N-terminal beta-propeller binding F-actin, but with unique sequences specific to each family member, and

a C-terminal coiled-coil domain which binds to the Arp2/3 complex (Gandhi et al., 2009) and whose trimerization is necessary for protecting macrophages (BoseDasgupta and Pieters, 2014). The hallmark beta-propeller domain is also called a WD-repeat, which symmetrically contains four or more units composed of 40 amino acids and ending with a tryptophan-aspartic acid (WD). WD has been shown to contact with plasma membrane. All these structures provide the possibilities for coronin to participate in actin related activities such as endocytosis and axon growth.

Coronin-1 (57kDa), a member of the Coronin family is often referred to as Coronin-1A, Coro1a and Taco. Coronin-1 possesses phospholipase activity (Suzuki et al, 1995) and in neutrophils, Coronin-1 can bind to F-actin and the NADPH oxidase (Grogan et al., 1997). In T-cells, Coronin-1 has been reported to bind the actin-related protein (Arp2/3) complex to inhibit cellular F-actin formation, during chemokine-mediated migration (Föger et al., 2006). Beyond its actin binding role in T cells, Coronin-1 appears to play an important role in pathogen-host interactions. Coronin-1 is associated with the pathogenic bacterium *M. tuberculosis*, where bacterial interaction with Coronin-1 enables the bacteria to evade phagosome fusion with lysosomes after engulfment by macrophages (Ferrari et al., 1999). More recently Pieter and colleagues found that recruitment of Coronin-1A to the pathogenic endosome elicited calcium/calcineurin signaling (Figure 8), which also seemed to be critical for preventing lysosomal fusion (Jayachandran et al., 2007). Based on microarray

data, Coronin-1 was also identified as a NGF-dependent gene. This dissertation addresses whether Coronin-1 plays a similar protective role on retrograde signaling endosomes and whether Coronin-1 is involved in actin related activities, such as target innervation during sympathetic neuron development.

Figure 8 Putative roles for Coronin-1 in NGF-TrkA signaling interact with PLC-gamma to induce possible calcium dependent signaling

Our hypothesis is that with help of Coronin-1, calcium induce signaling pathways that block lysosome fusion and activate CREB

Overview

Neurons have several features compared with unpolarized cells such as fibroblasts. Perhaps the most obvious difference is the comparatively long length of neurons ranging from less than a millimeter to over a meter in humans. Establishing a functional neural circuit is complicated because individual neurons (Deppmann *et al.*, 2008), axons (Zweifel *et al.*, 2005), axon branches (Singh *et al.*, 2008) and synapses (Sharma *et al.*, 2010) are known to compete with, and restrict each other, in order to achieve optimal communication in the network. The molecular basis for long distance communication can be explained by the signaling endosome model, but it is not clear how these endosomes maintain their signaling over such a long distance. Logic dictates that consistent neuron-target communication and some effector systems are necessary for this process (Hendry 1975). However how effector systems coordinate with signaling endosomes is still not clear. The specific question I explore is the role of Coronin-1 is an effector, including its mechanism of action. In Chapter 2, I will discuss the role of Coronin-1 as a retrograde signaling endosome effector that facilitates this long distance signaling. Evidence supporting the conclusion that Coronin-1 stabilizes NGF-TrkA signaling endosome to maintain high signal fidelity and also facilitate signaling endosome-dependent signaling via calcium, such as calcineurin and CREB.

Target innervation is indispensable in nascent neurons during early neural system development. As neurons develop, their axons tend to follow the path of intermediate targets (e.g. blood vessels) and progress along these until they terminate on their final target organs (e.g. hearts) (Glebova and Ginty, 2004). Neurotrophin dependent axon growth is a local response, because previous experiments showed that the axon was degenerating when anti-NGF antibody was applied to the distal axon and NGF applied in cell body (Campenot, 1977). NT3 has been implicated in intermediate target innervation in the developing sympathetic system, while NGF appeared to be important for innervation of final targets (Crowley et al., 1994; Levi-Montalcini, 1987). Interestingly, in the sympathetic system both NT3 and NGF signal through the same receptor, TrkA, to regulate axon growth and branching (Oriike 2001, Markus 2002), as well as inducing a similar P-TrkA level (Kuruvilla 2004). Given these common features between NT3 and NGF, how does sympathetic axons switch its preference from NT3, derived from an intermediate target to NGF derived from a final target? The distinct functions of NGF and NT3 are well known, but it remains unclear, how early neurons respond to NT3 and NGF differently and transition from NT3 to NGF during proper target innervation. To be specific, the question is whether Coronin-1 can make an impact on target innervation related activities, such as neuronal axon growth and branching. In Chapter 3 and 4, I demonstrate that Coronin-1 serves as a possible switch for intermediate and final target

innervation. Evidence will be provided for the observation that Coronin-1 has distinct functions on NGF-and NT3-dependent axon growth and branching.

Chapter II Coronin-1 is a neurotrophin endosomal effector required for developmental competition for survival

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Abstract

Retrograde communication from axonal targets to neuronal cell bodies is critical for both development and function of the nervous system. Much progress has been made in recent years linking long-distance, retrograde signaling to a signaling endosome, yet the mechanisms governing the trafficking and signaling of these endosomes remain mainly uncharacterized. Here we report that in mouse sympathetic neurons the target-derived NGF-TrkA signaling endosome, upon arrival at the cell body, induces the expression and recruitment of a novel effector protein known as Coronin-1. In the absence of Coronin-1, the NGF-TrkA signaling endosome fuses to lysosomes 6-10 fold faster than when Coronin-1 is intact. We also define a novel Coronin-1-dependent trafficking event where signaling endosomes recycle and re-internalize upon arrival at the cell body. Beyond influencing endosomal trafficking, Coronin-1 is also required for several NGF-TrkA dependent-signaling events including calcium release, calcineurin activation, and CREB phosphorylation. These results establish Coronin-1 as an essential component of a novel feedback loop mediating NGF-TrkA endosome stability, recycling, and signaling as a critical mechanism governing developmental competition for survival.

Introduction

Neurons are endowed with several features that distinguish them from unpolarized cells. One of the most obvious differences is their comparatively long length. With this extended distance comes several distinct challenges involving proper trafficking and maintenance of signal integrity. This form of communication is particularly important in the development and maintenance of the peripheral nervous system (PNS) where the assembly of neural circuits is coordinated by the target organs they innervate and control.

Amongst the best characterized of these long-distance signals are the structurally related family of target-derived growth factors, the neurotrophins. These factors convey their signal from the distal tip of the axon to the cell body and dendrites, which in turn coordinates the development of functional circuits (Dabrowski and Umemori, 2011; Harrington and Ginty, 2013). Neurotrophins: nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT3), and neurotrophin-4/5 (NT4/5), signal through two distinct receptor systems, the Trk family of receptor tyrosine kinases (RTKs), and p75-NGF receptor (p75-NGFR) (Lewin and Barde, 1996b). “Pro-building” events such as synapse formation and survival, are generally mediated by neurotrophin-Trk “signaling endosomes” that are formed at distal axons/growth cones in the periphery and travel back to neuronal cell bodies (Delcroix et al., 2003a; Hendry et al., 1974b; Sharma et al., 2010a; Ye et al., 2003a).

In recent years several effector proteins have been found to confer unique properties to long-distance, retrograde signaling endosomes. In particular, phospholipase C-gamma (PLC- γ 1), rap1, pincher, phosphatidylinositol 3-kinase (PI3K), ERK5, and cofilin have been shown to associate with the NGF-TrkA signaling endosome and that they are functionally significant in the context of *in vitro* survival assays (Delcroix et al., 2003b; Grimes et al., 1996b; Harrington et al., 2011b; Valdez et al., 2005b; Watson et al., 2001; Wu et al., 2001b). An emergent principle for endosomal-associated effectors is to play multiple roles, not only in signaling to promote developmental events, but also in trafficking and maturation. For example, it has recently been found that association of the actin modifying protein cofilin is necessary for NGF-TrkA retrograde trafficking (Harrington et al., 2011b). Several questions remain about this process including: Which proteins/signaling pathways are essential for trafficking events such as internalization, recycling, long-distance transport, or lysosomal fusion? Are there endosomally-associated proteins that confer a unique signaling ability at a particular time and place?

In this study we identify Coronin-1 as a novel effector protein for the NGF-TrkA signaling endosome. Coronin-1 is part of a family of structurally related proteins known for interacting with cytoskeletal proteins, such as F-actin (Chan et al., 2012; Gatfield et al., 2005; Suzuki et al., 1995b) (Supplementary Figs.1A-C). Although Coronin family members share similar structure and neuronal expression patterns, they do not appear to be functionally redundant. While the

most widely studied function of Coronin-1 is in the context of cytoskeletal dynamics, perhaps more relevant to the NGF-TrkA signaling endosome is its role in pathogen-host interactions. Previous reports concluded that *Mycobacterium tuberculosis* recruits Coronin-1 upon engulfment by macrophages, in order to avoid subsequent lysosomal fusion and evade phagocytic degradation (Ferrari et al., 1999b). In the absence of Coronin-1 or when the bacteria are heat killed, the pathogenic phagosome rapidly fuses to lysosomes. More recently it has been shown that recruitment of Coronin-1 to the pathogenic endosome confers an ability to elicit calcium/calcineurin signaling, which also seems to be critical for preventing lysosomal fusion (Jayachandran et al., 2007b). We hypothesized that Coronin-1 could stabilize the NGF-TrkA signaling endosome in much the same way that it stabilizes the *M. tuberculosis* pathogenic endosome, thereby sustaining signaling integrity between target organ and neuronal cell body.

Here we find that Coronin-1 expression and association with the signaling endosome are induced by neuronal exposure to NGF. We find Coronin-1 to be necessary for NGF-dependent calcium release, which, through activation of calcineurin, allows the NGF-TrkA signaling endosome to evade lysosomal fusion and degradation at the cell body. We also identify a novel role for Coronin-1 in mediating signaling endosome transcytosis. We find that the majority of long-distance retrograde NGF-TrkA endosomes undergo Coronin-1 dependent recycling and re-internalization at the cell body. Coronin-1 loss of function uncouples neurotrophin signaling from calcium release and CREB activation

resulting in a destabilization of the signaling endosome and impaired survival signaling. We conclude that this novel NGF-Coronin-1 feedback loop is critical for competition for survival during development.

Results

NGF is necessary and sufficient for Coronin-1 expression in developing sympathetic neurons

We previously identified a positive feedback loop in which neuronal exposure to target-derived NGF enhanced its own signal duration during developmental competition for survival (Deppmann et al., 2008). Based on this, we speculated that NGF would induce a putative factor responsible for extending signal duration. To identify one of these factors, we analyzed a previously published microarray, which identified NGF-dependent genes in the superior cervical ganglia (SCG) (Deppmann et al., 2008). Our criteria for this directed screen were not only that NGF have the capacity to induce the factor, but also that it have an ontology consistent with membrane or vesicle association. We found 77 genes that met this criteria displaying a more than 2-fold reduction in expression in SCGs from *NGF^{-/-};Bax^{-/-}* compared to controls *Bax^{-/-}* mice at postnatal day 0 (P0) (Fig. 1A and Supplementary Table1). The *Bax^{-/-}* background is necessary in these experiments to circumvent the massive loss of neurons observed in the absence of NGF (Crowley et al., 1994; Deckwerth et al., 1996). Coronin-1 (Coro1a) met the criteria of being associated with vesicles and was identified as being reduced 7.34 fold in SCGs from NGF null mice (*NGF^{-/-};Bax^{-/-}* P0 mice) compared to

controls (*Bax*^{-/-}) (Fig. 1A).

The expression pattern of Coronin family members broadly overlaps in the peripheral nervous system. In particular, TrkA positive peripheral neurons of the dorsal root ganglion show expression of all Coronin family members examined (Supplementary Fig. 2A). In the CNS, it appears that, while Coronin family members are highly expressed (e.g. cortical plate, hippocampus) they are not highly expressed in structures that are classically and discretely TrkA positive (e.g. Basal Forebrain) (Supplementary Fig. 2B).

To confirm that Coronin-1 expression is dependent on NGF, we performed *in situ* hybridization and immunohistochemistry on cryosectioned SCGs isolated from P0 *NGF*^{-/-};*Bax*^{-/-} or *Bax*^{-/-} mice. Antibody specificity was verified by comparing immunostained cryosections from wild-type and *Coronin-1*^{-/-} mice (Supplementary Fig. 1D). Consistent with microarray data, Coronin-1 mRNA and protein levels are dramatically reduced in P0 SCGs from *NGF*^{-/-};*Bax*^{-/-} relative to *Bax*^{-/-} (Fig. 1B and C). We next sought to determine whether NGF is sufficient to induce *Coronin-1* expression *in vitro*. Sympathetic neurons were deprived of NGF in the presence of the broad spectrum caspase inhibitor, Boc-Asp-FMK (BAF), to prevent cell death; NGF (45 ng/mL) was reapplied for the indicated amounts of time and mRNA and protein levels were analyzed via RT-PCR and immunoblot, respectively (Fig. 1D-F). In the absence of NGF, *Coronin-1* transcript and protein levels were low or undetectable and rapidly induced upon

NGF re-addition (Figs. 1D-F). We next defined the developmental timing of Coronin-1 expression via immunohistochemistry for Coronin-1 on cryosections containing SCGs from E15, E18, P0, and P5 mice. (Fig. 1G). Consistent with the notion that NGF is required for Coronin-1 expression, a dramatic increase in the level of Coronin-1 protein was observed after final target innervation (represented by P0) as compared to stages prior to, and at the beginning of, target innervation (represented by E15 and E18, respectively). These data indicate that final target innervation and exposure to NGF is necessary and sufficient for Coronin-1 expression in the sympathetic nervous system.

Figure 1: Coronin-1 expression is regulated by NGF in sympathetic neurons

(A) Candidates for the putative NGF signal duration enhancing factor. Top candidate genes with reduced expression in SCGs from *NGF^{-/-};Bax^{-/-}* P0 mice compared to *Bax^{-/-}* controls and are known to associate with membrane and/or vesicles. All 76 genes are represented in Supplementary Table1.

(B) NGF is necessary for *Coronin-1* mRNA expression. Trunks of P0 *Bax^{-/-}* or *NGF^{-/-};Bax^{-/-}* mice were cryo-sectioned and Coronin-1 message was visualized in the SCG by *in situ* hybridization. Carotid artery is marked as CA and the white arrow indicates the position of the SCG.

(C) NGF is necessary for Coronin-1 protein expression. Trunks of P0 *Bax^{-/-}* or *NGF^{-/-};Bax^{-/-}* mice were cryo-sectioned and Coronin-1 protein was visualized in the SCG by immunostaining. Carotid artery is marked as CA and the white arrow indicates the position of the SCG. Scale bar on right corner is 100 μ m.

(D) NGF is sufficient for induction of Coronin-1 mRNA expression. RT-PCR for Coronin-1 and GAPDH. Sympathetic neurons from P0 rats were cultured for 3 days, deprived of NGF for 16 hours and treated with 45 ng/mL NGF for indicated times. “no RT” is the group without adding reverse transcriptase.

(E) NGF is sufficient for Coronin-1 protein expression. Sympathetic neurons from P0 rats were cultured for 1 day, deprived of NGF for 36 hours and treated with 45 ng/mL NGF for indicated times.

(F) Quantification of E by densitometry using ImageJ. Three experiments were averaged and standard error of the mean was calculated (n=3). Error bars, s.e.m.

(G) Coronin-1 protein expression during development. Trunks of E15, E18, P0 and P5 WT mice were cryosectioned and Coronin-1 or β III tubulin proteins were visualized in the SCG by immunostaining. The carotid artery is used as a landmark and is marked as CA and the white arrow indicates the position of the SCG. Scale bar=100 μ m.

* $p < 0.05$ using unpaired two-tailed Student's t-test

Uncropped gels and blots are shown in Supplementary Figure 7.

Coronin-1 associates with TrkA and the signaling endosome in an NGF-dependent manner

Previous studies on pathogen-host interactions demonstrated a physical association between Coronin-1 and the *M. tuberculosis* phagosome (Ferrari et al., 1999b). We sought to determine whether there is an analogous interaction between Coronin-1 and the NGF-TrkA signaling endosome. In order to clearly visualize post-endocytic retrogradely transported NGF-TrkA, we used sympathetic neurons isolated from P0 *TrkA^{Flag}* mice where a Flag epitope has been knocked in frame with the extracellular domain of TrkA (Sharma et al., 2010b). These neurons were grown in microfluidic devices and distal axons were pulsed with anti-Flag antibody (M1) and NGF for 30 minutes at 37°C (Park et al., 2006a) (Figs. 2A, B). At different time points after the NGF/M1 pulse, neurons were fixed and Flag-TrkA localization was detected using immunofluorescence. Imaging the cell body compartment ensures that the punctum observed represent long-distance, retrogradely transported signaling endosomes.

We first used this assay to examine co-localization between the signaling endosome and Coronin-1. We observed that roughly 70% of signaling endosomes co-localized with Coronin-1 in cell bodies one hour after NGF/ α -Flag treatment on distal axons (Figs. 2C, D). This persists for at least 6.5 hours followed by a gradual decrease to roughly 40% at 24 hours post-NGF/ α -Flag pulse. Notably, at 2.5 hours post-NGF/M1 pulse, co-localization between

endosomal TrkA and Coronin-1 is 3-fold higher in cell bodies ($59.27\% \pm 6.66\%$) compared to axons ($19.49\% \pm 5.01\%$) indicating that this may be a subcellular region-dependent interaction. A lower magnification visualization of Coronin-1 in both dissociated neurons and electroporated chick spinal cord/DRG reveals that Coronin-1 is also highly localized in axons and the growth cones (Supplementary Figs. 1E-F). Coronin-1 association with the signaling endosome was also tested using PC12 cells stably expressing a chimeric Trk receptor containing the extracellular portion of TrkB and the intracellular domain of TrkA (TrkB/A). This receptor is activated and internalized following BDNF treatment, but retains TrkA downstream signaling (Harrington et al., 2011b). Upon ligand stimulation, Coronin-1 and TrkB/A are both shifted into fractions containing early endosomes. While a relatively small amount of TrkB/A is detected in late endosome fractions after BDNF treatment, Coronin-1 remains in early endosome fractions, suggesting a role for Coronin-1 at the early endosome stage (Fig. 2E).

To further explore whether Coronin-1 and TrkA exist on endosomes of a similar maturation stage, we magnetically enriched BDNF-containing endosomes as described previously (Glebov et al., 2006). Briefly, cultured TrkB/A PC12 cells were treated with a BDNF/ferrofluid mixture allowing BDNF-TrkB/A containing endosomes to be isolated by magnetic purification. TrkB/A, Coronin-1 and the early endosome marker, Rab5, were enriched in conditions where NGF and the ferrofluid were added together consistent with the notion that Coronin-1 is physically associated with the NGF-TrkA signaling endosome (Fig. 2F). These

data demonstrate that in response to neurotrophin signaling, Coronin-1 associates with TrkA on internalized membranes similar to early endosomes. Next we examined whether Coronin-1 can interact with TrkA in conditions where endosomal integrity is disrupted with detergent. We performed a co-immunoprecipitation (co-IP) between myc-tagged Coronin-1 and TrkA from PC12 cells lysed in Triton X-100 buffer in the presence or absence of NGF. Coronin-1 co-purifies with TrkA in both of these conditions (Fig. 2G). Together with the gradient sedimentation, colocalization, and magnetic purification experiments, these data suggest that not only does NGF regulate *Coronin-1* expression, but it also drives Coronin-1 association with the NGF-TrkA signaling endosome.

Figure 2: Coronin-1 associates with TrkA and the signaling endosome

(A) Schematic of the microfluidic device, which allows separation of cell bodies and axons.

(B) Schematic of retrograde Flag-TrkA feeding assay. Sympathetic neurons from P0 *TrkA^{Flag}* mice were established in microfluidic devices. Distal axons were labeled with α -Flag antibody at 4°C for 30 minutes followed by NGF treatment and incubation at 37°C for the indicated period of time.

(C) Coronin-1 co-localization with the NGF-TrkA signaling endosome. The Flag feeding assay was performed followed by a 1 hour NGF treatment. Coronin-1 and signaling endosomes were then visualized by immunostaining with anti-Coronin-1 antibody followed by application of fluorescent secondary antibodies to detect Coronin-1 and internalized Flag antibody. Scale bar = 10 μ m.

(D) Quantification of co-localization between Coronin-1 and the signaling endosome. This represents the percentage of signaling endosomes positive for Coronin-1 as a function of time after NGF feeding. Error bars, s.e.m.

(E) Following a 15 minute BDNF treatment of TrkB/A stable cells, both TrkB/A and Coronin-1 are enriched in the early endosome fraction (25/35) of a discontinuous sucrose gradient.

(F) Co-purification of Coronin-1 on TrkA positive endosomes. Magnetic purification of endosomes present after BDNF treatment of TrkB/A PC12 cells. Western blot analysis of TrkB/A, Coronin-1 and the early endosome marker Rab5. Right panels represent input material and left panels represent magnetic purification.

(G) Myc-Coronin-1 co-immunoprecipitates with TrkA in an NGF-dependent manner. Co-immunoprecipitation between endogenous TrkA and overexpressed myc-Coronin-1 in PC12 cells treated with or without NGF.

* $p < 0.05$ using unpaired two-tailed Student's t-test

Uncropped gels and blots are shown in Supplementary Figure 7.

Coronin-1 stabilizes the signaling endosome by preventing lysosomal fusion

Coronin-1 is known to be a requisite host factor for *M. tuberculosis* survival in macrophages (Ferrari et al., 1999b). They concluded that this protection involves preventing engulfed *M. tuberculosis* from undergoing lysosomal fusion. These previous studies, along with our finding that Coronin-1 associates with NGF-TrkA, suggest that Coronin-1 might serve a novel non-pathogenic role in preventing the neurotrophin signaling endosome from lysosomal fusion. Consistent with this, in sympathetic neurons, we found less than 10% co-localization between Coronin-1 and lysosomes as measured by immunocytochemistry for Coronin-1 and LysoTracker staining (Fig. 3A).

