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Supplementary Materials for

Neuronal Growth Cone Retraction Relies on Proneurotrophin Receptor Signaling Through Rac

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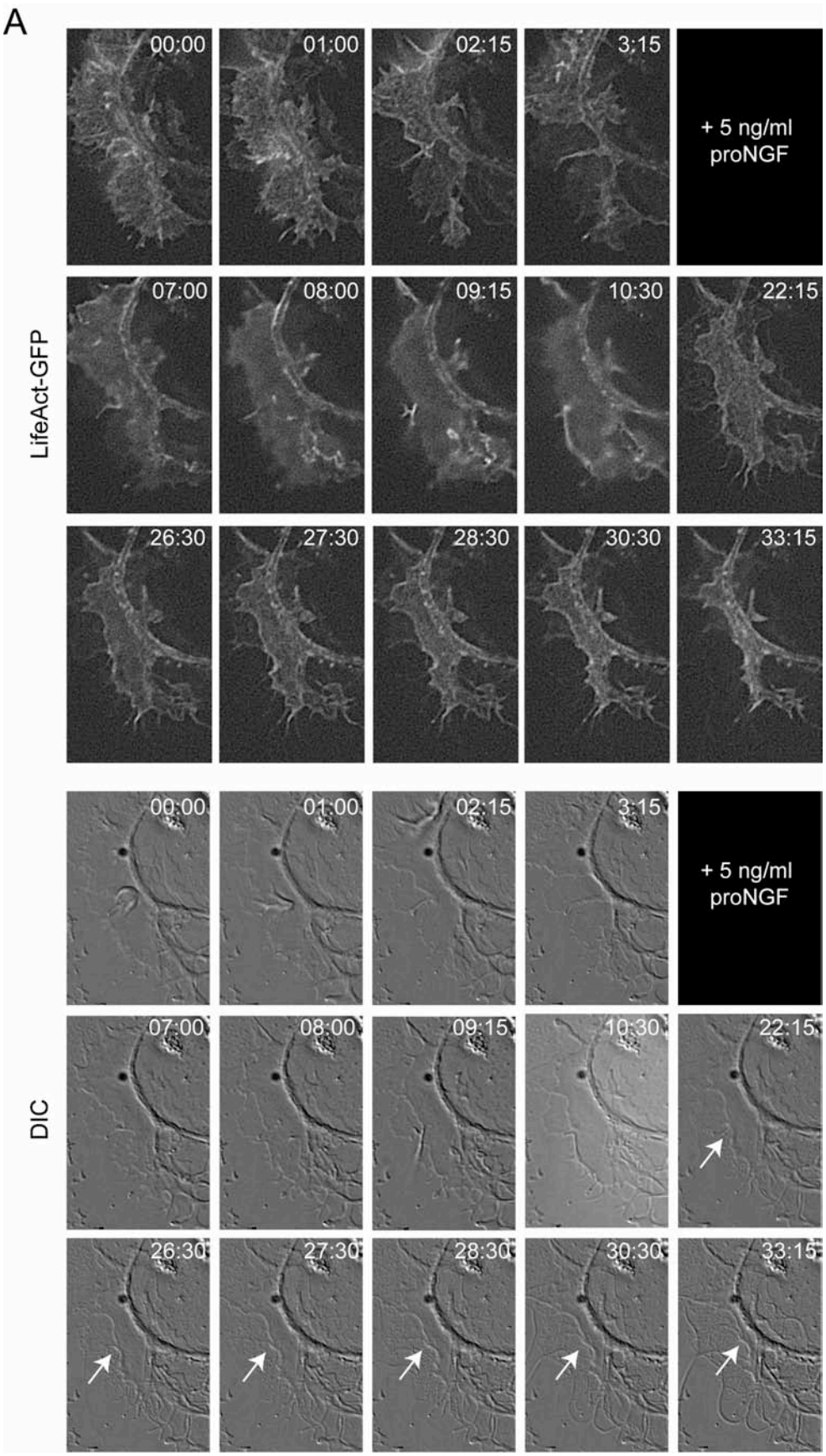
Supplementary Methods

Purification of GST-PAK-CRIB. GST and GST-PAK-CRIB proteins were expressed in *E.coli* BL21DE3 cells at 25°C for 3 hours. Bacteria were disrupted by incubation with PBS supplemented with lysozyme on ice for 30 min and subsequent addition of 1 mM $MgCl_2$, 0.1% TritonX-100 and 0.1 mg/ml DNAse for an additional 30 min. Bacterial lysates were cleared by centrifugation for 5 min at 3000g and cleared lysates were incubated with glutathione sepharose. Beads were washed and the purity of the recombinant proteins was analyzed by Coomassie blue staining. 20 μg of sepharose-coupled purified protein was used per reaction.

