Background: Neurons extend their dendrites and axons to build functional neural circuits, which are regulated by both positive and negative signals during development. Brain-derived neurotrophic factor (BDNF) is a positive regulator for neurite outgrowth and neuronal survival but the functions of its precursor (proBDNF) are less characterized.

Methodology/Principal Findings: Here we show that proBDNF collapses neurite outgrowth in murine dorsal root ganglion (DRG) neurons and cortical neurons by activating RhoA via the p75 neurotrophin receptor (p75NTR). We demonstrated that the receptor proteins for proBDNF, p75NTR and sortilin, were highly expressed in cultured DRG or cortical neurons. ProBDNF caused a dramatic neurite collapse in a dose-dependent manner and this effect was about 500 fold more potent than myelin-associated glycoprotein. Neutralization of endogenous proBDNF by using antibodies enhanced neurite outgrowth in vitro and in vivo, but this effect was lost in p75NTR−/− mice. The neurite outgrowth of cortical neurons from p75NTR deficient (p75NTR−/−) mice was insensitive to proBDNF. There was a time-dependent reduction of length and number of filopodia in response to proBDNF which was accompanied with a polarized RhoA activation in growth cones. Moreover, proBDNF treatment of cortical neurons resulted in a time-dependent activation of RhoA but not Cdc42 and the effect was absent in p75NTR−/− neurons. Rho kinase (ROCK) and the collapsin response mediator protein-2 (CRMP-2) were also involved in the proBDNF action.

Conclusions: proBDNF has an opposing role in neurite outgrowth to that of mature BDNF. Our observations suggest that proBDNF collapses neurites outgrowth and filopodial growth cones by activating RhoA through the p75NTR signaling pathway.

Introduction

Neuronal polarization involving neurite outgrowth and axonal elongation is essential for building functional neural circuits during brain development [1,2]. Both positive and negative signals regulate the neurite outgrowth and guide axons to their appropriate destinations. Mature neurotrophins (NTs) including nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF) and NT-3, NT-4/5 are well characterized positive signals promoting neurite outgrowth, axonal extension, filopodial protrusion and synaptogenesis [3,4].

Proneurotrophins are proteolytically cleaved to form biologically active mature molecules. Recent studies illustrate that the neurotrophin precursors, proNGF, proBDNF, and proNT3 trigger apoptosis of sympathetic and sensory neurons to antagonize the effects of mature neurotrophins [5,6,7,8]. ProBDNF is found to be a negative regulator of synaptic plasticity and regulates long-term depression via p75NTR [9,10]. In addition, it negatively regulates the migration of cerebellar granule cells during development and the infiltration of macrophages during spinal cord injury [11,12]. ProBDNF has distinct functions on different populations of neurons, reducing the number of cholinergic fibers and hippocampal dendritic spines without affecting the survival of these neurons [10]. However, the proBDNF dependent regulation of neurite outgrowth and the underlying signaling are not known.

A number of factors and signal pathways have been identified to negatively regulate neurite outgrowth or repulse the growth cones to cause neurite collapse during development and after nerve injury in the central nervous system (CNS). These include the myelin associated factors Nogo, myelin-associated glycoprotein (MAG) and oligodendrocyte-myelin glycoprotein (OMgp) which activate Nogo receptors (NgR) and its coreceptor p75NTR in RhoA dependent manner [13,14]. Additional neurite growth inhibitory factors such as semaphorin3A, ephrin-B3 or repulsive guidance molecule b repulse the regeneration of CNS neurons.
or mature BDNF (307.8 ng/ml) increased in presence of anti-proBDNF antiserum (326.3 ng/ml). A single band at 42 kD was used as an internal loading control. The application of proBDNF (30 ng/ml) or MAG (30 μg/ml) decreased the neurite length by 21.7% and 23.6% in primary cultured p75NTR+/−/− DRG neurons (p<0.01, Fig. 3E–G). However, the treatment of p75NTR+/−/− DRG neurons with proBDNF or MAG had no effect on neurite outgrowth (p>0.05, Fig. 3H–K).

### Results

**ProBDNF Collapses Neurites in a Dose-dependent Manner on Cortical and DRG Neurons**

To demonstrate a role of proBDNF in neurite outgrowth, we first investigated its effects on DRG neurons. Live imaging clearly showed the collapse of neurites in response to proBDNF (30 ng/ml, Figure S1) and the enhanced neurite growth in response to mature BDNF (50 ng/ml, Figure S2, Fig. 1A). ProBDNF caused a 30.6% decrease in the neurite length after 6 min (p<0.05) which was maintained throughout the 30 min treatment (Fig. 1B). To investigate whether the proBDNF-dependent neurite collapse also occurs in CNS neurons, we examined its effect on cultured cortical neurons. The treatment with proBDNF induced in cortical neurons a decrease in neurite length similar to DRG neurons (Fig. 1C). To investigate if the effect could be mediated via endogenous release of proBDNF and subsequent interaction with its receptors, we determined the protein expression levels of proBDNF, p75NTR and sortilin in primary cultured cortical and DRG neurons [19]. ProBDNF was detected as a single band at 35 kD, p75NTR at 75 kD and sortilin at 110 kD. B-actin with a single band at 42 kD was used as an internal loading control (Fig. 1D).

