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Phospho-regulation of soma-to-axon transcytosis of neurotrophin receptors

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Summary

Axonal targeting of signaling receptors is essential for neuronal responses to extracellular cues. Here, we report that retrograde signaling by target-derived Nerve Growth Factor (NGF) is necessary for soma-to-axon transcytosis of TrkA receptors in sympathetic neurons, and define the molecular underpinnings of this positive feedback regulation that enhances neuronal sensitivity to trophic factors. Activated TrkA receptors are retrogradely transported in signaling endosomes from distal axons to cell bodies, where they are inserted on soma surfaces and promote phosphorylation of resident naive receptors resulting in their internalization. Endocytosed TrkA receptors are then dephosphorylated by PTP1B, an ER-resident protein tyrosine phosphatase, prior to axonal transport. PTP1B inactivation prevents TrkA exit from soma and causes receptor degradation, suggesting a “gate-keeper” mechanism that ensures targeting of inactive receptors to axons to engage with ligand. In mice, PTP1B deletion reduces axonal TrkA levels and attenuates neuron survival and target innervation under limiting NGF ($NGF^{+/-}$) conditions.

eTOC Blurb

Yamashita et. al., describe a positive feedback mechanism where retrogradely transported TrkA signaling endosomes control the anterograde transcytosis of naive somatic TrkA receptors to enhance receptor availability in sympathetic axons. Furthermore, an ER-resident protein tyrosine phosphatase acts specifically in neuronal soma to positively regulate TrkA axon targeting and trophic functions.

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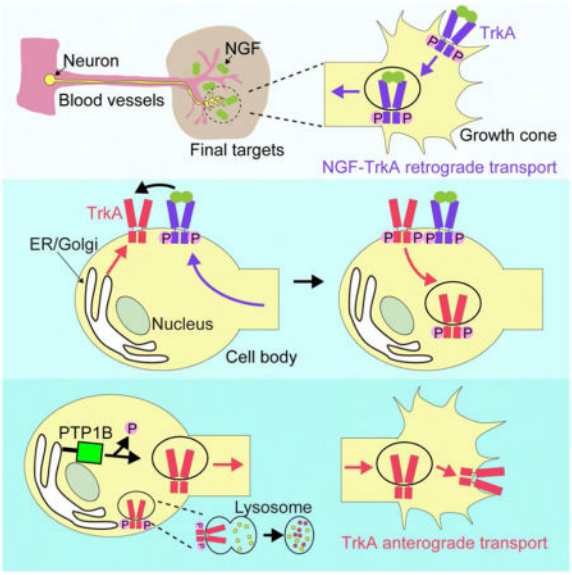

Introduction

Axonal targeting of membrane proteins controlling guidance, adhesion, neuronal survival, and synaptic connectivity is a critical determinant in the establishment of neuronal circuits during development. After synthesis in neuronal soma, delivery of membrane proteins to axons has been proposed to occur via several modes, including direct transport after sorting at the trans-golgi network, non-polarized delivery to axons and dendrites followed by selective retention in axons, or transcytosis where initial delivery of newly synthesized membrane proteins to somato-dendritic compartments is followed by endocytosis and anterograde transport (Horton and Ehlers, 2003; Winckler and Mellman, 2010). Discrete steps in the axonal targeting process including exit from neuronal soma, anterograde trafficking along microtubules, and local insertion into growth cones present regulatory nodes for precise control of surface receptor levels in response to cell-extrinsic factors or neuronal activity. Ligand-mediated local insertion of guidance and trophic receptors has been documented in growth cones (Bouchard et al., 2004; Dequidt et al., 2007; Du et al., 2000; Tojima et al., 2007). However, whether, and how, extracellular cues impinging on axon terminals can influence the long-range anterograde delivery of signaling receptors from neuronal cell bodies has been poorly defined.

The family of neurotrophins provides one of the best examples of target-derived diffusible cues that govern neuron survival, target innervation, and synaptic plasticity in the vertebrate nervous system (Huang and Reichardt, 2001). The prototypical neurotrophin, Nerve Growth Factor (NGF), is essential for neuron viability and axon growth of select neuronal populations in the peripheral nervous system during development, and is also a critical mediator of inflammatory pain during adulthood (Sofroniew et al., 2001). Since NGF is produced by target fields of peripheral neurons, a key mode of NGF action is ligand binding to TrkA receptors at axon terminals followed by receptor endocytosis and long-distance retrograde transport of NGF-TrkA-containing signaling endosomes back to neuronal cell

bodies (Yamashita and Kuruvilla, 2016). Receptor internalization in distal axons and retrograde transport must be balanced by anterograde targeting of newly synthesized receptors to ensure continued responses to target-derived ligand. Compared to the intense focus on retrograde trafficking of Trk receptors, relatively little is known about their anterograde delivery. Limited studies conducted so far favor the model of constitutive Trk transport through the secretory pathway, and have shed some light on the molecular components involved, including sortilin, a Vps10 domain family member that regulates receptor sorting at the Golgi apparatus (Vaegter et al., 2011), the plus-end directed motors, KIF1A and kinesin-1 (Arimura et al., 2009; Tanaka et al., 2016), and an adaptor complex consisting of the Rab27 GTPase, it's effector, Slp1, and CRMP-2, a microtubule-binding protein (Arimura et al., 2009).

Recent studies suggest that ligand-triggered signaling at axon terminals also plays a critical role in recruiting neurotrophin receptors to axons (Ascano et al., 2009; Cheng et al., 2011). In particular, in sympathetic neurons, TrkA receptors are transported to axons by transcytosis, where receptors embedded in the plasma membrane of neuronal soma are endocytosed and redirected to axons via Rab11-positive recycling endosomes (Ascano et al., 2009). Anterograde TrkA transcytosis is necessary for optimal signaling and growth responses to NGF, and intriguingly, is augmented by NGF itself acting on distal axons. These findings suggest a trafficking-based positive feedback mechanism that enhances neuronal sensitivity to limiting concentrations of target-derived neurotrophin. However, how NGF acting at axon terminals, promotes the long-range anterograde trafficking of its receptors remains unknown. Here, using compartmentalized cultures of sympathetic neurons and genetic mouse models, we reveal that retrograde NGF-TrkA signaling endosomes are necessary for anterograde transcytosis of TrkA receptors, and identify an ER-associated dephosphorylation mechanism that ensures delivery of inactive receptors to axon terminals.

Results

Retrograde transport of Trk receptors is necessary for anterograde transcytosis

How is the retrograde NGF signal, initiated in distal axons, relayed to naive TrkA receptors resident on soma surfaces, to mediate anterograde transcytosis? Retrograde communication of NGF trophic signaling occurs via ligand-triggered endocytosis of TrkA receptors in axon terminals and retrograde trafficking of NGF-TrkA signaling endosomes back to cell bodies (Yamashita and Kuruvilla, 2016). To address if NGF-TrkA endosomes are necessary for soma-to-axon transcytosis, we first asked whether Trk tyrosine kinase activity in distal axons, a prerequisite step in initiating retrograde neurotrophin signaling (Kuruvilla et al., 2000), is necessary for anterograde transport. To visualize transcytosis of Trk receptors originating from soma surfaces, we employed a chimeric Trk receptor-based live-cell antibody feeding assay in compartmentalized cultures of sympathetic neurons (Figure 1A) (Ascano et al., 2009). Neurons were infected with an adenoviral vector that expresses FLAG-tagged chimeric receptors that have the extracellular domain of TrkB and transmembrane and intracellular domains of TrkA (FLAG-TrkB:A). Sympathetic neurons do not normally express TrkB receptors, and chimeric Trk receptors respond to the TrkB ligand, Brain-Derived Neurotrophic Factor (BDNF), but retain the signaling properties of TrkA

(Ascano et al., 2009). Infected neurons were identified by GFP fluorescence where GFP was co-expressed with FLAG-TrkB:A receptors using a self-cleaving 2A peptide sequence. Surface chimeric receptors were live-labeled with anti-FLAG antibody exclusively in cell body compartments. BDNF stimulation of axons (100 ng/ml, 4 hr) significantly increased the intracellular accumulation of FLAG antibody-bound TrkB:A receptors originating from neuronal soma surfaces in cell bodies, and also promoted their anterograde transport to axons, as revealed by labeling with a fluorescent secondary antibody (Figures 1A–C, H and I), and consistent with our previous findings (Ascano et al., 2009). These results indicate that ligand, acting on distal axons, first triggers the internal accumulation of soma surface-derived Trk receptors that are then anterogradely transported to distal axons via transcytosis. To address if Trk receptor kinase activity in distal axon compartments is required for anterograde transcytosis, we took advantage of a F592A mutation in FLAG-TrkB:A receptors that renders them sensitive to a small molecule membrane-permeable inhibitor, 1NMPP1 (Chen et al., 2005). Addition of 1NMPP1 (100 nM) to distal axons of compartmentalized neurons suppressed the BDNF-induced intracellular accumulation in cell bodies and anterograde transport of FLAG-bound Trk receptors (Figures 1A–D, H and I). These results indicate that Trk receptor activation and initiation of retrograde neurotrophin signaling in distal axons is necessary for the intracellular accumulation of soma surface-derived receptors and their anterograde transport.

Previously, we reported that NGF-induced TrkA endocytosis in sympathetic axons depends on TrkA-mediated recruitment of Phospholipase C-γ (PLC-γ), which then stimulates calcineurin, a calcium-dependent phosphatase, to dephosphorylate the endocytic GTPase, dynamin1 (Bodmer et al., 2011). To further probe the retrograde Trk signaling mechanisms that mediate transcytosis, we sought to interfere with Trk receptor endocytosis in distal axons. We added specific pharmacological inhibitors of the PLC-γ-calcineurin-dynamin endocytic pathway to distal axons in compartmentalized cultures, and monitored the transcytosis of soma surface-derived Trk receptors using FLAG antibody feeding. We found that ligand-induced Trk internalization in cell bodies and anterograde transport was completely abrogated by addition of a PLCγ inhibitor (U73122, 10 μM), the calcineurin phosphatase inhibitors (FK506, 0.2μg/ml + cyclosporin A, 2 μg/ml), or dynasore (80 μM), an inhibitor of dynamin-mediated endocytosis, to axon compartments (Figures 1A, E–I). These results indicate that Trk receptor endocytosis initiated in distal axons is necessary for soma-to-axon transcytosis.

Axon- and soma surface-derived Trk receptors interact in neuronal soma

We next asked how the retrograde transport of active TrkA receptors influences anterograde transcytosis. Previously, retrogradely transported signaling endosomes have been observed to recycle between the soma cell surface and interior upon reaching cell bodies (Suo et al., 2014). Thus, one plausible mechanism for interactions between axon- and soma surface-derived receptor populations is that retrogradely transported TrkA receptors are exocytosed to the soma membrane, bringing them in close proximity to resident naive receptors. To visualize exocytosis of retrogradely transported receptors in soma, we performed a modified FLAG feeding assay where surface Trk receptors in axons, live-labeled with anti-FLAG antibody and retrogradely transported, were detected on soma surfaces of non-permeabilized

neurons using a fluorescent secondary antibody (Figure 2A). We found that, compared to un-stimulated neurons, BDNF treatment of distal axons elicited a 4-fold increase in FLAG punctae that appeared on soma surfaces (3 ± 0.48 FLAG punctae per neuron in un-stimulated condition *versus* 13 ± 1 with BDNF treatment) (Figures 2B–D). Because antibody-tagged receptors could only have originated from axonal surfaces, these results provide direct evidence for insertion of retrogradely transported Trk receptors on soma surfaces in response to ligand stimulation of distal axons.