To determine whether Coronin-1 can prevent NGF-TrkA signaling endosome degradation, we used an α -Flag antibody feeding assay in combination with LysoTracker staining to identify TrkA-containing lysosomes. Co-localization between these markers was performed following a 30-minute pulse of NGF/ α -Flag on distal axons and a chase period for the indicated periods of time (Figs. 3B-C). In neurons isolated from *TrkA^{Flag}* mice, co-localization remains below 10% until more than 24 hours post-NGF/ α -Flag pulse (Fig. 3C). In contrast, neurons isolated from *TrkA^{Flag}; Coronin-1^{-/-}* mice showed signaling endosomes rapidly fusing with lysosomes, as reflected by a co-localization in the cell body of 3-8 fold higher than in control neurons at all time-points post-pulse (Figs. 3B-C). This is consistent with the finding that NGF signaling is long-lived and suggests

that the mechanism for this is at least in part through Coronin-1-dependent evasion of lysosomal fusion. Notably, this phenomenon appears to be restricted to the cell body, because loss of *Coronin-1* appears to have no effect on lysosomal fusion in axons (Fig 3D). Importantly, loss of *Coronin-1* does not influence the total number of lysosomes in cell bodies or axons in the context of these experiments. (Supplementary Fig. 3A).

To determine whether Coronin-1 broadly influences lysosomal fusion, we examined degradation of internalized EGF in the presence or absence of *Coronin-1*. Consistent with previous reports fluorescent EGF does not undergo retrograde transport (data not shown) (Ferguson et al., 1991). Therefore, these ligands were fed to sympathetic cell bodies for 30 minutes and internalized ligands and lysosomes were visualized 6 hours later. Consistent with previous observations, co-localization of EGF with lysosomes was $57.52\% \pm 3.8\%$ in neurons isolated from wild type animals (Philippidou et al., 2011) (Fig. 3E). Moreover, in the absence of Coronin-1 internalized EGF fuses to lysosomes in a manner similar to wild type ($55.87\% \pm 4.3\%$)(Fig.3E). The fact that Coronin-1 had no impact on EGF lysosomal fusion suggests that it is not a general effector of membrane trafficking. For direct comparison, we performed an anti-Flag antibody feeding assay on neurons isolated from *TrkA^{Flag}* mice where NGF was applied to the cell body instead of distal axons for 6.5h. In the presence of Coronin-1, $7.3\% \pm 5.33\%$ of lysosome puncta colocalized with Flag-TrkA whereas in the absence of Coronin-1 this colocalization is elevated to $40\% \pm$

12.64%. Taken together, these data suggest that Coronin-1 may specifically influence the stability of a particular subset of internalized receptors such as those involved in long-distance retrograde signaling.

We have previously reported a series of NGF-dependent positive feedback loops, which are critical for developmental competition for survival (Deppmann et al., 2008b). One of these feedback loops involves NGF-dependent extension of NGF's own downstream signal duration. Upon NGF deprivation, neurons that are grown in the presence of NGF for 4 days display prolonged phospho-Akt (P-Akt) signaling compared to neurons grown for 1 day in the presence of NGF (Deppmann et al., 2008b). To address whether this change in signal duration is due to Coronin-1 dependent stabilization of TrkA, we examined the decay of P-Akt upon NGF withdrawal in neurons isolated from wild type or *Coronin-1*^{-/-} mice (Fig. 3F,G). P-Akt levels from wild type neurons remain stable for over 4 hours after NGF deprivation, whereas neurons from *Coronin-1*^{-/-} mice display a much faster P-Akt decay rate. These data are consistent with the notion that Coronin-1 is required for a feedback loop where NGF modulates the persistence of its own signaling through up-regulation and recruitment of Coronin-1.

Figure 3: Coronin-1 prevents signaling endosome fusion with lysosomes at the cell body

(A) Coronin-1 does not co-localize with lysosomes. Rat sympathetic neurons were grown 3 days *in vitro* (DIV), immunostained for Coronin-1 (green) and lysosomes were visualized using LysoTracker (red). Co-localization was assessed manually (<10%) and is negligible. Scale bar = 10 μ m.

(B) Signaling endosome co-localization with lysosomes in the presence or absence of *Coronin-1*. Flag feeding was performed for 30 minutes followed by chase period for the indicated time in sympathetic neurons isolated from *TrkA^{Flag}* or *TrkA^{Flag};Coronin-1^{-/-}* mice. Internalized Flag antibody is detected using a fluorescently conjugated anti-mouse secondary antibody and lysosomes were stained with LysoTracker. Scale bar is 10 μ m.

(C) Quantification of the percentage of lysosomes positive for Flag, as a function of time after anti-Flag/NGF feeding (n=5 for all groups). Experiments were performed as described for panel (B). Curves were fit using variable slope regression parameters in graph pad prism. Error bars, s.e.m.

(D) Quantification of co-localization between the signaling endosome and lysosomes in neurons isolated from *TrkA^{Flag}* or *TrkA^{Flag};Coronin-1^{-/-}* mice (n=5 all groups). Cell bodies and axons were visualized at different times after anti-flag antibody/NGF feeding as described in panels B-C. Error bars, s.e.m.

(E) EGF lysosomal fusion in the presence or absence of *Coronin-1*. Scale bar is 10 μ m.

(F) P-Akt decay in the presence or absence of *Coronin-1*. Neurons were cultured

from WT or *Coronin-1^{-/-}* mice for 2-3 DIV, deprived of NGF (in the presence of anti-NGF function blocking antibody) for the indicated times, lysed and analyzed by immunoblot for P-Akt and Akt.

(G) Quantification of E. Experiments were repeated at least three times and quantified with densitometry. P-Akt signals were normalized to total Akt (n=3).

Error bars, s.e.m.

*p<0.05 using unpaired two-tailed Student's t-test

Uncropped gels and blots are shown in Supplementary Figure 7.

Coronin-1 facilitates recycling of retrogradely transported NGF-TrkA at the cell body

The above findings that retrogradely trafficked NGF-TrkA takes roughly 30 hours for significant lysosomal fusion to occur in the cell body, are inconsistent with previous findings demonstrating that PC12 cells downregulate TrkA within 2-3 hours (Jullien et al., 2002). This prompted us to examine NGF-TrkA endosome accumulation in the cell body as a function of time after anti-Flag antibody/NGF pulse on distal axons. Remarkably, we observed very little difference in the initial accumulation and clearance of endosomes after pulse, despite the roughly 6-fold difference in rate of lysosome fusion in the presence versus absence of *Coronin-1* (Fig. 4A). However, after the initial wave of Flag-TrkA accumulation at the cell body (>8hrs), a significant and sustained doubling of internalized Flag-TrkA was observed in wild-type over *Coronin-1*^{-/-} neurons (Fig. 4A).

If not lysosomal fusion, what might be the mechanism for clearance of NGF-TrkA? NGF-TrkA signaling endosomes have been reported to correspond to early and recycling endosome compartments marked by Rab5 and Rab11, respectively (Ascaño et al., 2009; Cui et al., 2007). Therefore, we examined Coronin-1 co-localization with these markers in different subcellular compartments. Notably, Coronin-1 preferentially associates with Rab11 over Rab5 in the cell body, but no significant differences were observed in the proximal or distal axon compartments (Fig. 4B). Does Coronin-1 influence NGF-TrkA association with Rab11? To address this, we performed Flag feeding

assays on neurons from *TrkA^{Flag}* or *TrkA^{Flag};Coronin-1^{-/-}* mice as described in Fig. 2A, and performed immunocytochemistry for internalized Flag-TrkA and Rab11. Remarkably, loss of *Coronin-1* displays a dramatic reduction in Flag-TrkA co-localization with Rab11 where $45.68\% \pm 6.75\%$ of Flag-TrkA puncta are Rab11 positive in neurons from *TrkA^{Flag}* mice compared to $4.58\% \pm 3.68\%$ in *TrkA^{Flag};Coronin-1^{-/-}* neurons (Fig. 4C). We next sought to determine whether Coronin-1 influences recycling of long-distance NGF-TrkA signaling endosomes. To this end, we took advantage of a derivation of the Flag feeding assay, where anti-Flag antibody/NGF are fed to distal axons and an anti-mouse Cy-3 antibody is applied to the cell body similar to an assay previously performed (Zhe-yu Chen, Ieraci, Tanowitz, & Lee, 2005). Cy-3 positive puncta only appeared if retrogradely transported Flag-TrkA traveled back to the soma, recycled to the plasma membrane, and re-internalized (Supplementary Fig. 3B). Indeed, when Coronin-1 was intact, these long-distance endosomes efficiently recycled and reinternalized at 2.5 and 6 hours after feeding, whereas this did not appear to be the case when *Coronin-1* was absent (Figs. 4D, E). This appears to apply uniquely to endosomes that have arrived at the soma, since we observed very little recycling when the Cy-3 antibody was applied to axons. Taken together, these data suggest that *Coronin-1* is dispensable for internalization and retrograde transport of NGF-TrkA, but essential for recycling and evading lysosomal fusion.

Figure 4: Coronin-1 mediates recycling of the signaling endosome

(A) Accumulation of signaling endosomes in the presence or absence of Coronin-1. Flag feeding was performed for 30 minutes followed by chase period for the indicated time in sympathetic neurons isolated from *TrkA^{Flag}* or *TrkA^{Flag};Coronin-1^{-/-}* mice as described for Fig. 3B. A 3-dimensional model was generated from z-stacks obtained for Figure 3B-C and the total number of Flag-TrkA endosomes per neuron, were counted (n=5 for 1hour group and 8 hour group; *TrkA^{Flag}* n=7 for 12 hour group; *TrkA^{Flag};Coronin-1^{-/-}* n=9 for 12 hour group; n=9 for 24 hour group).

(B) Coronin-1 co-localization with Rab5 or Rab11. Sympathetic neurons were immunostained for Coronin-1 and Rab5 or Rab11 and co-localization was assessed and is represented as a percentage of Coronin-1 puncta positive for Rab5 or Rab11 in the cell bodies (CB), proximal axons (PA) or distal axons (DA) (n=5 for all groups). Error bars, s.e.m.

(C) Coronin-1 is required for recruitment of Rab11 to post-endocytic NGF-TrkA. NGF feeding was performed for 30 minutes followed by 2.5h chase in neurons isolated from *TrkA^{Flag}* or *TrkA^{Flag};Coronin-1^{-/-}* mice. Flag and Rab11 were visualized via immunostaining.

(D) Flag-TrkA transcytosis/recycling in the presence or absence of Coronin-1. The recycling assay was performed as described in Panel E on neurons isolated from *TrkA^{Flag}* or *TrkA^{Flag};Coronin-1^{-/-}* mice grown in microfluidic devices. Anti-Flag antibody was fed to distal axons for 30 minutes followed by a chase period for the indicated amount of time. Recycled Flag-TrkA in cell bodies and axons

was marked by Cy3. Schematic for Flag-TrkA recycling assay is presented in Supplementary Fig. 3B. Scale bar = 10 μm .

(E) Quantification of D (n=5 for all groups). Error bars, s.e.m.

*p<0.05 using unpaired two-tailed Student's t-test

Coronin-1 is required for NGF-TrkA dependent calcium mobilization and CREB activation

A generalized influence on NGF-TrkA dependent signals might be expected based on our finding that Coronin-1 is involved in signaling endosome maturation, however it remains unclear whether Coronin-1 represents a modulator for specific NGF-TrkA dependent pathways. Therefore, we next sought to determine whether Coronin-1 influences downstream NGF-TrkA pathways, independent of its role in NGF-TrkA signal duration. Coronin-1 has previously been reported to be important for calcium mobilization in the context of T-cell development and pathogen-host interaction (Föger et al., 2006b; Jayachandran et al., 2007b). Therefore, we hypothesized that Coronin-1 links the neurotrophin signaling endosome to calcium signaling. To test this possibility, we examined NGF-induced calcium release in sympathetic neurons grown in microfluidic devices isolated from P0 wild type or *Coronin-1*^{-/-} mice. To visualize calcium, we loaded cells with Fluo-4 for 20 minutes followed by treatment of indicated NGF concentrations for 15 minutes. As expected, increasing concentrations of NGF led to increasing calcium levels in wild type neurons (Figs. 5A, B). In the absence of *Coronin-1*, NGF loses its ability to induce calcium release (Figs. 5A, B). NGF-TrkA signaling is also known to activate the pro-survival transcription factor, CREB, through a variety of upstream kinases including, PKA, CAMK2, and RSK (Lonze and Ginty, 2002). To assess whether Coronin-1 also influences NGF-dependent CREB activation, Coronin-1 was knocked down in PC12 cells and NGF-dependent phosphorylation of CREB and ERK was assessed. Loss of

Coronin-1 significantly dampened NGF induction of CREB but not ERK phosphorylation (Fig. 5C). Next, *Coronin-1* was knocked down and 3xCRE-luciferase reporter gene assays were performed in sympathetic neurons to assess CREB transcriptional activity (Fig. 5D and Supplementary Fig. 3C). Loss of *Coronin-1* completely abrogated NGF induction of 3xCRE luciferase activity, even at high NGF concentrations (Fig. 5D). Importantly, loss of Coronin-1 does not universally disrupt CREB activation since forskolin-induced increase of cAMP was able to induce 3xCRE luciferase activity with and without knockdown. Taken together, these data indicate that Coronin-1 is an effector for not only NGF-TrkA trafficking and recycling, but independent of this, signaling through calcium and CREB.

Coronin-1 stabilizes the signaling endosome via calcium/calcineurin signaling

Having established that Coronin-1 is necessary for NGF to induce calcium release, we sought to determine how this NGF-dependent calcium signaling is related to NGF-TrkA endosomal stability. We used the cell permeable calcium chelator, BAPTA-AM, to determine if lowering intracellular calcium levels causes increased fusion of signaling endosomes to lysosomes. We were surprised to find that the ratio of signaling endosome/lysosomal fusion in BAPTA-AM treated neurons from *TrkA^{Flag}* mice phenocopied that observed in the absence of *Coronin-1* (Fig. 5E). Moreover, increasing intracellular calcium levels with the calcium ionophore, calcimycin, partially rescued the *Coronin-1* null phenotype

(Fig. 5E). Taken together these data suggest that NGF-TrkA endosome stability is directly related to its ability to induce calcium release.

Given that pathogens like *M. tuberculosis* use the calcium-dependent protein phosphatase, calcineurin, to avoid degradation, we speculated that the signaling endosome may use a similar mechanism. We used either cyclosporin A (CsA) or FK506 to assess whether inhibition of calcineurin on neuronal cell bodies resulted in an increase in the ratio of signaling endosome/lysosomal fusion. Similar to BAPTA-AM treatment, CsA or FK506 treatment of neurons from *TrkA^{Flag}* mice resulted in elevated endosomal fusion to lysosomes, which phenocopied the fusion rates observed in *TrkA^{Flag};Coronin-1^{-/-}* neurons (Fig. 5F). Consistent with Figure 3E, inhibition of calcium or calcineurin signaling does not influence EGF-lysosomal fusion in wild type or *Coronin-1^{-/-}* neurons (Fig. 5G). From these data, we conclude that NGF-TrkA recruitment of Coronin-1 leads to calcium release and calcineurin activation, both of which are required to maintain signaling endosome stability.

Figure 5: Coronin-1 mediates NGF-dependent signaling: calcium mobilization and CREB phosphorylation

(A) NGF induction of calcium is Coronin-1 dependent. Sympathetic neurons isolated from wild type and *Coronin-1*^{-/-} mice were established in culture and calcium release was visualized with the calcium dye, Fluo-4. Images of single neuronal cell bodies (CB) were acquired 15 minutes after NGF treatment. Scale bar = 5µm.

(B) Quantification of calcium release described in panel A as a function NGF concentration. All fluorescence intensities (F) are relative to those collected for 0 ng/mL NGF (F0) and are represented as F/F0 (n=5 for all groups). Error bars, s.e.m.

(C) Coronin-1 is required for NGF dependent phosphorylation of CREB. PC12 cells were transfected with siRNA targeting Coronin-1 or lamin, cultured for 24 hours, treated with NGF for the indicated amount of time and P-CREB, Coronin-1, and GAPDH protein levels were assessed by immunoblot.

(D) Requirement of Coronin-1 for CREB transcriptional activity. Sympathetic neurons transfected with siRNA targeting Coronin-1 or lamin as well as a 3xCRE-luciferase reporter were cultured for 1 DIV and treated with the indicated concentrations of NGF or 5µM forskolin (FSK) for an additional 24 hours followed by a luminescence assay. Data are represented as relative light units (n=3 for all groups). Error bars, s.e.m.

(E) Influence of calcium on signaling endosome stability. Signaling endosome co-localization with the lysosome was examined as described in Fig. 3.

Reducing intracellular calcium release by BAPTA-AM treatment of neurons from *TrkA^{Flag}* mice phenocopies the rate of lysosomal fusion observed in *TrkA^{Flag};Coronin-1^{-/-}* mice. Likewise, increasing intracellular calcium with Calcimycin (50 nM) treatment of neurons from *TrkA^{Flag};Coronin-1^{-/-}* mice partially rescues the lysosome fusion phenotype(n=5 for all groups). Error bars, s.e.m.

(F) Influence of calcineurin on signaling endosome stability. Calcineurin inhibitors, CsA (100 nM) and FK506 (500 nM) added to neurons from *TrkA^{Flag}* mice phenocopy the rate of lysosomal fusion observed in *TrkA^{Flag};Coronin-1^{-/-}* (in *TrkA^{Flag}* mice , control n=10; CSA, Fk506 n=5). Error bars, s.e.m.

(G) EGF positive lysosome puncta (%) are not influenced by calcimycin (calcium ionophore) or cyclosporinA (CSA) and FK506 (calcineurin inhibitors). Fluorescent EGF was fed to neurons from wild type or *Coronin-1^{-/-}* mice for 30 minutes followed by a chase period of 6.5 hours. Error bars, s.e.m.

*p<0.05 using unpaired two-tailed Student's t-test and one-way ANOVA non parametric test

Uncropped gels and blots are shown in Supplementary Figure 7.

Modeling Coronin-1 protection of endosomal TrkA and its impact on competition for survival

We and others have previously built computational models describing competition for survival in developing neurons. This model describes how neurons with similar responsiveness and access to target derived trophic factor can distinguish themselves from one another (Deppmann et al., 2008b). This requires several feedback loops that promote a ‘bistability’ resulting in either survival or apoptosis. Two of these feedback loops are required for competition to occur and include: (1) NGF regulating the expression of its own receptor, TrkA, resulting in an enhanced ability to take up NGF, as well as increased robustness of downstream signaling pathways in neurons that ‘win’ the competition and (2) NGF regulation of its own signal duration, which we related to the degradation rate of active TrkA. We next sought to examine whether this second critical feedback loop corresponds to NGF dependent Coronin-1 expression.

In order to predict the impact of losing Coronin-1 on neuronal competition for survival we updated our previous model with empirical observations from Figure 3C(Deppmann et al., 2008b). We first fit a rate of active TrkA degradation in the presence or absence of Coronin-1, which matches the observed sharp sigmoidal experimental values for signaling endosome-lysosome fusion (Fig. 3C, (Supplementary Figs. 4-6)). In the absence of Coronin-1 the rate of endosome-lysosome fusion hastens dramatically. Yet even in the absence of Coronin-1, NGF-TrkA endosomes persist much longer than other growth factor “signaling

endosomes". For example, post-endocytic EGF degradation peaks within 2 hours after internalization³⁵. This suggests the existence of an additional, Coronin-1 independent, mechanism regulating NGF-TrkA stability. It stands to reason that this putative mechanism would be employed in the axon as the signaling endosome approaches the cell body, which would be required for engagement of the aforementioned NGF dependent competition feedback loops.

We predict the existence of at least 3 post-endocytic TrkA pools defined by their stability: (1) those with Coronin-1, which are very stable (protected), (2) those without Coronin-1 that are moderately stable (unprotected), and (3) those without Coronin-1 that are highly unstable (punished). We have modeled the impact of this putative Coronin-1-independent degradation pathway under conditions of low (Supplementary Fig. 4A-F) or high (Supplementary Fig. 4G-L) trophic factor availability. Notably, the only conditions that display multiple intersections representing a life/death bistable system are those that have Coronin-1-induced protection of endosomes and, independent of this, another mechanism representing a moderate probability of unprotected endosome being converted to "punished" endosomes resulting in rapid fusion with lysosomes (Supplementary Fig. 4).

Based on these observations, we sought to further improve our simulation of developmental competition by building in a third feedback loop representing the

conversion of unprotected endosomes to punished endosomes. In order for this parameter to be useful in the context of developmental competition, this probability must begin at a low level and rise to moderate levels by the end of competition. This is reminiscent of the BDNF-p75-NGFR ‘punishment’ feedback loop described previously, which was found to be essential to expedite competition¹⁹. Although punishment of unprotected endosomes may occur via a different mechanism than punishment via BDNF-p75-NGFR, their feedback loops are likely to increase on a similar time scale. For this reason, the parameter corresponding to the unprotected endosome punishment pathway is coupled with the previously described p75-NGFR dependent punishment pathway.

Simulations reveal that when all previously reported feedback loops are in play, including the newly revised NGF-dependent signal duration loop mediated by Coronin-1, 61% of neurons gain enough trophic signal strength to survive (Fig. 6A-B). In the absence of the Coronin-1 signal duration feedback loop, or when only 50% of NGF is available, only 30% of starting neurons gain sufficient trophic signal strength to survive (Figs. 6A-B). Finally, under conditions simulating loss of Coronin-1 and availability of 50% of endogenous NGF, only 17% of starting neurons survive (Fig. 6B). Notably, because the onset of the previously described p75-NGFR dependent paracrine punishment signaling is linked to trophic signaling, weakening trophic signaling by removing a percentage of NGF results in a delay in competition¹⁹ (Fig. 6B). These simulations are consistent with the target matching hypothesis where a drop to half of NGF production as

observe in *NGF* +/- mice leads to loss of half of the neurons, respectively. Scaling endosome protection as a function of NGF-TrkA uptake is essential, in order to recapitulate the linear quality of target matching. This requirement allows a range of TrkA concentrations over which the NGF uptake from the target tissue is similar for each 'winning' neuron, even though different percentages of neurons may ultimately survive as a function of the rate of NGF production.