Mass spectrometry. $p75^{NTR}$ was immunoprecipitated from HT1080 cells stably expressing $p75^{NTR}$ or $p75^{NTR}$ and sortilin, immunoprecipitated proteins were resolved by SDS-PAGE and gels were stained with Coomassie Brilliant Blue (Bio-Rad). Bands that were only present in immunoprecipitates from cells expressing both $p75^{NTR}$ and sortilin were excised from the gel along with a control band from the same location of the gel in the lane containing immunoprecipitate of $p75^{NTR}$ without sortilin co-expression. Gel bands were cut into small pieces and destained in 25 mm ammonium bicarbonate in 50% acetonitrile, dehydrated with acetonitrile, and dried. The gel pieces were rehydrated with 10 ng/µl trypsin solution in 25 mM ammonium bicarbonate and incubated overnight at 37°C. Peptides were extracted twice with 5% formic acid in 50% acetonitrile followed by a final extraction with acetonitrile. Extracts were pooled, dried by vacuum centrifugation, and reconstituted in 5 µl of 0.1% formic acid, 2% acetonitrile for HPLC sample injection. Resuspended samples were loaded onto a Symmetry 5µm particle, 180 µm x 20 mm C18 precolumn (Waters), then washed 5 min with 1% acetonitrile in 0.1% formic acid at a flow rate of 20 µL/min. After washing, peptides were eluted and passed through an Atlantis 3 µm particle, 75 µm x 100 mm C18 analytical column (Waters, Milford, MA) with a gradient of 1-80% Acetonitrile in 0.1% formic acid. The gradient was delivered over 120 min by a nanoACQUITY UPLC (Waters) at a flow rate of 250 nL/min, to a fused silica distal end-coated tip nano-electrospray needle (New Objective, Woburn, MA). Data were collected by a Q-TOF Premier mass spectrometer (Waters/Micromass) set for MS survey scans and automatic data-dependent MS/MS acquisitions. Raw LC-

MS/MS data were processed using ProteinLynx GlobalServer 2.2 software (Waters). A database containing the combined IPI mouse and rat sequences concatenated with their reverse sequences (186,384 total sequences) was searched using Mascot software (version 2.1, Matrix Science, London, United Kingdom) for protein identification. Search criteria included trypsin specificity with one missed cleavage allowed, methionine oxidation, and minimum precursor and fragment-ion mass accuracy of 50 ppm and 0.1 Daltons respectively.

Supplementary Movie 1: ProNGF leads to growth cone collapse. E15.5 DIV2 hippocampal neurons were transfected with LifeAct-RFP and imaged by time-lapse microscopy 24 h later. ProNGF addition leads to impaired actin dynamics followed by growth cone collapse. This video consists of 136 frames. Elapsed time (min:sec) is indicated in the top right corner. The image size is 37 x 41 μm.