We next asked if we could visualize retrogradely transported Trk receptors in close proximity to soma surface-derived receptors. Thus, we performed dual-color FLAG antibody feeding in compartmentalized neurons, where axon and soma surface Trk receptors were live-labeled with mouse and rabbit anti-FLAG antibodies, respectively (Figure 2E). After stimulating axons with BDNF for 1 hr to promote retrograde Trk transport, neurons were fixed and incubated with anti-mouse Alexa-546 and anti-rabbit Alexa-350 secondary antibodies to detect retrogradely transported and soma surface-resident receptors, respectively. In the absence of ligand, soma surface-resident Trk receptors were observed at the plasma membrane, and there was little retrograde accumulation of axonal Trk receptors, as expected (Figures 2F–H). In contrast, ligand treatment of distal axons elicited retrograde appearance of axonal Trk receptors that co-localized with soma surface-derived receptors at the cell periphery (Figures 2I–L). Quantification revealed that 49.5 ± 3.2% of soma surface-derived FLAG-TrkB:A receptors co-localize with axon-derived receptors, suggesting that a significant fraction of soma surface-derived Trk receptors encounter retrogradely transported receptors in cell bodies. We next asked if these two receptor populations are capable of associating with each other. Thus, we performed an *in situ* proximity ligation assay (PLA) where interactions between axon and soma-surface derived FLAG-TrkB:A receptors can be visualized as discrete fluoresecent spots using a DNA ligation and rolling circle amplification strategy (Soderberg et al., 2006). We observed a significant increase in interactions between the axon- and soma surface-derived receptors, primarily at the cell surface, upon ligand stimulation (2 ± 0.56 PLA punctae per neuron in un-stimulated condition *versus* 10.6 ± 1.4 with BDNF treatment), (Figures 2M, N). PLA signals were rarely detected intracellularly suggesting that receptor interactions at the soma plasma membrane are transient. Since the *in situ* PLA cross-links proteins that are <40 nm apart, these results suggest that retrogradely transported active Trk receptors dimerize with naive resident receptors at the soma surface.

We next sought to address the outcome of interactions between axon-derived active Trk receptors and naive soma surface receptors. Exocytosis of retrograde Trk signaling endosomes could elicit phosphorylation of naïve cell surface-resident receptors, either by locally increasing receptor density and transient receptor dimerization in membrane micro-domains and/or via extracellular release of ligand that is co-transported in signaling endosomes. To address whether soma surface Trk receptors undergo phosphorylation in a manner dependent on retrograde neurotrophin signaling, we employed a cell surface biotinylation assay where surface proteins in cell body chambers of compartmentalized cultures were biotin-labeled, followed by NGF stimulation of axons (100 ng/ml, 1 hr). Cell body lysates were subjected to neutravidin precipitation and immunoblotting with an anti-phospho-TrkA antibody (P-TrkA^{Y794}) (Figure 2O). NGF stimulation of sympathetic axons

resulted in a striking increase in phosphorylation of TrkA receptors derived from the soma surface (Figures 2P, Q). Since surface proteins in cell body chambers were biotinylated before adding NGF to axons, these results imply that the Trk receptors being phosphorylated in a manner dependent on retrograde NGF signaling, were resident on soma surfaces prior to the initiation of retrograde transport.

Together, these results suggest that surface insertion of retrogradely transported active TrkA receptors in neuronal cell bodies elicits the phosphorylation of neighboring naive receptors, which is necessary to promote endocytosis of receptors destined for anterograde transcytosis.

PTP1B, an ER-resident phosphatase, dephosphorylates endocytosed Trk receptors in soma

Previous observations of axonal transport of phosphorylated Trk receptors have noted that active receptors are exclusively trafficked in the retrograde, but not anterograde, direction (Bhattacharyya et al., 2002; Ye et al., 2003). If Trk receptors destined for transcytosis are phosphorylated in cell bodies, a key question then is how are these receptors inactivated prior to anterograde transport? Thus, we reasoned that a tyrosine phosphatase activity must exist in the soma to dephosphorylate Trk receptors prior to axonal targeting. In searching for candidate phosphatases, a clue came from previous findings that PTP1B, an endoplasmic reticulum (ER)-resident tyrosine phosphatase with its catalytic domain on the cytosolic face, dephosphorylates active Receptor Tyrosine Kinases (RTKs) harbored in endosomes as they transit past the ER (Stuible and Tremblay, 2010). Trk receptors harbor a putative PTP1B substrate recognition motif in their tyrosine kinase domain, and PTP1B associates with active TrkB receptors (Krishnan et al., 2015; Ozek et al., 2014).

PTP1B's interactions with its substrates are transient, and stable PTP1B-substrate complexes can only be detected by using substrate-trapping PTP1B variants (Haj et al., 2002). We found that FLAG-TrkA associates with Myc-tagged $PTP1B^{Y46F/D181A}$ and $PTP1B^{C215S}$ (Figure S1A), both of which function as substrate-trapping variants (Ozek et al., 2014), using co-immunoprecipitation analyses in HEK293T cells. $PTP1B^{Y46F/D181A}$ exhibited stronger binding for TrkA compared to $PTP1B^{C215S}$ (Figure S1A), consistent with previously reported interactions for TrkB (Ozek et al., 2014). Importantly, when we expressed $PTP1B^{WT}$, this suppressed the tyrosine auto-phosphorylation of TrkA elicited by receptor over-expression in heterologous cells, as detected by FLAG immunoprecipitation and immunoblotting with a pan anti-phosphotyrosine antibody (pY20). In contrast, robust TrkA auto-phosphorylation was maintained in HEK293T cells expressing the catalytically inactive $PTP1B^{C215S}$ or weakly active $PTP1B^{Y46F/D181A}$ substrate trapping mutants (Figures S1B, C). PTP1B preferentially dephosphorylates tyrosine residues in the sequence motif [E/D]-pY-pY-[R/K] (Myers et al., 2001), and all three Trk (A/B/C) receptors have a conserved PTP1B recognition motif (DYYR) in their kinase activation domain. Since the over-expression of $PTP1B^{WT}$ diminished the global tyrosine phosphorylation status of TrkA, these results suggest that PTP1B likely dephosphorylates Trk receptors in their active site.

To address if PTP1B interacts with endogenous TrkA receptors in sympathetic neurons, we expressed $PTP1B^{WT}$ or the $PTP1B^{Y46F/D181A}$ substrate-trapping mutant using adenoviral

vectors. In sympathetic neurons, endogenous TrkA receptors co-immunoprecipitated with the PTP1B$^{Y46F/D181A}$ substrate-trapping mutant, but not PTP1B^{WT} (Figure 3A), consistent with findings in HEK293T cells. Furthermore, TrkA associated with PTP1B$^{Y46F/D181A}$ only in the presence of NGF (Figure 3A), indicating that this association is dependent on ligand-induced activation of TrkA receptors in primary neurons. NGF-induced TrkA tyrosine phosphorylation, detected using an anti-P-TrkA^{Y794} antibody, was diminished by adenovirus-mediated over-expression of PTP1B^{WT} (Figures 3B, C). In contrast, expression of PTP1B$^{Y46F/D181A}$ mutant, that has reduced catalytic activity, enhanced tyrosine phosphorylation of TrkA (Figures 3B, C), suggesting that this mutant likely functions in a dominant-negative manner. Together, these results provide evidence that PTP1B binds to activated TrkA receptors resulting in their dephosphorylation.

PTP1B acts on receptor tyrosine kinases after endocytosis (Haj et al., 2002), or at sites of direct ER contacts with the plasma membrane at regions of cell-cell contact (Eden et al., 2010; Haj et al., 2012). To address where in sympathetic neurons, soma surface-derived Trk receptors encounter PTP1B, we performed FLAG antibody feeding in compartmentalized cultures infected with adenoviruses expressing FLAG-TrkB:A and mCherry-PTP1B$^{Y46F/D181A}$ substrate trapping mutant (Figure 3D). The mCherry-PTP1B$^{Y46F/D181A}$ signal was observed close to the cell periphery (Figures 3F, L), and showed near complete overlap with peripheral ER localization in sympathetic cell bodies as marked by GFP-Sec61 expression (Figures S1D–F). In the absence of ligand, FLAG antibody-bound Trk receptors found at the cell surface showed minimal co-localization with mCherry-PTP1B$^{Y46F/D181A}$ (Figures 3E–J). In contrast, significant co-localization was observed between soma surface-derived Trk receptors and the mCherry-PTP1B$^{Y46F/D181A}$ signal at the cell body perimeter in ligand-treated neurons (Figures 3K–Q). We next asked if Trk-PTP1B interactions occur post-endocytosis, as reported previously for PTP1B and several receptor tyrosine kinases including the insulin receptor, epidermal growth factor receptor and platelet-derived growth factor receptor (Boute et al., 2003; Haj et al., 2002). We assessed the degree of co-localization between FLAG-bound receptors and mCherry-PTP1B$^{Y46F/D181A}$ after stripping surface FLAG antibodies from soma membranes. Prominent co-localization was still observed, with 61±2% of the FLAG signal co-localizing with mCherry fluorescence, after removal of surface-bound FLAG antibodies (Figures S1G–R), indicating association between internalized Trk receptors and PTP1B. The peripheral localization of Trk receptors in BDNF-stimulated neurons (Figures 3K, N) suggests that association with the PTP1B$^{Y46F/D181A}$ substrate-trapping mutant likely restricts endocytosed receptors to the soma perimeter.

PTP1B activity in neuronal soma is required for transcytosis

To address the necessity of PTP1B for transcytosis, we monitored soma-to-axon transcytosis of FLAG-TrkB:A receptors in compartmentalized neurons treated with a cell membrane-permeable PTP1B-specific inhibitor (compound II, 200 nM) (Xie et al., 2003), added exclusively to cell body or axon compartments (Figure 4A). We confirmed that the PTP1B inhibitor suppresses the ability of PTP1B to dephosphorylate TrkA receptors (Figures S2A, B). Strikingly, selective inhibition of PTP1B in cell bodies abolished ligand-induced anterograde transcytosis of Trk receptors, whereas PTP1B inhibition in axons had no effect

(Figures 4B–F). Although PTP1B is expressed in sympathetic axons (Figure S2C), our data that axon-specific inhibition of PTP1B did not alter transcytosis suggests that axonal PTP1B activity is dispensable for the retrograde neurotrophin signaling that mediates transcytosis. Importantly, our results demonstrate that PTP1B activity is required in cell bodies to promote anterograde Trk transcytosis. In sympathetic neurons, TrkA is also dephosphorylated by the SHP family of tyrosine phosphatases (Marsh et al., 2003). However, local application of a membrane-permeable SHP-1/2 inhibitor, NSC-87877, either to cell body or axon compartments of sympathetic neurons did not affect ligand-induced transcytosis of Trk receptors (Figures S2D–H). Together, these results point to a specific requirement for PTP1B tyrosine phosphatase activity in mediating anterograde Trk transcytosis.

