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# Delineating neurotrophin-3 dependent signaling pathways underlying sympathetic axon growth along intermediate targets

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## Abstract

Postganglionic sympathetic neurons detect vascular derived neurotrophin 3 (NT3) via the axonally expressed receptor tyrosine kinase, TrkA, to promote chemo-attraction along intermediate targets. Once axons arrive to their final target, a structurally related neurotrophic factor, nerve growth factor (NGF), also acts through TrkA to promote final target innervation. Does TrkA signal differently at these different locales? We previously found that Coronin-1 is upregulated in sympathetic neurons upon exposure to NGF, thereby endowing the NGF-TrkA complex with new signaling capabilities (ie calcium signaling), which dampens axon growth and branching. Based on the notion that axons do not express functional levels of Coronin-1 prior to final target innervation, we developed an *in vitro* model for axon growth and branching along intermediate targets using *Coro1a*$^{-/-}$ neurons grown in NT3. We found that, similar to NGF-TrkA, NT3-TrkA is capable of inducing MAPK and PI3K in the presence or absence of Coronin-1. However, unlike NGF, NT3 does not induce calcium release from intracellular stores. Using a combination of pharmacology, knockout neurons and *in vitro* functional assays, we suggest that the NT3-TrkA complex uses Ras/MAPK and/or PI3K-AKT signaling to induce axon growth and inhibit axon branching along intermediate targets. However, in the presence of Coronin-1, these signaling pathways lose their ability to impact NT3 dependent axon growth or branching. This is consistent with a role for Coronin-1 as a molecular switch for axon behavior and suggests that Coronin-1 suppresses NT3 dependent axon behavior.

### Keywords

Neurotrophin 3 (NT3); TrkA; RAS/MAPK; PI3K; axon growth

## 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

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(Baljet and Drukker, 1980; Kummer, 1992; Makita et al., 2008; Pardini et al., 1989). Growth of sympathetic axons along vascular intermediate targets is mediated by Neurotrophin- 3 (NT3) and final target innervation is mediated by a paralog, nerve growth factor (NGF) (Brennan et al., 1999; Francis et al., 1999; Harrington and Ginty, 2013; Kuruvilla et al., 2004). Both NT3 and NGF signal through the receptor tyrosine kinase (RTK), TrkA (Belliveau et al., 1997; Kuruvilla et al., 2004).

Neurotrophin-dependent signaling is at least as dynamic as the axonal behaviors it mediates. Axons growing along blood vessels likely experience signaling that suppresses branching while allowing growth (Carmeliet, 2003). Once axons reach their final target, these NT3 dependent axon behaviors must become muted, in order to accommodate growth properties consistent with rapid arborization within the final target (Shirasaki et al., 1998). In order to achieve this diversity of axon growth behaviors, we suggest that neurotrophin (NT3 versus NGF) signaling must be differentially interpreted at each stage of target innervation, especially between intermediate and final targets,.

Upon sympathetic final target innervation, one of the most robustly up-regulated genes is *Coronin-1* (Deppmann et al., 2008; Suo et al., 2014). Prior to NGF dependent Coronin-1 upregulation, we previously found that NGF-TrkA-PI3K signaling promotes robust axon growth and branching as axons first enter their final targets. Upon Coronin-1 upregulation, NGF-TrkA-calcium signaling dampens axon growth and branching, which is a behavior consistent with axons finding termination zones. In the absence of *Coro1a,* sympathetic neurons display excessive branching and overshoot their final targets (Suo et al., 2015). How NT3-TrkA signaling is different from these scenarios as axons grow along vasculature toward their final targets remains an open question. A comparison of the signaling pathways that NGF and NT3 can initiate via TrkA may shed light on this.

The finding that final target innervation (NGF exposure) is required for Coronin-1 expression in developing sympathetic neurons also implies that Coronin-1 is not expressed as sympathetic axons move along their intermediate targets (NT3 exposure) (Suo et al., 2015). Indeed, immunohistochemistry in the developing superior cervical ganglia (SCG) 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). While it is possible to dissect sympathetic neurons prior to final target innervation, establishing these sympathetic neurons in microfluidic devices in the absence of NGF results in neuron death. Interpretation of NT3 signaling must account for the upregulation of Coronin-1 upon NGF exposure (Suo et al., 2014). Based on these findings and limitations, we reasoned that *Coro1a*$^{-/-}$ neurons grown in NT3 would represent a more accurate *in vitro* model for axon growth along intermediate targets compared to neurons isolated from wild-type mice. In this model, *Coro1a*$^{-/-}$ neurons emulate neurons prior to NGF exposure while wild-type neurons having already been exposed to NGF *in vivo* emulate neurons in a later stage of development. Using this model, we were able to uncover fundamental differences between NT3-TrkA and NGF-TrkA dependent axon growth.

Here we show that the NT3-TrkA complex, in the absence of Coronin-1, enabled axon behaviors suited for growth along intermediate targets exhibiting: larger growth cones, moderate axon growth, and suppressed branching. This is in contrast to what we have previously reported for NGF-TrkA in the absence of Coronin-1, which results in smaller growth cones, increased axon growth, and exuberant branching (Suo et al., 2015). We further characterized the signaling pathways underlying these behaviors. Unlike NGF, NT3 is unable to evoke a calcium release regardless of Coronin-1 expression. Similar to NGF-TrkA, NT3-TrkA is capable of inducing MAPK and PI3K, however these pathways appear to be used in a different manner in the context of growth and branching. For instance, NT3-TrkA requires the MAPK pathway to promote axon growth and suppress axon branching, but only in the absence of Coronin-1; whereas MAPK signaling was dispensable for NGF-TrkA axon behaviors (Suo et al., 2015). In the absence of Coronin-1, NGF dependent PI3K signaling promotes exuberant axon branching and growth whereas NT3 dependent PI3K activity suppresses branching while retaining the ability to promote growth (Suo et al., 2015). These data support a model whereby NT3 drives axon growth and branch suppression in a MAPK/PI3K-dependent manner. However, when Coronin-1 is present NT3 dependent MAPK/PI3K no longer effects axon growth. These findings re-enforce the notion that Coronin-1 represents a molecular switch that allows differential interpretation of neurotrophin signaling capable of driving 3 distinct profiles of axon growth behavior.