A
LifeAct-GFP
DIC
00:00
01:00
02:15
3:15
+ 5 ng/ml proNGF
07:00
08:00
09:15
10:30
22:15
26:30
27:30
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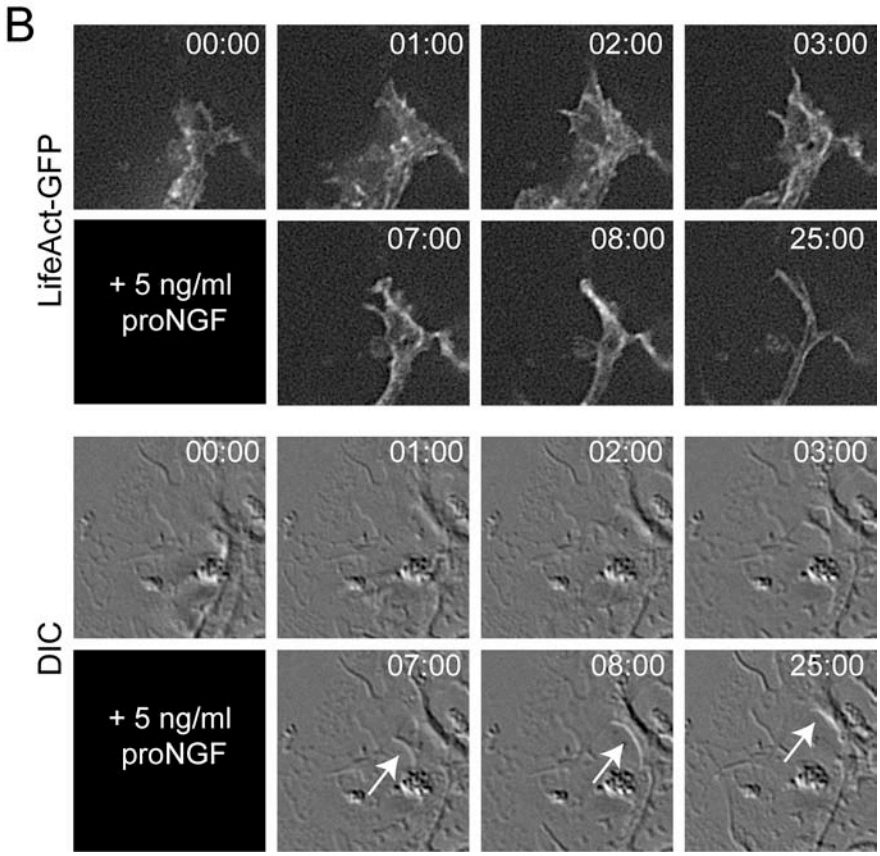


Fig. S1: Two additional examples showing proNGF-induced growth cone collapse. Cells were imaged as described in Fig. 1A. Shown are the LifeAct (top) and DIC (bottom) images of a slow (A) and a fast (B) collapsing growth cone. Times are indicated in the top right corners in (min:sec). The image sizes are 61.4 x 100 μm (A) and 52.4 x 55.1 μm (B).