To address if the effect of PTP1B on TrkA transcytosis was direct, we sought to specifically abolish TrkA-PTP1B interactions. Thus, we generated mutant TrkA^{R685A} receptors with a point mutation in the conserved PTP1B recognition motif (DYYR) located in the TrkA kinase activation domain (Figure S2I). The TrkA^{R685A} mutation prevented receptor binding to PTP1B and suppressed PTP1B-induced TrkA dephosphorylation, but did not disrupt TrkA kinase activity or downstream signaling (Figures S2J–M). We found that chimeric FLAG-TrkB:A^{R685A} receptors, when expressed in sympathetic neurons, exhibited impaired transcytosis in response to axon-applied BDNF (Figures 4G–K), similar to the findings with soma-specific PTP1B inhibition. Since receptor kinase activity is unaffected by the point mutation, we reason that the mutant TrkA^{R685A} receptors are likely to be internalized in axons and retrogradely transported. Together, these results support that specific interactions between TrkA and PTP1B, and PTP1B-mediated dephosphorylation of TrkA receptors dictate anterograde transcytosis.

PTP1B inactivation results in lysosomal degradation of soma surface-derived Trk receptors

We next addressed the cellular fate of soma surface-derived Trk receptors that are unable to undergo axonal transcytosis upon PTP1B inactivation. One clue came from our observations that, in compartmentalized cultures, we did not observe significant intracellular accumulation of FLAG-TrkB:A receptors originating from soma surfaces upon cell body-specific inactivation of PTP1B or expression of mutant FLAG-TrkB:A^{R685A} receptors (Figures 4E, J, and S3A, B). Furthermore, prolonged PTP1B inhibition (90 minutes) induced a pronounced down-regulation of surface TrkA receptors under steady state conditions, without altering total TrkA protein levels in mass cultures of sympathetic neurons (Figures S3C, D). Based on these results, we reasoned that surface TrkA receptors, that are hyper-phosphorylated upon PTP1B inactivation, might undergo degradation. To test this prediction, we performed FLAG antibody feeding in compartmentalized cultures to follow soma surface-derived Trk receptors in combination with immunostaining for LAMP1, a lysosomal marker (Figure 5A). After BDNF stimulation of axons for 2 hr, 8.9 ± 1.4% of FLAG-TrkB:A punctae co-localized with LAMP-1 in the absence of PTP1B inhibitor, whereas in the presence of the inhibitor added to cell bodies, this co-localization was significantly increased to 31 ± 2.1% (Figures 5B–D). Note, that with a shorter duration of PTP1B inhibition (2 hr), intracellular punctae for soma surface-derived Trk receptors are still observed in cell bodies

(Figure 5C), compared to 4 hr of inhibitor treatment (Figure 4E). Treatment of cell bodies with the PTP1B inhibitor also reduced co-localization of soma surface-derived Trk receptors with Rab-11 (Figures S3E–G), that marks recycling endosomes responsible for anterograde transcytosis of TrkA receptors (Ascano et al., 2009). Together, these results suggest that PTP1B inhibition results in the diverting of soma surface-derived Trk receptors to lysosomes, contributing to their failure to be transported to axons. Consistent with this notion, we found that exposure of neuronal cell bodies to the PTP1B inhibitor in the presence of chloroquine, a lysosomotropic agent that prevents lysosome acidification and endosome-lysosome fusion (de Duve et al., 1974), promoted robust intracellular accumulation of soma surface-derived Trk receptors in cell bodies and even elicited aberrant localization of receptors in axons (Figures 5E–K). These results suggest that inhibition of lysosome function protects soma surface-derived Trk receptors from a degradative fate upon PTP1B inhibition and allows the internally accumulated receptors to escape to axons.

PTP1B deletion in mice decreases axonal TrkA levels and impairs NGF-mediated trophic functions

To assess the role of PTP1B in TrkA trafficking *in vivo*, we examined TrkA expression in sympathetic neuron cell bodies and nerve terminals in conditional mutant mice lacking the *PTPN1* gene that encodes for PTP1B. Selective disruption of PTP1B in sympathetic neurons was accomplished by crossing mice harboring a floxed *PTPN1* allele (*PTPN1*$^{f/f}$ mice) (Bence et al., 2006) with *TH-Cre* transgenic mice (Gong et al., 2007). Quantitative polymerase chain reaction (qPCR) and immunoblotting analyses of superior cervical ganglia (SCG) showed significant loss of PTP1B transcript and protein in new-born *TH-CRE;PTPN1*$^{f/f}$ mice (Figures S4A–C). Inducible deletion of *PTPN1* from compartmented cultures of *PTPN1*$^{fl/fl}$ sympathetic neurons, using a membrane-permeable CRE recombinase (TAT-CRE), also markedly attenuated soma-to-axon Trk transcytosis, similar to the results with pharmacological inhibition of PTP1B (Figures S4D–I). We next examined TrkA protein expression *in vivo* in sympathetic neuron cell bodies residing in the SCG, and in the salivary glands, a target tissue that is densely innervated by sympathetic axon projections from the SCG. TrkA protein expression was reduced by ~45% in salivary gland tissue with conditional *PTPN1* loss, whereas TrkA levels in sympathetic ganglia were unaffected (Figures 6A, B). The decrease in axonal TrkA expression is not a result of fewer sympathetic nerves in the target area in *PTPN1*-deficient mice since Tyrosine Hydroxylase (TH), a noradrenergic marker, was comparable between the two genotypes (Figure 6A). *PTPN1* deletion had no effect on levels of the p75 neurotrophin receptor in sympathetic axons, suggesting a specific need for PTP1B in regulating axonal expression of TrkA receptors (Figures S4J, K). These results suggest that PTP1B is essential for anterograde transport of TrkA receptors in sympathetic axons.

Mice lacking one copy of the *TrkA* allele have normal neuron numbers and target innervation during sympathetic nervous system development (Ghasemlou et al., 2004; Vaegter et al., 2011). Consistent with these findings, the 45% depletion in axon TrkA levels elicited by PTP1B loss did not significantly impact sympathetic neuron viability (23,077 ± 903 neurons in *TH-CRE;PTPN1*$^{f/f}$ mice *vs.* 22,939 ± 1,236 in control *PTPN1*$^{f/f}$ litter-mates) at postnatal day 0.5 (P0.5) (Figures 6C, D, G). Similarly, no deficits in target

innervation were observed in new-born *TH-CRE;PTPN1$^{f/f}$* mice (Figures 6H, I, L). To unmask a potential role for PTP1B in neurotrophin-mediated functions, we asked if *PTPN1* loss would exacerbate neuron deficits in *NGF* heterozygous mice, a scenario where sympathetic neurons are engaged in a developmental competition for sub-optimal NGF concentrations. Thus, we crossed *TH-CRE;PTPN1$^{f/f}$* mice with *NGF* heterozygous mice that have one functional copy of *NGF*. Consistent with previous studies reporting on the haplo-insufficiency of NGF (Brennan et al., 1999; Ghasemlou et al., 2004), we found that loss of one *NGF* allele results in a significant decrease (31% reduction) in neuronal number in the P0.5 SCG (15,807 ± 508 neurons in *PTPN1$^{f/f}$;NGF$^{+/-}$* mice *vs.* 22,939 ± 1,236 in control *PTPN1$^{f/f}$* litter-mates) (Figures 6E, G). New-born *PTPN1$^{f/f}$;NGF$^{+/-}$* mice also exhibited reduced sympathetic axon innervation of salivary glands relative to control *PTPN1$^{f/f}$* litter-mates (Figures 6J, L). We found that loss of *PTPN1* in sympathetic neurons aggravated the phenotypes induced by the absence of one *NGF* allele, with sympathetic neuron numbers and axon innervation being further diminished in *TH-CRE;PTPN1$^{f/f}$;NGF$^{+/-}$* mice compared to the *PTPN1$^{f/f}$;NGF$^{+/-}$* mice (Figures 6F, G, K, L). These results provide evidence of genetic interactions between *PTPN1* and *NGF*, and support the notion that PTP1B-dependent transcytosis of Trk receptors provides a competitive advantage for neurons when concentrations of target-derived NGF are limited.

Discussion

During embryonic development, sympathetic neurons are produced in excess, and their survival and innervation of target tissues is dictated by limiting amounts of target-derived NGF. Retrograde NGF-TrkA signaling in developing peripheral neurons provides one of the best examples of the significance of endosomal signaling from receptor tyrosine kinases in neurobiology. TrkA receptors, activated in nerve terminals upon ligand binding, are internalized into signaling endosomes that are retrogradely transported to neuronal cell bodies to regulate cytoplasmic and transcriptional pathways underlying neuron survival, growth, and synaptic connectivity (Yamashita and Kuruvilla, 2016). Here, we describe a new function for signaling endosomes in instructing the soma-to-axon transcytosis of newly synthesized TrkA receptors, and we define the molecular underpinnings (Figure 7). We show that active TrkA receptors, retrogradely transported in signaling endosomes, are inserted on soma surfaces where they elicit phosphorylation and subsequent endocytosis of naive soma surface-resident TrkA receptors. However, the trade-off for receptor phosphorylation and internalization in cell bodies is a potential to send active receptors to axons, which would prevent new interactions with ligand at nerve terminals. We identify a gate-keeping mechanism in which TrkA receptors, destined for transcytosis, are dephosphorylated in neuronal soma by the ER-resident tyrosine phosphatase, PTP1B. TrkA-PTP1B interactions occur in a ligand-dependent manner, and PTP1B activity in soma is necessary for axonal targeting. Thus, PTP1B activity in cell bodies ensures that inactive receptors are transported to engage with target-derived NGF. In the absence of PTP1B activity, TrkA receptors internalized in cell bodies are re-routed to lysosomes for degradation. We provide *in vivo* evidence that PTP1B is necessary for sufficient TrkA levels in axons, and genetic interaction analyses demonstrate that PTP1B is necessary for neuron survival and axon innervation when concentrations of target-derived NGF are attenuated. Together, these results provide

mechanistic insight into a positive feedback loop by which target-derived NGF recruits its own receptors to nerve terminals to amplify neuronal responsiveness.

How do retrogradely transported NGF-TrkA signaling endosomes elicit phosphorylation of naive receptors at the soma surface? A plausible mechanism is that exocytosis of retrograde signaling endosomes to soma surfaces elicits spontaneous auto-phosphorylation of soma surface-resident Trk receptors due to local increases in receptor density and receptor clustering on membrane micro-domains (Sawano et al., 2002; Verveer et al., 2000). It is also possible that extracellular release of NGF, co-transported in the retrograde endosomes, assists in receptor dimerization and phosphorylation. However, we reason that the majority of Trk receptors internalized from soma surfaces are likely to be un-liganded, because the amount of NGF that is retrogradely transported from axons and released from soma is not enough to be associated with the endocytosed receptors. Only ~2–2.5% of NGF bound to axon terminals undergoes retrograde transport (Tsui-Pierchala and Ginty, 1999; Ure and Campenot, 1997) and the majority of signaling endosomes are estimated to house a single NGF dimer (Cui et al., 2007).