## Materials and Methods

### Reagents

Antibodies were previously validated for the applications used. The dilutions and applications were as follows: Coronin-1a (Abcam, ab53395, 1:400 for immunohistochemistry); Tubb3 (Covance, MMS-435P- 250, 1:400 for immunohistochemistry); Rhodamine Phalloidin (Life Technologies, R-415 1:400 for immunohistochemistry); Phospho-p44/42 MAPK (Erk1/2) Mouse mAb (Cell signaling, #9106, 1:1000 for western blot); pan-p44/42 MAPK(Erk1/2) antibody(Cell signaling, #9102s, 1:1000 for western blot); Anti-Tyrosine Hydroxylase (Millipore, AB1542 1:130 for immunohistochemistry); horseradish peroxidase-conjugated donkey anti-sheep IgG (Fisher/Jackson Immuno Research, NC9754415 1:250); 3,3′-Diaminobenzidine tetrahydrochloride tablet (Sigma, D5905-50TAB, 1 tablet: 20ml for staining); Neurotrophin 3 (Millipore, GF031); U-73122 (Sigma, U6756); GSK3β Inhibitor XIX (Millipore, 361565); Ionomycin (Sigma, I9657); BAPTA-AM (Invitrogen, B-1205); LY294002 (Sigma, L9908); BEZ235 (Selleckchem, S1009); PD0325901 (Selleckchem, S1036).

### Animals

All animal protocols followed are as described previously (Suo et al., 2014) and were conducted in accordance with Association for Assessment of Laboratory Animal Care Policies and approved by the University of Virginia Animal Care and Use Committee. Sprague Dawley rats were purchased from Harlan. Sympathetic neurons were dissected from P0–P2 rats or mice of either sex as previously described {Zareen, 2009 #39}(Zareen and Greene, 2009). All mice were in a C57BL/6 background and J. Pieters generously provided

the *Coro1a−/−* mice. Genotyping was performed as described previously (Jayachandran et al., 2007).

### Tissue culture

Sympathetic neuron cultures were plated as previously described (Deppmann et al., 2008). Mass culture and microfluidic culture media was Dulbecco's modified Eagle medium (DMEM) containing 10% fetal bovine serum (FBS), penicillin-streptomycin (1 U ml−1), 45ng/ml of mouse salivary glands purified NGF. Aphidicolin was applied for 48h after plating to remove glia contamination.

### Immunocytochemistry

**I**mmunocytochemistry and immunohistochemistry was performed as previously described (Sharma et al., 2010). At room temperature samples were fixed by 4% paraformaldehyde and blocked for 30min using 5% goat serum and 0.05% Triton X-100 in PBS. Next, primary antibody was applied overnight at 4°C. Secondary antibody was applied for 1 hour at room temperature. Samples were washed three times using 1X PBS and mounted in Vectashield fluoromount. Images were collected using confocal microscopy.

### Immunoblot analysis

Immunoblot analysis was performed as previous described (Mandai et al., 2009). Briefly, sympathetic neurons were harvested by boiling in 2× Laemmli buffer for 10 min. SDS-PAGE followed by western blot analysis was performed using the indicated antibodies and LI-COR for visualization.

### Microfluidic devices

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

### Axon growth assay

P0–P3 sympathetic superior cervical ganglia neurons were plated in the cell body chamber of a microfluidic device in the presence of NGF and aphidicolin (5 μM). After 2–3 days axons emerged in the distal axon chamber of the microfluidic device at which time NGF was deprived for 17 hours by adding anti-NGF and Bocaspartyl(OMe)-fluoromethylketone (BAF; 5 μM). The axon chamber media was then changed to anti-NGF (1μg/ml) or NT3 (100ng/ml) and images of the entire distal axon chamber field were acquired at this 'zero time point'. After 24 hours, images of the same axon were reacquired and axon growth rates were quantified as the difference between the two datasets.

### Growth cone staining

Staining protocol was adapted from previous studies by Letourneau and colleagues (Marsick and Letourneau, 2011). Coverslips were coated with laminin (40μg/ml) for 2 hours before plating. P0–P3 sympathetic neurons were established in mass culture as described above and

were then treated with either anti-NGF (1μg/ml) with BAF (5 μM) or NT3 (100ng/ml) for 24 hours. Neurons were fixed with 4% PFA containing $Ca^{2+}$ (1.5mM) and $Mg^{2+}$ (1mM). Double immunostaining was then performed with Tuj1 and Phalloidin. Images were acquired using 63X confocal microscopy.

### Branch assay

P0–P3 sympathetic neurons were plated in mass culture with anti-NGF or other treatment as indicated. After 1 day in culture neurons were fixed with 4% PFA. Double immunostaining was then performed with Tuj1. Images were taken using 10X confocal microscopy and analyzed by Sholl analysis as previously described (Suo et al., 2015). The area under the curve of each Sholl analysis curve was determined in Prism 7 and compared.