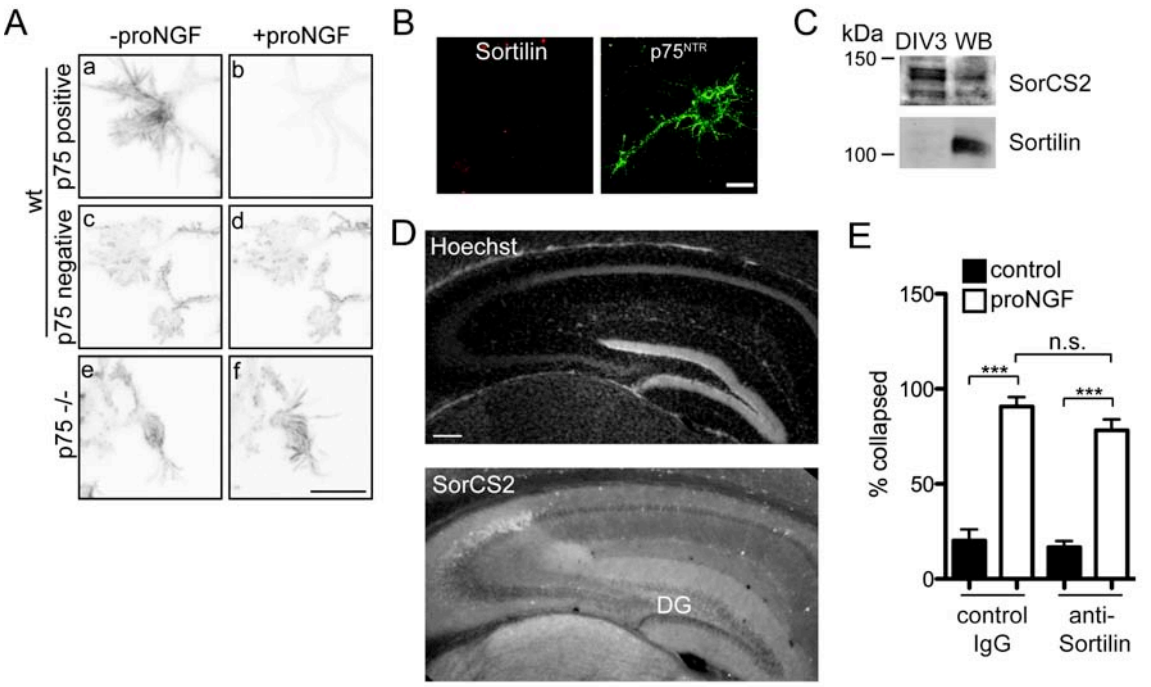
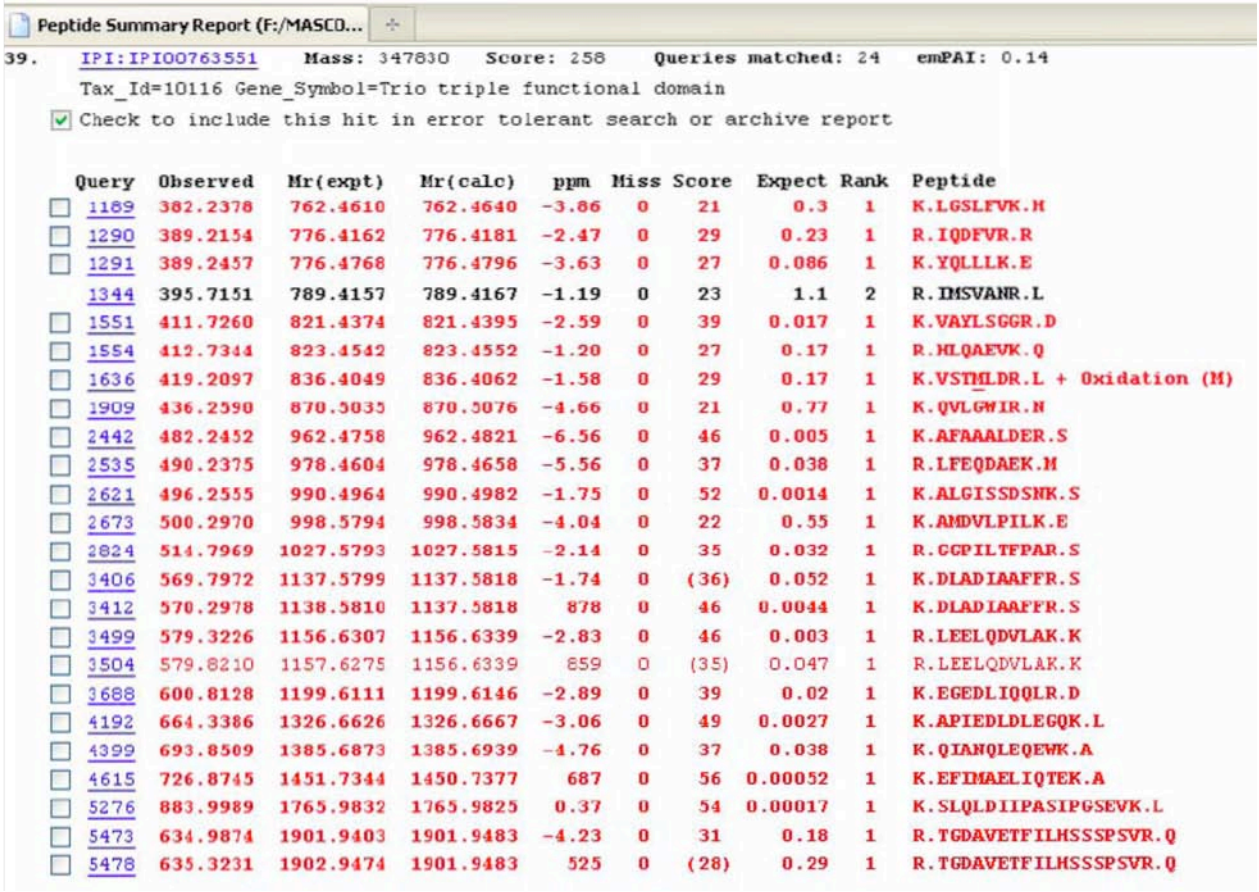


