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Neuronal growth cone retraction relies upon proneurotrophin receptor signaling through Rac

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Abstract

Growth of axons and dendrites is a dynamic process that involves guidance molecules, adhesion proteins, and neurotrophic factors. Although neurite extension during development has been extensively studied, the intracellular mechanisms that mediate neurite retraction are poorly understood. Here, we show that the proneurotrophin, proNGF, induces acute collapse of growth cones of cultured hippocampal neurons. This retraction is initiated by an interaction between p75^{NTR} and the sortilin family member, SorCS2 (sortilin-related VPS10 domain containing receptor 2). Binding of proNGF to the p75^{NTR}-SorCS2 complex induced growth cone retraction by initiating the dissociation of the guanine-nucleotide exchange factor Trio from the p75^{NTR}-SorCS2 complex, resulting in decreased Rac activity, and consequently, growth cone collapse. The actin-bundling protein fascin also became inactivated, contributing to the destabilization and collapse of actin filaments. These results identify a bifunctional signaling mechanism by which proNGF regulates actin dynamics to modulate neuronal morphology acutely.

Introduction

The formation of neuronal networks in the developing nervous system depends on the navigation of axons and dendrites, which is controlled by attractive and repulsive guidance

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Supplementary Methods

Legend for Supplementary Movie 1: ProNGF leads to growth cone collapse.

Fig. S1: Two additional examples showing proNGF-induced growth cone collapse.

Fig. S2: p75^{NTR} and SorCS2 are the receptors mediating growth cone retraction.

Fig. S3: Identification of Trio.

Fig. S4: Generation of the kinase-dead Trio mutant.

Fig. S5: Decrease of Rac- but not RhoA activity induces growth cone collapse.

Fig. S6: Fascin and p75^{NTR} form a complex in embryonic brain lysates.

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Competing interests: Cornell University has an issued patent and pending patent applications related to the work reported herein.

cues (1, 2). Although much attention has focused on the signaling mechanisms that promote the branching and extension of dendrites and axons, the extrinsic signals that restrict the size and extent of growth of neuronal processes remain incompletely defined. In particular, the molecular mechanisms by which extrinsic signals are translated to intracellular pathways mediating retraction are not well established.

Growth cones at the tip of extending neurites are rich in actin structures such as lamellopodia and filopodia, which in turn are stabilized by the actin bundling protein fascin (3). The dynamic extension and retraction of these actin structures is thought to control growth cone motility and is regulated by Rho family GTPases (4).

In addition to ephrins, neuropilins, and semaphorins (5, 6), the outgrowth of dendrites and axons is influenced by neurotrophins (7, 8). Neurotrophins, which include nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin (NT)-3, and NT-4, are required for neuronal survival and differentiation, synapse formation, and synaptic plasticity (9). These secreted proteins act through two classes of receptor, the tropomyosin-related kinase (Trk) receptor, which transduces survival and differentiation signals, and the p75 neurotrophin receptor (p75^{NTR}), which encodes an intracellular death domain. Neurotrophins are synthesized as precursors, or proneurotrophins, that can be cleaved intracellularly by furin or prohormone-convertases, or extracellularly by plasmin or matrix metalloproteases, to produce mature forms (10–12). Mature NGF activates TrkA to mediate survival and differentiative signaling. However, proneurotrophins, including proNGF, are not inactive; they bind to a receptor complex of p75^{NTR} and sortilin, a Vps10p (vacuolar protein sorting 10 protein) domain-containing transmembrane protein, to initiate apoptosis (10, 12, 13).

Here, we have identified proNGF as a ligand that initiates acute collapse of growth cones in hippocampal neurons. This action required p75^{NTR} and SorCS2 (sortilin-related VPS10 domain containing receptor 2), another Vps10p domain family member that served as a co-receptor with p75^{NTR}. ProNGF induced displacement of the guanine-nucleotide exchange factor (GEF) Trio from p75^{NTR} and SorCS2, thereby decreasing local activity of the small GTPase Rac and filopodia formation. ProNGF binding also led to phosphorylation of the actin bundling protein fascin by protein kinase C (PKC), and thereby to fascin inactivation and destabilization of existing actin filaments. These results thus identify two mechanisms by which an extracellular cue, proNGF, initiates intracellular pathways to regulate neuronal morphology by inducing rapid growth cone collapse.

