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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 (i.e. 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.

1. 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 (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

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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.

2. Materials and methods

2.1. 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: 20 ml 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).

2.2. 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).

2.3. 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}), 45 ng/ml of mouse salivary glands purified NGF. Aphidicolin was applied for 48 h after plating to remove glia contamination.

2.4. Immunocytochemistry

Immunocytochemistry and immunohistochemistry was performed as previously described (Sharma et al., 2010). At room temperature samples were fixed by 4% paraformaldehyde and blocked for 30 min 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 h at room temperature. Samples were washed three times using 1× PBS and mounted in Vectashield fluoromount. Images were collected using confocal microscopy.

2.5. 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.

2.6. 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 (50 μg/ml) and laminin (1 μg/ml). A 100 μl volume difference was maintained between two compartments to ensure fluidic isolation.

2.7. 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 h by adding anti-NGF and Bocaspartyl(OMe)-fluoromethylketone (BAF; 5 μM). The axon chamber media was then changed to anti-NGF (1 μg/ml) or NT3 (100 ng/ml) and images of the entire distal axon chamber field were acquired at this 'zero time point'. After 24 h, images of the same axon were reacquired and axon growth rates were quantified as the difference between the two datasets.