Coronin-1 is required for NGF-dependent sympathetic neuron survival

Testing these predictions in sympathetic neurons, we first examined retrograde NGF-dependent survival in neurons from *Coronin-1*^{-/-} and wild type mice. These neurons were established in microfluidic devices and the indicated NGF concentrations were applied to distal axons for 36 hours at which time neuronal death was assessed by Hoechst staining. Increasing the concentration of NGF exclusively on distal axons results in increased survival in wild type neurons. *Coronin-1*^{-/-} neurons display low levels of survival relative to wild type controls even at 100 ng/mL of NGF indicating an essential role for Coronin-1 in long-distance survival signaling (Fig. 6C). Notably, 10 ng/mL NGF applied to the cell body of *Coronin1*^{-/-} neurons is sufficient to support full survival relative to wild type controls. However, lower concentrations of NGF (0.1 and 1 ng/mL) applied to cell bodies fail to support the same levels of survival as in wild type controls (Supplementary Fig.3D). These data point to an essential role for Coronin-1 in mediating long-distance NGF-dependent survival.

We next sought to determine whether Coronin-1 also influences sympathetic neuron survival *in vivo* by counting the number of neurons in P0 SCGs from the indicated genotypes as described previously (Deppmann et al., 2008). Consistent with modeling data, in the absence of *Coronin-1* we observe a dramatic reduction in sympathetic neurons similar to that observed in SCGs from *NGF^{+/-}* mice (Fig. 6D, E). Moreover in SCG neurons from *NGF^{+/-};Coronin1^{-/-}* P0 mice we observed a further reduction of neuron number compared to *NGF^{+/-}* and *Coronin-1^{-/-}* neurons. These findings correlate very well with the survival predicted by simulations (Fig. 6F) and suggest that Coronin-1 is a critical NGF-TrkA effector protein that is required for developmental competition for survival *in vivo*. Modeling suggests that although these genotypes yield significantly different outcomes with respect to number of surviving neurons, at the conclusion of the competition, the neurons that survive have comparable abundances of protected, unprotected, and punished endosomes. This indicates that although different numbers of neurons ‘win’ in the presence of different concentrations of NGF, the ‘winners’ ultimately have a similar trophic state, which we suggest is a key feature of the target-matching hypothesis.

Figure 6: Coronin-1 is required for competition for survival and target matching

(A) Simulations showing the normalized TrkA concentration during development in a random set of 20 neurons (out of 100 simulated) for WT and *Coronin-1^{-/-}*. Parameters derived from *in vitro* experiments predict that in the absence of Coronin-1, fewer neurons survive the competition relative to WT neurons.

(B) Simulations of the time dependence of the surviving neurons for four genotypes: wild type, *Coronin-1^{-/-}*, *NGF^{+/-}* and *NGF^{+/-};Coronin-1^{-/-}*.

(C) Coronin-1 is required for long-distance retrograde NGF-dependent survival *in vitro*. P0 sympathetic neurons from wild type and *Coronin-1^{-/-}* mice were grown in microfluidic chambers. The indicated concentrations of NGF were applied to cell bodies (CB) or distal axons (DA) and cell survival was determined via Hoechst stain. Chambers that did not receive NGF received anti-NGF function blocking antibody. Each experiment was performed at least 3 times and survival is expressed relative to 100 ng/mL NGF on distal axons of wild type neurons (n=6 for all groups). Error bars, s.e.m.

(D) Coronin-1 is required for sympathetic neuron survival *in vivo*. SCGs were taken from at least 4 different P0 mice of the indicated genotypes. Trunks were cryo-sectioned and stained using the Nissl protocol. The carotid artery is marked as CA and the white arrow indicates the position of the SCG. Scale bar=100 μ m.

(E) Quantification of total number of neurons per ganglia from panel D (WT n=4; *NGF^{+/-}* n=6; *Cor^{-/-}* n=4; *NGF^{+/-};Cor^{-/-}* n=7). Error bars, s.e.m.

(F) A comparison between predicted versus experimental competition for survival

outcomes of following conditions: wild type, *Coronin-1*^{-/-}, *NGF*^{+/-} and *NGF*^{+/-};*Coronin-1*^{-/-}.

*p<0.05 using unpaired two-tailed Student's t-test

Discussion:

We demonstrate that Coronin-1 participates in a novel NGF-TrkA feedback loop that is required for proper development of the sympathetic nervous system. Target-derived NGF is necessary and sufficient for *Coronin-1* expression in sympathetic neurons. We also find that Coronin-1 directly interacts with the signaling endosome and is essential for several core NGF-dependent signaling events such as calcium release, calcineurin activation, and CREB phosphorylation. Coronin-1 is also critical for at least two NGF-TrkA endosomal trafficking events associated with endosome arrival at the cell body including: 1) a novel transcytosis event and 2) avoidance of lysosomal fusion. Loss of Coronin-1 results in abnormal neurotrophin signaling and trafficking which results in a diminished capacity of neurons to compete with one another, manifesting in excess developmental neuron death (Supplementary Figs 4-6). This defines a novel framework for modulating neurotrophin signaling in the context of developmental competition for survival (Supplementary Fig. 3E).

The notion of a long-distance signaling endosome sparks several questions related to how this form of signaling differs from signaling at the plasma membrane. These questions include: Does the nature of endosomal signaling change as a function of subcellular locale? How do endosome-derived signals influence endosome trafficking and what are the functional consequences of these trafficking events? The discovery that Coronin-1 is a novel endocytic TrkA effector protein provides a foothold for answering these questions.

Over the past decade, several NGF-TrkA signaling endosome effector proteins have been identified (Harrington and Ginty, 2013). To our knowledge, none of these effectors has been found to preferentially associate with the endosome in a particular subcellular compartment. Here we find that Coronin-1 preferentially associates with endosomal NGF-TrkA in the cell body (Fig. 2D). It is established that sympathetic neuron survival requires long-distance endosomal transport and signaling from distal axons to the cell body (Kuruville et al., 2004b; Ye et al., 2003b). Therefore it is not surprising that the endosome would associate with an effector like Coronin-1 upon arrival at the cell body, which would endow NGF-TrkA-containing endosomes with several specific pro-survival signaling properties, including modulation of calcium release, calcineurin activation, and CREB-dependent transcription.

An emergent property of the NGF-TrkA endosome is that its trafficking is directed by its own signaling. This has been shown for several aspects of trafficking including: TrkA phosphorylation of dynamin which is required for internalization, NGF-TrkA dependent PI3K activity which is required for retrograde transport but not internalization, and NGF-TrkA dependent MAPK activity, which is required for endosomal trafficking into dendrites (Bodmer et al., 2011; Kuruville et al., 2000b; Sharma et al., 2010b). The recruitment of Coronin-1 to the NGF-TrkA signaling endosome seems to fit into this theme, since it regulates two key trafficking events at the cell body: transcytosis/recycling and avoidance of lysosomal fusion.

The requirement of recycling is becoming recognized as a critical component of neurotrophin signaling. Lee and colleagues demonstrated that in mass cultures, TrkA undergoes a relatively high rate of recycling and that by mutating the TrkA juxtamembrane region responsible for recycling, cell survival is impaired (Chen et al., 2005c). In future studies it will be interesting to determine whether Coronin-1 associates with this juxtamembrane region to facilitate TrkA recycling.

By definition, internalization of the signaling endosome at the distal axon followed by recycling at the somatic/dendritic plasma membrane is considered transcytosis. While our study is the first report of retrograde transcytosis for NGF-TrkA, it has been previously found that as part of a maturation process, *de novo* synthesized TrkA must be recycled to the neuronal cell surface prior to being anterogradely transported to the growth cone (Ascaño et al., 2009). Similar to our study, they found that these recycled endosomes are associated with Rab11 and expression of dominant negative Rab11 blocked recycling and transcytosis resulting in defects in axon growth. Rab11-dependent recycling has also been implicated in BDNF-TrkB dependent dendrite formation and synaptic plasticity (S. Huang et al., 2013; Lazo et al., 2013).

A role for Coronin-1 in preventing lysosomal fusion has previously been described in the context of pathogen-host interaction (Ferrari et al., 1999). However, this is the first example of Coronin-1 protecting a growth factor signaling endosome from lysosomal fusion. Beyond the specific calcium-

dependent signaling pathways that Coronin-1 mediates, we suggest that it more generally influences duration of signals emanating from the NGF-TrkA endosome like PI3K or ERK (Figs. 3D, 6C).

NGF-dependent Coronin-1 expression represents a classic development feedback loop. What are the implications of this feedback loop on competition for survival? From our previous work, we know that modulation of neurotrophin signal duration is critical during the developmental competition for survival first described by Hamburger and Levi-Montalcini (Levi-Montalcini and Hamburger, 1949). Mathematical modeling suggests that a feedback loop consisting of an NGF-dependent change in NGF-TrkA signal duration, is required for developmental competition for survival (Deppmann et al., 2008). This study suggests that the Coronin-1 feedback loop may represent this critical element of competition regulating NGF-dependent signal duration. We have updated our mathematical model (Deppmann et al., 2008) to include new parameters from the empirical observations made in this study (Supplementary Figs. 4-6). Indeed, a Coronin-1 feedback loop fits well with the previous model and predicts *in vivo* survival phenotypes in *Coronin-1*^{-/-} animals (Fig. 6F, Supplementary Figs. 4-6). Future studies examining how the constituents of the signaling endosome change with each spatio-temporal trafficking step promise to lend insight into the logic underlying target-driven development of the peripheral nervous system.

Author Contributions:

C.D.D. and D.S. designed experiments. D.S. performed: Coronin-1 expression analysis by immunostaining and immunoblot, all immunocytochemistry, signaling analysis of AKT, CREB, and ERK, calcium signaling experiments, and *in vivo* neuron counts of the SCG. C.D.D. built plasmid constructs, performed *in situ* hybridization, RT-PCR, coimmunoprecipitation, luciferase assays, and chick electroporation. J.P. quantified Flag-TrkA accumulation/disappearance and performed *in vitro* neuron death assays. L.S.Z. and A.W.H. performed endosomal fractionation biochemistry experiments. S.M. performed computational modeling. C.D.D., S.M., and D.S. wrote the manuscript. C.D.D. supervised the project.

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SUPPLEMENTAL TABLE & FIGURE:

Fold Change (Reduced in NGF-/-;Bax-/- compared to Bax-/-)	Gene Symbol	Gene Title
13.45	Pcdh11x	protocadherin 11 X-linked
9.03	Chst15	carbohydrate (N-acetylgalactosamine 4-sulfate 6-O) sulfotransferase 15
8.72	Vgf	VGF nerve growth factor inducible
7.34	Coro1a	coronin, actin binding protein 1A
7.34	Jakmip1	janus kinase and microtubule interacting protein 1
7.09	Slc17a6	solute carrier family 17 (sodium-dependent inorganic phosphate cotransporter), member 6
7.09	Zdhhc9	zinc finger, DHHC domain containing 9
6.73	Lrrtm4	leucine rich repeat transmembrane neuronal 4
6.06	Htr3b	5-hydroxytryptamine (serotonin) receptor 3B
5.37	Cntn3	contactin 3
5.37	Tspan8	tetraspanin 8
4.92	Sgcb	sarcoglycan, beta (dystrophin-associated glycoprotein)
4.59	Accn3	amiloride-sensitive cation channel 3
4.52	Grm7	glutamate receptor, metabotropic 7
4.44	Dpp10	dipeptidylpeptidase 10
4.44	Islr2	immunoglobulin superfamily containing leucine-rich repeat 2
4.44	Unc5b	unc-5 homolog B (C. elegans)

4.14	Ntrk2	neurotrophic tyrosine kinase, receptor, type 2
4.14	Snca	synuclein, alpha
4.14	St6galnac5	ST6 (alpha-N-acetyl-neuraminyl-2,3-beta-galactosyl-1,3)-N-acetylgalactosaminide alpha-2,6-sialyltransferase 5
3.86	Chst8	carbohydrate (N-acetylgalactosamine 4-O) sulfotransferase 8
3.80	Sgpp2	sphingosine-1-phosphate phosphatase 2
3.73	Lypd1	Ly6/Plaur domain containing 1
3.54	1500015O10Rik	RIKEN cDNA 1500015O10 gene
3.42	Atp7a	ATPase, Cu ⁺⁺ transporting, alpha polypeptide
3.31	Dsg2	desmoglein 2
3.31	Fos	FBJ osteosarcoma oncogene
3.25	Fxyd7	FXYD domain-containing ion transport regulator 7
3.08	Wnt7a	wingless-related MMTV integration site 7A
3.03	H2-D1	histocompatibility 2, D region locus 1
3.03	Ngfr	nerve growth factor receptor (TNFR superfamily, member 16)
2.98	Pcdh17	protocadherin 17
2.98	Ret	ret proto-oncogene
2.93	Atp1a1	ATPase, Na ⁺ /K ⁺ transporting, alpha 1 polypeptide
2.88	Akap7	A kinase (PRKA) anchor protein 7
2.88	Atp1b1	ATPase, Na ⁺ /K ⁺ transporting, beta 1 polypeptide
2.83	Cx3cr1	chemokine (C-X3-C) receptor 1
2.83	Ntng1	netrin G1
2.78	Atl3	atlastin GTPase 3
2.78	Gpr135	G protein-coupled receptor 135

2.73	Grik1	glutamate receptor, ionotropic, kainate 1
2.73	Olfr78	olfactory receptor 78
2.64	Mab21l1	mab-21-like 1 (C. elegans)
2.64	Synpr	synaptoporin
2.55	Sybu	syntabulin (syntaxin-interacting)
2.51	Chl1	cell adhesion molecule with homology to L1CAM
2.51	Rasgrp1	RAS guanyl releasing protein 1
2.46	Negr1	neuronal growth regulator 1
2.46	Nrn1	neuritin 1
2.42	Cntn4	contactin 4
2.38	Olfr1	olfactomedin 1
2.38	Pcdh10	protocadherin 10
2.34	Dner	delta/notch-like EGF-related receptor
2.34	Nefl	neurofilament, light polypeptide
2.34	Ptpk	protein tyrosine phosphatase, receptor type, K
2.30	Ewsr1	Ewing sarcoma breakpoint region 1
2.26	Scn11a	sodium channel, voltage-gated, type XI, alpha
2.26	Slc6a15	solute carrier family 6 (neurotransmitter transporter), member 15
2.26	Syt9	synaptotagmin IX
2.22	Atf6b	activating transcription factor 6 beta
2.22	Fbxo2	F-box protein 2
2.22	Kitl	kit ligand
2.18	AB099516 /// Higd1c	cDNA sequence AB099516 /// HIG1 domain family, member 1C
2.18	Slc6a2	solute carrier family 6 (neurotransmitter transporter,

		noradrenalin), member 2
2.14	Atp6v1b2	ATPase, H ⁺ transporting, lysosomal V1 subunit B2
2.14	Atrnl1	attractin like 1
2.14	Cckar	cholecystokinin A receptor
2.14	Cntn1	contactin 1
2.07	P2rx3	purinergic receptor P2X, ligand-gated ion channel, 3
2.03	2900062L11Rik	RIKEN cDNA 2900062L11 gene
2.03	Asl	argininosuccinate lyase
2.03	Cacna2d3	calcium channel, voltage-dependent, alpha2/delta subunit 3
2.03	Htr3a	5-hydroxytryptamine (serotonin) receptor 3A
2.03	Ptprg	protein tyrosine phosphatase, receptor type, G
2.03	Th	tyrosine hydroxylase
2.00	F2r	coagulation factor II (thrombin) receptor
2.00	Itga6	integrin alpha 6

Supplemental Table 1: Identification of NGF induced membrane-associated proteins

Full list of all 77 genes that met our criteria of displaying a more than 2-fold reduction in expression in SCGs from NGF null mice (*Ngf*^{-/-}; *Bax*^{-/-} P0 mice) compared to controls (*Bax*^{-/-} P0 mice), from which Coronin-1 was identified.

a.

coronin-1a	coronin-1b	coronin-1c	coronin-2a-1	coronin-2a-2	coronin-2b	coronin-6-1	coronin-6-2	coronin-6-3	coronin-7	
100%	81%	83%	68%	68%	70%	82%	81%	75%	52%	coronin-1a
	100%	88%	69%	69%	68%	83%	82%	75%	53%	coronin-1b
		100%	65%	65%	65%	85%	85%	78%	53%	coronin-1c
			100%	100%	77%	65%	65%	60%	50%	coronin-2a-1
				100%	77%	65%	65%	60%	50%	coronin-2a-2
					100%	65%	65%	61%	49%	coronin-2b
						100%	98%	92%	51%	coronin-6-1
							100%	92%	51%	coronin-6-2
								100%	47%	coronin-6-3
									100%	coronin-7

b. **c.** **d.** **e.** **f.** **g.**

Supplemental Figure 1: Comparison of Coronin-1 paralogs

(A) Amino acid sequence similarity between all known Coronin paralogs.

(B) Phylogenetic analysis of coronin family members. Protein sequences were obtained from NCBI <http://www.ncbi.nlm.nih.gov/protein> and phylogeny was generated via <http://phylogeny.lirmm.fr/>. Numbers on the line illustrates how close the two units are.

(C) Schematic showing the key domains in Coronin responsible for its cytoskeletal interactions and association with the plasma membrane.

(D) Coronin-1 antibody specificity. Immunostaining for Coronin-1 on cryosectioned trunks from P0 WT and *Coronin-1*^{-/-} mice. Scale bar=100 µm

(E) Coronin-1 subcellular localization in dissociated sympathetic neurons isolated from WT mice grown 1DIV. Immunostained images were captured at 63X and tiled to reconstitute the length of the neuron. Insets represent magnified images of the growth cone or cell body. Scale bar=100 µm

(F) Schematic of the myc-Coronin-1-IRES-GFP construct (top) and an example of this construct introduced by injection and *in ovo* electroporation of the chick neural tube.

(G) P-Akt decay in the presence or absence of *Coronin-1*. Neurons were cultured from WT or *Coronin-1*^{-/-} mice for 2-3 DIV, deprived of NGF (in the presence of anti-NGF function blocking antibody) for 640 min, lysed and analyzed by immunoblot for P-Akt, Akt, and Tuj1.

Supplemental Figure 2: *Coronin* isoform expression in the developing PNS and the adult CNS

(A) *In situ* hybridization representing *Coronin* isoform expression from sections of the P4 spinal cord, (Allen Spinal Cord Atlas [Internet]. Seattle (WA): Allen Institute for Brain Science. ©2009. Allen Spinal Cord Atlas [Internet]. Seattle (WA): Allen Institute

(B) *In situ* hybridization representing *Coronin* isoform expression from p53 coronal sections of the brain, (Allen Spinal Cord Atlas [Internet]. Seattle (WA): Allen Institute for Brain Science. ©2009. Allen Spinal Cord Atlas [Internet]. Seattle (WA): Allen Institute

Supplemental Figure 3: Effect of Coronin-1 on lysosome number, survival, and EGF lysosomal fusion.

(A) Total number of lysosomes in cell bodies (CB) and distal axons (DA) from Fig3D data were quantified. Number of lysosomes per 120 μ m of proximal axon is represented (n=5 for all groups). Error bars, s.e.m.

(B) Schematic for Flag-TrkA recycling assay. Flag feeding assays were performed as described for Fig. 3B followed by a chase period of 2.5 or 6 hours. 30 minutes prior to the end of the chase period, anti-mouse Cy3 was added to the cell body or distal axon compartment of the microfluidic device. Neurons are washed fixed and fluorescence is visualized using confocal microscopy. Cy3 positive puncta represent Flag-TrkA that has travelled back to the indicated compartment, recycled to the plasma membrane and reinternalized.

(C) Western blot of Coronin-1 knockdown. Amaxa/Lonza nucleofection was used to introduce siRNA against *Coronin-1* or *Lamin* (control) mRNA into sympathetic neurons isolated from wild type rats. Neurons are cultured for 36hrs and harvested for immunoblot analysis.

(D) Coronin-1 is required for NGF dependent survival in sympathetic neurons grown in mass culture *in vitro*. P0 sympathetic neurons from wild type and Coronin-1^{-/-} mice were grown in mass culture. The indicated concentrations of NGF were applied for 36 hours and cell survival was determined via Hoechst stain. Neurons that did not receive NGF, receive anti-NGF function neutralizing antibody. Each experiment was performed at least 3 times and survival is expressed relative to those treated with 10 ng/ml NGF (WT n= 16; *Cor*^{-/-} n=6).

Error bars, s.e.m.

(E) Schematic of Coronin-1 function in NGF-TrkA signaling. Target innervation and exposure to NGF triggers an up-regulation of Coronin-1 message. Prior to Coronin-1 up-regulation, the NGF-TrkA signaling endosome is unstable by virtue of rapid lysosomal fusion and impaired recycling; consequently, the neuron is poorly responsive to NGF. Once Coronin-1 is up-regulated, NGF-TrkA signaling endosomes gain the capacity to induce calcium release, activate CREB-dependent transcription, evade lysosomal fusion, and recycle. This represents an NGF-dependent positive feedback loop, which is essential for proper competition for survival during sympathetic nervous system development.

* $p < 0.05$ using unpaired two-tailed Student's t-test

Supplemental Figure 4: Analysis of TrkA expression and degradation in low or high NGF concentration at different paracrine punishment levels for WT and Cor^{-/-} cells.