## Results

### Establishing *Coro1a*$^{-/-}$ neurons as a model for axon behavior along intermediate targets

We first sought to explore the role of Coronin-1 in NT-3 dependent axon growth of sympathetic neurons *in vitro*. We established sympathetic neurons from *Coro1a*$^{-/-}$ or WT (*Coro1a*$^{+/+}$) mice in microfluidic devices to separate axons from cell bodies and provide easily identifiable landmarks to find the same axon from day to day (Figure 1A) (Park et al., 2006). All neurons were established in 45ng/ml NGF until axons traversed the microgrooves. NGF was then washed out and 100ng/ml NT3 or 1μg/ml anti-NGF were added exclusively to distal axons under fluidic isolation, while the cell bodies and proximal axons were treated with anti-NGF and a broad spectrum caspase inhibitor, bocaspartyl(OMe)-fluoromethylketone (BAF), to prevent apoptosis. Positive and negative growth of individual axons was measured over a 24-hour period. The presence of Coronin-1 had no effect on NT3-dependent axon growth rate (Figure 1B). This is in contrast to what is observed for NGF treated axons from wild type and *Coro1a*$^{-/-}$ neurons, which grow at approximately 75 and 150 microns/day, respectively (Suo et al., 2015).

Coronin-1 is also required for the ability of NGF to produce large growth cones, which are associated with slower axon growth rate and greater turning/branching capacity (Argiro et al., 1984; Bray and Chapman, 1985; Suo et al., 2015). Therefore, we next examined growth cone area in NT3 treated neurons from *Coro1a*$^{-/-}$ and WT mice. Remarkably, we observed that growth cones of NT3-treated WT neurons were small and compact consistent with previous findings (Kuruvilla et al., 2004) whereas NT3-treated *Coro1a*$^{-/-}$ neurons displayed large bulbous tips (Figure 1C, D). This is in contrast to our previous observation that NGF treated growth cones are small in the absence of Coroinin-1 (Suo et al., 2015).

We next examined the role of Coronin-1 in NT3-dependent axon branching. Sympathetic neurons from P0 *Coro1a*$^{-/-}$ or WT mice were sparsely cultured for 1 day *in vitro* (DIV) in the presence of NT3. Neurons were immunostained for β3-tubulin and branching was assessed via Sholl analysis as previously described (Magarinos et al., 2006; Suo et al., 2015). We found that NT3-induced branching was depressed in the absence of Coronin-1 compared to in WT neurons (Figure 1E, F).

Taken together, the axon behaviors that we observe in NT3 treated neurons from *Coro1a*$^{-/-}$ mice are consistent with growth along intermediate targets; steady axon growth, large growth cones for pathfinding, and minimal branching to maintain fascicles. The presence of Coronin-1 reveals a unique NT3 dependent axon growth profile with collapsed growth cones and increase branching, which would be consistent with axons transitioning into final target organs. However this condition likely plays a limited physiological role since neurons that express Coronin-1 are also exposed to NGF, which preferentially binds to TrkA over NT3. Herein, we model growth along the intermediate targets using NT3 treated axons from *Coro1a*$^{-/-}$ mice.

## Coronin-1 is dispensable for NT3-TrkA induced signaling

We have previously found that Coronin-1 influences several NGF-TrkA dependent pathways relevant to axon growth properties including GSK3β phosphorylation and calcium release (Suo et al., 2015). We next sought to determine whether this is also true for NT3-TrkA signaling. We began by examining whether Coronin-1 influences NT3-TrkA-dependent ERK, PI3K, and GSK3β activation. To this end we cultured sympathetic neurons from *Coro1a*$^{-/-}$ and WT mice in order to assess NT3 (100ng/ml) dependent p-ERK, p-AKT, and GSK3β levels (Figure 2A). Importantly, NT3 induced similar levels of p-ERK, p-AKT, and p-GSK3β in both *Coro1a*$^{-/-}$ and WT neurons, suggesting that *Coro1a−/−* is not necessary for NT3-TrkA activation of these pathways (Figure 2A–D). The dispensability of Coronin-1 for NT3-TrkA induced p-AKT and p-ERK is similar to what is observed in NGF-TrkA activation of these pathways (Suo et al, 2015). However, NT3-TrkA is capable, while NGF-TrkA is not capable, of inducing an inactivating phosphorylation event on GSK3β in the presence of Coronin-1. Inactivation of GSK3β has been associated with increased axon growth rate and branching (Suo et al., 2015; Zhou et al., 2004).

We have previously found that Coronin-1 is required for NGF dependent calcium release from intracellular stores (Suo et al., 2014). Therefore, we next sought to determine whether NT3 influences calcium release in the presence or absence of Coronin-1. 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 (Suo et al., 2014), NT3 failed to promote calcium release in both WT and *Coro1a−/−* neurons (Fig 2E, F). Taken together, these data suggest that NT3 dependent processes like axon growth may involve activation of Ras-MAPK and PI3K-AKT pathways as well as inhibition of GSK3β. However, calcium signaling is likely not used to mediate NT3-TrkA dependent processes.

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

We next sought to determine whether MAPK signaling downstream of NT3-TrkA is required in our model for axon growth and branching along intermediate targets using neurons from *Coro1a*$^{-/-}$ mice. We first performed axon growth assays as described in Figure 1, on NT3 treated neurons from *Coro1a*$^{-/-}$ or WT mice in the presence or absence of the MEK inhibitors, PD98059 or PD0325901. In *Coro1a*$^{-/-}$ neurons treated with NT3, both PD98059 and PD0325901 reduced the rate of NT3-dependent axon extension by 40% (Figure 3A). However, in WT neurons neither PD98059 nor PD0325901 had an effect on

NT3 dependent axon growth. We next examined the role of MEK in NT3-dependent branching. Interestingly, both PD98059 and PD0325901 enhanced branching of NT3 treated *Coro1a*$^{-/-}$ neurons, with no effect on WT neurons (Figure 3B–E). Taken together, these data suggest that the presence of Coronin-1 masks a role for RAS-MAPK signaling in NT3 dependent axon growth and branching. In this model of axon growth along intermediate targets (*Coro1a*$^{-/-}$), NT3-TrkA signaling to RAS/MAPK promotes axon growth and inhibits branching (Figure 3F).