Fig. S2: p75^{NTR} and SorCS2 are the receptors mediating growth cone retraction. (A) Neurons of indicated genotypes were transfected with LifeAct-RFP, imaged before and after proNGF addition, fixed and counterstained for p75^{NTR} expression. Shown are stacked difference views of images taken every 15 s over the course of 10 min. Signal in these images is derived from changes between individual frames and therefore represents motion. Only cells that express p75^{NTR} were found to stall actin dynamics in response to proNGF and collapse (top panel), while p75^{NTR}-negative cells were insensitive to proNGF treatment (middle panel). No growth cone collapse was observed in cells derived from p75$^{NTR-/-}$ mice (bottom panel). N= 13 neurons from 7 different embryos, derived from 3 litters. Scale bar, 10 μm. (B, C) Sortilin is not expressed in E15 DIV3 hippocampal cultures. N= three independent experiments each. (B) Cells were fixed at DIV3 and stained for Sortilin and p75^{NTR}. Scale bar, 20 μm. (C) DIV3 hippocampal cultures (DIV3) or E15 whole embryonic brains (WB) were lysed and analysed by Western blot for Sortilin and SorCS2 expression. (D) SorCS2 is expressed in the hippocampus in vivo. Brain sections from 6-week old C57/BL6 mice were stained for SorCS2. Scale bar, 200 μm. (E) Anti-Sortilin antibodies do not prevent growth cone collapse. Neurons were preincubated with anti-Sortilin antibodies or control IgG prior to proNGF addition for 20 min, and collapse was quantified. ***, $p<0.001$; n.s., not significant; one way ANOVA. N = 3 independent experiments.



Peptide Summary Report (F:/MASCO...

39. IPI:IPI00763551 Mass: 347830 Score: 258 Queries matched: 24 emPAI: 0.14

Tax_Id=10116 Gene_Symbol=Trio triple functional domain

Check to include this hit in error tolerant search or archive report

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1290	389.2154	776.4162	776.4181	-2.47	0	29	0.23	1	R.IQDFVR.R
1291	389.2457	776.4768	776.4796	-3.63	0	27	0.086	1	K.YQLLLK.E
1344	395.7151	789.4157	789.4167	-1.19	0	23	1.1	2	R.IMSVANR.L
1551	411.7260	821.4374	821.4395	-2.59	0	39	0.017	1	K.VAYLSGGR.D
1554	412.7344	823.4542	823.4552	-1.20	0	27	0.17	1	R.MLQAEVK.Q
1636	419.2097	836.4049	836.4062	-1.58	0	29	0.17	1	K.VSTMLDR.L + Oxidation (M)
1909	436.2590	870.5035	870.5076	-4.66	0	21	0.77	1	K.QVLGWIR.N
2442	482.2452	962.4758	962.4821	-6.56	0	46	0.005	1	K.AFAAALDER.S
2535	490.2375	978.4604	978.4658	-5.56	0	37	0.038	1	R.LFEQDAEK.M
2621	496.2555	990.4964	990.4982	-1.75	0	52	0.0014	1	K.ALGISSDSNK.S
2673	500.2970	998.5794	998.5834	-4.04	0	22	0.55	1	K.AMDVLPILK.E
2824	514.7969	1027.5793	1027.5815	-2.14	0	35	0.032	1	R.GGPILTFPAR.S
3406	569.7972	1137.5799	1137.5818	-1.74	0	(36)	0.052	1	K.DLADIAAFFR.S
3412	570.2978	1138.5810	1137.5818	878	0	46	0.0044	1	K.DLADIAAFFR.S
3499	579.3226	1156.6307	1156.6339	-2.83	0	46	0.003	1	R.LEELQDVLAK.K
3504	579.8210	1157.6275	1156.6339	859	0	(35)	0.047	1	R.LEELQDVLAK.K
3688	600.8128	1199.6111	1199.6146	-2.89	0	39	0.02	1	K.EGEDLIQQLR.D
4192	664.3386	1326.6626	1326.6667	-3.06	0	49	0.0027	1	K.APIEDLDLEGQK.L
4399	693.8509	1385.6873	1385.6939	-4.76	0	37	0.038	1	K.QIANQLEQEWK.A
4615	726.8745	1451.7344	1450.7377	687	0	56	0.00052	1	K.EFIMAELIQTEK.A
5276	883.9989	1765.9832	1765.9825	0.37	0	54	0.00017	1	K.SLQLDIIPASIPGSEVK.L
5473	634.9874	1901.9403	1901.9483	-4.23	0	31	0.18	1	R.TGDAVETFILHSSSPSVR.Q
5478	635.3231	1902.9474	1901.9483	525	0	(28)	0.29	1	R.TGDAVETFILHSSSPSVR.Q

Fig. S3: Identification of Trio.
Mascot database search results showing the high-confidence identification of Trio from immunoprecipitates of $p75^{NTR}$-interacting proteins from HT1080 cells stably expressing $p75^{NTR}$ and sortilin but not found in immunoprecipitates of cells expressing $p75^{NTR}$ alone.