A-F Computations. This simulation was conducted at concentrations of NGF corresponding to 1/10 of the dissociation constant of TrkA for NGF. Plots show the relationship between Trk production versus degradation. Left hand plots represent production versus degradation in the presence of Coronin-1, there is only one stable equilibrium concentration at a very low trophic level for both WT (left column elements; A,C,E) while right hand graphs (B,D,F) depict this relationship in the absence of Coronin-1 (0%) i.e knockouts. Different levels of conversion probability between unprotected and punished endosomes are represented top to bottom; low or absent (A,B, corresponding to low endosome/lysosome fusion probability), medium (C,D, corresponding to medium endosome/lysosome fusion probability) and high or saturating (E,F, corresponding to high endosome/lysosome fusion probability). Circles represent point of intersection, where production matches consumption. G-L This simulation was conducted at concentrations of NGF corresponding to 10 times the dissociation constant of TrkA for NGF. All other parameters are identical to the above, with left hand graphs representing wild-type where Coronin-1 is present at 100% (G,I,K) and right hand graphs represent Coronin-1 KO or 0% (H,J,L). Solid circles represent point of intersection where the system stabilizes with production matching degradation. Broken line circles represent an unstable point of intersection.

Supplemental Figure 5: Simulation of competition during development for four different genotypes: This simulation plots in the right hand column of graphs, trophic signal strength against time, and measures survival of a random set of 20 neurons over a 50 day period. Parameters were set based on data from *in vitro* experiments and four different genotypes were evaluated; wild-type equivalent to 100% Coronin-1 (A,E), Coronin-1 knockouts (KO or 0% - B,F), NGF heterozygous knockouts (Het - C,G) and the combination, Coronin-1 KO/NGF Het (D,H). The levels of five global parameters are depicted in each of the four left hand column graphs. These global parameters are; red line - the concentration of NGF in the target tissue (relative to its dissociation constant from TrkA), blue line - the fraction of neurons which survived to that time (in which a neuron is considered surviving by having an TrkA concentration below 5% of the population mean or 5% of the initial value), black line - total and brown line - active TrkA concentrations averaged over all the neurons in the population and green line - the level of unprotected endosomal degradation which is linked to paracrine punishment.

Supplemental Figure 6:Parameter analysis for computational model

A-D: Dependence of neuronal competition on initial distribution of TrkA concentration normalized to the half-activation value of the downstream cascades. **A** The individual trophic levels of 20 randomly chosen neurons when the initial TrkA concentration is half of that used for all figures in the main text. **B** Surviving neuron fraction (blue), NGF concentration in the target tissue normalized by its K_d from TrkA (red), total TrkA concentration divided by initial neuron number (black), endosomal TrkA concentration divided by initial neuron number (gray), punishment signal (green) when the initial TrkA concentration is halved. **C and D** Same as **A** and **B** including the random seeds such that the same 20 neurons are plotted, when the initial TrkA concentration is doubled.

E-H: Dependence of neuronal competition on initial NGF concentration normalized to the half-activation value of the downstream cascades. **E** The individual trophic levels of 20 randomly chosen neurons when the initial NGF concentration is half of that used for all figures in the main text. **F** Surviving neuron fraction (blue), NGF concentration in the target tissue normalized by its K_d from TrkA (red), total TrkA concentration divided by initial neuron number (black), endosomal TrkA concentration divided by initial neuron number (gray), punishment signal (green) when the initial NGF concentration is halved. **G and H** Same as **E** and **F** including the random seeds such that the same 20 neurons are plotted, when the initial NGF concentration is doubled.

I-L: Dependence of neuronal competition on the parameter characterizing TrkA positive feedback loop at the level of its expression. **I** The individual trophic levels of 20 randomly chosen neurons when the dependence of TrkA expression on its concentration is decreased by 20%. **J** Surviving neuron fraction (blue), NGF concentration in the target tissue normalized by its Kd from TrkA (red), total TrkA concentration divided by initial neuron number (black), endosomal TrkA concentration divided by initial neuron number (gray), punishment signal (green) when TrkA expression on its concentration is decreased by 20%. **K and L** Same as **I** and **J** including the random seeds such that the same 20 neurons are plotted, when TrkA expression on its concentration is increased by 20%.

M-P: Dependence of neuronal competition on the basal TrkA expression. **M** The individual trophic levels of 20 randomly chosen neurons when basal TrkA expression is halved. **N** Surviving neuron fraction (blue), NGF concentration in the target tissue normalized by its Kd from TrkA (red), total TrkA concentration divided by initial neuron number (black), endosomal TrkA concentration divided by initial neuron number (gray), punishment signal (green) when basal TrkA expression is halved. **O and P** Same as **M** and **N** including the random seeds such that the same 20 neurons are plotted, when basal TrkA expression is doubled

Q-T: Dependence of neuronal competition on the parameters characterizing punishment signal production. **Q** The individual trophic levels of 20 randomly chosen neurons when the rate constant of punishment production is decreased by 20%. **R** Surviving neuron fraction (blue), NGF concentration in the target tissue normalized by its K_d from TrkA (red), total TrkA concentration divided by initial neuron number (black), endosomal TrkA concentration divided by initial neuron number (gray), punishment signal (green) when the rate constant of punishment production is decreased by 20%. **S and T** Same as **Q** and **R** including the random seeds such that the same 20 neurons are plotted, when the rate constant of punishment production is increased by 20%.

U-X: Dependence of neuronal competition on the basal free TrkA depletion. **U** The individual trophic levels of 20 randomly chosen neurons when basal free TrkA depletion is halved. **V** Surviving neuron fraction (blue), NGF concentration in the target tissue normalized by its K_d from TrkA (red), total TrkA concentration divided by initial neuron number (black), endosomal TrkA concentration divided by initial neuron number (gray), punishment signal (green) when basal free TrkA depletion is halved. **W and X** Same as **U** and **V** including the random seeds such that the same 20 neurons are plotted, when basal free TrkA depletion is doubled.

Y-B2: Dependence of neuronal competition on the parameters characterizing the effect of punishment signal on TrkA depletion. **Y** The individual trophic levels of 20 randomly chosen neurons when the maximal lysosomal activation by the punishment signal is decreased by 20%. **Z** Surviving neuron fraction (blue), NGF concentration in the target tissue normalized by its Kd from TrkA (red), total TrkA concentration divided by initial neuron number (black), endosomal TrkA concentration divided by initial neuron number (gray), punishment signal (green) when the maximal lysosomal activation by the punishment signal is decreased by 20%. **A2 and B2** Same as **Y** and **Z** including the random seeds such that the same 20 neurons are plotted, when the maximal lysosomal activation by the punishment signal is increased by 20%.

Supplemental Figure 7: Full-length blots/gels

- (A) Full-length blots for Figure 1D
- (B) Full-length blots for Figure 1E
- (C) Full-length blots for Figure 2F
- (D) Full-length blots for Figure 2G
- (E) Full-length blots for Figure 2H
- (F) Full-length blots for Figure 3F
- (G) Full-length blots for Figure 5C
- (H) Full-length blots for supplementary Figure 1G
- (I) Full-length blots for supplementary Figure 3B

Chapter III Coronin-1 and calcium signaling governs sympathetic final target innervation

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ABSTRACT

Development of a functional peripheral nervous system requires axons to rapidly innervate and arborize into final target organs and then slow but not halt their growth to establish stable connections while keeping pace with organ growth. Here we examine the role of the Nerve Growth Factor (NGF)-TrkA effector protein, Coronin-1, on postganglionic sympathetic neuron final target innervation. In the absence of *Coronin-1* we find that NGF-TrkA-PI3K signaling drives robust axon growth and branching in part by suppressing GSK3 β . In contrast, the presence of Coronin-1 (wild type neurons) suppresses but does not halt NGF-TrkA dependent growth and branching. This relative suppression in axon growth behaviors is due to Coronin-1 dependent calcium release via PLC- γ 1 signaling, which releases PI3K dependent suppression of GSK3 β . Finally, we demonstrate that *Coro1a*^{-/-} mice display sympathetic axon overgrowth and overbranching phenotypes in developing heart and stomach. Taken together with previous work demonstrating the Coronin-1 expression is NGF dependent, this work suggests that periods before and after NGF-TrkA induced Coronin-1 expression (and likely other factors) defines two distinct axon growth states, which are critical for proper circuit formation in the sympathetic nervous system.

Introduction

Wiring a functional nervous system during development requires axons to make several choices and change behaviors as they transition between intermediate targets (e.g. blood vessels) and eventually into a final target (e.g heart, muscle, and eye). The sympathetic nervous system represents a relatively simple paradigm to study how these axons change their behavior en route to their final destination. Sympathetic axons grow along their intermediate targets in fascicles indicating that they likely experience signaling that suppresses branching and turning (Carmeliet, 2003). However, upon arrival to their final targets, axons must become much more dynamic in order to achieve proper coverage of the organ indicating another switch in axonal signaling properties. After this robust period of NGF-TrkA dependent growth and branching in sympathetic final targets these processes are dampened suggesting another change in axonal signaling (Kohn et al., 1999; Manousiouthakis et al., 2014; Singh et al., 2008a). Importantly, NGF-TrkA signaling appears to be required for axon growth and ramification during both early and late stages of final target innervation but not for growth along the intermediate target (Glebova and Ginty, 2004a; Kuruvilla et al., 2004a; Nam et al., 2013). Herein, we explore the molecular basis by which NGF-TrkA dependent sympathetic axon growth properties change during final target innervation.

What is the mechanism by which neurons change their axon growth properties during different stages of final target innervation? The answer may lie in the

spatial and temporal relationship between local and long distance NGF-TrkA signaling. It has been suggested that TrkA dependent axon growth processes can be mediated through local signaling at the plasma membrane via players such as phosphoinositide 3-kinase (PI3K), Glycogen synthase kinase 3- β (GSK3 β), and small G proteins (Atwal et al., 2000; Harrington et al., 2011a; Huo et al., 2004; Kuruvilla et al., 2004a; Ng et al., 2002). This is in contrast to the long-distance endosome mediated retrograde signaling, which is required for several developmental events including competition for survival and synapse formation (Howe and Mobley, 2005; Sharma et al., 2010a; Taniuchi and Johnson, 1985). Local signaling may not engage transcriptional programs, whereas long-distance NGF-TrkA signaling activates transcription factors such as CREB and SRF (Riccio et al., 1999; Rivera et al., 1993). Interestingly, activation of these transcriptional factors has been shown to influence axon growth and final target innervation (Knöll et al., 2006; Wickramasinghe et al., 2008). Therefore, we speculate that the time it takes to undergo long-distance signaling and engage NGF dependent transcriptional programs related to axon growth may demarcate the border between early and late stages of final target innervation.

We hypothesize that a candidate molecular switch that changes axon growth at the early and late stages of final target innervation should display the following characteristics: 1) exhibit specific, inducible expression after final target innervation and exposure to NGF, 2) endow NGF-TrkA complexes with unique signaling abilities thereby changing the behavior of growing axons in early versus

late stages of final target innervation, and 3) alter growth programs to instruct axons to slow growth and branching once they arrive at their final destination.

Coronin-1 is an NGF dependent gene, dynamically changing from undetectable prior to sympathetic final target innervation to high expression after neurons encounter NGF (Deppmann et al., 2008a; Suo et al., 2014). Coronin-1 is known to regulate both calcium and cAMP dependent signaling pathways in response to stimuli ranging from pathogen infection to learning paradigms (Jayachandran et al., 2007a, 2014). Calcium and cAMP have also been reported to influence axon growth behavior (Gomez and Zheng, 2006; Henley and Poo, 2004; Song et al., 1997). The remarkable NGF dependence of Coronin-1 expression taken together with the notion that Coronin-1 regulates several signaling pathways known to influence axon growth makes this an attractive candidate for a molecular switch influencing final target innervation. Moreover, the finding that loss of *Coronin-1* uncouples NGF-TrkA from calcium signaling provides a powerful tool as we seek to dissect the signaling pathways underlying sympathetic nervous system development (Suo et al., 2014).

In this study, we provide *in vitro* and *in vivo* evidence that Coronin-1 meets all of the aforementioned qualities of a molecular switch required for sympathetic axons to change their growth properties in final target organs: 1) its expression changes from undetectable to robust levels upon final target innervation and exposure to NGF (Suo et al., 2014), 2) its up-regulation corresponds to a switch

from primarily PI3K- to calcium-influenced axon growth, branching, and growth cone morphology, and, 3) the NGF-TrkA-Coronin-1-calcium signaling axis is required to slow axon growth and repress branching via disinhibition of GSK3 β as axons approach their final destination.

Results

Coronin-1 acts as a molecular switch that dampens NGF- dependent axon growth, growth cone morphology, and branching.

We investigated whether Coronin-1 plays a role in governing axon behavior *in vitro*. Toward this end sympathetic neurons from WT (Coro1a⁺) or *Coro1a*^{-/-} mice were established in microfluidic devices to separate axons from cell bodies and provide easily identifiable landmarks to find the same axon from day to day (Figure 1B, Figure 1a) (Park et al., 2006b). All neurons were established in 45ng/ml NGF, which is sufficient to support Coronin-1 expression (Suo et al., 2014), until axons traversed the microgrooves. Under fluidic isolation, 2ng/ml NGF was added exclusively to distal axons, while the cell bodies and proximal axons were treated with anti-NGF and a broad spectrum caspase inhibitor, boc-aspartyl-(OMe)-fluoromethyl-ketone (BAF) to prevent apoptosis. Positive and negative growth of individual axons was measured over a 24-hour period. Remarkably, neurons from *Coro1a*^{-/-} mice display a roughly 2-fold increase in NGF-dependent axon growth compared to WT (Coro1a⁺) neurons (Figure 1C).

NGF has also been shown to regulate growth cone size, which relates to axon

growth rate (Argiro et al., 1984; Bray and Chapman, 1985). Therefore, we next examined growth cone area in NGF treated neurons from WT and *Coro1a*^{-/-} mice. These neurons were grown in mass culture for 1 day *in vitro* (DIV) followed by immunocytochemistry for β 3-tubulin and actin. As reported previously, growth cones of WT neurons without neurotrophin were collapsed consistent with a retraction bulb, whereas NGF treated neurons displayed larger bulbous tips (Figure 1D and E) (Harrington et al., 2011a; Kuruvilla et al., 2004a; Seeley and Greene, 1983). In contrast, neurons from *Coro1a*^{-/-} mice displayed a roughly 4-fold decrease in growth cone area (Figure 1D and E). It has been previously suggested that smaller, simpler growth cones appear to correspond with faster axon growth, while larger growth cones appear to be more dynamic, often paused and poised to make choices with respect to directionality and termination in response to instructive environmental cues (Godement et al., 1994). This notion is consistent with accelerated axon growth observed in the absence of *Coronin-1* (Figure 1C).

We next examined the role of Coronin-1 in NGF-dependent axon branching. Sympathetic neurons from P0 WT or *Coro1a*^{-/-} mice were sparsely cultured for 1DIV in the presence of 2ng/ml NGF. Neurons were immunostained for β 3-tubulin and branching was assessed via Sholl analysis as previously described (Magariños et al., 2006). Similar to axon growth and growth cone area analyses, the ability of NGF to induce branching is dramatically increased in the absence of *Coronin-1* relative to WT neurons (Figure 1F and G). This apparent role for

Coronin-1 in regulating NGF dependent branching does not appear to be dependent on neurotrophin concentration, since we observe a similar trend using 45ng/ml NGF (Figure 1F and H). Taken together, these data suggest that Coronin-1 is required to regulate sympathetic axon growth rate, growth cone size, and branching (Figure 1I, J).

Figure 1. Coronin-1 is a molecular switch for NGF dependent axon growth, growth cone morphology and branching behaviors

(A) Schematic of the microfluidic device for compartmentalized treatment of cell bodies and axons (Done by Juyeon Park).

(B) Distal axon chamber pictures showing the axons at time 0 and 24hrs later (day 1) in neurons cultured in the presence of anti-NGF (1 μ g/ml), NGF (2ng/ml). Scale bar = 30 μ m(Done by Juyeon Park).

(C) Quantification of the rate of axon growth from panel B. WT with NGF (n=66), WT with anti-NGF (n=17). *Coro1a*^{-/-} with NGF (n=59), *Coro1a*^{-/-} with anti-NGF (n=13). (Done by Juyeon Park)

(D) Neurons were cultured in the presence of anti-NGF (1 μ g/ml) or NGF (2ng/ml). Neurons from WT or *Coro1a*^{-/-} mice were cultured for 1DIV with NGF or anti-NGF and stained for β 3-tubulin (green) and actin (red). Scale bar = 10 μ m.

(E) Quantification of growth cone area from panel D. (n=15).

(F) NGF-induced axon branching patterns in the presence or absence of Coronin-1. Neurons from WT or *Coro1a*^{-/-} mice were cultured for 1DIV in the presence of NGF (2ng/ml) or NGF (45ng/ml). Tuj1 immunostaining was performed after 1 day of plating. Scale bar = 150 μ m.

(G-H) Sholl analysis quantification of panel F. (n=7)

(I) Neurons from *Coro1*^{-/-} mice grown in NGF represent a novel axon growth niche that displays exuberant axon growth and branching:

(J) In the presence of Coronin-1 (WT) axon growth and branching activity are reduced.

Error bars represent s.e.m.

* $p < 0.05$ using unpaired two-tailed Student's t-test

PI3K signaling in promotes axon growth and branching in the absence of Coronin-1

We next sought to determine whether Coronin-1 influences classic TrkA-dependent pathways known to be involved in axon growth and branching including Ras-MAPK, PI3K, and/or PLC- γ 1-calcium (Arévalo and Wu, 2006). NGF dependent PI3K signaling has also been shown to promote axon growth in TrkA⁺ sensory neurons (Zhou et al., 2004). We asked whether Coronin-1 influences NGF-TrkA dependent PI3K activation by assessing phospho-AKT levels in cultured sympathetic neurons as described in Figures 1A, B. NGF induces p-AKT to similar extents in both WT and *Coro1a*^{-/-} neurons (Figures 2A and B). We then examined whether PI3K signaling is required for regulating axon behaviors in the absence or presence of Coronin-1. We first performed axon growth assays as described in Figure 1B, C in the presence or absence of PI3K inhibitor, LY290002 (50 μ M). Remarkably, LY290002 suppresses the robust NGF dependent axon growth and branching observed in *Coro1a*^{-/-} neurons but had no effect on WT neurons (Figures 2C-H). These data suggest that PI3K drives the exuberant axon growth and branching that we observe in the absence of Coronin-1 but has no effect in the presence of Coronin-1 (WT neurons) (Figure 2I). Because NGF activates PI3K to similar extents in WT and *Coro1a*^{-/-} neurons, we speculate that Coronin-1 blocks axon growth downstream of AKT phosphorylation but upstream of adenomatous polyposis coli (APC), a microtubule plus end binding protein known to regulate axon growth in sensory neurons (Zhou et al., 2004).

Figure 2. PI3K signaling drives exuberant axon growth and branching in the absence of *Coronin-1*

(A) NGF dependent P-AKT induction in sympathetic neurons cultured from WT or *Coro1a*^{-/-} mice for 2–3 DIV. Neurons were deprived of NGF for 17 hours then treated with anti-NGF or NGF for 20min followed by immunoblot analysis for P-AKT and AKT.

(B) Quantification of the data in A. Experiments were quantified with densitometry and P-AKT signals were normalized to total AKT, respectively (n=3).

(C) PI3K signaling is required for NGF induced axon growth in *Coro1a*^{-/-} but not WT neurons. Axon growth assays were performed on neurons from WT or *Coro1a*^{-/-} mice grown in NGF (2ng/ml) with or without LY294002 (50μM) as described for Figure 1B. Scale bar = 30μm. (Done by Juyeon Park)

(D) Quantification of axon growth from c. WT control (n=46), WT with LY294002 and NGF (n=41), *Coro1a*^{-/-} control (n=15), *Coro1a*^{-/-} with LY294002 and NGF (n=25). (Done by Juyeon Park)

(E, G) PI3K signaling is required to promote exuberant branching in the transition window axon growth niche. Branching of WT or *Coro1a*^{-/-} mice grown in NGF (2ng/ml) with or without LY294002 (50μM) as described for Figure 1F. Scale bar = 150μm.

(F, H) Sholl analysis quantification of panel E and G. (n=7)

(I) The role of PI3K on axon growth and branching in *Coro1a*^{-/-} and WT.

Error bars represent s.e.m.

*p<0.05 using unpaired two-tailed Student's t-test

Ras-MAPK signaling does not influence axon growth or branching in sympathetic neurons

Ras-MAPK signaling is known to regulate axon growth and branching in TrkA+ sensory neurons, we examined whether Coronin-1 influences TrkA-dependent ERK activation (Markus et al., 2002; Newbern et al., 2011). We cultured sympathetic neurons from wild type and *Coro1a*^{-/-} mice and assessed NGF dependent p-ERK levels via immunoblot analysis (Figure 3A). Similar to p-AKT induction, WT and *Coro1a*^{-/-} neurons displayed similar levels of NGF dependent p-ERK induction (Figure 3B). We next examined whether RAS-MAPK signaling is required for the axon growth and branching phenotypes observed in our *in vitro* models for axon behavior in the presence or absence of Coronin-1. To this end, we performed axon growth assays as described in Figure 1, on NGF treated neurons from WT or *Coro1a*^{-/-} mice in the presence or absence of the MEK inhibitor, PD98059 (50μM). In neurons from both WT and *Coro1a*^{-/-} mice MEK signaling is not required for axon growth or branching (Figure 3C-H). Taken together, these data suggest that NGF-TrkA-Ras-Erk signaling is dispensable for sympathetic axon growth and with or without Coronin-1 (Figure 3I).

Figure 3. NGF dependent axon branching requires MAPK signaling in the presence but not absence of *Coronin-1*

(A) NGF dependent P-ERK induction in sympathetic neurons cultured from WT or *Coro1a*^{-/-} mice for 2–3 DIV. Neurons were deprived of NGF for 17 hours then treated with anti-NGF or NGF for 20min followed by immunoblot analysis for P-ERK and ERK.

(B) Quantification of the data in A. Experiments were quantified with densitometry and P-Erk signals were normalized to total ERK, respectively (n=3).

(C) MEK signaling is dispensable for NGF dependent axon growth in all growth niches. Axon growth assays were performed on neurons from WT or *Coro1a*^{-/-} mice grown in NGF (2ng/ml) with or without PD98059 (50μM) as described for Figure 1C. Scale bar = 30μm. (Done by Juyeon Park)

(D) Quantification of axon growth from C. WT control (n=26), WT with PD98059 and NGF (n=19), *Coro1a*^{-/-} control (n=23), *Coro1a*^{-/-} with PD98059 and NGF (n=40). (Done by Juyeon Park)

(E, G) MEK signaling is required to suppress branching in the final destination axons growth niche. WT or *Coro1a*^{-/-} mice grown in NGF (2ng/ml) with or without PD98059 (50μM) as described for Figure 1F. Scale bar = 150μm.