## NT3-TrkA-dependent PI3K signaling is required to promote axon growth and suppress branching in absence of Coronin-1

We next examined the role of PI3K signaling in regulating axon behaviors in the absence and presence of Coronin-1. The PI3K inhibitor, LY29002, inhibited axon growth of NT3-treated neurons from *Coro1a*$^{-/-}$ mice but exhibited no effect on the growth of WT neurons (Figure 4A). Additionally, LY29002 enhanced branching of neurons from *Coro1a*$^{-/-}$ mice grown in NT3, but had no effect on WT neurons grown in NT3 (Figure 4B–E). 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 and are required for NT3- dependent axon growth in this niche where Coronin-1 levels are below the level of detection (Figure 4F).

## Calcium signaling is dispensable for NT3-TrkA dependent axon growth

In neurons from both *Coro1a*$^{-/-}$ and WT mice, we observe an inability of NT3 to induce calcium release. However, it is formally possible calcium signaling is still required for axon growth and branching. The calcium chelator, BAPTA-AM (1μM), had no effect on growth in NT3-treated *Coro1a*$^{-/-}$ neurons, but suppresses axon growth of NT3-treated WT neurons (Figure 5A). This is in contrast to our previous observation that BAPTA-AM doubles the axon growth rate of NGF treated WT neurons (Suo et al., 2015). BAPTA-AM had no effect on NT3-dependent branching in neurons from *Coro1a*$^{-/-}$ or WT mice (Figure 5B–E). Taken together with our finding that NT3 is unable to induce calcium release (Figure 2D, E), these data suggest that calcium signaling plays little role in NT3 dependent growth and branching (Figure 5F).

## NT3-TrkA-dependent GSK-3β inhibition is required for axon growth

We previously showed that NGF-TrkA-Coronin-1 suppressed axon growth and branching downstream of PI3K signaling by disinhibiting GSK3β (Suo et al., 2015). Thus, we sought to discern whether GSK3β inhibition (analogous to the phosphorylated state) influences NT3 dependent axon growth and branching. We assessed the axon growth of NT3-treated neurons from *Coro1a*$^{-/-}$ or WT mice with or without GSK3β inhibitor XIX (200nm:GSKi). Both *Coro1a*$^{-/-}$ and WT neurons displayed a decrease in NT3 dependent axon growth when GSK3β was inhibited (Figure 6A). However, GSKi caused no change in the already depressed NT3 dependent branching in *Coro1a*$^{-/-}$ or WT neurons (Figure 6B–E). These data contrast previous work, which suggests that inhibition of GSK3β via phosphorylation is required for NGF dependent axon growth (Suo et al., 2015; Zhou et al., 2004). Here we find the opposite. Inhibition of GSK3β presumably via phosphorylation suppresses NT3 dependent axon growth. Moreover, the fact that GSK3β inhibition does not further depress

branching underscores the notion that unlike NGF, NT3 does not engage a pro-branching signaling program (Figure 6F).

## Discussion

Here, we have examined the signaling underlying NT3-TrkA dependent axon growth. We and others have previously identified genes that are upregulated in sympathetic neurons upon final target innervation (i.e. Coronin-1, TrkA, and p75NTR) (Deppmann et al., 2008; Kuruvilla et al., 2004; Suo et al., 2014). This previous work reveals a flaw in current *in vitro* models of growth along intermediate targets. Because sympathetic neurons are typically dissected from mice after they have encountered NGF *in vivo* (p0–p3) and are then established *in vitro* in NGF, they express many final target dependent genes. Our previous finding that Coronin-1 is a critical molecular switch between final target axon transition and termination niches allowed us to develop an *in vitro* model system that more accurately reflects the molecular state of sympathetic axons growing along intermediate targets (neurons from *Coro1a*$^{-/-}$ mice grown in NT3). With this model system, we uncover key differences between NT3-TrkA and NGF-TrkA dependent signaling and axon growth. These differences reveal at least 3 distinct niches of axon growth: along intermediate targets, entering the final target prior to Coronin-1 upregulation, and terminating in final targets after Coronin-1 upregulation (Figure 7).

As sympathetic axons grow along the intermediate target, NT3-TrkA activates MAPK as well as PI3K and inhibits GSK3β (Figure 2). Inhibition of GSK3β likely occurs downstream of PI3K as has been previously reported (Saijilafu et al., 2013). In this intermediate target niche, both MAPK and PI3K are critical for promoting axon growth and inhibiting branch formation, which is consistent with the idea that while it is important for fascicles to pathfind along blood vessels, they should not arborize (Figure 3, 4). Intriguingly, the presence of Coronin-1 appears to uncouple MAPK and PI3K from NT3-TrkA dependent axon growth and branching behaviors. We suggest that upon exposure to NGF and upregulation of *Coronin-1,* NT3-TrkA signaling is masked with respect to promoting growth and branching. This could be via an unknown Coronin-1 dependent mechanism or a coincident Coronin-1 independent event. For example, the up regulation of p75NTR expression that accompanies final target innervation depresses the ability of NT3 to signal through TrkA (Kuruvilla et al., 2004). Unlike MAPK and PI3K pathways, the inhibition of GSK3β via phosphorylation depresses axon growth but has no effect on branching (Figure 5). This signaling is consistent with previous work showing drastic reduction in the axon growth of DRG neurons exposed to GSK3β inhibitors (Kim et al., 2006).