A key question is how does PTP1B distinguish between soma- *versus* axon-derived TrkA receptors? We found that PTP1B is present in axons, but axon-specific inhibition of PTP1B did not affect the ability of axon-derived Trk receptors to mediate soma-to-axon transcytosis (Figure 4), suggesting that retrogradely transported active TrkA receptors are immune to the actions of PTP1B. It is likely that the presence of ligand in NGF-TrkA-signaling endosomes ensures the continuous phosphorylation of retrogradely transported TrkA receptors. In contrast, the soma surface-derived receptors that are un-liganded are susceptible to PTP1B-mediated dephosphorylation. Previously, PTP1B has been shown to distinguish between basally versus ligand-bound phosphorylated EGFRs (Baumdick et al., 2015).

Previous studies have reported on other tyrosine phosphatases that attenuate basal and/or ligand-stimulated TrkA activity (Faux et al., 2007; Marsh et al., 2003; Shintani and Noda, 2008; Zhang et al., 2016). Our findings that PTP1B enhances neuronal responsiveness to target-derived NGF emphasize that protein tyrosine phosphatases do not simply attenuate signaling responses to extracellular signals, but can act as positive regulators of receptor and receptor-associated tyrosine kinase signaling. Moreover, a notable aspect of our study is the unusual mechanism by which a tyrosine phosphatase positively regulates trophic factor signaling via exerting control of the long-distance trafficking of receptors to axonal growth cones.

Our observations that conditional *PTPN1* deletion in sympathetic neurons diminished axonal levels of TrkA by half without obvious impairment of neuron viability or axon growth are in agreement with previous studies of normal sympathetic neuron numbers and target innervation in mice lacking one functional allele of *TrkA* (Ghasemlou et al., 2004; Vaegter et al., 2011). However, PTP1B deficiency significantly attenuated sympathetic neuron numbers and target innervation specifically under conditions of NGF haplo-insufficiency, indicating that PTP1B activity is crucial for trophic support when NGF concentrations are sub-optimal. We do not exclude the possibility that PTP1B has additional substrates in sympathetic neurons. However, the observed genetic interactions between *NGF* and *PTPN1* argue that

the sympathetic neuron phenotypes observed in *TH-CRE;PTPN1$^{f/f}$;NGF$^{+/-}$* mice most likely arise from the loss of positive regulation of TrkA receptors, and not from global changes in tyrosine phosphorylation. Furthermore, there were no gross impairments in sympathetic nervous system development in *PTPN1* conditional mutant mice. These findings, together with previous observations that genetic ablation of *PTPN1* does not elicit global changes in tyrosine phosphorylation patterns in mouse embryonic fibroblasts (Myers et al., 2001), are consistent with the notion that PTP1B has limited and exquisite functional specificity *in vivo*. A mechanism that could contribute to functional specificity is the anchorage of PTP1B to the cytosolic face of the ER that topologically restricts access to specific substrates.

Our findings raise the intriguing possibility that different modes of axonal targeting are utilized during distinct stages of sympathetic nervous system development. Constitutive delivery via the direct secretory pathway might serve to deliver TrkA receptors to growing sympathetic axons during the initial phases of outgrowth from sympathetic ganglia and extension along intermediate targets, which are NGF-independent (Fagan et al., 1996; Glebova and Ginty, 2004; Wickramasinghe et al., 2008). However, when sympathetic axons reach final NGF-expressing targets, retrograde NGF signaling actively recruits the transcytosis machinery and PTP1B activity to augment anterograde delivery of TrkA receptors to gain a competitive advantage in the developmental competition for target-derived growth factor. A key question for future studies is whether regulation of the transcytosis machinery is a general mechanism for target-derived NGF to recruit additional membrane proteins necessary for axon growth and synapse maturation. Target-derived NGF is a potent trophic factor for sympathetic neurons, and it is feasible that NGF promotes the anterograde delivery of adhesion molecules, ion channels, synaptic components, and other membrane proteins, co-localized in the same transcytosing vesicles as TrkA, and necessary for fully functional nerve terminals. For example, the growth-promoting adhesion molecules, NgCAM and β-1-integrins, are transported to axons via transcytosis (Eva et al., 2010; Wisco et al., 2003), although it is unknown if their transport is regulated by target-derived NGF. The role of transcytosis in axonal targeting may have implications that extend beyond early neural development to the pathogenesis of some neurodegenerative disorders. Impaired soma-to-axon transcytosis of amyloid precursor protein (APP) and low-density lipoprotein receptors observed in human iPSC-derived neurons harboring mutations in familial Alzheimer's Disease-associated genes has been postulated to contribute to pathological deficits in synapse maintenance and function (Woodruff et al., 2016). In future studies, biochemical characterization of a "transcytosome" and identification of specific signaling pathways and endocytic machinery will be critical to fully characterizing this newly described mode of axonal targeting, and will help clarify how extracellular signals regulate long-distance communication between the axon and somato-dendritic membrane domains.

STAR METHODS

CONTACT FOR REAGENT AND RESOURCE SHARING

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Rejji Kuruvilla (rkuruvilla@jhu.edu).

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Animals—All procedures relating to animal care and treatment conformed to Johns Hopkins University Animal Care and Use Committee (ACUC) and NIH guidelines. Animals were housed in a standard 12:12 light-dark cycle. Postnatal day 0.5 (P0.5) pups of both sexes were used for analyses.

Neuronal cultures—Sympathetic neurons were harvested from P0.5 Sprague-Dawley rats or *PTPN1*$^{f/f}$ mice and grown in mass cultures or compartmentalized cultures as described previously (Bodmer et al., 2011). Cells were maintained in culture with high-glucose DMEM media supplemented with 10% fetal bovine serum (FBS), penicillin/streptomycin (1U/ml), and NGF (100 ng/ml). NGF was prepared as previously described (Mobley et al., 1976). For NGF deprivation, neurons were placed in high-glucose DMEM containing 0.5% FBS with 1:1000 anti-NGF and boc-aspartyl(O-methyl)-fluoromethylketone (BAF) (50 μM) for 36 hr. For adenoviral infections, neuronal cultures were infected with high-titer CsCl-purified pAdenoX-Tet3G adenoviruses for 36 hr. Adenovirus-mediated protein expression was induced by adding doxycycline (Sigma, 100 ng/ml) to culture media. For CRE-mediated deletion of PTP1B, cultured sympathetic neurons from *PTPN1*$^{f/f}$ mice were treated with TAT-CRE (1 μM) for 3 days. Neurons were then infected with adenoviruses as described above. In Figures S1D–F, GFP-Sec61 and mCherry-PTP1B$^{Y46F/D181A}$-Myc expressing vectors were electroporated by using P3 Primary Cell 4D-Nucleofector® X Kit (program number: CA-137) according to the manufacturer's instructions.

METHOD DETAILS

Adenoviral and plasmid constructs—PTP1B-Myc was generated by amplifying *PTP1B* by RT-PCR from mouse brain cDNA, and inserting into pcDNA3.1 Myc-His vector. PTP1B$^{Y46F/D181A}$-Myc, PTP1B^{C215S}-Myc, FLAG-TrkA^{R685A} constructs were generated from PTP1B-Myc or FLAG-TrkA using PCR-based site-directed mutagenesis. Mutant plasmids were verified by DNA sequencing. mCherry cDNA was then inserted into PTP1B constructs at their N-termini. Recombinant adenoviruses expressing mCherry-PTP1B^{WT}-Myc, mCherry-PTP1B-Myc$^{Y46F/D181A}$, FLAG-TrkB/A^{F592A}-P2A-GFP, or FLAG-TrkB/A$^{F592A/R685A}$-P2A-GFP were generated by sub-cloning into pAdenoX-Tet3G using the Adeno-XTM Adenoviral System 3 kit. Recombinant adenoviral backbones were packaged into infectious adenoviral particles by transfection into HEK 293 cells using Polyethyleneimine (PEI). High-titer viral stocks were purified using a CsCl gradient.

Live-cell antibody feeding

Soma-to-axon transcytosis in compartmentalized cultures: Live-cell antibody feeding assays to monitor Trk receptor transcytosis were performed as previously described (Ascano et al., 2009). Sympathetic neurons were grown in compartmentalized culture chambers, assembled on collagen-coated glass cover-slips for 9 days in vitro (DIV), until axonal projections were evident in the side compartments. Neurons were infected with an adenoviral vector expressing FLAG-TrkB:A chimeric receptors. Infected neurons were identified by GFP that is co-expressed with FLAG-TrkB:A using a self-cleaving P2A peptide sequence (GSGATNFSLLKQAGDVEENPGP). After withdrawing NGF from the

culture media, surface chimeric receptors residing in cell body compartments were labeled under live-cell conditions with a mouse anti-FLAG antibody (1:500) in Phosphate Buffered Saline (PBS) for 30 minutes at 4°C. Excess antibody was washed off and cultures were pre-treated with various pharmacological inhibitors locally applied to axon compartments (1NMPP1, U73122, FK506 + Cyclosporin A, Dynasore, PTP1B inhibitor, or NSC-87877) or to cell body compartments (PTP1B inhibitor, NSC-87877, or PTP1B inhibitor + Chloroquine) at 37°C for 30 minutes, followed by stimulation with BDNF (100 ng/ml) added to axon compartments for 4 hr. Neurons were then washed quickly with PBS, fixed with 4% paraformaldehye (PFA) in PBS for 30 minutes at room temperature, permeabilized with 0.1% Triton X-100/5% normal goat serum/PBS and incubated with anti-mouse Alexa-546 secondary antibody (1:1000; Thermo Scientific) for 2 hr, followed by mounting on slides with Fluoromount Aqueous Mounting Medium (Sigma) containing 100 μg/ml DAPI.

Cell body images were acquired using a Zeiss AxioImager Z1 imaging microscope with Hamamatsu Orca Flash 4.0 CMOS camera and Apotome 2. The same acquisition settings were applied to all images taken from a single experiment. Cell bodies were analyzed by using ZEN 2012 (blue edition) software. Intracellular accumulation of chimeric receptors in cell bodies were quantified as the number of FLAG-immunopositive punctae per neuron. Cell bodies were visualized using the GFP signal and FLAG signals overlapping with GFP fluorescence were defined as internalized soma surface-derived receptors. For all imaging, 15 cell bodies were analyzed per condition per experiment. Results are expressed as means ± SEM and expressed relative to the “no ligand” condition.

Axon-derived Trk receptors exocytosed to soma surfaces were quantified as the number of FLAG-immunopositive punctae per soma. 20 neurons were analyzed per condition per experiment. Axon images representing 0.8 μm optical slices were acquired using a Zeiss LSM 700 confocal scanning microscope with 405, 488, and 555nm lasers. The same confocal acquisition settings were applied to all images taken from a single experiment. Axons were analyzed by taking z-stacks and creating a 3-dimensional reconstruction using ZEN 2012 SP1 (black edition) software. Transcytosed chimeric receptors in axons were quantified as the number of FLAG-immunopositive punctae per square micrometer of distal axons. Axons were visualized using the GFP signal and measurements were taken from a stretch of axons 100 μm from the axon tip. For all imaging, 20 axons were analyzed per condition per experiment. Results are expressed as means ± SEM and expressed relative to the “no ligand” condition.