Results

ProNGF induces growth cone collapse

Neurotrophins have been classically defined as trophic factors involved in growth cone turning and extension (14). However, treatment of primary mouse hippocampal neurons with 0.1 nM proNGF led to rapid freezing of growth cone movement, followed by growth cone collapse over a period of minutes. We imaged actin dynamics in live neurons by using a peptide that labels filamentous actin (F-actin) without disrupting the cytoskeleton (15). Under basal conditions, filopodial movement in the growth cone was dynamic (Fig. 1A; see also Movie S1 and fig. S1). However, in a subset (~25%) of neurons, the motion of the growth cone froze within 2 min of proNGF addition and the now rigid actin structures subsequently collapsed. This collapse was not limited to LifeAct-labeled filaments, as judged by differential interference contrast (DIC) microscopy images or by staining for endogenous actin, fascin, and p75^{NTR} (Fig. 1B, lower panel; see also fig. S1). The remaining neurons (~75%) were unaffected by proNGF.

ProNGF binds to a p75^{NTR} and sortilin receptor complex (10, 12). To determine if the subpopulation of cells responsive to proNGF had these receptors, cultures were treated with proNGF for 20 min, and then fixed and stained for endogenous actin, the actin-bundling protein fascin, and p75^{NTR}. Untreated cells displayed extended, fan-like growth cones and filopodia rich in actin and fascin (Fig. 1B, top panel, C). Cells positive for p75^{NTR} responded to proNGF treatment with growth cone collapse (Fig. 1B, lower panel, see arrows, C). Growth cones of p75^{NTR}-negative cells did not collapse after addition of proNGF, however, nor did NGF elicit actin rearrangement in p75^{NTR}-positive cells (Fig. 1B, lower panel, see asterisks, C). Cultures from p75-deficient embryos failed to respond to proNGF (fig. S2A), indicating that p75^{NTR} was required for proNGF-mediated collapse of actin- and fascin-rich protrusions.

We were not able to detect sortilin in neurons of this age [neurons collected at embryonic day 15 (E15) and stained at 3 days in vitro (DIV3), fig. S2B, C]. However, we detected a related protein, SorCS2, in p75^{NTR}-positive cells in vitro (Fig. 1D, fig. S2C) and in the hippocampus (fig. S2D) and immunoprecipitation of transfected cells indicated that, like sortilin, SorCS2 interacted with proNGF (Fig. 1E). To determine if SorCS2 was involved in retraction of actin-rich structures, we pre-incubated cultures with an antibody directed against the SorCS2 ectodomain before adding proNGF. Then, we fixed the cells and visualized actin, fascin, and p75^{NTR} localization. Pre-incubation with anti-SorCS2 had no independent effect on neuronal morphology, but it blocked the ability of proNGF to induce growth cone collapse, whereas control IgG or antibodies directed against the sortilin ectodomain did not impair the proNGF response (Fig. 1F and fig. S2E). These data suggest that p75^{NTR} and SorCS2 act as co-receptors for proNGF to mediate acute alterations in actin morphology.

Trio interacts with the p75^{NTR} and SorCS2 receptor complex

To identify signaling proteins downstream of the proNGF-activated p75^{NTR} and SorCS2 complex, we performed a proteomic analysis of proteins that specifically associated with p75^{NTR} and the closely related SorCS2 family member, sortilin, as compared to p75^{NTR} alone. Among the proteins detected by mass spectrometry, we identified Trio, a scaffold protein containing Rac and Rho GEF domains (16) (Fig. 2A and fig. S3). We confirmed the interaction between p75^{NTR} and Trio by co-immunoprecipitation. Co-expression of p75^{NTR} and sortilin, or p75^{NTR} and SorCS2 in cells containing Trio or individual Trio domains resulted in a stable complex containing p75^{NTR} and Trio (Fig. 2B), an interaction that required the Trio putative serine and threonine kinase (kin) domain (Fig. 2C–E). To determine if ligand binding altered the interaction of Trio with p75^{NTR} and SorCS2, we treated cells co-expressing these receptors with proNGF, and then immunoprecipitated the lysates with anti-p75^{NTR}. Addition of proNGF markedly reduced the basal interaction of p75^{NTR} with Trio (Fig. 2F, G), indicating that proNGF induces the displacement of Trio from the p75^{NTR} and SorCS2 complex.