NGF emanating from final target organs has a roughly 10 fold greater affinity for TrkA than NT3 (Belliveau et al., 1997). As these axons transition from the intermediate target to the final target, NGF outcompetes NT3 for TrkA binding. In this transition zone niche, NGF-TrkA activates the same pathways as NT3-TrkA, however, axon growth and branching no longer rely on RAS-MAPK signaling (Suo et al, 2015). PI3K appears to be the principal TrkA dependent pathway used to drive axon growth in this niche, which manifests as robust axon growth and branching to rapidly arborize the final target (Suo et al, 2015).

Modulation of calcium levels within growing axons is inextricably tied to axonal behavior (Mattson and Kater, 1987; Song et al., 1997). Rapidly growing axons with minimal turning or branching exhibit lower levels of calcium while slower axonal growth correlates with higher levels of calcium (Robles et al., 2003; Song et al., 1997). As sympathetic axons are exposed to a threshold concentration of NGF for a threshold period of time, *Coronin-1* is upregulated, which endows the NGF-TrkA complex with the ability to induce calcium release, in concert with PLC-gamma signaling (Suo et al, 2014). This calcium signaling inhibits pathways downstream of PI3K in order to disinhibit GSK3β leading to a suppression of NGF dependent axon growth and branching (Suo et al, 2015). In the absence of Coronin-1*,* axons over-branch and overshoot their final target fields, *in vivo*. While NGF signaling results in robust calcium release as part of its control over axonal growth and branching (Suo et al., 2014; Suo et al., 2015), we found that NT3 does not cause calcium release regardless of whether or not Coronin-1 is present (Figure 2D, E). Consistent with this, blocking calcium signaling in the absence of Coronin-1 has no effect on NT3-TrkA dependent axon growth or branching (Figure 5).

How can NT3 and NGF signaling through the same receptor result in such dramatically different axon growth properties? It has been reported that while NGF-TrkA can internalize into persistent signaling endosomes capable of trafficking long distances, NT3-TrkA cannot (Kuruvilla et al., 2004). Although, NT3-TrkA is capable of internalizing, the complex recycles back to the surface rapidly and does not traffic long-distances (Harrington et al., 2011). This is because NT3-TrkA dissociates in the acidic lumen of the endosome, whereas NGF-TrkA is stable under these conditions (Harrington et al., 2011). This transient versus persistent internalization of TrkA is likely the critical determinant for differences in axon growth and branching properties across the 3 niches that we describe (Figure 7). Indeed, it is known that the TrkA signaling endosome adds effector proteins (*e.g.* rac1, Erk5, Cofilin, Coronin-1) as a function of trafficking and maturation (Barford et al., 2016; Harrington et al., 2011; Suo et al., 2014; Watson et al., 2001). It is likely that these effector proteins endow NGF-TrkA with the ability to induce axon growth properties distinct from NT3-TrkA.

Future work will be needed to discern whether other Type I Coronin family members also act as molecular switches in these neurons. Additionally, it will be important to determine whether this is specific to sympathetic neurons or a more general principle of neurons as they grow toward their final target. We expect that while the molecular players may not be identical across neuron types, the phases of growth that we describe here and previously will be broadly applicable.

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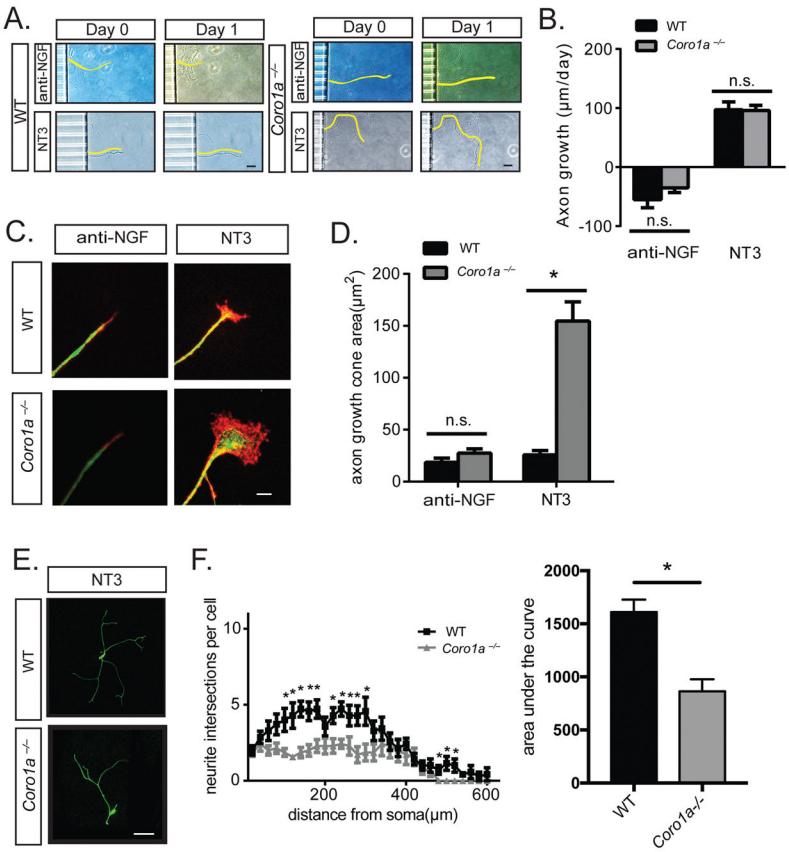



**Figure 1. Coronin-1 masks NT3 dependent axon behaviors**

(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 (1μg/ml) or NT3 (100ng/ml). Scale bar = 30μm.