Exocytosis of axon-derived Trk receptors in soma: Chimeric FLAG-TrkB:A receptors in distal axon compartments of compartmentalized cultures were live-labeled with a mouse anti-FLAG antibody (1:500). Excess antibody was washed off, and axons were treated with BDNF (100 ng/ml, 1 hr, 37°C) or left un-stimulated. Neurons were fixed, and incubated with anti-mouse Alexa-546 secondary antibody without permeabilization. Images were acquired using a Zeiss AxioImager Z1 imaging microscope with Hamamatsu Orca Flash 4.0 CMOS camera and Apotome 2. The same acquisition settings were applied to all images taken from a single experiment. Axon-derived Trk receptors exocytosed to soma surfaces were

quantified as the number of FLAG-immunopositive punctae per soma. 20 neurons were analyzed per condition per experiment.

Dual color antibody feeding assay: Surface chimeric receptors residing in distal axon and cell body compartments were live-labeled with a mouse anti-FLAG antibody (1:500) and a rabbit anti-FLAG antibody (1:100), respectively. Excess antibodies were washed off and axons were stimulated with BDNF (100 ng/ml, 1 hr, 37°C) or left un-stimulated. Neurons were fixed, permeabilized using 0.1% Triton X-100/5% normal goat serum/PBS, and incubated with anti-mouse Alexa-546 and anti-rabbit Alexa-350 secondary antibodies. Images were acquired using a Zeiss AxioImager Z1 imaging microscope with Hamamatsu Orca Flash 4.0 CMOS camera and Apotome 2. Co-localization of axon and soma surface-derived Trk receptors were quantified as the number of punctae double-positive for Alexa-350 and Alexa-546 and expressed as a percentage of the Alexa-350-positive punctae in soma (total Trk receptors derived from soma surfaces). To investigate the interactions between axon and soma surface-derived Trk receptors, neurons were fixed, permeabilized, and interacting signals were visualized by Duolink *in situ* PLA kits according to the manufacturer's instructions. Number of PLA punctae per neuron were then counted.

Co-localization of FLAG-TrkB:A with mCherry-PTP1B-Myc$^{Y46F/D181A}$: Sympathetic neurons were infected with FLAG-TrkB:A and mCherry-PTP1B-Myc$^{Y46F/D181A}$ adenoviruses. Surface chimeric receptors in cell body compartments were live-labeled with a mouse anti-FLAG antibody, followed by stimulation of axons with BDNF (100 ng/ml) for 30 minutes. Neurons were fixed, permeabilized, and incubated with anti-mouse Alexa-350 secondary antibody. Images were acquired using a Zeiss AxioImager Z1 imaging microscope with Hamamatsu Orca Flash 4.0 CMOS camera and Apotome 2. Co-localization between FLAG-TrkB:A with mCherry-PTP1B-Myc$^{Y46F/D181A}$ was quantified as the % of FLAG punctae in cell bodies that co-localized with mCherry fluorescence. In Figures S1G–R, neurons were quickly stripped of surface-bound FLAG antibodies by three quick washes in an ice-cold acidic buffer (0.23N acetic acid, 0.53M NaCl, pH 3.0) followed by assessing co-localization as described above.

Co-localization of FLAG-TrkB:A with lysosomes or Rab11: Surface chimeric receptors in cell body compartments were live labeled with a mouse anti-FLAG antibody, and then treated with PTP1B inhibitor (200 nM) added to cell body compartments. Neurons were stimulated with BDNF on axons for 2 hr, then fixed and immunostained for anti-LAMP1 antibody (1:500) to assess the co-localization with lysosomes. To investigate the co-localization with Rab11, neurons were infected with FLAG-TrkB:A and mCherry-Rab11a adenovirus followed by antibody feeding. Images were acquired using a Zeiss AxioImager Z1 imaging microscope with Hamamatsu Orca Flash 4.0 CMOS camera and Apotome 2. The number of punctae double-positive for FLAG and LAMP1 or Rab11 were counted and expressed as a percentage of the total FLAG signal in cell bodies.

The co-localization signal, visualized in white color, in Figures 3G, 3J, 3M, 3P, 5B, 5C, S1I, S1L, S1O, S1R, S3E, and S3F were presented using co-localization highlighter (ImageJ).

Biochemical analyses

Cell-surface biotinylation: To examine the effect of retrograde neurotrophin signaling on phosphorylation of soma surface TrkA receptors, neurons were grown in compartmentalized cultures and surface proteins in cell body compartments biotinylated with a reversible membrane-impermeable form of biotin (EZ-Link NHS-S-S-biotin, 2 mg/ml in PBS) at 4°C for 25 minutes. Neurons were washed briefly with PBS containing 50mM glycine (Sigma-Aldrich) to remove remaining unconjugated biotin. Axons were stimulated with NGF (100 ng/ml, 1 hr, 37°C), following which cell body lysates were prepared with 300 μl of immunoprecipitation buffer (20mM Tris-HCl (pH 8.0), 150mM NaCl, 10mM NaF, 1mM Na_3VO_4, 1mM EDTA, 1% NP-40, cOmplete Mini protease inhibitor cocktail (Roche)), and subjected to precipitation using 40 μl immobilized neutravidin agarose beads (Life Technologies), followed by immunoblotting with anti-pTrkA^{Y794} (1:1000) and stripping and reprobing for anti-TrkA (1:1000) for normalization of protein amounts.

To examine surface levels of TrkA receptors, surface proteins in mass cultures of sympathetic neurons were biotinylated with 2 mg/ml of EZ-Link NHS-S-S-biotin at 4°C for 25 minutes. Neurons were washed briefly with PBS containing 50 mM glycine, and were stimulated with NGF (100 ng/ml) in the presence or absence of the PTP1B inhibitor (1 μM) for 90 minutes. Neurons were lysed with 500 μl of immunoprecipitation buffer and subjected to neutravidin precipitation and immunoblotting with anti-TrkA antibody, followed by stripping and reprobing for anti-TfR (1:1000) for protein normalization. Supernatants were immunoblotted with anti-TrkA antibody to assess total TrkA levels, followed by stripping and reprobing of blots for p85 (1:1000) for normalization.

Co-immunoprecipitation and immunoblotting analyses: HEK293T cells were seeded at 1.0×10^6 cells/dish in a 6-cm dish. After 24 hr, cells were transfected with a FLAG–TrkA expression vector together with PTP1B-Myc^{WT}, PTP1B-Myc$^{Y46F/D181A}$, or PTP1B-Myc^{c215S} plasmids using PEI. After 48 hr, cells were lysed with immunoprecipitation buffer (excluding Na_3VO_4), and then immunoprecipitated using mouse anti-FLAG antibody (1 μg) and Protein-G agarose beads (Santa Cruz). In Figure S2A, PTP1B inhibitor (1 μM) or NSC-87877 (10 μM) were added into culture media after plasmid transfection. Immunoprecipitated samples were immunoblotted with mouse anti-FLAG (1:1000), anti-Myc (1:1000), or anti-pTyr (1:5000) antibodies, while input lysates were subjected to anti-FLAG or anti-Myc western blotting.

To investigate interactions between endogenous TrkA and PTP1B, sympathetic neurons grown in mass cultures in NGF containing media for 6 DIV were infected with mCherry-PTP1B^{WT}-Myc or mCherry-PTP1B$^{Y46F/D181A}$-Myc adenoviruses. Neurons were subjected to NGF deprivation for 36 hr, and then stimulated with NGF (100 ng/ml) for 30 minutes. Lysates were subjected to immunoprecipitation using anti-Myc antibody and immunoblotted with anti-TrkA or anti-Myc antibodies. Supernatants were subjected to immunoblotting for TrkA, Myc or P-TrkA^{Y794}.

To detect endogenous PTP1B in sympathetic neurons, rat sympathetic neurons were grown in compartmentalized cultures for 9 DIV in the presence of NGF (100 ng/ml) added only to distal axons. Lysates prepared separately from cell body and distal axon compartments were

subjected to PTP1B immunoblotting (1:1000). To detect TrkA, PTP1B, or p75 in mouse tissues, SCG and salivary glands were dissected from mouse P0.5 pups. SCG lysates were directly subjected to immunoblotting for TrkA, PTP1B (1:2000), p75 (1:1000), TH (1:1000) or p85. Salivary gland lysates were subjected to immunoprecipitation using an anti-pan Trk (Santa Cruz Biotechnology; sc-7268, 1μg) antibody. Immunoprecipitated samples were immunoblotted with mouse anti-TrkA antibody, while input lysates were subjected to anti-p75 or anti-TH western blotting. Normalization for protein amounts was done by anti-TH or anti-p85 immunoblotting. All immunoblots were visualized with ECL Plus Detection Reagent (GE Healthcare).

Neuronal cell counts—P0.5 mouse torsos were fixed in PBS containing 4% PFA, and then cryoprotected in 30% sucrose-PBS. SCG sections (123μm) were stained with a solution containing 0.5% cresyl violet (Nissl). Cells with characteristic neuronal morphology and visible nucleoli were counted in every fifth Nissl-stained section.

Immunohistochemistry—P0.5 mouse sections (12 μm) were washed in PBS, permeabilized in PBS containing 0.1% Triton X-100, and blocked using 5% goat serum in PBS + 0.1% Triton X-100. Sections were then incubated with a rabbit anti-TH antibody (1:200) overnight. Following PBS washes, sections were incubated with anti-rabbit Alexa-488 secondary antibody (1:200; Thermo Scientific). Sections were then washed in PBS and mounted in Fluoromount Aqueous Mounting Medium containing 100 μg/ml DAPI. Images representing 1.8 μm optical slices were acquired using a Zeiss LSM 700 confocal scanning microscope with 405, 488. The same confocal acquisition settings were applied to all images taken from a single experiment. Quantification of sympathetic innervation density in the salivary glands was done by calculating integrated TH fluorescence density per unit area (ImageJ) from multiple random images.

RT-PCR analyses—Total RNA was prepared from dissected SCG using RNeasy mini purification kit (QIAGEN). RNA was then reverse transcribed using a M-MLV Reverse Transcriptase (Promega). Real-time qPCR was performed using a Maxima SYBR Green/Rox Q-PCR Master Mix (Thermo Scientific), in a StepOnePlus™ Real-Time PCR Systems (Thermo Scientific). PTP1B mRNA (NM_011201.3) levels were measured by using primers targeting nucleotides 701-890. GAPDH was used as a control. Each sample was analyzed in triplicate reactions. Fold change in *PTP1B* transcript levels was calculated using the $2^{(-\Delta\Delta Ct)}$ method, normalizing to GAPDH transcript.

QUANTIFICATION AND STATISTICAL ANALYSIS

Sample sizes were similar to those reported in previous publications (Ascano et al., 2009; Bodmer et al., 2011; Patel et al., 2015). Data were collected randomly. For practical reasons, analyses of neuronal cell counts and axon innervation in mouse tissues were done in a semi-blinded manner such that the investigator was aware of the genotypes prior to the experiment, but conducted the staining and data analyses without knowing the genotypes of each sample. All Student's t tests were performed assuming Gaussian distribution, two-tailed, unpaired, and a confidence interval of 95%. One-way or two-way ANOVA analyses with post hoc Tukey test were performed when more than two groups were compared.