**Endogenous Probdn Inhibits Neurite Growth Through p75NTR**

In this assay, cultured primary adult DRG neurons were treated with different factors (Fig. 2A–E). The neurite length was significantly reduced in presence of proBDNF (82.7±9 μm) but increased in presence of anti-proBDNF antiserum (326.3±14 μm) or mature BDNF (307.8±12 μm) compared to the IgG control (139.4±8 μm, p<0.05, Fig. 2F). The results suggest that both exogenous and endogenous proBDNF inhibits neurite outgrowth on DRG neurons while mature BDNF enhances the neurite growth.

The application of proBDNF resulted in dose-dependent decrease in sensory neuron neurite length compared to untreated DRG neurons (Fig. 3A, C) with IC50 is about 10 ng/ml. The p75NTR is also the co-receptor for Nogo receptor which binds the inhibitory molecule MAG. This allowed us to directly compare the potency of proBDNF and MAG on the inhibition of the neurite outgrowth in primary cultured DRG neurons. The application of MAG (10, 30 μg/ml) resulted in a small but significant decrease in neurite length (100±7 μm, 46±4 μm) compared with the control group (118.4±6 μm, p<0.05), whereas a lower concentration of MAG (3 μg/ml) to DRG neurons had no effect (Fig. 3B, D). The concentration of 30 ng/ml MAG-Fc (MW: 120,000, 250 nM) has an equivalent effect of, 30 ng/ml proBDNF (dimer MW: 64,000~70,000, 0.42~0.46 nM). Thus proBDNF is 53.3~58.3 fold more potent than MAG.

To further investigate whether the p75NTR signaling is involved in proBDNF effects we applied proBDNF to neurons dissected from p75NTR+/−/− and p75NTR−/− mice. The application of proBDNF (30 ng/ml) or MAG (30 μg/ml) decreased the neurite length by 21.7% and 23.6% in primary cultured p75NTR+/−/− DRG neurons (p<0.01, Fig. 3E–G). However, the treatment of p75NTR−/− DRG neurons with proBDNF or MAG had no effect on neurite outgrowth (p>0.05, Fig. 3H–K).

**RhoA Activity is Increased After ProBDNF Treatment but Lost in p75NTR−/− mice**

It is known that MAG and Nogo cause neurite collapse by activating RhoA and this effect is mediated by a direct interaction of the Rho GDP dissociation inhibitor [Rho-GDI] with p75NTR [20]. Because proBDNF binds p75NTR with high affinity, we next tested the hypothesis that proBDNF collapses neurites by activating RhoA. After treatment with proBDNF for 10 and 20 min, RhoA activity increased 3.9±1.2 and 4.9±0.4 fold (Fig. 4A–B). Interestingly, no change in the level of activated Cdc42 was seen in response to proBDNF (Fig. 4C). Based on these observations we used the pull-down activity assay to compare RhoA activity in cortical neurons with or without proBDNF from p75NTR+/−/− (n=12) and p75NTR−/−/− mice (n=6). The results showed that, in contrast to cells from p75NTR−/−/− mice, proBDNF increased RhoA activation in cells from p75NTR+/−/− mice over untreated cells (2.6±0.2 fold, p<0.01, Fig. 4D).

**ProBDNF Activates RhoA and Induces the Collapse of Growth Cone Filopodia**

We detected activated RhoA using specific GTP-RhoA antisera. As shown in Fig 5A–D, proBDNF treatment caused a time-dependent increase in GTP-RhoA immunoreactivity in growth cones. After treatment with proBDNF for 10 and 20 min, GTP-RhoA immunoreactivity increased 3.7±0.9 and 5.5±0.4 fold in growth cones (Fig. 5I) whereas the immunoreactivity for GTP-Cdc42 antibody was similar at the different time points (Fig. 5E–H, 3J).

We next determined whether proBDNF regulates filopodial length and number (Fig. 5K–N). Filopodia collapsed in response to proBDNF and subsequent interaction with its receptors, we determined the protein expression levels of proBDNF, p75NTR and sortilin in primary cultured cortical and DRG neurons [19]. ProBDNF was detected as a single band at 35 kD, p75NTR at 75 kD and sortilin at 110 kD. B-actin with a single band at 42 kD was used as an internal loading control (Fig. 1D).

**ProBDNF Induced Inhibition of Neurite Outgrowth Depends on the Activation of Rhoa and ROCK**

Next, we asked whether Rhoa and ROCK signaling is involved in proBDNF-mediated inhibition of neurite outgrowth. C3-transferase (a molecule that ADP ribosylates Rhoa) and Y27632 (a well characterized ROCK inhibitor) were used to inactivate Rhoa and inhibit ROCK, respectively. Functional studies using the neurite outgrowth assay showed that short-term incubation (20 min) of cultured DRG neurons with proBDNF significantly inhibited neurite outgrowth compared with control IgG (Fig. 6A, B). The extent of inhibition induced by pharmacological inhibitors of Rhoa and ROCK were slightly different (Fig. 6C, D). C3-transferase reversed the inhibitory effects by 95%, whereas Y27632 abolished the inhibitory effect of proBDNF completely (Fig. 6E). GTP-bound Rhoa or phosphorylation levels of the
collapsin response mediator protein-2 (CRMP-2) were increased in response to proBDNF but their responses were abolished by preincubation of these cells with C3 or Y27632 for 30 min and subsequent stimulation with proBDNF for 20 min (Fig. 6F).