(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).

(C) Neurons were cultured in the presence of anti-NGF (1μg/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).

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

(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 NGF (2ng/ml) or NT3 (100ng/ml). Tuj1 immunostaining was performed after 1 day of plating.

(F) Sholl analysis quantification of panel E and quantification of the area under the curve (n=7).

Error bars represent s.e.m.

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

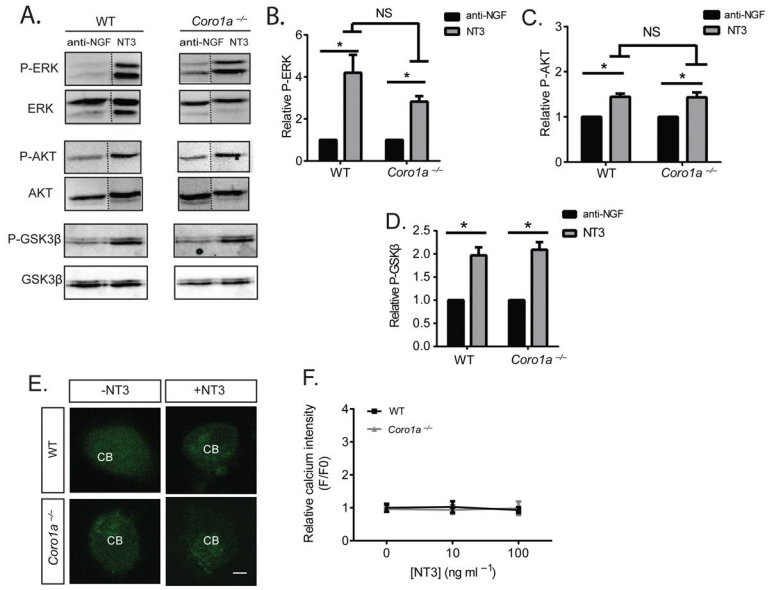



**Figure 2. Coronin-1 alters NGF- and NT3-induced calcium release but has no effect on P-Erk or P-Akt signaling**

(A) NT3 dependent P-ERK3β, P-AKT, or P-GSK3β 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 immunoblot analysis for P-ERK, ERK, P-AKT, AKT, P-GSK3β, and GSK3β.

(B–D) 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).

(E) NT3 is unable to induces 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 treatment. Scale bar = 5μm.

(F) Quantification of calcium release described in panel D 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

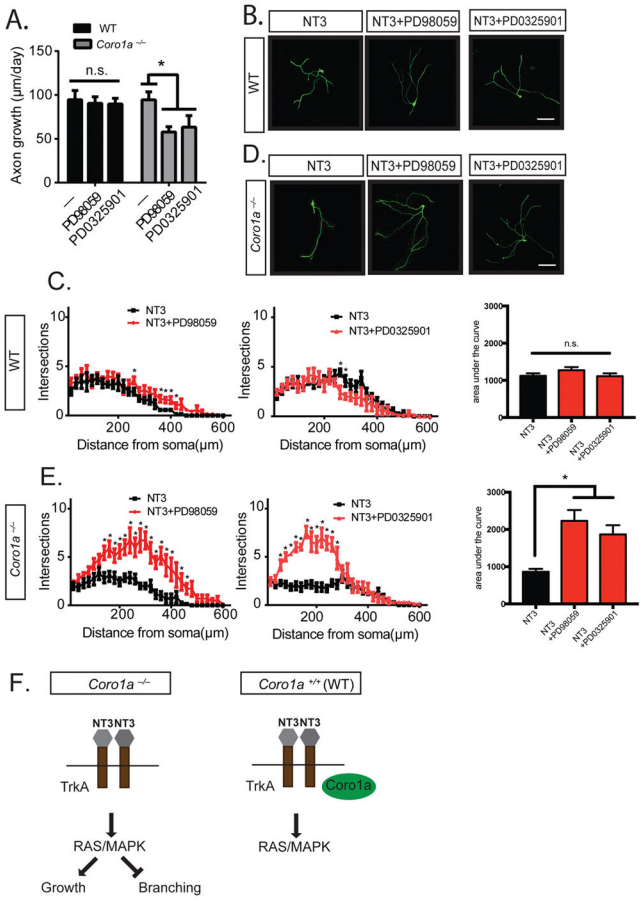



**Figure 3. NT3 dependent axon growth and suppression of branching requires MAPK signaling**

(A) MEK signaling is required for NT3 induced axon growth in *Coro1a*$^{-/-}$ but not wild-type neurons. Axon growth was examined as described for Figure 1B with the addition PD98059 (50μM) or PD0325901 (1μM) to indicated groups. WT with NT3 (n=22), WT with PD98059 and NT3 (n=30), *Coro1a*$^{-/-}$ with NT3 (n=17), *Coro1a*$^{-/-}$ with PD98059 and NT3 (n=26), WT with PD0325901 (n=30), Coro1a−/− with PD0325901 (n=40).

(B, D) 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.

(C, E) Sholl analysis quantification of panel B and D and quantification of the area under the curve (n=7).

(F) 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 (Sholl analysis curve) and an ordinary one-way ANOVA using Tukey's multiple comparisons test with a single pooled variance (area under the curve).