Statistical analyses were based on at least 3 independent experiments, and described in the figure legends. All error bars represent the standard error of the mean (s.e.m).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights

- Retrograde TrkA signaling endosomes instruct soma-to-axon transcytosis of naive TrkA
- Soma surface TrkA is phosphorylated and endocytosed by axon-derived active TrkA
- Endocytosed somatic phospho-TrkA is dephosphorylated by ER-resident PTP1B
- PTP1B activity in soma is necessary for TrkA axon targeting and trophic functions

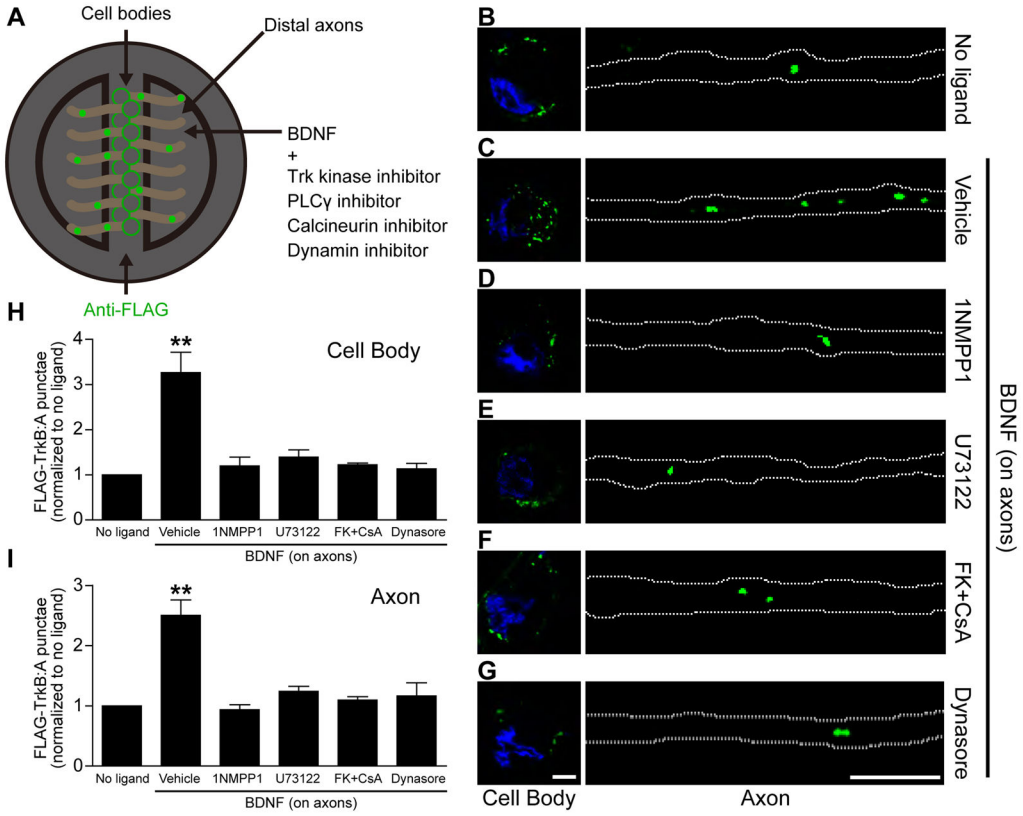

Figure 1. Trk activity and endocytosis in distal axons is necessary for transcytosis

(A) Antibody feeding to monitor soma-to-axon transcytosis of FLAG-TrkB:A receptors in compartmentalized cultures. Cell body compartments were live-labeled with FLAG antibody and distal axons were stimulated with BDNF (100 ng/ml, 4 hr) in the presence of inhibitors of Trk receptor kinase activity or endocytosis added to axon compartments. (B–G) Ligand-induced transcytosis of FLAG antibody-bound Trk receptors is suppressed by inhibition of Trk activity (1NMPP1) or PLCγ-calcineurin-dynamin-mediated endocytosis in distal axons. Arrowheads indicate soma surface-derived FLAG-labeled receptors in axons. Nuclei were stained by DAPI (blue). Scale bars, 5 μm. (H, I) Quantification of FLAG-Trk punctae in cell bodies (H) or axons (I). **p<0.01 relative to “no ligand” condition, two-way ANOVA and Tukey-Kramer post-hoc test. Results are means ± SEM from 3 independent experiments. 15 cell bodies (H) or 20 axons (I) were counted per experiment.

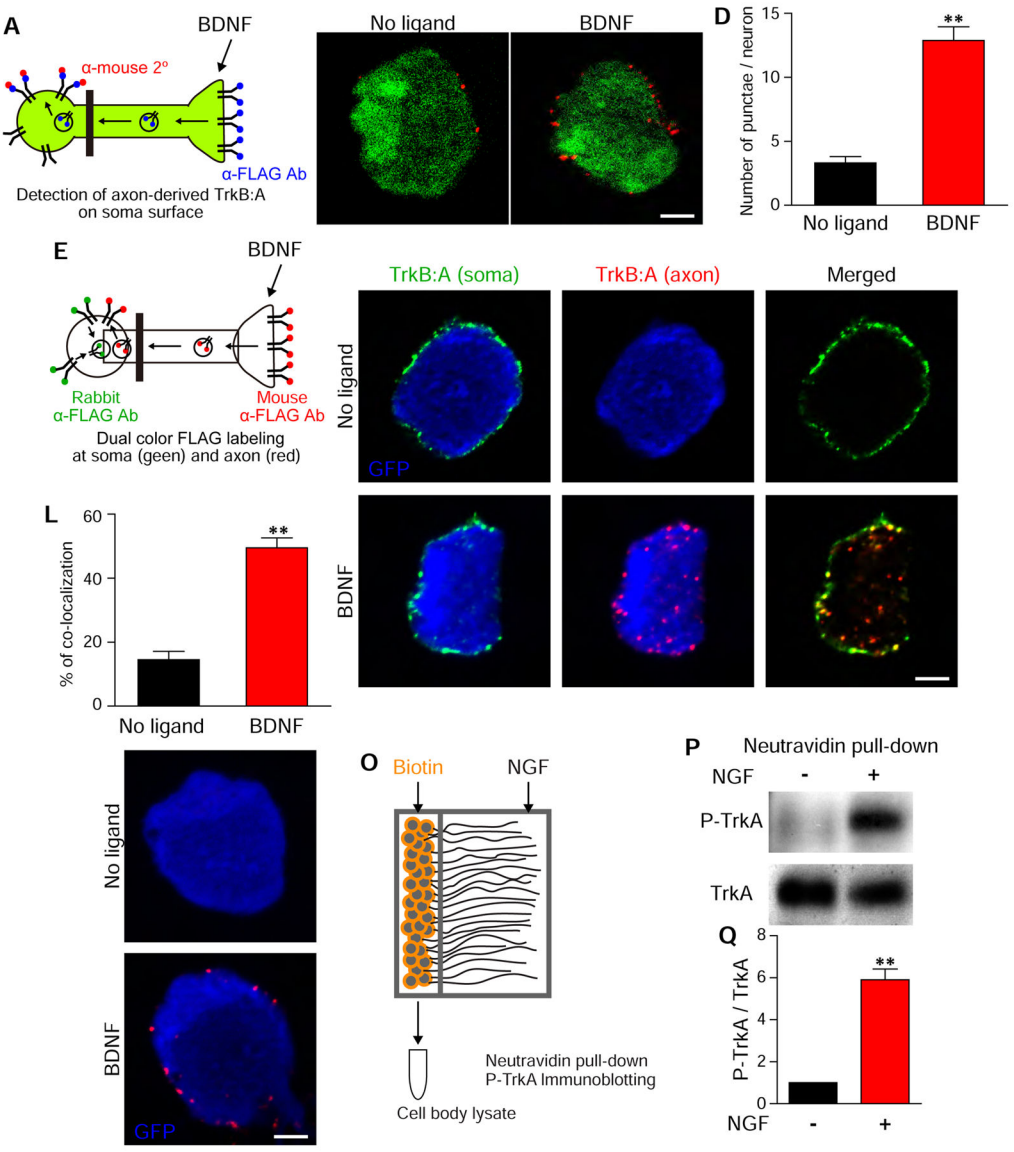

Figure 2. Axon- and soma surface-derived Trk receptors interact in cell bodies

(A) Antibody feeding to detect exocytosis of axon-derived FLAG-TrkB:A receptors in cell bodies. Axon compartments were live-labeled with FLAG antibody, distal axons stimulated with BDNF (100 ng/ml, 1hr), and neurons incubated with fluorescent secondary antibody without permeabilization. (B,C) Retrogradely transported Trk receptors undergo exocytosis on soma membranes. GFP is co-expressed with FLAG-TrkB:A. Scale bar, 5 μm. (D) Quantification of FLAG punctae per neuron. **p<0.01, *t*-test. Results are means ± SEM from 3 independent experiments. 20 neurons were analyzed per condition per experiment. (E) Dual color antibody feeding for simultaneous detection of axon and soma surface Trk receptors. Axon and cell body compartments were live-labeled with mouse anti-FLAG antibody (red) and rabbit anti-FLAG antibody (green), respectively and distal axons were stimulated with BDNF (100 ng/ml, 1hr). (F–K) Axon- and soma surface-derived Trk receptors co-localize near the cell body perimeter. GFP is co-expressed with FLAG-TrkB:A.

Scale bar, 5 μm. (L) Quantification of co-localization of axon and soma surface-derived Trk receptors. **p<0.01, *t*-test. n=45 neurons each for un-stimulated and ligand treatments, from 3 independent experiments. (M, N) Interactions between axon- and soma surface-derived Trk receptors are visualized by *in situ* PLA. GFP is co-expressed with FLAG-TrkB:A. Scale bar, 5 μm. Representative images are from at least 20 neurons analyzed per treatment, from 3 independent experiments. (O–Q) Soma surface-resident TrkA receptors are phosphorylated by retrograde NGF signaling. Cell bodies in compartmentalized cultures were biotin-labeled before stimulating distal axons with NGF (100 ng/ml, 1 hr). Cell body lysates were subjected to neutravidin precipitation and immunoblotting using anti-P-TrkA^{Y794}. P-TrkA immunoblots were stripped and reprobed for TrkA for normalization of protein amounts. (Q) Densitometric quantification of P-TrkA levels normalized to total TrkA. Results are means ± SEM from 3 independent experiments, and expressed relative to the un-stimulated condition. **p<0.01 *t*-test.