In hippocampal neurons, endogenous Trio localized to actin-rich protrusions (Fig. 3A, top panel and fig. S4A), as previously shown (17). ProNGF led to the loss of Trio from the neurite tip in p75^{NTR}-positive cells (Fig. 3A, bottom panel, arrows), whereas Trio remained enriched in intact growth cones of p75^{NTR}-negative cells (Fig. 3A, bottom panel, asterisk). We confirmed the presence of the p75^{NTR}, SorCS2, and Trio trimeric complex in embryonic rat brain lysates in vivo (Fig. 3B). We expressed the Trio kinase domain, or a kinase-dead mutant (trio kin^{K2921A}; fig. S4B), in cultured hippocampal neurons to displace endogenous Trio (and thereby its GEF1 and GEF2 activities) from the p75^{NTR} complex. This led to collapse of actin structures in absence of proNGF (Fig. 3C, D), indicating that Trio displacement is sufficient to induce growth cone collapse.

ProNGF binding leads to Rac inactivation

The *Drosophila* Trio protein promotes axon growth and guidance through its ability to act as a GEF for Rac (18). To assess whether proNGF-induced growth cone collapse is a consequence of the inactivation of Trio and thereby Rac, we used the Cdc42/Rac interactive binding (CRIB) domain of the Rac effector p21-activated kinase (PAK-CRIB) to isolate and purify activated Rac (19). ProNGF exposure led to a significant decrease in Rac activity in primary hippocampal neurons compared to that in untreated neurons (Fig. 4A, B). To determine whether decreased Rac activity resulted in growth cone collapse, we incubated hippocampal neurons with the Rac inhibitor EHT 1864, or the Trio GEF1 domain inhibitor ITX3 (20). Both drugs markedly decreased Rac activity in primary neuronal cultures (fig. S5A) and promoted growth cone collapse in all treated cells (Fig. 4C), consistent with the notion that Rac inactivation may underlie the proNGF-induced retraction of actin-rich structures. Finally, we evaluated whether expression of the Trio GEF1 domain, which promotes Rac activation, could overcome proNGF-induced growth cone collapse. Indeed, expression of the Trio GEF1 domain abolished proNGF induced growth cone retraction (Fig. 4D, E). In contrast, inactivation of RhoA with C3 transferase or exposure to the ROCK (Rho-associated kinase) inhibitor Y-27632 neither induced growth cone collapse nor did these treatments interfere with proNGF-mediated growth cone collapse (Fig. 4F, fig. S5B, C). Together, these results suggest that proNGF binding to the p75^{NTR} and SorCS2 receptor complex leads to displacement of Trio, reduced activation of Rac, and collapse of actin-rich protrusions.

ProNGF-dependent collapse requires fascin phosphorylation

Live imaging of neurons treated with proNGF revealed an initial reduction in the extension and retraction of actin filaments, which was followed by their overt collapse (Fig. 1A, fig. S1; Movie S1). Therefore, we investigated a potential role for the actin filament stabilizing protein, fascin, in mediating growth cone retraction. Fascin is highly enriched in growth cones (3), although the mechanisms that regulate fascin bundling of filamentous actin in neurons are unclear. However, fascin interacts directly with p75^{NTR} in non-neuronal cells, (21) and inactivation of fascin by protein kinase C (PKC)-dependent phosphorylation at Ser³⁹ (22) abrogates proNGF-dependent melanoma cell migration in a PKC-dependent manner (21).