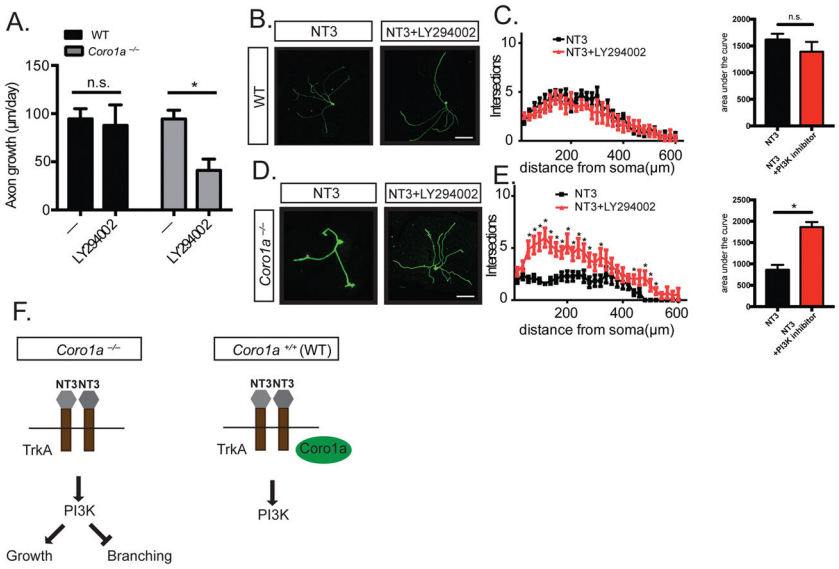



**Figure 4. PI3K signaling pathway plays a Coronin-1 dependent role in axon branching**

(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. WT with NT3 (n=35), WT with LY294002 and NT3 (n=19), *Coro1a*$^{-/-}$ with NT3 (n=38), *Coro1a*$^{-/-}$ with LY294002 and NT3 (n=23).

(B, D) 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.

(C, E) Sholl analysis of panel B and D and quantification of the area under the curve (n=7).

(F) 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

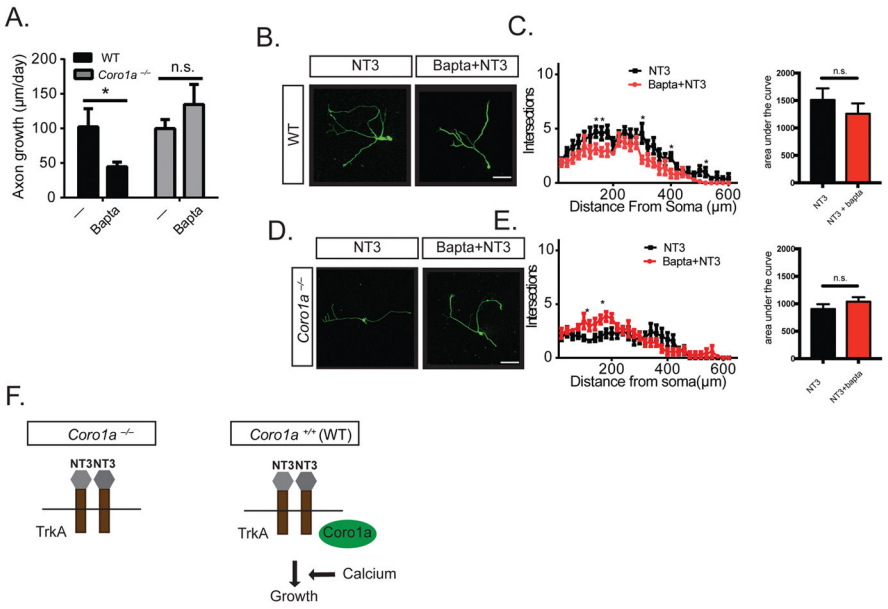



**Figure 5. Coronin-1 dependent calcium signaling suppresses axon growth and branching plays in the final destination axon growth niche**

(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 (1μM) WT (n=57), WT with Bapta and NT3 (n=54), *Coro1a*$^{-/-}$ (n=57), *Coro1a*$^{-/-}$ with BAPTA-AM and NT3 (n=49).

(B, D) 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 (1μM).

(C, E) Sholl analysis quantification of panel C and E and quantification of the area under the curve (n=7).

(F) The role of calcium signaling 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