(F, H) Sholl analysis quantification of panel E and G. (n=7)

(I) The role of MEK on axon growth and branching in neurons from *Coro1a*^{-/-} and WT mice.

Scale bar = 30μm. Error bars represent s.e.m.

*p<0.05 using unpaired two-tailed Student's t-test

Coronin-1 dependent calcium release represses NGF dependent axon growth and branching

Given that Coronin-1 does not influence the ability of NGF to induce PI3K, the question remains, is there an NGF-TrkA dependent signal that requires Coronin-1 and in turn changes how PI3K driven axon growth and branching is interpreted? We have previously shown that Coronin-1 is essential for NGF-dependent calcium release (Suo et al., 2014). We have repeated these calcium-imaging studies in sympathetic neurons from WT and *Coro1a*^{-/-} mice to again demonstrate that calcium release is completely dependent on Coronin-1 (Figure 4A,B). Taken together, these data suggest that while Ras-MAPK and PI3K-AKT pathways are likely to be active throughout final target innervation, TrkA dependent calcium signaling is likely to occur exclusively after Coronin-1 expression is induced.

To determine whether Coronin-1 influences NGF-TrkA dependent axon growth and branching via calcium signaling we used a cell permeable calcium chelator or ionophore in neurons from WT and *Coro1a*^{-/-} mice to inhibit or activate calcium signaling, respectively. Chelation of intracellular calcium using BAPTA-AM (1μM) had no effect on axon growth or branching in neurons from *Coro1a*^{-/-} mice presumably because calcium levels are already low (Figure 4C-F). However, in WT neurons BAPTA-AM increases NGF dependent axon growth and branching to levels similar to those observed in neurons lacking *Coronin-1* (Figure 4C-H). In contrast, the calcium ionophore, ionomycin (10μM), reduced NGF dependent

axon growth and branching in NGF treated neurons from *Coro1a*^{-/-} mice to levels similar to those observed in WT neurons (Figure 4I-L). However, ionomycin had no effect on axon growth and branching in WT neurons presumably because NGF-TrkA-Coronin-1 is already driving sufficient calcium signaling to suppress these processes (Figure 4I-N). Taken together these findings suggest that the mechanism by which Coronin-1 influences axon growth and branching is by governing NGF dependent calcium release (Figure 4O).

Figure 4

Figure 4. Coronin-1 dependent calcium signaling suppresses axon growth and branching

(A) NGF induces calcium release in a Coronin-1 dependent manner. Sympathetic neurons isolated from WT or *Coro1a*^{-/-} mice were established in culture and calcium release in response to NGF of indicated concentrations was visualized with the calcium dye, Fluo-4. Images of single neuronal cell bodies (CB) were acquired 15 minutes after NGF treatment. Scale bar = 5μm.

(B) Quantification of calcium release described in panels A as a function of NGF concentration. All fluorescence intensities (F) are relative to those collected for 0 ng/mL NGF (F0) and are represented as F/F0 (n=10 for all groups).

(C) Chelation of Calcium increases NGF induced axon growth in WT but not *Coro1a*^{-/-} neurons. Axon growth assays were performed on neurons from WT or *Coro1a*^{-/-} mice grown in NGF (2ng/ml) with or without BAPTA (1μM) as described for Figure 1B. Scale bar = 30μm. (Done by Juyeon Park)

(D) Quantification of axon growth from C. WT control (n=39), WT with BAPTA-AM and NGF (n=65), *Coro1a*^{-/-} control (n=20), *Coro1a*^{-/-} with BAPTA-AM and NGF (n=36). (Done by Juyeon Park)

(E, G) Chelation of calcium increases branching in WT but not *Coro1a*^{-/-} neurons. Branching of WT or *Coro1a*^{-/-} mice grown in NGF (2ng/ml) with or without BAPTA-AM (1μM) as described for Figure 1F. Scale bar = 150μm.

(F, H) Sholl analysis quantification of panel E and G. (n=7)

(I) Elevated Calcium suppresses NGF induced axon growth in *Coro1a*^{-/-} but not wild type neurons. Axon growth assays were performed on neurons from WT or

Coro1a^{-/-} mice grown in NGF (2ng/ml) with or without Ionomycin (10μM) as described for Figure 1B. Scale bar = 30μm. (Done by Juyeon Park)

(J) Quantification of axon growth from I. WT control (n=39), WT with Ionomycin and NGF (n=34), *Coro1a*^{-/-} control (n=20), *Coro1a*^{-/-} with Ionomycin and NGF (n=42). (Done by Juyeon Park)

(K, M) Elevated calcium suppresses branching in *Coro1a*^{-/-} but not wild type neurons. Branching of WT or *Coro1a*^{-/-} mice grown in NGF (2ng/ml) with or without Ionomycin (10μM) as described for Figure 1F. Scale bar = 150μm.

(L, N) Sholl analysis quantification of panel K and M. (n=7)

(O) The role of calcium signaling on neurons from *Coro1a*^{-/-} and WT mice.

It has been previously shown that NGF-TrkA dependent calcium release requires PLC- γ 1 (Obermeier, 1991; Vetter, 1991). Pieters and colleagues have previously shown in thymocytes that Coronin-1 interacts with and is required for PLC- γ 1 activation in response to engagement of T cell receptor (Mueller et al., 2008). Consistent with a similar role in neurons, we found that inhibition of PLC- γ 1 with U73122 (1 μ M) had no effect on NGF dependent axon growth and branching in neurons from *Coro1a*^{-/-} mice (Figure 5A-D). However, similar to BAPTA-AM treatment, U73122 treatment of WT neurons phenocopies the elevated NGF dependent axon growth and branching observed in neurons lacking *Coronin-1* (Figure 5A-F). This supports the notion that PLC- γ 1 and Coronin-1 work together to support NGF dependent calcium signaling and suppress axon growth and branching (Figure 5G).

Figure 5. Coronin-1 and PLC- γ work in concert to support calcium dependent inhibition of axon growth and branching

(A) PLC- γ inhibitor has a similar effect as BAPTA-AM on NGF induced axon growth. Axon growth assays were performed on neurons from WT or *Coro1a*^{-/-} mice grown in NGF (2ng/ml) with or without U73122 (1 μ M) as described for Figure 1B. Scale bar = 30 μ m. (Done by Samuel Young)

(B) Quantification of axon growth from A. WT control (n=28), WT with U73122 and NGF (n=31), *Coro1a*^{-/-} control (n=29), *Coro1a*^{-/-} with LY294002 and NGF (n=35).

(C, E) PLC- γ inhibitor has a similar effect as BAPTA-AM on NGF induced axon branching. Branching of WT or *Coro1a*^{-/-} mice grown in NGF (2ng/ml) with or without LY294002 (50 μ M) as described for Figure 1F. Scale bar = 150 μ m.

(D, F) Sholl analysis quantification of panel C and E. (n=7)

(G) The role of PLC- γ on axon growth and branching in neurons from *Coro1a*^{-/-} and WT neurons.

Error bars represent s.e.m.

*p<0.05 using unpaired two-tailed Student's t-test

Coronin-1 dependent calcium signaling disinhibits GSK3 β to repress axon growth and branching.

Taken together with our finding that loss of Coronin-1 does not impact the ability of NGF to induce PI3K activity, we suggest that the point of cross-talk between these two pathways lies several steps downstream of PI3K activation. GSK3 β is an excellent candidate for this. PI3K activation results in an inhibitory phosphorylation event on GSK3 β , resulting in disinhibition of APC microtubule polymerization activity and promotion of axon growth and branching activity (Jiang et al., 2005; Zhou et al., 2004). Therefore, we sought to assess the role of Coronin-1 on inhibition of GSK3 β by phosphorylation. Toward this end, we cultured sympathetic neurons from WT and *Coro1a*^{-/-} mice and assessed NGF dependent p-GSK3 β levels via immunoblot analysis (Figure 6A). Remarkably, NGF elevates p-GSK3 β in *Coro1a*^{-/-} but not WT neurons (Figure 6B). We next examined whether GSK3 β signaling is required for NGF dependent axon growth and branching in the presence or absence of Coronin-1. To this end, we performed axon growth assays on NGF treated neurons from WT or *Coro1a*^{-/-} mice in the presence or absence of the GSK3 β Inhibitor XIX (200nM) (GSKi), which is analogous to inhibitory phosphorylation of GSK3 β . The GSK3 β inhibitor had no effect on axon growth or branching in neurons from *Coro1a*^{-/-} mice but elevated these properties in WT neurons (Figure 6C-H). Taken together, these data suggest that NGF-TrkA-Coronin-1-PLC- γ 1-Calcium signaling suppresses axon growth and branching by disinhibiting GSK3 β (Figure 6I).

Figure 6. GSK3 β signaling halts axon growth and branching in the presence of *Coronin-1*

(A) NGF dependent P- GSK3 β induction in sympathetic neurons cultured from WT or *Coro1a*^{-/-} mice for 2–3 DIV. Neurons were deprived of NGF for 17 hours then treated with anti-NGF or NGF for 20min followed by immunoblot analysis for P-GSK3 β and GSK3 β .

(B) Quantification of the data in A. Experiments were quantified with densitometry and P- GSK3 β signals were normalized to total GSK3 β , respectively (n=3).

(C) GSK3 β signaling is required for inhibiting NGF induced axon growth in WT but not *Coro1a*^{-/-} neurons. Axon growth assays were performed on neurons from WT or *Coro1a*^{-/-} mice grown in NGF (2ng/ml) with or without GSKi (GSK3 β Inhibitor XIX 200nM) as described for Figure 1B. Scale bar = 30 μ m. (Done by Samuel Young)

(D) Quantification of axon growth from c. WT control (n=23), WT with GSKi and NGF (n=23), *Coro1a*^{-/-} control (n=14), *Coro1a*^{-/-} with GSKi and NGF (n=16).

(E, G) GSK3 β signaling is required to inhibiting NGF induced axon branching in WT but not *Coro1a*^{-/-} neurons. Branching of WT or *Coro1a*^{-/-} mice grown in NGF (2ng/ml) with or without GSKi (GSK3 β Inhibitor XIX 200nM) as described for Figure 1F. Scale bar = 150 μ m.

(F, H) Sholl analysis quantification of panel E and G. (n=7)

(I) The role of GSK3 β on axon growth and branching in *Coro1a*^{-/-} and WT.

Error bars represent s.e.m.

*p<0.05 using unpaired two-tailed Student's t-test

Coronin-1 regulates NGF-dependent axon growth and branching *in vivo*

These *in vitro* data provide a clear prediction for axon growth and branching *in vivo* in mice lacking *Coronin-1*. If initiation of dampened axon growth and branching is associated with induction of Coronin-1 expression, we would expect sympathetic overgrowth and excessive branching in final target organs of mice lacking *Coronin-1*. To test this prediction, we performed whole-mount tyrosine hydroxylase staining to label sympathetic axons originating from the stellate ganglia as they innervate the heart. We examined hearts taken from WT and *Coro1a^{-/-}* animals at E17.5, E18.5 and P0, times that are 1-2 days after axons first come in contact with the heart and NGF (Manousiouthakis, 2014; Nam, 2013). In hearts from E17.5 WT animals, the majority of axons appear at the dorsal surface of the ventricular chamber of the heart. However, in the absence of *Coronin-1*, axons grow much further towards the lateral walls and the apex of the heart (Figure 7A, Supplemental Figure 1A and B). At all ages examined, we observed a significant increase in branch number in hearts from *Coro1a^{-/-}* mice (Figure 7A-D). By P0, *Coro1a^{-/-}* mice have a 50% reduction in sympathetic neurons (Suo et al., 2014) making this overgrowth phenotype even more remarkable. We next compared sympathetic innervation of stomachs from E17.5 WT and *Coro1a^{-/-}* mice. Consistent with the phenotype observed in the heart, we also observed axon overgrowth in stomachs lacking *Coronin-1* (Supplemental Figure 2 A and B). Although *in vitro* experiments were performed using neurons from the SCG, sympathetic axons that supply the heart and stomach are derived from Stellate and Celiac ganglia, respectively. This suggests that, just as NGF

regulates sympathetic neural growth and survival in all sympathetic end organs, the role of Coronin-1 is likely to be similarly broad.

Figure 7. Loss of *Coronin-1* results in excess growth and branching *in vivo*

(A) Whole-mount tyrosine hydroxylase immunostaining of sympathetic fibers in E17.5 hearts from WT and *Coro1a*^{-/-} mice. Higher magnification images are shown in the lower panels. White arrows indicate sympathetic axon terminals. White stars indicate sympathetic axon branches. Scale bar = 200µm.

(B) Whole-mount tyrosine hydroxylase immunostaining of sympathetic fibers in E18.5 hearts from WT and *Coro1a*^{-/-} mice. Higher magnification images are shown in the lower panels. White arrows indicate sympathetic axon terminals. White stars indicate sympathetic axon branches (n=3). Scale bar = 200µm.

(C) Whole-mount tyrosine hydroxylase immunostaining of sympathetic fibers in P0 hearts from WT and *Coro1a*^{-/-} mice. Higher magnification images are shown in the lower panels. White stars indicate sympathetic axon branches. Scale bar = 250µm.

(D) Quantification of axon branch points per region of interest from panels A, B and C (n=3).

Error bars represent s.e.m.

*p<0.05 using unpaired two-tailed Student's t-test

Discussion

Here we examine the role of Coronin-1 in sympathetic axon growth and branching. Because Coronin-1 expression in sympathetic neurons is dependent on NGF at the final target (Suo et al 2014), we speculate that Coronin-1 is a physiological switch involved in interpreting NGF-TrkA signaling in at least 2 different axon growth states during final target innervation: 1) The early innervation stage when axons experience local NGF-TrkA signaling prior to Coronin-1 expression and display smaller growth cones, accelerated axon growth, and increased branching in a PI3K-dependent manner (Figure 8A). As neurons effectively compete for target derived neurotrophic factors, they begin to engage transcriptionally mediated feedback loops (Deppmann et al., 2008a; Suo et al., 2014) and neurons that are 'winning' the competition for survival up-regulate Coronin-1. We suggest that the duration of this early stage of final target innervation is dependent on the lag between initial exposure to target derived NGF and expression of Coronin-1 (and perhaps other genes). Indeed this interval may be as long as 2-4 days since sympathetic axons begin to infiltrate many of their final targets by E14.5 and begin expressing Coronin-1 between E15.5 and E18.5 followed by peak expression at P0 (Suo et al, 2014). 2) The late final target innervation stage corresponds to Coronin-1 up-regulation which increases NGF-induced calcium release and depresses axon growth and branching by suppressing signaling downstream of PI3K resulting in disinhibition of GSK3 β (Figure 8B). The presence of Coronin-1 slows but does not completely halt growth and branching which is likely important to support continued coverage of

end organs as they grow. We speculate that axon growth and branching in neurons that are 'losing' the competition for neurotrophic factor will be slower to induce Coronin-1 expression and will experience a longer early stage remaining highly active until they either find a sufficient concentration of NGF to support survival or are eliminated via apoptosis between E18.5 and P0. This may provide a general logic for how axons switch their behaviors during developmental wiring of the nervous system.

Axon growth in sympathetic axons can be driven through local signaling in distal axons (Campenot, 1977). Based on previously reported kinetics of NGF-TrkA internalization, we speculate that much of the NGF dependent local signaling emanates from an endosomal platform in distal axons (Chen et al., 2005c; Harrington et al., 2011a; Kuruvilla et al., 2004a). The notion that Coronin-1 expression and association with the NGF-TrkA endosome are both NGF dependent (Suo et al., 2014) suggests that the constituents of the signaling endosome platform are quite dynamic. Coronin-1 is likely to recruit or influence several other endosomal proteins. For example, our data taken together with previous studies suggest that although PLC- γ 1 may be immediately recruited to activated TrkA via its SH2 domain, it is not functional until Coronin-1 is recruited during the late stage of final target innervation (Mueller et al., 2008; Ohmichi et al., 1991). It is intriguing to speculate that neurotrophin-induced calcium release occurs as a function of effector proteins associated with post-endocytic TrkA. It has been shown that calcium modulation is directly linked to axon growth rate

and turning behavior (Mattson and Kater, 1987; Song et al., 1997). Low calcium levels correspond to rapid growth and decreased turning while high calcium levels correspond to slowed growth, consistent with Coronin-1 function in sympathetic neurons and is suggestive of a general principal in axon growth (Robles et al., 2003; Song et al., 1997).

Beyond influencing signaling, endosomally associated Coronin-1 may directly influence cytoskeleton dynamics by inhibiting actin nucleation protein(s) Arp2/3 (Rodal et al., 2005). Coronin family members have also been reported to associate with the actin severing protein cofilin which interacts with the signaling endosome and regulates proper NGF-TrkA trafficking (Gandhi et al., 2009; Harrington et al., 2011a). Whether and how Coronin-1 recruits and/or regulates endosomal proteins involved in cytoskeletal modification will be the subject of future inquiry.

Despite similar trophic dependencies between sympathetic and sensory neurons, they appear to use classic TrkA dependent signaling pathways differently to control axon growth and branching. Compelling studies in TrkA+ sensory neurons, have demonstrated that RAS-MAPK and PI3K control growth and branching (Markus et al., 2002; Newbern et al., 2011; Zhou et al., 2004). Inhibition of either of these pathways in WT sensory neurons results in suppressed growth and branching (Atwal et al., 2000; Jiang et al., 2005; Zhou et al., 2004). This is in contrast to our findings in sympathetic neurons, where

inhibition of RAS-MAPK or PI3K had no effect on these properties in WT neurons. Rather, PI3K activity appears to be critical for driving sympathetic axon growth and branching only in the absence of Coronin-1-calcium signaling. It has been shown that while NGF sensitizes calcium channels like TRPV1, NGF alone does not induce calcium release in sensory neurons despite expressing Coronin-1 (Shu and Mendell, 2001; Suo et al., 2014; Wheeler et al., 2014; Zhang et al., 2005). This suggests differential usage of Coronin-1 and perhaps calcium by NGF-TrkA in sympathetic and sensory neurons. It is possible that sensory axon growth and branching are not influenced by calcium signaling, however, this seems unlikely as pharmacological manipulation of calcium levels has been demonstrated to influence axon growth rate and branching in many neuron types (Ramakers et al., 2001; Song et al., 1997). We also observed that in contrast to sensory neurons Ras-MAPK has little effect on axon growth or branching in the presence or absence of Coronin-1 (Markus et al., 2002; Newbern et al., 2011). Future studies on these differences in what have been widely assumed to be relatively similar PNS neuron populations will provide an opportunity to dissect key downstream pathways and better understand the logic of axon behavior as they transition from path finding to target innervation.

Figure 8. Model for Coronin-1 function in defining two stages of final target innervation

(A) Model for axon behavior in the early innervation stage: NGF-TrkA signaling in the absence of Coronin-1. NGF-TrkA acts through PI3K to promote exuberant axon branching and axon growth.

(B) Model for axon behavior in the late innervation stage: NGF-TrkA signaling in the presence of Coronin-1 increase calcium presumably through PLC- γ 1, which inhibits axon growth and branching by suppressing signaling downstream of PI3K.

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SUPPLEMENTAL FIGURE AND LEGENDS:

Supplemental Figure 1 Loss of coronin-1 prevents closure of late stage heart innervation resulting in excess growth and branching *in vivo*

(A) Schematic of heart. SV, sinus venosus; LV, left ventricle; RV, right ventricle; RA, right atrium.

(B) Quantification of e17.5 axon branch points per left and right ventricles from Figure 7A (n=3).

(C) Quantification of e18.5 axon branch points per left and right ventricles from Figure 7B (n=3).

(D) Quantification of p0 axon branch points per left and right ventricles from Figure 7C (n=3).

* $p < 0.05$ using unpaired two-tailed Student's t-test

Supplemental Figure 2 Loss of coronin-1 prevents closure of late stage stomach innervation resulting in excess growth and branching *in vivo*

(A) Whole-mount tyrosine hydroxylase immunostaining of sympathetic fibers in E17.5 stomach from WT and *Coro1a*^{-/-} mice. Higher magnification images are shown in the lower panels. White arrows indicate sympathetic axon terminals. Scale bar = 400µm.

(B) Quantification of axon length per region of interest from panel A. WT n=7 and *Coro1a*^{-/-} n=6.

Error bars represent s.e.m.

*p<0.05 using unpaired two-tailed Student's t-test

**Chapter IV The role of Coronin-1 in Neurotrophin-3
dependent signaling pathways related to sympathetic
neurons axon behaviors**

Abstract

Early developing axons need to move in fascicles/bundles along the intermediate target in order to reach the final target. In postganglionic sympathetic neurons, this process is driven by vascular-derived neurotrophin 3 (NT3) acting through its receptor tyrosine kinase, TrkA. We want to determine how NT3 affects this signaling process to control axon growth and branching. NT3 is capable of inducing MAPK and PI3K through TrkA; however, it does not induce the calcium release. In our *in vitro* experiments, we showed that NT3 increases axon growth through NT3-TrkA-RAS/MAPK signaling and inhibits axon branching through NT3-TrkA-RAS/MAPK and NT3-TrkA-PI3K in the absence of Coronin-1. Once Coronin-1 was unregulated upon reaching final target, these signaling pathways made no impact on axon growth and branch. Although relevant *in vivo* data has not been found, *in vitro* Coronin-1 dependent NT3 signaling experiments give us hint that Coronin-1 might serve as a possible molecular switch during the early innervation stage to transit from intermediate target to final target.