We found that fascin also interacted with p75^{NTR} in embryonic brain lysates (fig. S6). Therefore, we hypothesized that proNGF-induced fascin phosphorylation and its dissociation from actin filaments may contribute to growth cone destabilization and facilitate their collapse. To test this hypothesis, we preincubated hippocampal neurons with the PKC inhibitor Gö6976 or the small peptide PKC inhibitor 20–28 before exposing them to proNGF. In both cases, proNGF failed to induce collapse of actin-rich structures (Fig. 5A, C), suggesting that PKC-dependent phosphorylation was required for this process. To confirm whether fascin was the PKC target, we expressed fascin, or a fascin phosphorylation mutant [fascin^{S(36,38,39)A}, in which serines 36, 38, and 39 are substituted with alanines] that mimics the active, actin-bundling form of the protein (23). Addition of proNGF to hippocampal neurons expressing fascin^{S(36,38,39)A} failed to induce growth cone collapse, whereas overexpression of wildtype fascin did not interfere with the proNGF response (Fig. 5B, C). This suggests that fascin inactivation contributes to growth cone collapse downstream of proNGF.

Collectively, these observations suggest that proNGF signaling through the p75^{NTR} and SorCS2 receptor complex elicits rapid growth cone collapse. Growth cone collapse is mediated by the dissociation of Trio from a p75^{NTR} and SorCS2 complex to diminish Rac activity, and by the phosphorylation of fascin, leading to its dissociation from actin filaments

in the growth cone (Fig. 5D). We therefore propose that proNGF uses dual, synchronized mechanisms to elicit neurite retraction by stimulating growth cone collapse.

Discussion

Here, we showed that the precursor protein form of NGF acutely effects neuronal morphology. Remodeling of neuronal processes by proNGF-induced activation of p75^{NTR} differs from the effects of mature NGF, which promote neurite elongation through TrkA. Although many growth factors are produced as precursor proteins, the differential biological functions of the precursor and mature forms of growth factors are still poorly defined. Our results imply that conversion of proneurotrophins to mature forms may control acute morphological responses in central neurons.

The proNGF receptor, p75^{NTR}, acts as a co-receptor for multiple partners, including Trk receptors, the Nogo receptor, ephrin A, and sortilin (24). Of these, sortilin is primarily an intracellular sorting receptor, which only localizes efficiently to the cell membrane in the presence of NRH2 (25). Therefore, the presence of different co-receptors with p75^{NTR}, permitting the binding of different ligands, can lead to diverse biological outcomes, such as apoptosis, survival (10, 13, 24), and process retraction that depend upon the selective activation of distinct signaling mechanisms. Here, we identified two signaling mechanisms downstream of a complex of the p75^{NTR} and the sortilin family member SorCS2 that act to reduce process outgrowth. ProNGF induced dissociation of Trio from p75^{NTR} and SorCS2 to decrease Rac activity. Concomitantly, PKC is activated in an unknown manner to induce fascin phosphorylation and its dissociation from actin filaments, permitting rapid collapse of the growth cone (Fig. 5D). Trio can promote axon guidance and enhance neurite outgrowth by coupling to netrin and deleted in colorectal cancer (DCC) (26), or by associating with Trk receptors through the scaffolding protein ankyrin-rich membrane spanning protein (ARMS) (Kidins220) to promote Rac activation (27). Here, we observe that existing complexes of Trio with p75^{NTR} and SorCS2 undergo proNGF-induced dissociation to decrease Rac activation at the leading edges of growth cones, resulting in growth cone retraction. Together, these data indicate that Trio localization provides a switch to facilitate, or impede filopodial extension at the growth cone in response to mature or pro- neurotrophins, respectively. Trio is a modular protein, containing spectrin repeats that mediate protein-protein interactions, such as with ARMS (Kidins220), a GEF1 domain that can stimulate RhoG and Rac to facilitate axon outgrowth, a GEF2 domain that can stimulate RhoA to limit neurite outgrowth, and a kinase domain with poorly defined substrates [Fig. 2E; (16, 28)]. As such, Trio may act as a platform to locally modulate Rac and RhoA activity in response to neurotrophins, wherein the mature form of NGF preferentially activates TrkA to promote the interaction of ARMS and Trio at the plasma membrane and Rac-dependent neurite outgrowth (27). Conversely, proNGF induces the dissociation of Trio from p75^{NTR} to inactivate Rac locally, and initiates growth cone retraction. Thus, recruitment of Trio to the plasma membrane by Trk promotes actin filament extension, whereas dissociation of Trio from p75^{NTR} precipitates growth cone collapse. This provides an attractive model to regulate Rac activation and inactivation locally through differential use of neurotrophin ligands.