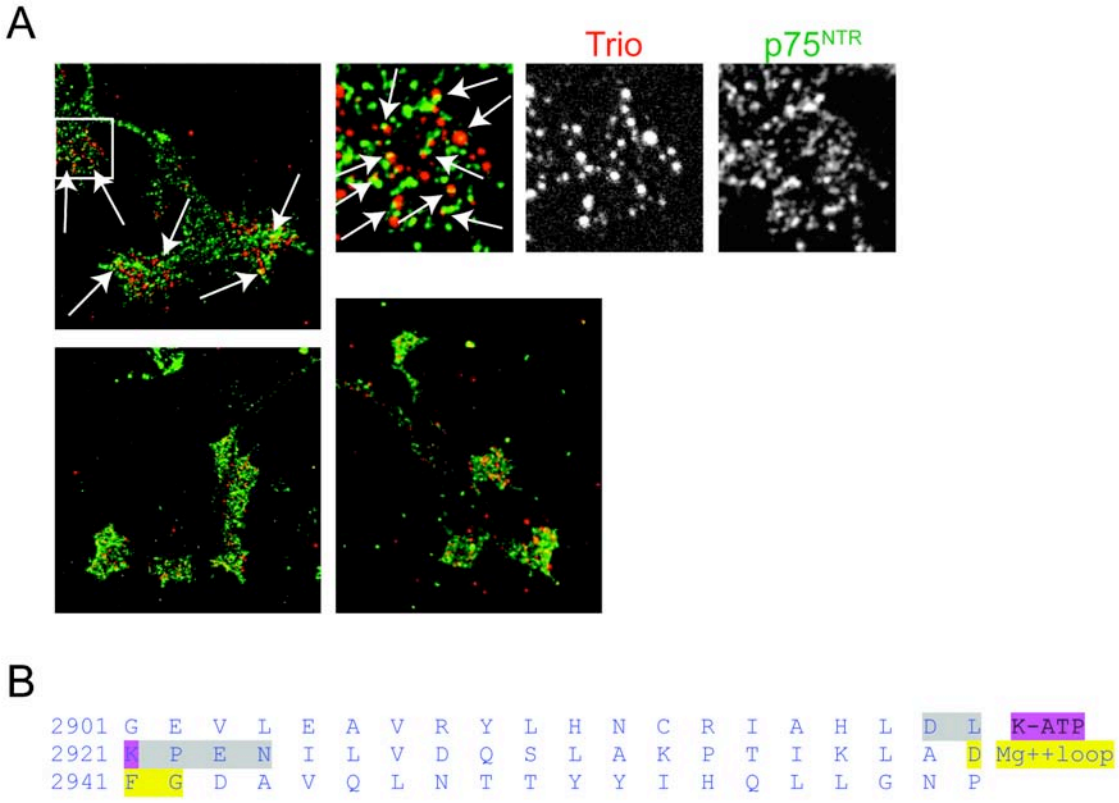

Fig. S4: Generation of the kinase-dead Trio mutant.
(A) Trio partially colocalises with p75^{NTR}. Shown is the merge of Trio and p75^{NTR} staining from Fig. 3A, including a higher magnification inset with merged image and individual channels, as well as two other examples of Trio and p75^{NTR} costaining. Arrows highlight examples of colocalisation. (B) Sequence of parts of the Trio kinase domain. The lysine residue highlighted in purple is predicted to be critical for nucleotide binding and was mutated to alanine to generate the kinase-dead mutant. Single-letter abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.