Introduction

During sympathetic nervous system development, nascent axons grow along vascular intermediate targets and then into final target organs (e.g. heart, eye) where they terminate (Balijet and Drukke, 1980; Kummer et al., 1992; Makita et al., 2008; Pardini et al., 1989). Intermediate target innervation is thought to be mediated by neurotrophic factor signaling, where vascular-derived neurotrophin-3 (NT3) mediates growth of sympathetic neurons along intermediate targets (Francis et al., 1999; Harrington et al., 2011; Kuruvilla et al., 2004). In sympathetic neurons NT3 signals through the receptor tyrosine kinase (RTK), TrkA (Belliveau et al., 1997; Kuruvilla et al., 2004a). Because intermediate target is a transition spot before axon reaches its final target, it remains to be determined how NT3-TrkA signaling controls axon growth properties as they grow along intermediate targets. What is more, how does sympathetic axons respond to both NT3 and NGF within the boundary between intermediate target and final target is also not clear.

Neurotrophin-dependent signaling is likely to be at least as dynamic as the axonal behaviors it mediates which change as axons pass by intermediate targets. Axons growing along blood vessels would likely experience signaling that suppresses branching and turning, while increasing growth (Carmeliet, 2003). Once axons reach their final target, these NT3 dependent axon behaviors must become muted to accommodate growth properties consistent with rapid arborization within the final target (Shirasaki, 1998). We suggest that

neurotrophin (NT3 versus NGF) signaling must be differentially interpreted at each stage of target innervation, especially in the boundary between intermediate target and final target to achieve this diversity of axon growth behaviors.

One of the most robustly up-regulated genes in late stage of developing sympathetic neurons is Coronin-1, whose expression increases after neurons encounter NGF during final target innervation (Deppmann et al., 2008a; Suo et al., 2014) (Chapter III). In Chapter II, we demonstrated that Coronin-1 is required for NGF dependent calcium release, implying that calcium could play a significant role in axon development (Song et al., 1997; Suo et al., 2014). We provide *in vitro* evidence that Coronin-1 acts as a molecular switch required for axons changing their growth properties as they move beyond intermediate targets into their final destinations. NGF dependent Coronin-1 up-regulation corresponds to a switch from primarily MAPK/PI3K-influenced axon development to a mutated state, during which MAPK/PI3K is no longer effecting axon growth or development. In total, at least according to our *in vitro* data, we suggest that the up regulation of Coronin-1 controls the switching from intermediate target to final target preference in boundary between NGF and NT3. This is critical for proper circuit formation in the sympathetic nervous system.

Results

We have previously demonstrated that final target innervation and exposure to NGF is necessary and sufficient for Coronin-1 expression in developing sympathetic neurons (Suo et al., 2014). These findings also suggest that as

sympathetic axons move along their intermediate targets and experience NT3-TrkA signaling, Coronin-1 is not expressed (Chapter II 1B and C). Indeed, immunohistochemistry in the developing superior cervical ganglia reveals undetectable Coronin-1 protein expression until times after axons have reached their final target at roughly E16.5 (Coronin-1 expression is present at E18.5 and peaks at P0) (Glebova and Ginty, 2004; Manousiouthakis et al., 2014; Suo et al., 2014) (Chapter III Figure 1A). To investigate whether Coronin-1 is involved in axon signaling pathways at intermediate targets, we want to test whether Coronin-1 is necessary for intermediate target derived factor, NT3, dependent signaling pathways which are related to axon growth and branching. It is important to note that our *in vitro* protocol necessitates early neuronal exposure to NGF both prior to dissection at P0 and during neuron reestablishment *in vitro*; therefore, all cultured WT sympathetic neurons are positive for Coronin-1. Although we have previously demonstrated that long-term NGF deprivation can deplete Coronin-1 protein (Suo et al., 2014), we chose not to use such lengthy deprivation to avoid axonal degeneration and soma atrophy. In this case we aim to test NT3 dependent axon growth and branching through culturing WT (Coronin-1 positive) and *Coro1a*^{-/-} *in vitro*.

Coronin-1 acts as a molecular switch with opposing effects toward NT3-induced growth cone morphology, and branching

We first sought to explore the role of Coronin-1 in axon growth of sympathetic neurons *in vitro*. Toward this end sympathetic neurons from WT (Coro1+) or

Coro1a^{-/-} mice were established in microfluidic devices to separate axons from cell bodies and provide easily identifiable landmarks to find the same axon from day to day (Chapter III Figure 1A) (Park et al., 2006). All neurons were established in 45ng/ml NGF until axons traversed the microgrooves. Under fluidic isolation, 100ng/ml NT3 were added exclusively to distal axons, while the cell bodies and proximal axons were treated with anti-NGF and a broad spectrum caspase inhibitor, boc-aspartyl-(OMe)-fluoromethyl-ketone (BAF) to prevent apoptosis. Positive and negative growth of individual axons was measured over a 24-hour period. Loss of Coronin-1 had no effect on NT3-dependent axon growth (Figure 1B).

NT3 has been shown to differentially regulate growth cone size, which may be related to axon growth rate (Argiro et al., 1984; Bray and Chapman, 1985). Therefore, we next examined growth cone area in NT3 treated neurons from WT and *Coro1a*^{-/-} mice. These neurons were grown in mass culture for 1 day *in vitro* (DIV) followed by immunocytochemistry for β 3-tubulin and actin. We observed growth cones of NT3-treated WT neurons were small and compact, whereas NT3 treated *Coro1a*^{-/-} neurons displayed larger bulbous tips (Figure 1C and D), which is opposite of what we previously observed in NGF treated neurons (Chapter III Figure 1 D and E).

We next examined the role of Coronin-1 in NT3- dependent axon branching. Sympathetic neurons from P0 WT or *Coro1a*^{-/-} mice were sparsely cultured for

1DIV in the presence of NT3 or NGF. Neurons were immunostained for β 3-tubulin and branching was assessed via Sholl analysis as previously described (Magariños et al., 2006). Similar to axon growth cone area analyses, we found that NT3-induced branching was depressed in neurons lacking *Coronin-1* (Figure E and F).

Taken together, these data are consistent with a role for Coronin-1 is dispensable for axon growth but critical for the regulation of axon growth cone size and branching. We speculate that this change in axon behavior might be important for patterning of intermediate target innervation.

Figure 1. Coronin-1 is a molecular switch for NT3-dependent growth cone morphology and branching behaviors

(A) Distal axon chamber pictures showing the axons at time 0 and 24hrs later (day 1) in neurons cultured in the presence of anti-NGF (1 μ g/ml), NT3 (100ng/ml). Scale bar = 30 μ m. (Done by Jueyon Park)

(B) Quantification of the rate of axon growth from panel B. WT with NT3 (n=57), WT with anti-NGF (n=17). *Coro1a*^{-/-} with NT3 (n=69), *Coro1a*^{-/-} with anti-NGF (n=13). (Done by Jueyon Park)

(C) Neurons were cultured in the presence of anti-NGF (1 μ g/ml) and NT3 (100ng/ml). Neurons from WT or *Coro1a*^{-/-} mice were cultured for 1DIV with NT3 or anti-NGF and stained for B3-tubulin (green) and actin (red). Scale bar = 10 μ m.

(D) Quantification of growth cone area from panel D. (n=15).

(E) NGF- and NT3-induced axon branching patterns in the presence or absence of Coronin-1. Neurons from WT or *Coro1a*^{-/-} mice were cultured for 1DIV in the presence of NT3 (100ng/ml). Tuj1 immunostaining was performed after 1 day of plating. Scale bar = 150 μ m.

(F) Sholl analysis quantification of panel E. (n=7)

Error bars represent s.e.m.

*p<0.05 using unpaired two-tailed Student's t-test

The role of Coronin-1 in gating NT3-TrkA induced signaling

Assays examining growth cone size and branching indicate that Coronin-1 is critical to some aspects of axon growth behaviors. Given the diversity of niche dependent axon growth behaviors we sought to determine whether Coronin-1 influences several classic TrkA-dependent pathways, Ras-MAPK and PI3K, in presence of NT3 (Arévalo and Wu, 2006). We began by examining whether Coronin-1 influences NT3-TrkA-dependent ERK or PI3K activation. To this end we cultured sympathetic neurons from wild type and *Coro1a*^{-/-} and assessed NT3 (2ng/ml) dependent p-ERK and p-AKT levels (Figure 2A). Importantly, NT3 yielded similar levels of p-ERK and p-AKT induction in both WT and *Coro1a*^{-/-} neurons (Figure 2A-C).

We next sought to examine the role of Coronin-1 in NT3 versus NGF induction of calcium release. To this end we loaded cells with the calcium indicator, Fluo-4, for 20 minutes followed by treatment with increasing concentrations of NT3 for 15 minutes. In contrast to NGF (Chapter II), NT3 failed to promote calcium release in WT neurons and loss of Coronin-1 had no effect on this (Fig 2D, E). Taken together, these data suggest that while Ras-MAPK and PI3K-AKT activity may be utilized in NT3 dependent signaling, TrkA dependent calcium signaling might be dispensable for NT3 signaling.

Figure 2. Coronin-1 alters NGF- and NT3-induced calcium release but has no effect on P-Erk or P-Akt signaling

(A) NT3 dependent P-ERK or P-AKT induction in sympathetic neurons cultured from wild-type or *Coro1a*^{-/-} mice for 2–3 DIV. Neurons were deprived of NGF for 17 hours then treated with anti-NGF or NT3 for 20min followed by immunoblotting analysis for P-ERK, ERK, P-AKT, and AKT.

(B, C) Quantification of the data in A. Experiments were quantified with densitometry and P-Erk and P-AKT signals were normalized to total ERK and AKT, respectively (n=3).

(D, E) NT3 is not able to induce calcium release in a Coronin-1 dependent manner. Sympathetic neurons isolated from wild-type or *Coro1a*^{-/-} mice were established in culture and calcium release in response to NT3 of indicated concentrations was visualized with the calcium dye, Fluo-4. Images of single neuronal cell bodies (CB) were acquired 15 minutes after NT3 or NGF treatment. Scale bar = 5µm.

(F, G) Quantification of calcium release described in panels D and E as a function of NT3 concentration. All fluorescence intensities (F) are relative to those collected for 0 ng/mL NGF (F0) and are represented as F/F0 (n=10 for all groups).

Error bars represent s.e.m.

*p<0.05 using unpaired two-tailed Student's t-test

NT3-TrkA-dependent MAPK/MEK signaling is required to promote axon growth and suppress branching in absence of Coronin-1

Although we did not see a change of P-Erk level in presence and absence of Coronin-1, it is possible Coronin-1 still modify the outputs of MAPK/MEK signaling. Therefore, we examined the role of MAPK/MEK signaling *in vitro* for axon growth and branching. To examine the intermediate target niche, we performed axon growth assays as described in Figure 1, on NT3 treated neurons from WT or *Coro1a*^{-/-} mice in the presence or absence of the MEK inhibitor, PD98059. In *Coro1a*^{-/-} neurons treated with NT3, PD98059 inhibited NT3-dependent axon extension (Figure 3A, B). However, in WT (*Coro1*⁺) neurons PD98059 had no effect on growth. We next examined the role of MEK in NT3-dependent branching. Interestingly, PD98059 enhanced branching of NT3 treated *Coro1a*^{-/-} neurons, while nothing happened in WT neurons (Figure 3C-F). Taken together, these data suggest that Coronin-1 can uncouple RAS/MAPK signaling from its effect on axon behaviors, which is increasing axon growth and inhibiting branching in absence of Coronin-1 (Figure 3G).

Figure 3. NT3-dependent axon growth and suppression of branching requires MAPK signaling

(A) MEK signaling is required for NT3 induced axon growth in *Coro1a*^{-/-} but not wild-type neurons. Axons growth was examined as described for Figure 1B with the addition PD98059 (50μM) to indicated groups. Scale bar = 30μm.

(B) Quantification of axon growth rate from A. WT with NT3 (n=22), WT with PD98059 and NT3 (n=30), *Coro1a*^{-/-} with NT3 (n=17), *Coro1a*^{-/-} with PD98059 and NT3 (n=26). (Done by Jueyon Park)

(C, E) MEK signaling suppressed NT3-induced axon branching in intermediate targets. Neurons from wild-type or *Coro1a*^{-/-} mice were grown in NT3 (100ng/ml) and with or without PD98059 (50μM). Axons were visualized via immunostaining for Tuj1 after 1DIV. Scale bar = 150μm.

(D, F) Sholl analysis quantification of panel C and F. (n=7)

(G) The role of MEK on axon growth and branching in absence and presence of Coronin-1.

Scale bar = 30μm. Error bars represent s.e.m.

*p<0.05 using unpaired two-tailed Student's t-test

PI3K signaling is required to suppress branching in absence of Coronin-1

We next examined the role of PI3K signaling in regulating axon behaviors in absence and presence of Coronin-1. The PI3K inhibitor, LY29002, had no effect on axon growth of NT3 treated neurons from WT or *Coro1a*^{-/-} mice (Figure 4A, B). However, LY29002 enhanced branching of neurons from *Coro1a*^{-/-} mice grown in NT3, but had no effect on WT (Coro1+) neurons grown in NT3 (Figure 4C-F). Taken together with MEK inhibitor experiments (Figure 3), these data suggest that both NT3-TrkA-MAPK and NT3-TrkA-PI3K signaling suppresses branching along intermediate targets but only MAPK is required for NT3-dependent axon growth in this niche where Coronin-1 levels are absent (Figure 4M).

Figure 4. PI3K signaling pathway plays a Coronin-1 dependent role in axon branching

(A) PI3K signaling is not required for NT3 induced axon growth in both wild-type and *Coro1a*^{-/-} neurons. Axons growth was examined as described for Figure 1B with the addition LY294002 (50μM) to indicated groups. Scale bar = 30μm.

(B) Quantification of axon growth rate from A. WT with NT3 (n=29), WT with LY294002 and NT3 (n=28), *Coro1a*^{-/-} with NT3 (n=10), *Coro1a*^{-/-} with LY294002 and NT3 (n=20). (Done by Jueyon Park)

(C, E) PI3K signaling suppresses axon branching in absence of Coronin-1. Neurons from wild-type or *Coro1a*^{-/-} mice were grown in NT3 (100ng/ml) with or without LY294002 (50μM). Branching was visualized as described in Figure 1F. Scale bar = 150μm.

(D, F) Sholl analysis of panel C and F (n=7).

(G) The role of PI3K on axon growth and branching in absence and presence of Coronin-1.

Error bars represent s.e.m.

*p<0.05 using unpaired two-tailed Student's t-test

Calcium signaling is dispensable to NT3-TrkA and Coronin-1 dependent axon growth and branching

In WT and *Coro1a*^{-/-}, the separable roles of TrkA dependent MEK and PI3K pathways reveal that Coronin-1 (or lack thereof) is responsible for differential interpretation of these signals. We next examined the role of calcium signaling in axon growth and branching in absence and presence of Coronin-1. The calcium chelator, BAPTA-AM suppresses axon growth of NT3 treated WT (Coro1+) neurons but had no effect on growth in NT3 treated *Coro1a*^{-/-} neurons (Figure 5A, B). While this suppression in WT neurons is interesting it is likely not developmentally relevant because axons growing along intermediate targets do not express Coronin-1. BAPTA-AM also had no effect on NT3-dependent branching in neurons from WT or *Coro1a*^{-/-} mice (Figure 5C-F). Although calcium can increase NT3 induced axon growth, taken together with data from Figure 2, which showed NT3 is not able to induce calcium release, we suggest that calcium signaling plays little role in growth and branching in absence and presence of Coronin-1.

Figure 5. Calcium signaling suppresses axon growth and branching plays in presence of Coronin-1

(A) Coronin-1 dependent calcium signaling is required to suppress NT3 dependent axon growth in the final destination axon growth niche. Axons growth was examined as described for Figure 1B with the addition the Ca^{2+} chelator, Bapta ($1\mu\text{M}$). Scale bar = $30\mu\text{m}$.

(B) Quantification of the speed of axon growth from A. WT control (n=57), WT with Bapta and NT3 (n=54), *Coro1a*^{-/-} control (n=57), *Coro1a*^{-/-} with BAPTA-AM and NT3 (n=49). (Done by Jueyon Park)

(C, E) Coronin-1 dependent calcium release is required to axon branching in the final destination axon growth niche. Neurons from wild-type or *Coro1a*^{-/-} mice were grown in NT3 (100ng/ml) with or without BAPTA ($1\mu\text{M}$). Scale bar = $150\mu\text{m}$.

(D, F) Sholl analysis quantification of panel C and E. (n=7)

(G) The role of calcium signaling on axon growth and branching in absence and presence of Coronin-1.

Discussion

Our *in vitro* data showed that Coronin-1 is necessary for NT3-dependent signaling to control axon behaviors. In absence of Coronin-1, NT3 increases axon growth through TrkA-RAS/MAPK signaling pathway, but inhibits axon branching through TrkA-RAS/MAPK and TrkA-PI3K signaling pathways. On the other hand in presence of Coronin-1, NT3 is not able to effect axon growth through the same signaling pathways even if these signaling pathways are still active. Moreover, we report that Coronin-1 is dispensable for the induction of NT3-TrkA-dependent calcium signaling and activation of other pathways like ERK and AKT. Our observation that NGF (Chapter III Figure 3), but not NT3 (Figure 2), can induce calcium increase underscores the notion that Coronin-1 is an effector specific to NGF-TrkA but not NT3-TrkA signaling pathways. Taken together, these data showed the critical role of Coronin-1 on uncoupling NT3 dependent signaling pathways from the axon behaviors *in vitro*.

As we begin to understand the complexities of nervous system development, we also appreciate the need to re-use individual signaling components in multiple ways to achieve a functional circuit. The sympathetic nervous system is ideal for delineating the basis for these signaling pathways. It is intriguing to speculate that neurotrophin-induced calcium release occurs as a function of effector proteins associated with post-endocytic TrkA. Calcium is particularly important for axon growth behavior. It has been shown that calcium modulation is directly linked to axon growth rate and turning behavior (Mattson and Kater, 1987; Song

et al., 1997). However NT3 cannot induce any calcium release. These systems may include the re-use of RTKs like Ret, TrkC, or Met, at multiple stages of sensory, sympathetic, or motor neuron development. Moreover, understanding this process in peripheral axons with well-defined and relatively simple trajectories will inform more complex path-finding programs and trophic dependencies of neurons in the CNS.

Although we are still missing some key evidence, our in vitro data provides us some clues to understand the process when sympathetic neurons move across from intermediate target to final target. Given our previous research, Coronin-1 expression is not induced until final target innervation and exposure to NGF occurs, therefore axons traveling along their intermediate targets (i.e. blood vessels) express little or no *Coronin-1* (Suo et al., 2014). Combining with our finding in Chapter III, herein we propose a model where Coronin-1 acts as a switch to ensure the transition from intermediate target to final target. We use *Coro1a*^{-/-} neurons treated with NT3 to model axon behavior prior to induction of Coronin-1 expression. When axons are growing along intermediate targets or within the boundary between intermediate targets and final targets, Coronin-1 is not unregulated. NT3-TrkA promotes axon growth through the Ras-Raf-MAPK pathway and suppresses branching through the Ras-Raf-MAPK and PI3K pathway before Coronin-1 is unregulated (Figure 6A). Then we use WT neurons treated with NT3 to model axon behavior after induction of Coronin-1. Once neurons arrive at final target, while Coronin-1 expression is increasing, these

signaling pathways no longer have control of axon growth or branching, which can make sure that axons do not go back intermediate target (Figure 6B).

Although our working model explains well how neurons switch from intermediate targets to final targets, there is still evidence missing to support this model. 1) We definitely need *in vivo* evidence. For instance in *Coro1a*^{-/-}, does the neuron return to intermediate even after it reach the final target? How long will the axon stay in the transition area between an intermediate target and a final target? 2) We have indirect evidence showing that NT3 can not induce Coronin-1 expression. However we need more direct evidence such as RT-PCR or protein induction. 3) We showed Coronin-1 upcoupled NT3 dependent signaling from axon behavior *in vitro* by comparing WT and *Coro1a*^{-/-}, but we do not know how much Coronin-1 protein expression is needed. A gradient transfection to *Coro1a*^{-/-} might give us some hint. 4) Beside Coronin-1, is there any other effector that can also modify this process?

Figure 6. Working model for NT3-TrkA signaling and Coronin-1 expression during innervating from intermediate target to final target

(A) Early innervation stage signaling in absence of Coronin-1. NT3-TrkA signals through RAS/MAPK and PI3K to control axon growth and branching. In this period the NT3 can still support axon to move forward through these signaling pathways.

(B) Late stage signaling in the presence of Coronin-1. NT3-TrkA still activate RAS/MAPK and PI3K, but have uncoupled with axon growth and branching from these signaling pathways. In this way NT3 loss control of axon growth and branching, while NGF still has control (Chapter III).

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Chapter V Discussion

Relationship of Coronin-1 with other signaling endosome factors

Over the past decade, several NGF-TrkA signaling endosome effector proteins have been identified (Harrington and Ginty, 2013); however, to our knowledge, none of these effectors have been found to associate preferentially with the endosome in a particular subcellular region. We found that Coronin-1 preferentially associates with endosomal NGF-TrkA in the cell body (Suo et al., 2014). It is established that sympathetic neuron survival requires long-distance endosomal transport and signaling from distal axons to the cell body (Kuruvilla et al., 2004; Ye et al., 2003). Therefore it is not surprising that the endosome would associate with an effector like Coronin-1 upon arrival at the cell body, which would endow NGF-TrkA-containing endosomes with several specific pro-survival signaling properties, including modulation of calcium release, calcineurin activation, and CREB-dependent transcription. The actin-severing protein cofilin and the small G proteins Rac1 and Rap1 specifically associate with the NGF-TrkA signaling endosome (Harrington et al., 2011; Wu et al., 2001). Interestingly, Coronin family members have been suggested to associate with cofilin and the cofilin phosphatase, slingshot, all of which may interact to influence signaling from the endosome and actin dynamics (Gandhi et al., 2009; Tsujita et al., 2010). In sensory neurons, signaling effectors, such as MAPK, PI3K and phospholipase C, associate with NGF-TrkA signaling endosome and a similar situation may exist in sympathetic neurons (Delcroix et al., 2003). It will be interesting to examine Coronin-1 in other neuron populations that require NGF-TrkA signaling.