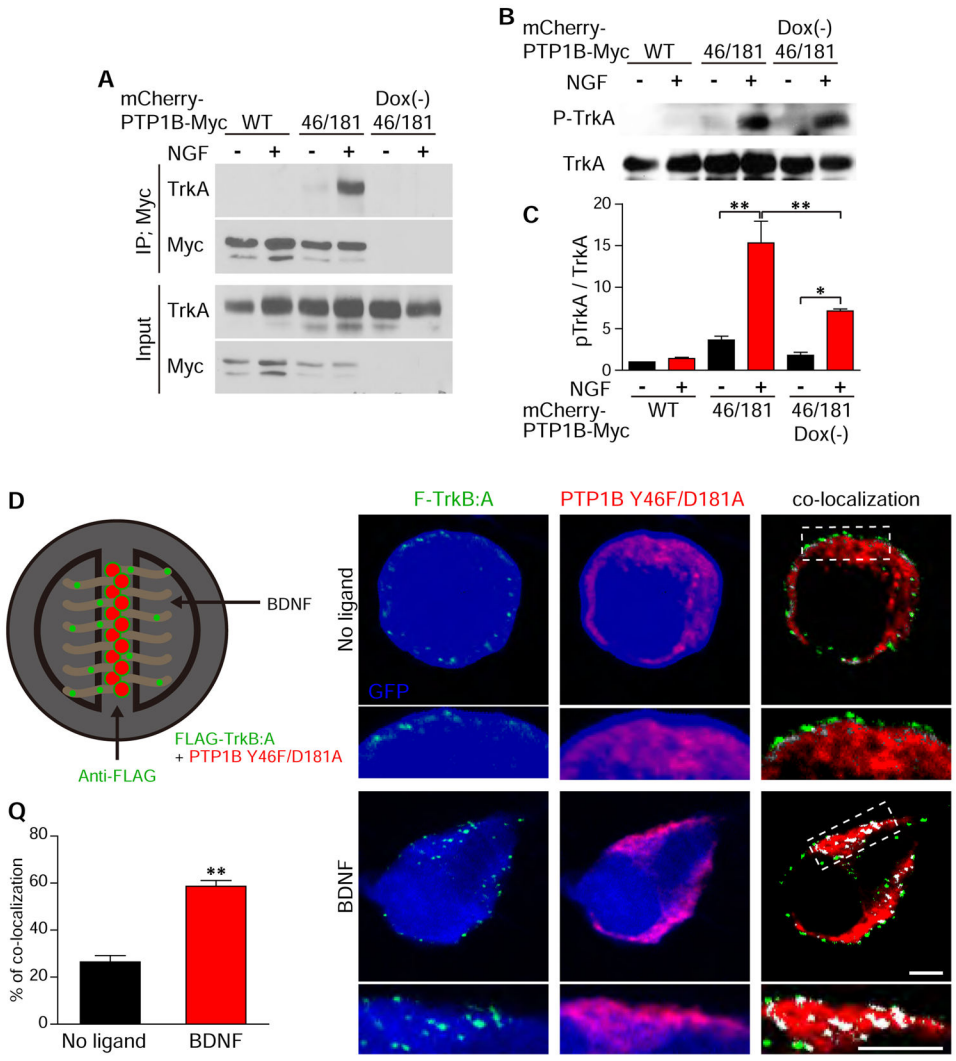

Figure 3. PTP1B dephosphorylates endocytosed soma surface-derived Trk receptors

(A) NGF-induced association of TrkA with PTP1B$^{Y46F/D181A}$ substrate trapping mutant, but not PTP1B^{WT}, in sympathetic neurons. Adenoviruses under Tet-On regulation were used to express PTP1B^{WT}-Myc or PTP1B$^{Y46F/D181A}$-Myc in sympathetic neurons. No exogenous PTP1B-Myc expression is observed in absence of doxycycline (Dox). Immunoprecipitation was done with anti-Myc, and immunoblotting with anti-Myc or anti-TrkA antibodies. (B) NGF-induced tyrosine phosphorylation of TrkA is suppressed by PTP1B^{WT} over-expression, and enhanced by PTP1B$^{Y46F/D181A}$ substrate-trapping mutant. (C) Densitometric quantification of P-TrkA levels normalized to total TrkA. **p<0.01, *p<0.05, two-way ANOVA and Tukey-Kramer post-hoc test. Results are mean ± SEM from 3 independent experiments. (D) Visualization of soma surface FLAG-TrkB:A receptors and mCherry-PTP1B$^{Y46F/D181A}$ in compartmentalized neurons. (E–P) Peripheral co-localization of intracellular Trk receptors, originating from soma surfaces, and mCherry-PTP1B$^{Y46F/D181A}$ in BDNF-stimulated neurons. Magnified images of dashed boxed areas in (G) and (M) are shown in lower panels (H–J and N–P). Co-localization of FLAG-TrkB:A and PTP1B is shown in white, using Image J co-localization highlighter (G, J, M, P). GFP is co-expressed

with FLAG-TrkB:A. Scale bars, 5 μm. (Q) Quantification of co-localization of TrkB:A and mCherry-PTP1B$^{Y46F/D181A}$. **p<0.01 *t*-test. Results are mean ± SEM from 3 independent experiments. Total of 45 neurons per condition were analyzed.

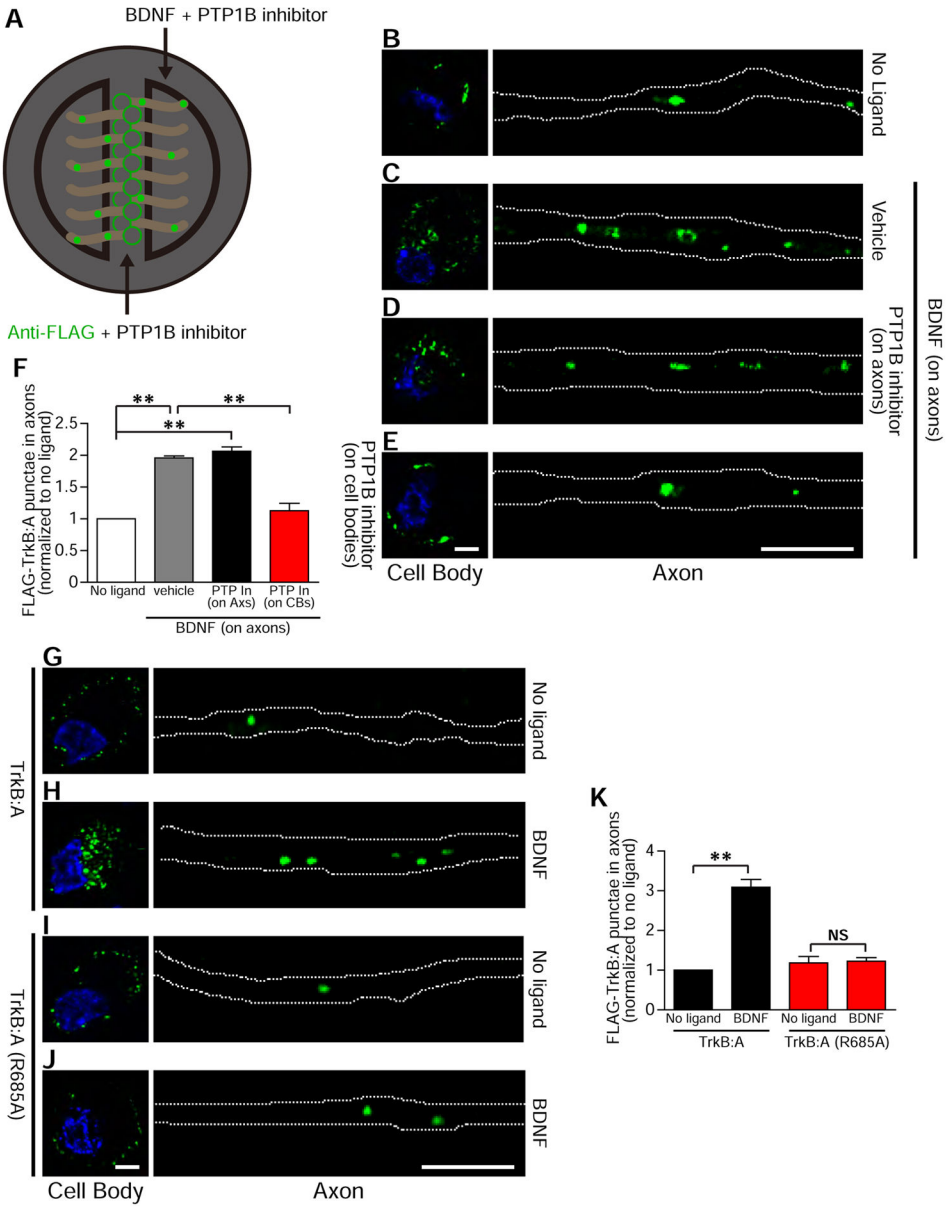

Figure 4. PTP1B activity in cell bodies is required for soma-to-axon Trk transcytosis

(A) Antibody feeding to monitor transcytosis of FLAG-Trk receptors in compartmentalized cultures with PTP1B inhibitor (200 nM) added either to cell body or axon compartments. Distal axons were stimulated with BDNF (100 ng/ml, 4 hr). (B–E) PTP1B activity in cell bodies, but not distal axons, is required for Trk soma-to-axon transcytosis. Arrowheads indicate soma surface-derived FLAG-labeled receptors in axons. Nuclei were labeled by DAPI (blue). Scale bars, 5 μm. (F) Quantification of FLAG-Trk punctae in axons. **p<0.01 relative to “no ligand” condition, two-way ANOVA and Tukey-Kramer post-hoc test. Results are means ± SEM from 3 independent experiments. 20 axons per condition were counted per experiment. (G–J) Mutant FLAG-TrkB:A^{R685A} receptors, unable to bind PTP1B and undergo dephosphorylation, show impaired soma-to-axon transcytosis. Compartmented sympathetic neurons were infected with adenoviruses for FLAG-TrkB:A^{R685A} or control

FLAG-TrkB:A receptors. Arrowheads indicate soma surface-derived FLAG-labeled receptors in axons. Nuclei were labeled by DAPI (blue). Scale bars, 5 μm. (K) Quantification of FLAG-Trk punctae in axons. **$p<0.01$ relative to "no ligand for FLAG-TrkB:A" condition, two-way ANOVA and Tukey-Kramer post-hoc test. Results are means ± SEM from 3 independent experiments. 20 axons were counted per experiment.

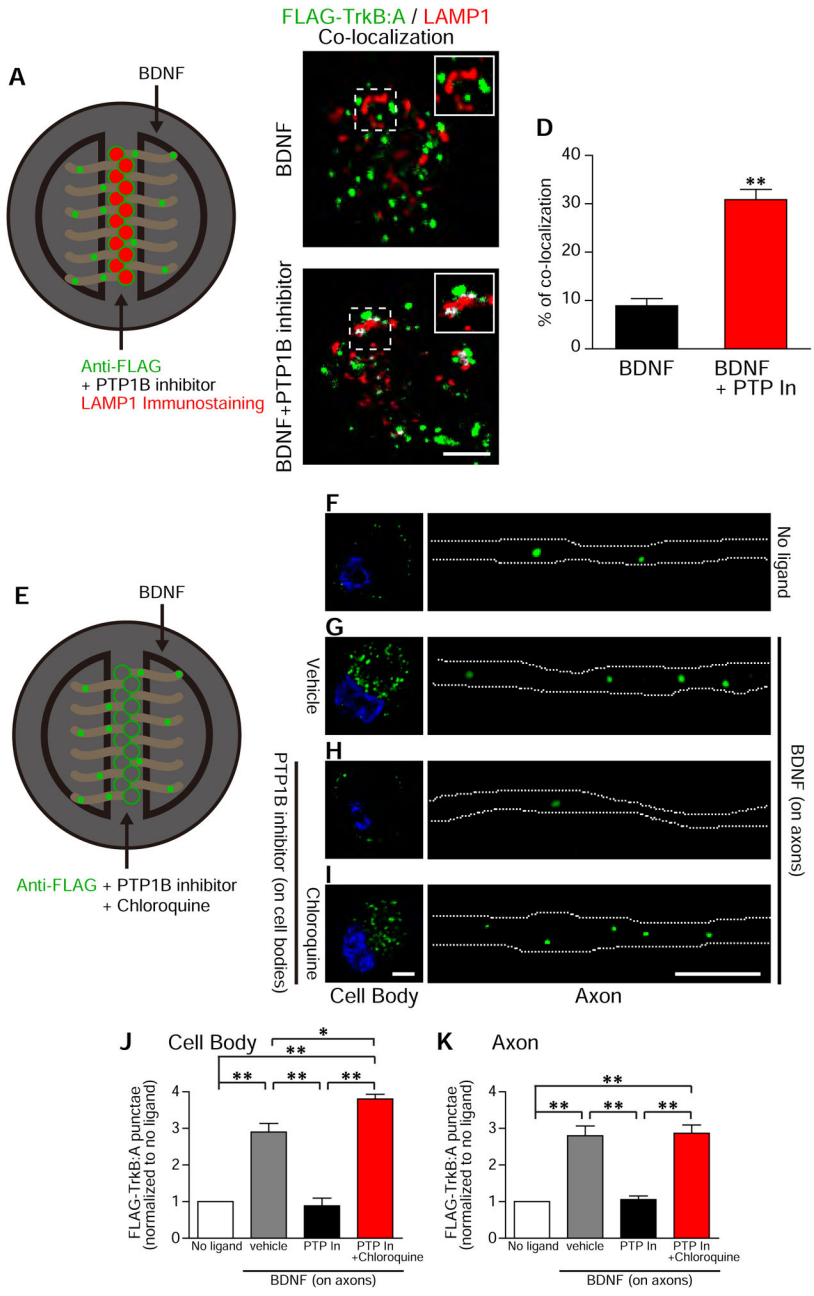

Figure 5. Soma surface-derived Trk receptors undergo lysosomal degradation upon PTP1B inhibition