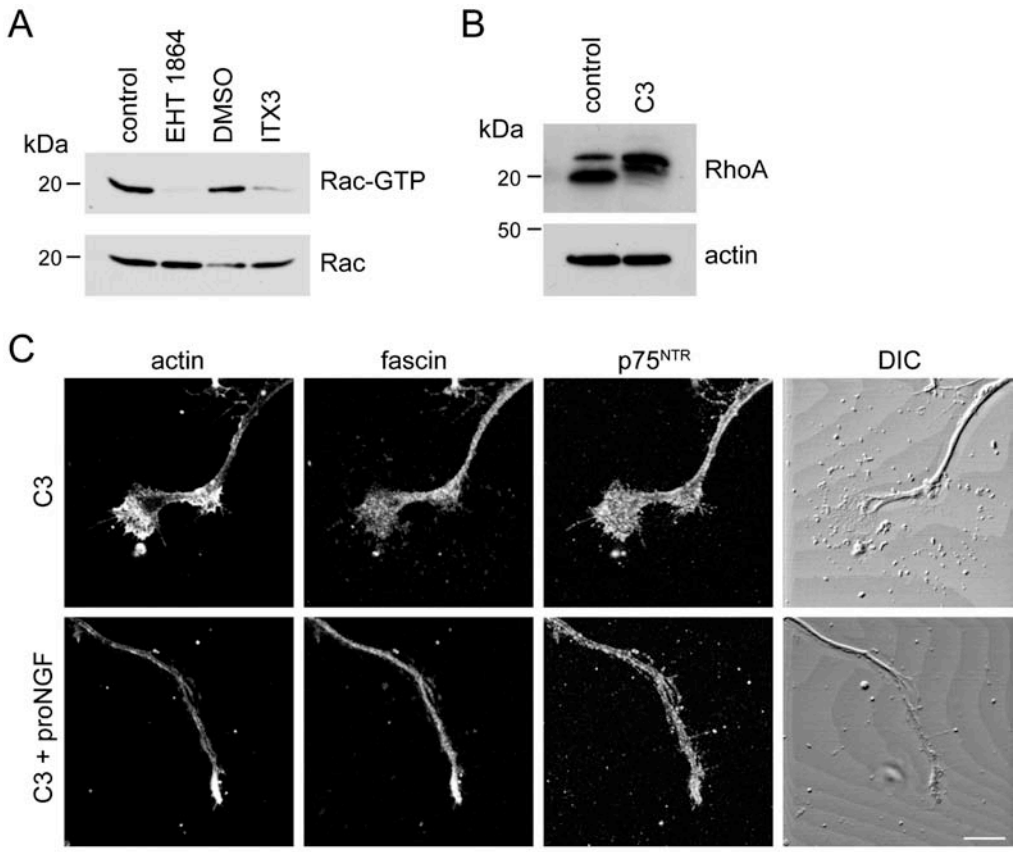

Fig. S5: Decrease of Rac but not RhoA activity induces growth cone collapse. (A) Treatment with the Rac inhibitor EHT 1864 or the Trio GEF1 inhibitor ITX3 leads to decreased Rac activity in primary neuronal cultures. Neurons were treated with EHT 1864 or ITX3, and active Rac was isolated as described in Fig. 4A. N= 3 independent experiments. (B, C) Inactivation of RhoA does not induce or prevent growth cone collapse. (B) Treatment of neurons with C3 transferase efficiently ribosylated and therefore inactivated RhoA within 4 hrs of treatment, as marked by an upward shift of the band. (C) C3-treated neurons have intact growth cones that are still able to collapse in response to proNGF treatment. N= 4 independent experiments. Scale bar, 10 μm.

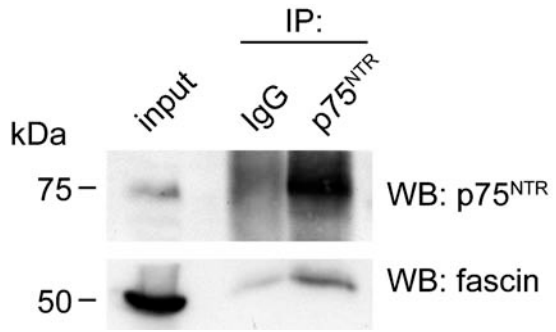

Fig. S6: Fascin and p75^{NTR} form a complex in embryonic brain lysates. E15 embryonic brains were lysed and lysates were immunoprecipitated with anti-p75^{NTR} antibody or control rabbit IgG crosslinked to the beads. After separation by SDS-PAGE and Western blotting, membranes were crosslinked with glutaraldehyde before blocking and probing with indicated antibodies to minimize background from the antibody heavy chain. N = 4 independent experiments.