Coronin-1 and the recycling mechanism

By definition, internalization of the signaling endosome at the distal axon followed by recycling at the somatic/dendritic plasma membrane is considered transcytosis, and the need for recycling is becoming recognized as a critical component of neurotrophin signaling. Lee and colleagues demonstrated that in mass cultures, TrkA undergoes a relatively high rate of recycling and that by mutating the TrkA juxtamembrane region responsible for recycling, cell survival is impaired (Chen et al., 2005b). The persistent internalization of NGF-TrkA into a signaling endosome versus the principally cell surface signaling (or rapidly recycling) of NT3-TrkA may account for the apparent differential engagement of downstream pathways (Harrington *et al.*, 2011). While our study is the first report of retrograde transcytosis for NGF-TrkA, it has been previously found that as part of a maturation process, *de novo* synthesized TrkA must be recycled to the neuronal cell surface prior to its anterogradely transport to the growth cone (Ascaño *et al.*, 2009). Similar to our study, this previous work found that these recycled endosomes are associated with Rab11 and that expression of dominant negative Rab11 blocked recycling and transcytosis resulting in defects in axon growth. Rab11-dependent recycling has also been implicated in BDNF-TrkB-dependent dendrite formation and synaptic plasticity (Huang et al., 2013; Lazo et al., 2013). In future studies it will be interesting to determine whether Coronin-1 associates with this juxtamembrane region to facilitate TrkA recycling. In addition besides going back to the cell body, where else does the recycling TrkA go? One possibility is that TrkA returns to the axon via anterograde transport or is routed

to neighboring neurons after secretion via exosomes (Platta and Stenmark, 2011) (Figure 1). If NGF-TrkA exosomes exist, it is worthy to test the destination of the exosomes and whether Coronin-1 binds to them.

Figure 1 Possible output of NGF-TrkA signaling endosome- exosome

After endocytosis (1), the endocytic vesicle fuses with the early endosome (2). The recycling endosomes can be produced from the early endosome and then TrkA is re-expressed on cell surface (3). Alternatively the early endosome can form multivesicular bodies (4). The multivesicular bodies can fuse with the

lysosome to be degraded (5). In addition multivesicular bodies can also fuse with the plasma membrane to release the exosomes (6).

Possible interaction of Coronin-1 with Arp2/3 complex and cofilin for Axon growth

The coronin family of proteins is well known for its role in actin-related cellular functions such as migration and cell division (Föger et al., 2006, 2011; Gandhi et al., 2009). Based on our observation that Coronin-1 modifies signaling endosome stability and neurotrophin-dependent axon growth, it is possible that Coronin-1 cooperates with other actin modifiers within the actin network to impact axon growth.

The first candidate for a Coronin-1 interacting protein is the actin binding protein, Arp2/3 complex. It is a key component in modifying actin dynamics, being able to assemble the monomeric actin to actin filaments. It has been reported to contribute to clathrin-mediated endocytosis (Merrifield et al., 2004) and vesicular trafficking collaborating with the nucleation-promoting factor, WASH (Duleh and Welch, 2010). In neurons, it has been shown that inhibition of Arp2/3 decreases actin polymerization in the growing tip, the growth cone. Arp2/3 is critical for growth, protrusion and stabilization of the leading edge of the growing/protracting neuron (San Miguel-Ruiz and Letourneau, 2014).

In tissues other than neurons, the interaction of Coronin-1 and Arp2/3 has been shown. Coronin-1 has been shown to partly co-purify with Arp2/3 from neutrophils (Machesky et al., 1997), and Coronin-1B has been shown to bind with Arp2/3 in Rat2 fibroblasts (Cai et al., 2005). In mammalian T cells, Coronin-1 had a negative effect on F-actin formation through an Arp2/3-dependent mechanism (Föger et al., 2006a). In addition Arp2/3 complex is required for Coronin-1-dependent chemokine-mediated migration (Föger et al., 2006). This previous research alluded to the possibility of potential interactions in neurons. Additionally, because both signaling endosome stability and axon outgrowth require prompt and precise responsiveness of the dynamic actin cytoskeleton, it would be interesting to test whether Coronin-1 cooperates with Arp2/3 to modify the actin network in neurons (Figure 2). For example we can add an Arp2/3 inhibitor (CK-666) to our axon growth assay in absence of Coronin-1. If we see an axon growth rate like that seen in *Coro*^{-/-}, it means that Coronin-1 is independent of Arp2/3 complex for axon growth.

Another candidate to interact with Coronin-1 is ADF/cofilin, which contributes to dynamic actin turnover. ADF/cofilin plays a critical role in actin severing and depolymerization (Bamburg, 1999). However it does not regulate actin depolymerization alone, requiring other actin binding proteins for this process, including Aip1, twinfilin and Coronin (Kueh *et al.*, 2008). Depending on the ATP/ADP state of the actin filament, Coronin can influence the turnover of cofilin severing functions in yeast (Gandhi et al., 2009). In rat fibroblast, Coronin family

member, Coronin-1B, has been reported to associate with cofilin and the cofilin phosphatase, slingshot, all of which may interact to influence signaling from the endosome and actin dynamics (Cai et al, 2009). In the context of the signaling endosome formation and target innervation, actin depolymerization is indispensable during the process of signaling endosome formation and trafficking, as well as axon growth and branching (Harrington et al., 2011). Based on these previous data it is possible that Coronin-1 works with ADF/cofilin to promote various aspects of neurotrophin signaling during development (Figure 2). Additionally, it will be interesting to examine non-neurotrophin related functions of Coronin-1 in neurons such as adjusting endosome pH, because pH is related to the endosome stability (Harrington et al., 2011).

Figure 2 Regulation of actin during signaling endosome maturation hypothesis

Diagram shows the possible interactions between Arp2/3 complex, Coronin-1, slingshot and ADF/cofilin. Left diagram is the beginning process of endocytosis. Right diagram is the signaling endosome pinching off from the membrane. Yellow

spot is Coronin-1; grey spot is slingshot; brown square is cofilin; star is actin filaments; purple spot is neurotrophin.

Future directions

For the signaling endosome study, there are two aspects that should be explored in more detail. First we must examine how the constituents of the signaling endosome change with each spatio-temporal trafficking step. Understanding this promises to lend insight into the logic underlying the target-driven development of the peripheral nervous system. Secondly we must examine populations of neurons with more complex trophic dependencies as well as complex trajectories toward their targets such as somatosensory or motor neurons. These systems may include the re-use of RTKs like Ret, TrkC, or Met, at multiple stages of sensory, sympathetic, or motor neuron development. Moreover, understanding this process in peripheral axons with well-defined and relatively simple trajectories will inform more complex path-finding programs and trophic dependencies of neurons in the CNS.

NGF and NT3 have also been shown to differentially regulate growth cone size, which may be related to axon growth rate (Argiro et al., 1984; Bray and Chapman, 1985). As reported previously, growth cones of NT3-treated WT neurons were small and compact, whereas NGF-treated neurons displayed larger bulbous tips

(Harrington et al., 2011; Kuruvilla et al., 2004). Our current results are that *Coro1a*^{-/-} neurons treated with NT3 displayed large growth cones compared to very small growth cones observed in NT3-treated WT neurons. In contrast, we observe large NGF-dependent growth cone areas in WT neurons, which are decreased roughly 4-fold in neurons from *Coro1a*^{-/-} mice. These effects appear to be neurotrophin-dependent, because the absence of NT3 and NGF resulted in a lack of growth cones (commensurate with a retraction bulb) in both WT and *Coro1a*^{-/-} neurons. Given these preliminary data, further studies are warranted to better understand how Coronin-1 affects axon growth cone morphology and turning. These studies would address: 1) how different substrates affect axon growth cone morphology such as the cell adhesion molecule (CAM) L1 and laminin, and would 2) examine whether Coronin-1 mediates axon growth cone leading edge protrusion under NGF or NT3. Additionally, we would want to 3) examine whether NGF and NT3 stimulated axon growth involves Coronin-1 interaction with the Arp2/3 complex and what is 4) the role of Coronin-1 on actin retrograde flow in growth cone tips, using Dunn chambers and strip assays, to check the impact of Coronin-1 on growth cone turning and guidance. 5) The *in vitro* result, which Coronin-1 changes NT3 and NGF signaling pathways, is based on WT and *Coro*^{-/-} neurons, while *Coro*^{-/-} neurons have no Coronin-1 expression and WT neurons have Coronin-1 fully expressed. However it is highly possible that the situation will be different *in vivo*. I will determine the expression threshold of Coronin-1 for such signaling changes.

Chapter VI Methods

Antibodies

Antibodies were previously validated for the applications used, and the dilutions and applications were as follows: Coronin-1a (Abcam, Cambridge, UK, ab53395, 1:400 for immunohistochemistry); Tubb3 (Covance, Princeton, NJ, MMS-435P-250, 1:400 for immunohistochemistry); Phospho-p44/42 MAPK (Erk1/2) Mouse mAb (Cell Signaling, Danvers, MA, #9106, 1:1000 for western blot); pan-p44/42 MAPK(Erk1/2) antibody (Cell Signaling, Danvers, Massachusetts, #9102s, 1:1000 for western blot); Anti-Tyrosine Hydroxylase (Millipore, Billerica, MA, AB1542 1:130 for immunohistochemistry); horseradish peroxidase-conjugated donkey anti-sheep IgG (Fisher/Jackson ImmunoResearch, Waltham, MA NC9754415 1:250); anti-Flag m1 monoclonal antibody (Sigma, St. Louis, MO, F3040, 1:500)

Animals

Sprague Dawley rats were purchased from Harlan Laboratories (Indianapolis, IN). Sympathetic rat neurons were isolated from P0-P2 rat pups as previously described (Zareen and Greene, 2009).

All animals were maintained in a c57bl6 background. *NGF*^{-/-} (Crowley et al., 1994) and *Bax*^{-/-} (Knudson et al., 1995) genotyping and generation of *Bax*^{-/-}; *NGF*^{-/-} animals was described previously (Glebova and Ginty, 2004). *TrkA*^{Flag} animals were a generous gift from David Ginty and genotyping of this allele was

described previously (Sharma et al., 2010b). *Coronin1*^{-/-} mice were a generous gift from Jean Pieters and genotyping was performed as described previously (Jayachandran et al., 2007b). *TrkA*^{Flag};*Coronin-1*^{-/-} mice could be generated at Mendelian ratios and bred in the double homozygous state. 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.

Tissue culture and transfection

Sympathetic neuron cultures were established as described previously (Deppmann et al., 2008b). Briefly, neurons were obtained by dissociation of P0-P3 rat or mouse superior cervical ganglia. These neurons were plated in mass culture or compartmentalized microfluidic devices in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% Fetal Bovine Serum (FBS), penicillin/streptomycin (1U/mL), and 50 ng/mL of NGF purified from mouse salivary glands (Kuruvilla et al., 2004a; Park et al., 2006b). Glial contamination was removed from cultures using either 5 μ M cytosine arabinofuranoside (Ara-C) or aphidicolin for 48 hours and NGF concentrations were changed as indicated. For microfluidic devices, neurons were given time to project their axons to the outer chamber (2-4 day *in vitro*) and indicated treatments were performed for 36 hours. Sympathetic neurons were transfected using the Amaxa Nucleofector System (Lonza, Basel) according to manufacturer's protocol. Normal PC12 cells or PC12 cells stably expressing the TrkB/A chimeric protein (Harrington et al.,

2011b) were maintained in DMEM (Invitrogen, Carlsbad, CA), supplemented with 1 U/mL penicillin-streptomycin and 5% horse serum (Invitrogen, Carlsbad, CA). PC12 cells were transfected using lipofectamine 2000 (Promega, Madison, WI) according to manufacturer's instruction. All cells were cultured at 37 °C.

Co-immunoprecipitations and Immunoblot analysis.

Co-immunoprecipitation and immunoblot analysis was performed as previously described (Mandai et al., 2009). Briefly, sympathetic neurons or PC12 cells were harvested using RIPA (Tris 50mM, NaCl 150 mM, 0.1 % SDS, 0.5 % Na.Deoxycholate, 1% NP-40) containing protease inhibitor cocktail (Sigma, St. Louis, MO). Lysates were cleared by centrifugation at 14,000xg for 10 minutes at 4°C. Supernatants were then incubated with the indicated antibodies rocking at 4°C for 2 hours. Protein A/G sepharose was added and tubes were allowed to rock at 4°C for an additional 2 hours. Beads were washed 5 times with lysis buffer and protein was eluted by boiling in 2x Laemmli buffer (Boston Bioproducts, Ashland, MA, BP-110R) for 10 minutes. These samples were subjected to SDS-PAGE followed by western blot analysis using the indicated antibodies and either western blotting detection reagents ECL or Licor imaging system for visualization.

RT-PCR

RT-PCR was performed as described previously (Deppmann et al., 2008b). Briefly, RNA was isolated from mass sympathetic neuron cultures using Trizol (Invitrogen, Carlsbad, CA) as described by the manufacturer. First strand cDNA

was synthesized using random hexamers and the Superscript III system (Invitrogen, Carlsbad, CA). Primers that were used for RT-PCR are as follows:

GAPDH: F- CCCATCACCATCTTCCAGGA, R-
TTGTCATACCAGGAAATGAGC

Coronin-1: F: TTCCCTCAAGGATGGCTACGT R-
CCTCCAGCCTTGACACGGTAT

In Situ Hybridization

Probe preparation and *in situ* hybridization was performed as described previously (Deppmann et al., 2008b). Briefly, trunks from P0 mice of the indicated genotypes were snap frozen in Tissue-Tek CRYO-OCT compound (Thermo Fisher Scientific, Waltham, MA, 14-373-65). SCGs were cryo-sectioned at a thickness of 14 µm. Probes for Coronin-1, Coronin-2, and Coronin-3 were amplified from a cDNA library derived from SCGs and cloned into the pBK-CMV vector (Stratagene, La Jolla, CA). The primers used to clone Coronin-1, 2, and 3 *in situ* probes are as follows:

Coronin-1

F: GGCCTCGAGATGGCTCTGATCTGTGAGGC

R: GGCTCTAGAACGCTGTAGATTGTGTCCGG

Coronin-2

F: GGCCTCGAGAGCAGGGAGAACGTATCAGC

R: GGCTCTAGAACTTGGGAATTTGCCTTGG

Coronin-3

F: GGCCTCGAGTCAGGGCAGAGCATAGTATGG

R: GGCTCTAGAAGTTCACTGTCTTCCTCCGG

anti-Flag, EGF, Transferrin Feeding assays:

The TrkA-Flag feeding assay was performed as described previously (Sharma et al., 2010b). Briefly, the Flag M1 antibody was used to label distal axons of neurons isolated from *TrkA^{Flag}* mice for 20 minutes at 4°C. Unbound antibody was removed by rinsing distal axon compartments with growth medium and then medium containing NGF (50 ng/mL). Cells were placed at 37°C for the indicated amounts of time to induce internalization and retrograde transport. Cells were then fixed with 4% paraformaldehyde and internalized TrkA was visualized by anti-Flag immunostaining. A similar protocol was used to assess EGF and transferrin internalization. In this case fluorescent EGF or transferrin were used, which negated the need for further immunostaining. To assess lysosome localization, LysoTracker (Invitrogen, Carlsbad, CA, L-7528) was added 2 hours prior to fixation on the cell body side of microfluidic devices. Co-localization between endosomes and lysosomes was assessed via confocal microscopy (Zeiss 780 NLO System, 63X) and blinded, manual quantification.

Microfluidic devices

Microfluidic devices were generated as described previously (Park et al., 2006a). These chambers were fixed to coverglass which were coated with poly-D-lysine

(50 µg/mL) and laminin (1µg/mL) and washed with water. Total volume differential between the two compartments was maintained at 100 µL to ensure fluidic isolation.

In vitro survival assays

For cell survival assays in chambers, fluorescent microspheres (Invitrogen, Calsbad, CA) were added to distal axons 24 hours prior to scoring cell survival via Hoechst staining (Deppmann et al., 2008b). For experiments where NGF deprivation was required, anti-NGF and BOC-ASP(OMe)-FMK (BAF) (MP Biomedicals, Santa Ana, CA, 03FK01105) were used to neutralize remaining NGF and keep cells alive, respectively. For mass cultures, the same procedure is followed with no microspheres. Images of Hoechst staining were acquired and blinded for unbiased quantification. Dead versus alive neurons were scored as described previously (Deckwerth and Johnson, 1993). The survival rate of top concentration NGF on wild type neurons was set to 1 and all other conditions are relative to that.

Immunocytochemistry:

Immunocytochemistry and immunohistochemistry were performed as described previously (Sharma et al., 2010b). Neurons or sections were fixed with 4% PFA and blocked/permeabilized (blocking buffer: 5% goat serum, 0.05% Triton-x-100 in PBS) for 30 minutes at room temperature. Cells were then incubated overnight at 4°C with primary antibody diluted in blocking buffer. Cells were then

washed 3x with PBS and incubated with fluorescent-conjugated secondary antibody for 1 hour at room temperature followed by 3 washes in PBS and mounting in Vectashield Fluoromount (Thermo Fisher Scientific, Waltham, MA, H-1200). Fluorescence was visualized using inverted confocal microscopy (Zeiss 780 NLO System, 63X) and 3D reconstructed images were made in ImageJ (<http://imagej.nih.gov/ij/>).

Calcium imaging

Calcium imaging was performed as previously described (Gomez et al., 2001). Briefly, neurons grown on cover glass were loaded with Fluo-4 calcium indicator (Invitrogen, Carlsbad, CA, F14201) (3 μ M) for 20 minutes at 37°C (5% CO₂). The neurons were then moved to an environmental chamber (37°C, 5% CO₂) mounted on an inverted confocal microscope. Basal calcium levels were assessed by randomly imaging 6-10 cells per coverslip prior to treatment. Indicated concentrations of NGF or NT3 were added to neurons and allowed to incubate for 15 minutes. The previous 6-10 neurons were imaged again to measure their calcium levels after treatment. We determined the change calcium levels by F/F₀ to quantify the calcium intensity. F₀ is the 0ng/mL intensity and F is the intensity of different NGF treatment. Conditions without neurotrophin treatment were set to 1 and all other conditions are represented relative to that value.

In vivo sympathetic neuron counts

SCG neuron number was quantified as described previously (Deppmann et al., 2008b). Briefly, trunks from P0 mice were snap frozen in OCT and cryo-sectioned at a thickness of 10 μ m. Every 5th section was processed for Nissl staining. Images were acquired, blinded, and quantified as previously described (Chen et al., 2005a).

Biochemical analysis of endosomes

Magnetic isolation of newly internalized vesicles was performed using a colloidal suspension of ferric oxide particles having an average diameter of 10nm (Liquids Research Ltd., Bangor, UK). Approximately 20 million TrkB/A cells were suspended in 9 mL of DMEM and 1 mL ferrofluid suspension. To one sample, 50ng/mL BDNF was added and cells were incubated at 37°C for 15 minutes. Cells were then pelleted, homogenized and processed as described previously to obtain a crude endosomal fraction (Harrington et al., 2011b). Samples were then placed adjacent to a magnet for 1 hour and ferrofluid containing endosomes were isolated and prepared for SDS-PAGE by the addition of Laemmli sample buffer. Discontinuous sucrose gradients used to separate early and late endosomal compartments were performed exactly as described previously (Harrington et al., 2011).

Axon growth assay

P0-P3 sympathetic superior cervical ganglia neurons were plated in the cell body chamber of the microfluidic device in the presence of NGF and aphidicolin (5 μ M).

After 2-3 days axons emerged from the axon chamber of the microfluidic device at which time NGF was deprived for 17 hours by adding anti-NGF and BAF (5 μ M). The axon chamber medium was then changed to anti-NGF (1 μ g/ml), NGF (2ng/ml) or NT3 (100ng/ml) (Millipore, Billerica, MA, GF031); and images of the entire distal axon chamber field were acquired at this 'zero time point'. After 24 hours, images of the axons were repeated and axon growth rates were quantified as the difference between the two datasets (Figure 1A)

Growth cone staining

Coverslips were coated with laminin (40 μ g/ml) for 2 hours before plating. P0-P3 sympathetic cervical neurons were plated in mass culture with anti-NGF (1 μ g/ml), NGF (2ng/ml) or NT3 (100ng/ml) (Millipore, Billerica, MA, GF031). After 1 day in culture at 37°C neurons were fixed with 4% PFA containing Ca^{2+} (1.5mM) and Mg^{2+} (1mM). Double immunostaining was then performed with Tuj1 and Rhodamine Phalloidin (Life Technologies, Carlsbad, CA, R-415 1:400 for immunohistochemistry). Images were taken under confocal microscope fitted with 63X objective (Zeiss 780 NLO System).

Branch assay

P0-P3 sympathetic cervical neurons were plated in mass culture under NGF (2ng/ml), NT3 (100ng/ml) or other treatment as indicated. After 1 day culture at 37°C, neurons were fixed with 4% Paraformaldehyde (PFA). Immunostaining was then performed with Tuj1. Images were taken under confocal microscope fitted with 10X objective (Zeiss 510 Meta System) and analyzed by Sholl analysis

(Magariños et al., 2006).

Whole-Mount Tyrosine Hydroxylase Immunohistochemistry

Whole-mount tyrosine hydroxylase (TH) immunohistochemistry was performed as previously described (Enomoto et al., 2001). Briefly samples were incubated with sheep anti-TH affinity-purified polyclonal antibody (Millipore) at 0.5µg/ml for 72 hours at 4°C. Next horseradish peroxidase-conjugated donkey anti-sheep IgG (4µg/µl) was applied overnight at 4°C. Next 3,3'-Diaminobenzidine tetrahydrochloride (Sigma, St. Louis, MO, D5905-50TAB, 1 tablet :20ml for staining) was applied for visualization.

Microfluidic devices

The microfluidic devices were used as previously described (Park et al., 2006b). Chambers were attached to glass cover slips coated with poly-D-lysine (50µg/ml), laminin (1µg/ml) and washed with H₂O. A 100µl volume difference was maintained between two compartments to ensure fluidic isolation.

Statistical Analysis

Statistical analysis was performed in Graphpad Prism (www.graphpad.com/). Significance was determined by unpaired two-tailed Student's t-test. Error bars represent standard error of the mean.