(A) FLAG antibody feeding and LAMP-1 immunostaining to detect soma surface FLAG-TrkB:A receptors and lysosomes in compartmentalized neurons. Distal axons were stimulated with BDNF (100 ng/ml, 2 hr), and PTP1B inhibitor was locally applied to cell body compartments. (B–D) Soma-specific inhibition of PTP1B promotes lysosomal targeting of soma surface-derived Trk receptors. Co-localization of FLAG-TrkB:A and LAMP-1 is shown in white using Image J co-localization highlighter. Magnified images of dashed boxed areas in (B) and (C) are shown in inserts on top right. Scale bar, 5 μm. (D) Quantification of co-localization between TrkB:A and anti-LAMP1. **p<0.01, *t*-test.

Results are mean ± SEM from 3 independent experiments. Total 45 neurons were analyzed. (E) Inhibition of lysosome acidification prevents the degradation of Trk receptors elicited by PTP1B inactivation. Cell body compartments were live-labeled with FLAG antibody in the presence of chloroquine (50 μM) and PTP1B inhibitor (200 nM), while distal axons were stimulated with BDNF (100 ng/ml, 4 hr). (F–I) Chloroquine treatment of cell bodies antagonizes the PTP1B inhibitor-mediated disappearance of soma surface-derived Trk receptors. FLAG antibody-bound Trk receptors accumulate in neuronal cell bodies and are also transported to axons in neurons treated with chloroquine + PTP1B inhibitor locally on cell bodies and BDNF on distal axons. Arrowheads indicate soma surface-derived FLAG-labeled receptors in axons. Nuclei were stained by DAPI (blue). Scale bars, 5 μm. (J, K) Quantification of FLAG-Trk punctae in cell bodies (J) or axons (K). **$p<0.01$ relative to “no ligand” condition, two-way ANOVA and Tukey-Kramer post-hoc test. Results are means ± SEM from 3 independent experiments. 15 cell bodies (J) or 20 axons (K) were counted per experiment.

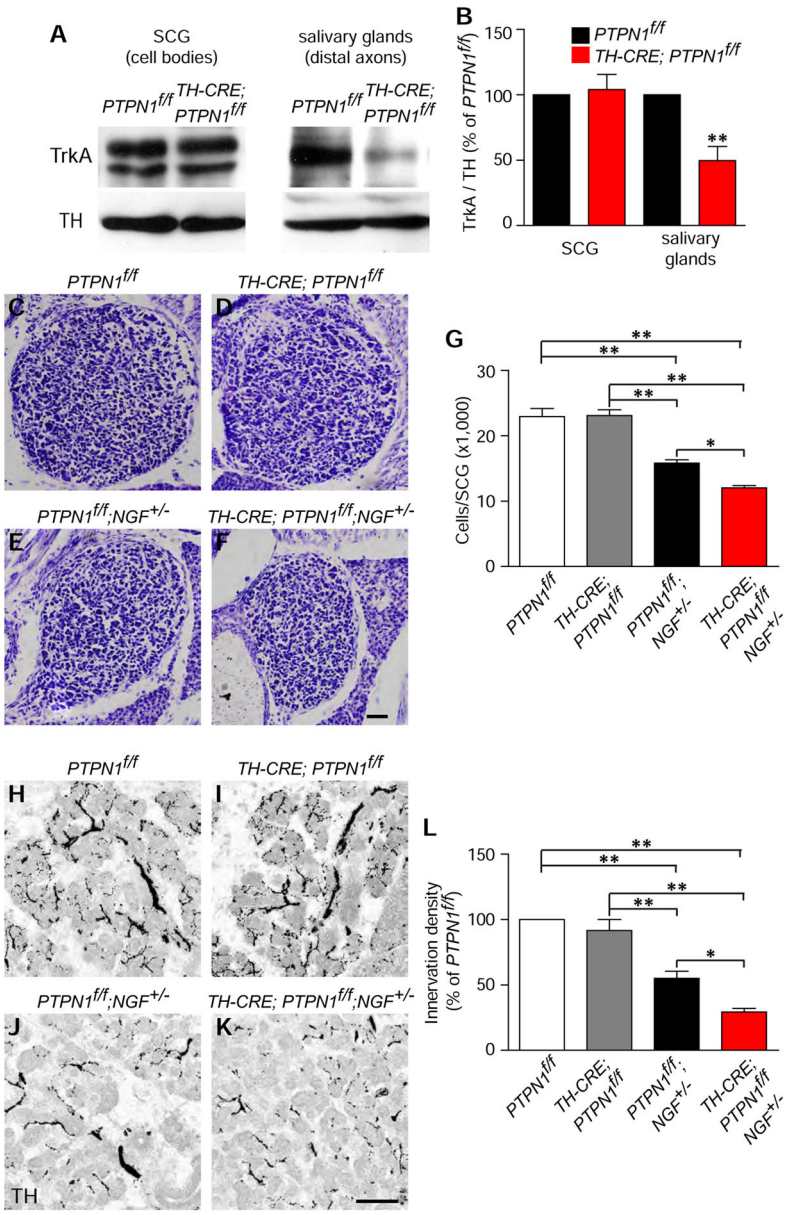

Figure 6. *PTPN1* deletion decreases axonal TrkA levels and disrupts NGF-dependent trophic functions

(A) Conditional *PTPN1* deletion in sympathetic neurons attenuates TrkA protein levels in axon terminals innervating salivary glands, but not in cell bodies residing in superior cervical ganglia (SCG). Sympathetic ganglia and salivary glands from P0.5 *TH-CRE;PTPN1$^{f/f}$* and control *PTPN1$^{f/f}$* mice were harvested and immunoblotted for TrkA, followed by stripping and reprobing for Tyrosine Hydroxylase (TH). (B) Densitometric quantification of TrkA levels normalized to TH. **p<0.01 *t*-test. Results are means ± SEM from 5 mice per genotype. (C–G) *PTPN1* deletion exacerbates sympathetic neuron loss in *NGF$^{+/-}$* mice. New-born *NGF$^{+/-}$* mice have a 31% decrease in SCG numbers which is further reduced by *PTPN1* loss. SCGs were visualized by Nissl staining and cell counts were performed on Nissl stained tissue sections. Scale bar, 200 μm. **p<0.01, *p<0.05, ANOVA

and Tukey-Kramer post-hoc test. Results are mean ± SEM from 4 mice per genotype. (H–L) *PTPN1* loss aggravates sympathetic innervation deficits in $NGF^{+/-}$ mice, assessed by TH immunohistochemistry of P0.5 salivary gland tissue sections. Normalized innervation density (% of $PTPN1^{f/f}$) is shown in (L). Scale bar, 100 μm. **p<0.01, *p < 0.05, one-way ANOVA. Results are mean ± SEM from 4 mice per genotype.

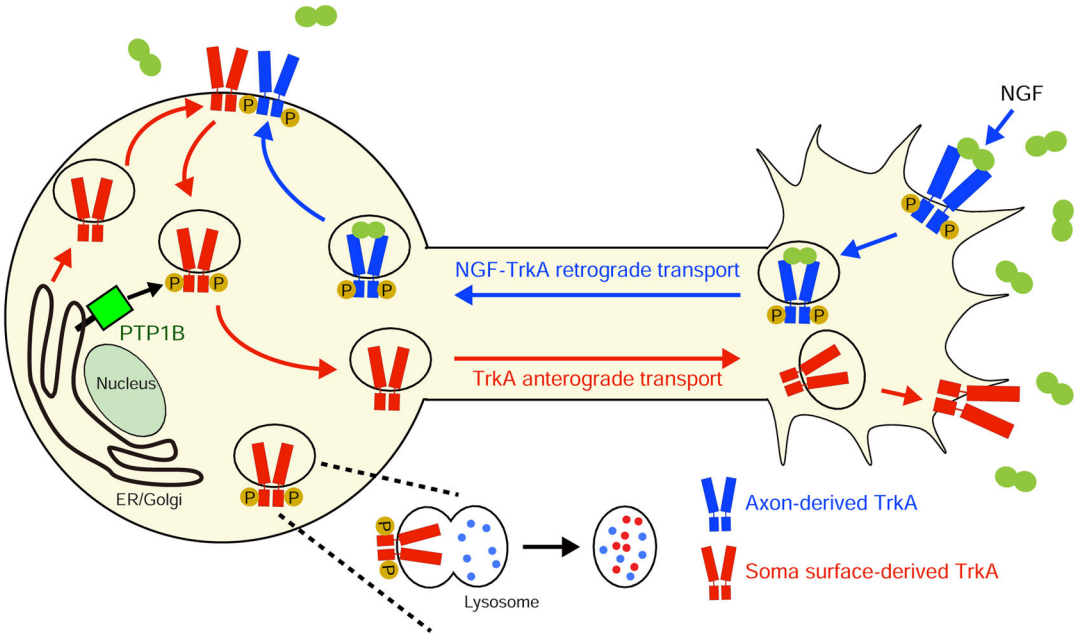

Figure 7. Retrograde control of soma-to-axon transcytosis of Trk receptors

NGF signaling, initiated in axon terminals, is necessary for anterograde transport of new TrkA receptors from soma surfaces. Active TrkA receptors, retrogradely transported in NGF-TrkA-harboring signaling endosomes, are inserted on soma surfaces where they elicit phosphorylation and subsequent endocytosis of naive soma surface-resident TrkA receptors. The endocytosed receptors are then dephosphorylated by PTP1B, an ER-resident protein tyrosine phosphatase, to ensure axon targeting of inactive receptors to engage with ligand. PTP1B inactivation in soma results in lysosomal degradation of TrkA receptors. *In vivo*, PTP1B is necessary for replenishing axonal TrkA levels and for NGF-mediated trophic support of sympathetic neurons under suboptimal NGF concentrations. These results identify phospho-regulatory mechanisms that underlie a positive feedback loop where target-derived NGF recruits its own receptors to nerve terminals to amplify neuronal responsiveness.