The finding that p75^{NTR} activation by proNGF also promoted fascin phosphorylation and its dissociation from actin filaments provides a dual mechanism to induce growth cone collapse. Dephosphorylated fascin bundles actin into stable filaments to permit filopodial extension (23), and fascin decorates the entire length of filamentous actin in the growth cone (3). Fascin cycles rapidly between the actin-bound, dephosphorylated state, and the actin-dissociated phosphorylated state (23). Prior studies have shown that p75^{NTR} binds fascin (21), that PKC mediates fascin phosphorylation (22), and that PKC activation induces

collapse of filopodia (29). Our current study provide a mechanistic link by which p75^{NTR} alters fascin localization and phosphorylation to permit rapid growth cone collapse.

Our data indicate that the precise repertoire of p75^{NTR} co-receptors on a growth cone, combined with exposure to particular ligands, will determine whether a collapse response occurs. Prior studies suggest that p75^{NTR} acts as a signaling partner with the semaphorin receptor plexin A3 or the ephrin receptor EphB to promote growth cone collapse in sympathetic axons, through activation of the Rho-ROCK pathway (30). However, in sympathetic axons, the binding of mature BDNF to p75^{NTR} alone is insufficient to promote acute collapse, and requires the concomitant activity of the ligands Sema3 or ephrin B2 (30). In contrast, our results indicate that engagement of p75^{NTR} and SorCS2 by proNGF is sufficient to mediate acute collapse, by means of the inactivation of Rac and fascin. The inability of mature NGF to induce collapse of hippocampal processes suggests that simultaneous engagement of p75^{NTR} and SorCS2 by proNGF, as compared to mature NGF, results in their differential signaling to the actin cytoskeleton so that only proNGF induces retraction.

The abundance of proNGF and p75^{NTR} is frequently increased under pathological conditions, such as under conditions of acute axonal injury, seizures, or spinal cord injury, which result in acute degeneration of neuronal projections. Our data provide insights into the mechanisms whereby neurite re-growth is impaired following acute injury, or in neurodegenerative disease states, such as Alzheimer's disease (31–33). The widespread increase in the abundance of p75^{NTR} in injured neurons, together with increase in the abundance of proNGF that occurs in acute and chronic central nervous system injury, suggest that p75^{NTR}-mediated axonal and dendritic degeneration may play a role in disease pathogenesis.

Materials and Methods

The LifeAct sequence(15) was cloned into the pEGFP-N1 backbone (Clontech) using the BglII and BamHI restriction sites. Trio constructs containing an IRES-GFP element were described previously (34); human sortilin cDNA or human SorCS2 cDNA was subcloned into the pcDNA3.1 hygro expression vector (Invitrogen). A Myc-tag was inserted three residues after the furin site using PCR. Wildtype and fascin^{S(36,38,39)A} cDNA was kindly provided by Prof. Xin-Yun Huang (35) and was subcloned into pcDNA 3.1 using the BamHI and EcoRI sites.

Primary antibodies included: anti-actin and anti-HA (Sigma), anti-fascin and anti-Rac1 (Millipore), anti-p75^{NTR} (9651 (36); and R&D Systems), anti-sortilin and anti-SorCS2 (specific for the ectodomain of these receptors; R&D Systems), anti-Trio (Santa Cruz C20; and CT-35 (37), anti-Myc and control IgGs (Santa Cruz). Fluorescent secondary antibodies were from Invitrogen, HRP-coupled secondary antibodies were from Sigma and Amersham. Anti-HA agarose was from Roche Diagnostics, anti-Myc (9E10) agarose and control IgG agarose was from Santa Cruz. All chemicals were from Sigma unless indicated otherwise. ProNGF was produced in Sf9 cells and purified as described (38).

Cell culture and transfection

Primary hippocampal neurons were isolated from E15 C57BL/6 mouse or E16 Sprague-Dawley rat embryos. Neurons were dissociated by incubation with 0.05% trypsin at 37°C for 8 min followed by trituration with fire-polished glass Pasteur pipettes. Cells plated on poly-D-lysine coated dishes were grown in Neurobasal medium containing B27, 0.5 mM glutamine (Invitrogen) and 10 μ M 5-fluorodeoxyuridine. Hippocampal neurons were transfected at DIV2 using Lipofectamine2000 (Invitrogen). For coverslips, 0.5 μ g plasmid

was mixed with 0.5 μ l Lipofectamine2000, and for glass bottom dishes, 1.5 μ g plasmid was mixed with 1.5 μ l Lipofectamine2000. Complexes were added for 30–45 min, and then neurons were placed into preconditioned media until analysis the following day.