Appendix I Phosphorylation of dynein intermediate chain is necessary for sympathetic neuron retrograde cell death

Dynein has been reported to play a role in long-distance neurotrophin survival signaling (Heerssen et al., 2004). The Pfister lab found that neurotrophin can stimulate dynein intermediate chain (IC) phosphorylation (Mitchell et al., 2012). When the IC is dephosphorylated, there was less dynein bound to Trk endosomes. In collaboration with Juyeon Park, I examined the role of this phosphorylation event (at serine 80) on NGF-dependent survival of sympathetic neurons. Toward this end, we made use of cultured sympathetic neurons whose survival requires transport of NGF-TrkA endosomes. When cells were transfected with WTIC-1B-mRFP, there was no effect on cell survival compared to control transfected cells (Figure 1). However, when cells were transfected with the dephosphomimic mutant IC-1B-S80A-mRFP, a significant decrease in NGF-dependent neuronal survival was observed. Expression of an IC that cannot be phosphorylated on this specific site has a dominant-negative effect on neuronal survival. This observation supports a role for phosphorylation of this site in recruiting dynein to Trk-containing signaling endosomes.

Figure 1. Expression of the dephospho-IC-1B S80A mutant in sympathetic neurons reduces

NGF-dependent cell survival. Sympathetic neurons were cultured as described and transfected with either the IC-1B WT or S80A dephospho mutant by nucleofection, and the survival of the transfected cells in the presence of antibody to NGF or presence of 20 ng/ml NGF was determined. When the S80A mutant was expressed, there was a 45% decrease in cell survival relative to WT transfected cells in the presence of NGF ($p < 0.003$, Student's *t* test, highly significant). Green, Control transfected cells; yellow, WT IC-1B-mRFP transfected cells; blue, IC-1B-S80A transfected cells. Error bars indicate SEM.

Appendix II Mathematical modeling

(Collaborated with Dr. Stefan Mihalas, Allen Brain Institute)

1 Model Description

We follow the same model for TrkA production and activation as that described previously (Deppmann et al., 2008b) and provide a new description for TrkA protection from degradation and expedited degradation linked to paracrine punishment. For ease of understanding, the essential modeling elements of TrkA activation and production described previously are reproduced here.

1.1 Model Equations

A simplified description of TrkA production, activation and signal duration in neurons is to consider only as two states for TrkA: free ($TrkAf$) and NGF-bound, or activated ($TrkAa$). TrkA is synthesized in the free or inactive form, is activated upon NGF binding, and degraded in both the active and inactive forms, which effects signal duration.

$$\begin{aligned}
 TrkAf_i'(t) &= Vsyn_i(t) - kon \times NGF(t) \times TrkAf_i(t) + koff \times TrkAa_i(t) - Vdepf_i(t) \times TrkAf_i(t) \\
 TrkAa_i'(t) &= kon \times NGF(t) \times TrkAf_i(t) - koff \times TrkAa_i(t) - Vdepa_i(t) \times TrkAa_i(t) \\
 NGF(t) &= pr - V_{depNGF} \times NGF(t) - volR \times \sum_i (kon \times NGF(t) \times TrkAf_i(t) - koff \times TrkAa_i(t))
 \end{aligned}$$

(1)

where:

$TrkAf_i$ - concentration of free TrkA in cell i

$TrkAa_i$ - concentration of active TrkA in cell i

NGF - concentration of NGF in the extracellular space

$Vsyn_i(t)$ - rate of TrkA production in cell i

$Vdepf_i(t)$ - rate constant of free TrkA degradation in cell i

$Vdepa_i(t)$ - rate constant of active TrkA degradation in cell i

kon - rate constant of NGF binding to TrkAf

$koff$ - rate constant of NGF dissociation from TrkAa

pr - rate of NGF production

V_{depNGF} - rate constant of NGF depletion

$volR$ - ratio between the volume of a neuron and the volume of the target tissue

Association and dissociation between NGF and TrkA are much faster processes than TrkA synthesis and degradation which leads to quasistatic equilibrium for free and active TrkA. If we assume that at every time the quasistatic equilibrium for active TrkA is reached for every neuron:

$$TrkAa_{i,adiab}(t) = \frac{NGF(t) \times kon}{koff + Vdepf} TrkAf_{i,adiab}(t)$$

To express free and active TrkA as a function of total TrkA in neuron i:

$$\begin{aligned} TrkA_{i,adiab}(t) &= \frac{TrkA_{i,adiab}(t)}{1 + \frac{koff + Vdepf}{NGF(t) \times kon}} \\ TrkA_{i,adiab}(t) &= \frac{TrkA_{i,adiab}(t)}{1 + \frac{NGF(t) \times kon}{koff + Vdepf}} \end{aligned} \quad (2)$$

Using (2) the system of equations (1) reduces to:

$$\begin{aligned} TrkA_i'(t) &= Vsyn_i(t) - TrkA_i(t) \times \left(\frac{Vdepf_i(t)}{1 + \frac{NGF(t)}{Kd}} + \frac{Vdepa_i(t)}{1 + \frac{Kd}{NGF(t)}} \right) \\ NGF(t) &= pr - V_{depNGF} \times NGF(t) - \frac{volR}{1 + \frac{Kd}{NGF(t)}} \times \sum_i (Vdepa_i(t) \times TrkA_i(t)) \end{aligned} \quad (3)$$

where: $Kd = koff/kon \cong (koff + Vdepa)/kon$

Every term in (3) has an intuitive meaning. The equation for TrkA time derivative consists of a term characterizing synthesis, and two terms characterizing depletion: the first for depletion of free and the second for depletion of active TrkA. The equation for free NGF time derivative consists of a production term, an NGF depletion term in the target tissue, and a term equal to the sum of all active TrkA degradation in all neurons. Active TrkA degradation contributes to total NGF depletion.

The approximation used in equation (3) is equivalent to neglecting the effect that active TrkA accumulation in neurons has on the free NGF concentration. This approximation is valid if the quantity of free NGF is large compared to the quantity of NGF bound to TrkA. A less stringent approximation for the quasi-static equilibrium can be used. At any time, the solution of (3) is a small perturbation from the quasi-static equilibrium:

$$TrkAa_i(t) = (1 + \alpha_i(t)) kon / (koff + Vdepf) \times NGF(t) \times TrkAf_i(t) \quad (4)$$

where $|\alpha_i(t)| \ll 1$. By using this relationship between $TrkAf$ and $TrkAa$, an equation for $\alpha_i(t)$ is obtained for each neuron i . Using the approximations that $|NGF'(t)/NGF(t)| \ll |TrkAf'_i(t)/TrkAf_i(t)|$ and $|\alpha'_i(t)| \ll |TrkAf'_i(t)/TrkAf_i(t)|$ this equation can be solved:

$$\alpha_i(t) = \left(\frac{Vsyn}{koff \times TrkAa_i(t)} + \frac{Vdepa - Vdepf}{kon \times NGF(t)} \right) / \left(1 + \frac{Kd}{NGF(t)} \right) \quad (5)$$

The set of equations describing the evolution of the system becomes:

$$\begin{aligned} TrkA'_i(t) &= Vsyn_i(t) - TrkA_i(t) \times \left(\frac{Vdepf_i(t)}{1 + \frac{Kd}{NGF(t)}} + \frac{Vdepa_i(t)}{1 + \frac{Kd}{NGF(t)}} \right) \\ NGF'(t) &= pr - V_{dep} NGF \times NGF(t) - \frac{volR}{1 + \frac{Kd}{NGF(t)}} \times \sum_i \left(Vsyn_i + \frac{Vdepa_i(t) - Vdepf_i(t)}{1 + \frac{Kd}{NGF(t)}} \times TrkA_i(t) \right) \end{aligned} \quad (6)$$

The system of equations (6) is similar (3), the only difference is in the term for NGF depletion. Equation (6), in addition to the NGF depletion term caused by degradation of active TrkA, also contains a term to account for NGF depletion caused by active TrkA accumulation. Intuitively, the change in free NGF caused by TrkA in neuron i in a time interval is equal to the amount of active TrkA which was degraded, and the change in the quantity of active TrkA in that time interval. Infinitesimally this is:

$$\begin{aligned}
 V_{depNGFTrkA_i} &= volR \times \left(\frac{V_{depa_i}(t) \times TrkA_i(t)}{1 + \frac{Kd}{NGF(t)}} + \frac{TrkA_i'(t)}{1 + \frac{Kd}{NGF(t)}} \right) = \\
 &= \frac{volR}{1 + \frac{Kd}{NGF(t)}} \left(V_{syn_i}(t) - TrkA_i(t) \times \left(\frac{V_{depf_i}(t)}{1 + \frac{Kd}{NGF(t)}} + \frac{V_{depa_i}(t)}{1 + \frac{Kd}{NGF(t)}} \right) - V_{depa_i}(t) \times TrkA_i(t) \right) = \\
 &= \frac{volR}{1 + \frac{Kd}{NGF(t)}} \times \left(V_{syn_i} + \frac{V_{depa_i}(t) - V_{depf_i}(t)}{1 + \frac{Kd}{NGF(t)}} \times TrkA_i(t) \right)
 \end{aligned}$$

which is exactly the term obtained in (6).

1.2 Synthesis of TrkA

We observed a positive feedback loop in which NGF activates the expression of its own receptor, TrkA. If TrkA directly activates its own expression, the production term will depend on the concentration of active TrkA as expressed via a Hill function.

$$V_{syn_i}(t) = V_{syn0} + \frac{V_{syn1}}{1 + \left(\frac{k_{syn}}{TrkAa_i(t)} \right)^h} \quad (7)$$

where

V_{syn0} - rate of TrkA synthesis in the absence of NGF

V_{syn1} - additional rate of TrkA synthesis in the presence of saturating NGF

k_{syn} - concentration of active TrkA at which the TrkA-dependent expression is at half-maximal rate

h - Hill coefficient

Using (7) the system of equations (6) describing N neurons becomes:

$$TrkA_i'(t) = V_{syn0} + \frac{V_{syn1}}{1 + \left(\frac{k_{syn} \left(1 + \frac{Kd}{NGF(t)} \right)}{TrkA_i(t)} \right)^h} - TrkA_i(t) \times \left(\frac{V_{depf_i}(t)}{1 + \frac{NGF(t)}{Kd}} + \frac{V_{depa_i}(t)}{1 + \frac{Kd}{NGF(t)}} \right)$$

$$NGF(t) = pr - V_{depNGF} \times NGF(t) - \frac{volR}{1 + \frac{Kd}{NGF(t)}} \times \sum_i (V_{depa_i}(t) \times TrkA_i(t))$$

(8)

In order to reduce the number of parameters $TrkAa$ and NGF were normalized by their half-activation values:

$$TrkAn_i'(t) = V_{syn0n} + \frac{V_{syn1n}}{1 + \left(\frac{1}{\frac{NGFn(t)}{TrkAn_i(t)}} \right)^h} - TrkAn_i(t) \times \left(\frac{V_{depf_i(t)}}{1 + NGFn(t)} + \frac{V_{depa_i(t)}}{1 + \frac{1}{NGFn(t)}} \right)$$

$$NGFn'(t) = prn - V_{depNGF} \times NGFn(t) - \frac{volRn}{1 + \frac{1}{NGFn(t)}} \times \sum_i (V_{depa_i(t)} \times TrkAn_i(t))$$

(9)

where:

$$\begin{aligned} TrkAn_i(t) &= TrkAa_i(t)/ksyn \\ V_{syn0n} &= V_{syn0}/ksyn \\ V_{syn1n} &= V_{syn1}/ksyn \\ NGFn(t) &= NGF(t)/Kd_{NGF} \quad (10) \\ prn &= pr/Kd_{NGF} \\ volRn &= volR \times ktr/Kd_{NGF} \end{aligned}$$

If the Hill coefficient h is strictly larger than 1 it is possible to obtain multiple stable solutions for TrkA concentrations from equation (9). However, TrkA expression levels during development are best fit using Levenberg-Marquardt if TrkA expression depends on its concentration via a Hill function with a coefficient 1.

1.3 TrkA Protection from Degradation

We consider a mechanism in which endosomal TrkA leads to expression of Coronin-1 with a half activation value of ka_{Trk} and a turnover number of kp_{Cor} . Coronin-1 enters a protective protein complex which can bind to endosomal TrkA with an on rate k_2 and unbinds with km_2 . Coronin-1 unbound to endosomal TrkA

and the protein complex degrades with a rate k_{depCor} . In the absence of the protective complex, $TrkAau(t)$ is depleted at a rate k_{lys} .

$$\begin{aligned}
 TrkAau'(t) &= -k_2 \times Cor(t) \times TrkAau(t) - k_{lys} \times TrkAau(t) + k_{m2} \times TrkAaCor(t) \\
 TrkAaCor'(t) &= k_2 \times Cor(t) \times TrkAau(t) - k_{m2} \times TrkAaCor(t) \\
 Cor'(t) &= -k_2 \times Cor(t) \times TrkAau(t) + k_{m2} \times TrkAaCor(t) \\
 &\quad - k_{depCor} \times Cor(t) + k_{pCor} \frac{TrkAau(t) + TrkAaCor(t)}{k_a Trk + TrkAau(t) + TrkAaCor(t)}
 \end{aligned} \tag{11}$$

Similarly to the TrkA production, we use an adiabatic approximation in which Coronin-1 protein complex binding and unbinding from endosomal TrkA is much more rapid than its depletion. The concentration of endosomal TrkA in the absence of the protective complex, $TrkAau$ can be expressed as a function of the endosomal concentration, $TrkAa$:

$$TrkAau = TrkAa \frac{k_a Trk + TrkAa}{k_a Trk + TrkAa + k_{Cor} \times TrkAa} \tag{12}$$

where:

$$k_{Cor} = \frac{k_2 \times k_{pCor}}{k_{m2} \times k_{depCor}} \tag{13}$$

Implementing this approximation (12) leads to a depletion term in (9)

$$V_{depa_f}(t) = k_{Lys} \frac{1 + k_a Trkn / TrkAn \times (1 + 1/NGFn)}{1 + k_{Cor} + k_a Trkn / TrkAn \times (1 + 1/NGFn)} \tag{14}$$

where $k_a Trkn = k_a Trk / k_{syn}$ represents the half activation value of Coronin-1 expression relative to the half activation value of TrkA expression.

1.4 Punishment Signal

One possible mechanism to expedite the competition is to consider a punishment mechanism in which the neurons with high trophic signal strength contribute to the death of those with low trophic signal strength. The punishment factors are produced by sympathetic neurons and we assume their production rate is proportional to their trophic signal strength while their depletion is linear with concentration:

$$PUN'(t) = \sum_i \left(\frac{Pr_{PUN}}{1 + \frac{Kd_{PUN}(1 + 1/NGFn(t))}{TrkAn_i(t)}} \right) - V_{PUN} \times PUN(t) \quad (15)$$

where: $PUN(t)$ - concentration of the punishment signal in the sympathetic ganglia. The punishment signals: BDNF, NT3, NT4 bind to and activate the p75 receptor. We normalize the concentration of punishment signals to its dissociation constant from the p75 receptor. This equation introduces three new free parameters:

Pr_{PUN} - maximal rate of punishment signal production by a neuron

Kd_{PUN} - concentration of active TrkA which leads to half-activation of punishment production

V_{PUN} - rate constant of punishment signal depletion.

1.5 lysosome activation

In the equation describing the protection from degradation by the Coronin-1 protein complex (14), the lysosomal activation is no longer considered a constant but rather it is assumed to be dependent on the punishment signal (15)

$$kLys(t) = Lys \left(kLys0 + (1 - kLys0) \times \frac{PUN(t)}{1 + PUN(t)} \right) \quad (16)$$

By adding the punishment term (16) and its protection (14) to TrkA equations (9), and complementing the system of equations with the time dependence of the punishment signal (15), the full set of equations characterizing the competition with punishment is:

$$\begin{aligned} TrkAn_i'(t) = & Vsyn0n + \frac{Vsyn1n}{1 + \left(\frac{1 + \frac{1}{NGFn(t)}}{TrkAn_i(t)} \right)^h} - TrkAn_i(t) \times \frac{Vdepf0}{1 + NGFn(t)} \\ & - Lys(kLys0 + (1 - kLys0) \frac{PUN(t)}{1 + PUN(t)}) \frac{TrkAn_i(t)/(1 + 1/NGFn(t)) + kaTrkn}{1 + kCor + (1 + 1/NGFn(t)) \times kaTrkn / TrkAn_i(t)} \\ NGFn'(t) = & prodn - V_{depNGF} \times NGFn(t) - \\ & - volRn \times Lys(kLys0 + (1 - kLys0) \frac{PUN(t)}{1 + PUN(t)}) \sum_i \left(\frac{TrkAn_i(t)/(1 + 1/NGFn(t)) + kaTrkn}{1 + kCor + (1 + 1/NGFn(t)) \times kaTrkn / TrkAn_i(t)} \right) \\ PUNn'(t) = & \sum_i \left(\frac{Pr_{PUNn}}{1 + \frac{Kd_{PUN}(1 + 1/NGFn(t))}{TrkAn_i(t)}} \right) - V_{PUN} \times PUNn(t) \end{aligned} \quad (17)$$

This set of equations is the basis for all in-vivo simulations.

2 Parameter Values

2.1 Initial concentrations

The final surviving neurons have a TrkA concentration close to the half activation value, and roughly 50% of the neurons survive, corresponding to an average *TrkAan* of 0.5. Measurement of TrkA levels following NGF treatment show a maximal change of a factor roughly 10. Therefore the initial distribution chosen for *TrkAan* has a mean of 0.05. We chose the initial distribution to be normal and with a standard deviation ten times smaller than the mean. Varying the initial distribution does not lead to large variations in the final neuron number (Figure S6 A-D), however to reproduce the developmental stage in which average *TrkAan* has an exponential growth it needs to start at values $\ll 1$. The time 0 of the simulation corresponds to embryonic day 15, when the axons of the sympathetic neurons arrive at target.

We assume that the concentration of NGF in the target tissue is on the same order of magnitude as its affinity for TrkA. Specifically, we considered [NGF] to be 0.5 times its dissociation constant from TrkA. Varying starting [NGF] does not lead to large variations in the final neuron number (Figure 6S E-H). The initial punishment signal concentration is assumed to be zero.

2.2 Regulation of TrkA Expression

For TrkA expression, the previously fit value for its dependence on its own concentration of $V_{syn}1n=4/day$ was used. The simulation results depend strongly on this value (Figure S6 I-L). Previous fits constrained the value of basal TrkA to

values near zero. We used $V_{syn0n}=10^{-3}/day$. While this value stays small, the simulation results are practically independent of its value (Figure S6 I-L).

2.3 NGF

The value used for normalized in/out volume ratio of one neuron to the target tissue is $vol/Rn=1/n_0$. The normalized rate of NGF production, $pr=2$ is chosen such that roughly 50% of the initial $n_0=100$ neurons survive. NGF production has a linear effect on the number of neurons which survive the competition (resulting in the target matching hypothesis which is discussed below).

2.4 Punishment Signal

The concentration of active TrkA which leads to half-activation of punishment signal expression is considered the same as the concentration of active TrkA which leads to half-activation of TrkA expression: $Kd_{PUN}=1$. The relative concentration of punishment signal is more important and thus we normalize its production: $Pr_{PUNn}/V_{PUNn}=1/n_0$. Changes in parameters characterizing the punishment signals produce only modest changes in final neuron number but substantial changes in the speed of competition (Figure S6 Q-T)

2.5 Punishment and Protection of TrkA

As with all TrkA dependent signals, the concentration of TrkA which leads to half-activation of the Coronin-1 expression is considered the same as the concentration of active TrkA which leads to half-activation of TrkA expression:

$k_a Trk_n = 1$. The fraction of lysosomal activation in the absence of punishment was chosen to be very small $lys_0 = 0.02$, but its value does not affect the competition as long as it remains small. The degradation of free TrkA is assumed to also be small $V_{dep} f_0 = 1/day$. Its value has little influence on the final neuronal survival fraction, but it does change the competition time (Figure S6 U-X).

To estimate the parameters for maximal lysosomal activation Lys and the protection mechanism $kCor$ the time course of endosome-lysosome binding was fit. Simulations show a highly nonlinear process, which matches the sharp sigmoidal experimental values. At the beginning of the experiment, after a strong NGF induction the number of endosomes is high, leading to both high lysosomal degradation and protection mechanisms. Since the protection levels are high, very few endosomes are degraded. When the number of endosomal TrkA drops below a critical value, a self-reinforcing process takes place: a lower endosomal TrkA leads to smaller protection signal which in turn leads to an increasing level of TrkA degradation. This exponential growth in the rate of degradation is apparent after approximately 24 hour in wild-type and 12 hours in Coronin-1 knockout neurons. The time until this exponential increase in endosomal degradation occurs is one of the most important parameters in the model. Interestingly even in the Coronin-1 knockouts the exponential decay does not start immediately, but rather after a 12 hour delay. Thus partial protection of endosomal TrkA exists even in the absence of Coronin-1. Under the adiabatic approximation (12) it is possible to implicitly fit them, using Levenberg-Marquadt

method the solution to the time dependence of the lysosomal fraction. The values for these parameters modestly depend on the chosen value for the plateau. If the inaccessible fraction is 0.2, the best fit values are $Lys=3.9/hour$ and $kCor=62$. If the inaccessible fractions is 0.25, the best fit values are $Lys=2.9/hour$ and $kCor=40$. Simulations were performed using the dynamic system without adiabatic approximation to test this rough estimates, and values which were consistent with both lysosomal fraction experiment and well as pAKT depletion were $Lys=3.5/hour$ and $kCor=40$. The maximal lysosomal activation by the punishment mechanism affects both the neuronal survival fraction as well as the speed of the competition (Figure S6 Y-B2). Simulations of the same dynamic system fitting the same data but from *Coronin-1*^{-/-} mice were consistent with both lysosomal fraction experiment and well as pAKT depletion produce $kCor_{CorKO}=20$. The influence of the protection parameters on neuronal survival fraction is large.

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