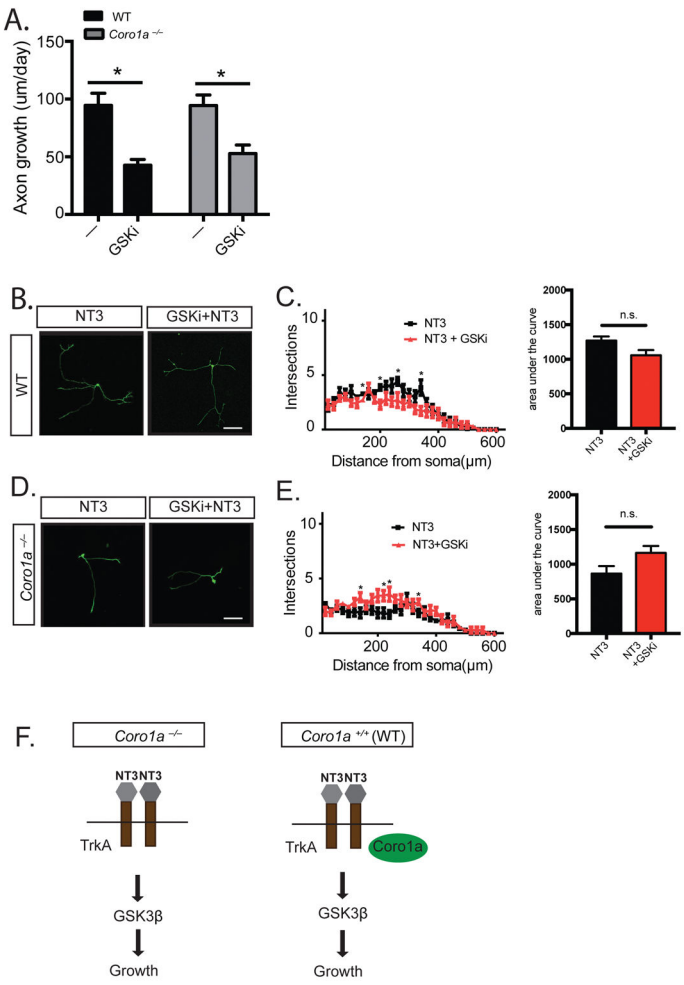



**Figure 6. GSK3β inhibition suppresses NT3 dependent axon growth but does not effect branching**

(A) P-GSK3β signaling is 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 GSKi XIX (200μM) to indicated groups. WT with NT3 (n=35), WT with GSKi XIX and NT3 (n=17), *Coro1a*$^{-/-}$ with NT3 (n=38), *Coro1a*$^{-/-}$ with GSKi XIX and NT3 (n=44).

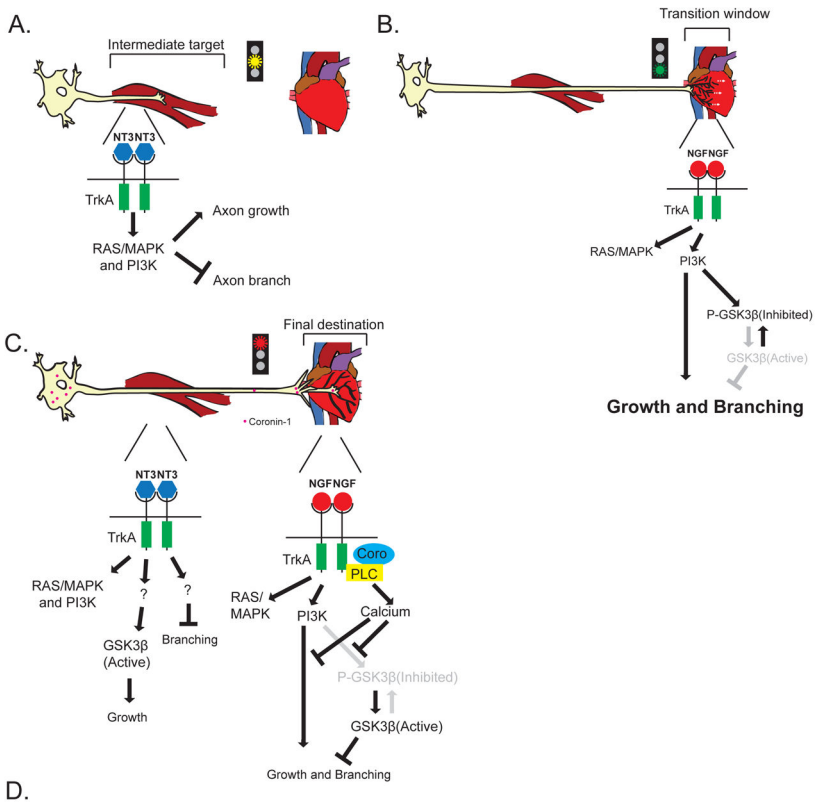
(B, D) P-GSK3β signaling suppresses axon branching in absence of Coronin-1. Neurons from wild-type or *Coro1a*$^{-/-}$ mice were grown in NT3 (100ng/ml) with or without GSKi XIX (200μM). Branching was visualized as described in Figure 1F.

(C, E) Sholl analysis of panel B and D and quantification of the area under the curve (n=7).

(F) The role of GSK3β 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




| | Intermediate target NT3-TrkA | | Transition Window NGF-TrkA | | Final Destination NGF-TrkA +Coronin-1 | |
|---|---|---|---|---|---|---|
| | growth | branching | growth | branching | growth | branching |
| RAS/MAPK | ↑ | ↓ | - | - | - | ↑ |
| PI3K | - | ↓ | ↑↑ | ↑↑ | - | ↓ |
| Coronin-1/ Calcium | - | - | - | - | ↓ | ↓ |

**Figure 7. Model for hierarchal neurotrophin signaling and Coronin-1 expression in defining spatiotemporally distinct axon growth environments**

(A) Early stage axon behavior and neurotrophic signaling during sympathetic neuron development. Diagram depicts sympathetic neuron growing along intermediate target (e.g. blood vessel) en route to final target (e.g. heart). NT3-TrkA signals through RAS/MAPK, PI3K, and GSK3β to control axon growth and branching.

(B) Middle stage axon behavior and neurotrophic signaling during sympathetic neuron development. The growing axon has reached its final target and is exposed to target-derived NGF. NGF-TrkA results in the expression of Coronin-1 in the sympathetic neuron.

(C) Late stage axon behavior and neurotrophic signaling during sympathetic neuron development. NT3-TrkA still activates RAS/MAPK and PI3K, but these pathways are uncoupled from axon growth and branching. NT3-TrkA activates P-GSK3β through an unknown pathway to control axon growth. NGF-TrkA-Coronin-1 signaling slows axonal growth and causes innervation of the final target.

(D) Role of NT3 and NGF in the developmental stages of sympathetic axon projection and innervation of final target. Comparison of pathways involved in axon growth and branching examined here and in Suo et al., 2015.