HT1080 cells were grown in DMEM supplemented with 10% fetal bovine serum (Invitrogen) and transfected using AMAXA nucleofection (Lonza) according to manufacturer's instructions. 293FT and Cos-7 cells were grown in DMEM supplemented with 10% fetal bovine serum and transfected with Lipofectamine2000.

Cell treatments

To assay which pathways are involved in filopodial collapse, 5–10 ng/ml proNGF or 5–100 ng/ml NGF (Harlan) was added to primary hippocampal neurons for 20 min before fixation. As indicated, cells were pretreated with 100 nM Gö6976 (Calbiochem) for 1 hour (39), 5 μ M EHT1864 (40) for 30 min or 100 nM ITX3 (20) (ChemBridge Corporation, cat. no. 6007262) for 45 min, or 8 μ M 20–28 (Calbiochem) (41). To block SorCS2, cells were incubated with 20 μ g/ml anti-SorCS2 or control IgG for 20 min on ice before proNGF addition at 37°C. To block RhoA activity, neurons were preincubated with 1 μ g/ml cell permeable C3 Transferase (Cytoskeleton Inc) for 4 h or 10 μ M Y-27632 (Calbiochem) for 45 min before proNGF exposure.

Immunofluorescence

DIV3 hippocampal neurons were fixed in 4% paraformaldehyde and 20% sucrose or ice-cold methanol for 10 min and processed for fluorescence microscopy. In the case of PFA fixation, the fixative was quenched with 50 mM NH_4Cl in PBS for 5 min and cells were permeabilized with 0.1% TritonX-100 in PBS for 2 min before blocking. Coverslips were blocked with 10% normal donkey serum, 2% bovine serum albumin and 0.25% fish skin gelatin in Tris-buffered saline for 30 min, incubated with primary antibodies diluted in blocking solution for 30 min, washed three times with Tris-buffered saline, 0.25% fish skin gelatin, incubated with secondary antibodies mixed with Hoechst in blocking buffer for 30 min, washed and mounted using Mowiol488. Cells were imaged using a LSM510 laser-scanning confocal microscope equipped with a 40x Plan Neofluor NA1.3 DIC oil-immersion objective (Carl Zeiss Microimaging). Images were processed using LSM510 software (Zeiss) and ImageJ (NIH).

Live imaging and data analysis

Cells plated on gridded glass bottom dishes (MatTek Corporation) were imaged at DIV3 in phenol red-free Neurobasal medium (Invitrogen) supplemented with 30 mM HEPES-NaOH, pH7.4 using an Olympus IX71 inverted microscope driven by IPLab software (BD Biosciences) and equipped with a 60x PlanApoN objective (NA 1.42), a Hamamatsu EM-CCD camera and a heated stage maintained at 37°C, or driven by softWoRX software (version 3.7.1, DeltaVision), and equipped with a CoolSnap HQ2 camera (Photometrics) and an environmental chamber maintained at 37°C. Images were taken every 15 seconds. After live imaging cells were fixed, counterstained for p75^{NTR}, located by grid number, and examined for p75^{NTR} expression. All movies were exported and processed using IPLab (BD Biosciences), softWoRX (version 3.7.1, DeltaVision) and ImageJ (NIH) software.

To quantify growth cone collapse, all neurites in at least 3–4 fields of view per experiment and at least three independent experiments were scored.

Co-immunoprecipitation

HT-1080 cells were transfected by AMAXA with HA-p75^{NTR} and myc-SorCS2. 24–48 hours later, cells were treated with 25 ng/ml proNGF for 20 min as indicated. Cells were

lysed with lysis buffer (50 mM Tris-HCl pH 8.0, 140 mM NaCl, 2 mM EDTA, 1% NP40, 10% glycerol) supplemented with protease inhibitors and complexes were immunoprecipitated using anti-HA agarose (Roche). Beads were washed four times with lysis buffer supplemented with 500mM NaCl.

To map the Trio domain required for the interaction with p75^{NTR}, 293T cells were transfected with HA-p75^{NTR} and constructs of the individual Trio domains. After 24 hours, cells were lysed, and complexes immunoprecipitated using anti-HA agarose or anti-Myc agarose as indicated.

To immunoprecipitate endogenous complexes, embryonic brains were lysed in lysis buffer (50 mM Tris-HCl pH 8.0, 140 mM NaCl, 1% NP40, 10% glycerol) using a dounce homogeniser. Lysates were cleared by centrifugation for 10 min at 20000g and precleared on proteinA beads for 45 min. 10 µg anti-p75^{NTR} antibody (9651) or rabbit IgG was crosslinked to proteinA beads using BS³ (Pierce), excess crosslinker was quenched using Tris-HCl pH 8.0, and complexes were immunoprecipitated from 10 mg total protein per condition over night, and washed in lysis buffer supplemented with 400mM NaCl. For detection of the p75^{NTR} and fascin interaction, the membrane was crosslinked with 2.5% glutaraldehyde in PBS for 30 min after transfer and before blocking to minimize background from the antibody heavy chain.

Rac activity assay

Rac activity assays were performed as described (27). Briefly, DIV2 hippocampal neurons were stimulated with 20 ng/ml proNGF for 20 min, cells were lysed in lysis buffer supplemented with 10 mM MgCl₂. Lysates were cleared by centrifugation at 9000g for 1 min, and cleared lysates were incubated with GST or GST-PAK-CRIB beads for 30 min at 4°C. Beads were washed and isolated active Rac was analyzed by Western blot. In parallel, extra lysates were incubated with 1 mM GDP or 0.1 mM GTPγS for 30 min at room temperature before incubation with GST-Pak-CRIB beads. Western blots were analyzed by densitometry using ImageJ software, and isolated active Rac was normalized to the input.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. ProNGF signaling through p75^{NTR} and SorCS2 induces growth cone retraction
 (A) E15.5 DIV3 hippocampal neurons transfected with LifeAct-RFP were imaged by time-lapse microscopy before (left panels) or after (right panels) treatment with proNGF. Time is indicated in (min:sec). See also Movie S1. (B) Neurons were incubated with proNGF for 20 min, fixed, and stained for actin, fascin and p75^{NTR}. Arrows indicate collapsed growth cones, asterisks indicate intact growth cones. Insets (1,2) are provided at higher magnification. (C) Quantification of (B). Control, untreated controls; proNGF, cells exposed to 10 ng/ml proNGF (n=12 independent experiments); NGF, cells incubated with 5 ng/ml NGF (n= 4 independent experiments). ***, p<0.001; n.s., not significant; one way ANOVA. (D) Neurons were fixed and stained for p75^{NTR} and SorCS2. Arrows highlight growth cones. (E) Cos-7 cells were transfected with indicated constructs, and lysates were immunoprecipitated with anti-HA antibodies and analyzed by Western blot (WB) with indicated antibodies. N = 4 independent experiments. (F) Neurons were preincubated with anti-SorCS2 antibodies or control IgG prior to proNGF addition for 20 min, and collapse was quantified. N= 4 independent experiments. ***, p<0.001; n.s., not significant; one way ANOVA. Scale bars, 10 μ m (A, B) and 20 μ m (D).

Figure 2. Trio interacts with the p75^{NTR} and SorCS2 receptor complex

(A) Coomassie Blue staining of proteins co-precipitating with p75^{NTR} in HT1080 cells transfected with constructs encoding p75^{NTR} and sortilin. Trio was identified by subsequent mass spectrometry (see also fig. S3). (B) Co-immunoprecipitation of endogenous Trio with p75^{NTR} from HT1080 cells expressing both p75^{NTR} and sortilin or p75^{NTR} and SorCS2, but not p75^{NTR} alone. Input= cell lysate prior to immunoprecipitation (IP). N= 5 independent experiments. (C, D) Myc-tagged Trio domains were co-expressed with HA-p75^{NTR} in 293T cells, immunoprecipitated with anti-Myc (C) or with anti-HA (D) antibodies, and Western blots probed with the indicated antibodies. N= 5 (C) and 4 (D) independent experiments. (E) Schematic representation of full-length Trio. (F, G) HT1080 cells expressing p75^{NTR} and SorCS2 were incubated with proNGF for 20 min, lysed and p75^{NTR} was immunoprecipitated. (F) Western blots were probed with indicated antibodies. (G) Co-precipitation of Trio with p75^{NTR} was quantified from 10 separate experiments using densitometry and normalized to the input. ***, p<0.001; Student's T-test.

Figure 3. Trio dissociation from the p75^{NTR} and SorCS2 complex leads to growth cone collapse (A) Hippocampal neurons were incubated with proNGF for 20 min, fixed and stained for Trio, actin, and p75^{NTR}. (B) Endogenous Trio and SorCS2 form a complex with p75^{NTR}. Embryonic brain lysates were immunoprecipitated with antibodies against p75^{NTR} or control IgGs and probed for indicated proteins. N = 4 independent experiments. (C, D) Hippocampal neurons were transfected at DIV2 with the Trio kinase domain containing an IRES-GFP element, or the kinase-dead mutant K2921A, and fixed at DIV3. (C) Growth cone collapse was quantified scoring 47 (Trio-kin) and 50 (Kin^{K2921A}) neurites in three independent experiments. ***, p<0.001; n.s., not significant; one way ANOVA. (D) Arrows indicate collapsed growth cones. Scale bars, 10 μ m.

Figure 4. Decreased Rac activity underlies growth cone collapse

(A) Hippocampal neurons were incubated with proNGF for 20 min, and cell lysates were incubated with GST-PAK-CRIB beads to isolate activated Rac. (B) Isolated activated Rac was measured by densitometry and normalized to total Rac in the input. Shown is the mean +SEM from four independent experiments. *, $p=0.018$; Student's T-test. (C) Inhibition of Rac or Trio GEF1 activities induces growth cone collapse. Hippocampal neurons were treated with EHT 1864 or ITX3, fixed, and stained for actin. Arrows indicate collapsed growth cones. $N=3$ independent experiments. (D) Expression of the Trio GEF1 domain rescues proNGF-induced collapse. Hippocampal neurons were transfected with the Trio GEF1 domain containing an IRES-GFP element at DIV2. The following day, cells were treated with proNGF, fixed, and stained with indicated antibodies. (E) Quantitation of (D). Shown is the mean+SEM of four independent experiments; ***, $p<0.001$; n.s., not significant, Student's t-test. (F) Inhibition of RhoA activity does not prevent proNGF-induced growth cone collapse. Neurons were pretreated with C3 Transferase for 4h or

Y-27632 for 45min prior to proNGF exposure for 20 min, fixed, stained and collapse was quantified in three independent experiments. Scale bars, 10 μ m.

Figure 5. PKC-dependent fascin inactivation contributes to growth cone retraction

(A) Hippocampal neurons were pretreated with the PKC inhibitor Go6976, followed by addition of proNGF, fixation, and staining. (B) Neurons were co-transfected with GFP and fascin^{S(36,38,39)A} and 24 h later were treated with proNGF, fixed, and stained for fascin, actin, and p75^{NTR}. (C) Quantification of proNGF-induced collapse in cells pretreated with Go6976, the inhibitory peptide 20–28, or expressing fascin or fascin^{S(36,38,39)A}. Shown is the mean+SEM of at least three independent experiments; n.s., not significant; ***, $p < 0.001$; one way ANOVA. Scale bars, 10 μ m. (D) Model of acute proNGF action on actin dynamics. The p75^{NTR} and SorCS2 receptor complex is associated with the Rac GEF Trio and localizes Rac activity (dark orange ovals: active Rac; light orange ovals: inactive Rac) to structures in dynamically expanding growth cones. Upon proNGF binding, Trio dissociates from the p75^{NTR}-SorCS2 complex and Rac activity decreases to impair

filopodial formation. In parallel, PKC is activated and phosphorylates and inactivates the actin bundling protein fascin (blue circles). This leads to a destabilization of existing actin filaments and their collapse.