ProBDNF Decreases the Nerve Innervations of Ventral Planta of the Hind Limb in Vivo Through p75NTR

Finally, we examined whether proBDNF had a similar effect on fiber growth in vivo. Postnatal day 5 (P5) p75NTR+/+ and p75NTR−/− mice were injected with proBDNF, anti-proBDNF or IgG via footpad for 3 days. At P10, the density of total nerve fibers in the ventral planta was immunostained and quantified (Fig. 7A–F). Interestingly, in p75NTR+/+ pups, more nerve fibers could be seen in the anti-proBDNF group (2.0 ± 0.2 fold, *p < 0.001) compared with the IgG group. In the proBDNF group, the fiber density was significantly decreased (0.4 ± 0 fold, *p < 0.001), indicating the fibers retracted after proBDNF treatment. However, this effect was absent when p75NTR−/− mice were treated with proBDNF (Fig. 7G). This suggests that endogenous proBDNF may play a negative role in the innervations of tissue in vivo through p75NTR.

Discussion

The morphology of axons and dendrites of neurons depends on the dynamics of the cytoskeleton which is regulated by diffusing factors from the environment, extracellular matrix, cell surface receptors and intracellular signals [21]. BDNF and other mature neurotrophins are important factors regulating neurite outgrowth and neuronal differentiation during development. However, the physiological function of proneurotrophins such as proBDNF in neurite outgrowth remains unclear. Our hypothesis is that endogenously released neuronal proBDNF may oppose functions of mature BDNF [22,23,24]. ProBDNF is highly expressed in DRG neurons and cortical neurons, can change transport

Figure 1. ProBDNF treatment decreases neurite length on DRG or cortical neurons. A, Time-lapse recordings show the collapsed neurite growth with proBDNF (left), and enhanced neurite growth with BDNF (right). Frames indicate 5, 10, 20, 30 min respectively. Scale bar, 20 μm. B, Treatment of DRG neurons with proBDNF decreased the rate of neurite extension within 3 min and remained decelerated through 30 min. n = 30 neurites/treatment. *p < 0.05, **p < 0.001, compared to BSA; Students t test. C, Treated cortical or DRG neurons with proBDNF caused similar collapse in neurite length. n = 30–85 neurons/treatment. *p < 0.05, **p < 0.001, significantly different from untreated neurons; Students t test. D, Expression of sortilin, p75NTR on the lysate of cultured cortical or DRG neurons processed for Western blot. Bands of 35 kDa of proBDNF, 75 kDa of p75NTR, and 110 kDa of sortilin were detected with their respective antibodies. β-actin (42 kD) antibody was used as internal protein loading control. n = 3 independent experiments.

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Figure 2. ProBDNF decreases neurite length on DRG neurons *in vitro*. Cultured DRG neurons were treated by different factors as shown, immunofluorescence stained by anti-MAP2 antibody. A, Normal sheep IgG. B, ProBDNF. C, Anti-proBDNF. D, BDNF. E, Anti-proBDNF. F, The neurite outgrowth assay were analyzed under the different culture conditions. Scale bar, 50 μm. n = 89–92 neurons/treatment from three independent dishes. *p<0.05, **p<0.01, compared to IgG; one-way ANOVA with least-significant difference *post hoc* test.
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not bind the p75NTR, works via p75NTR to cause neurite collapse.

To further confirm the inhibitory effect of proBDNF on neurite growth, we used MAG as the positive control [37, 38] to compare their effects. Both dose-dependently inhibit neurite growth but proBDNF is 533–583 fold more potent than MAG. As endogenous myelin-associated inhibitors are considered major impediments to regeneration after nerve injury, a myriad of strategies are applied to suppress these factors for promoting regeneration [39, 40, 41]. However the results are disappointing as the elimination of MAG, Nogo, OMgp or Semaphorin-mediated inhibition are not sufficient to promote extensive axon repair after spinal cord injury [41, 42]. These studies suggest that these inhibitors do not play a major role in CNS axon degeneration [43]. In the present study, we found that proBDNF is over 500 more potent than MAG in collapsing neurites, suggesting that proBDNF can be one of the potent inhibitors for nerve regeneration after CNS injury. In particular, when axons are injured, proBDNF is anterogradely transported to the injury sites then accumulates in the injured axonal bulb in the spinal cord [25]. It is likely that the accumulated proBDNF is released to collapse growth cones of CNS axons and prevent the regeneration of injured neurons. Thus the suppression of endogenous proBDNF and other proneurotrophins following CNS injury may promote regeneration of injured nerves.

Filopodia can detect environmental cues to transduce signals which then guide growth cone directions and dynamics [21, 44]. Growth cone turning was induced by the guidance cue, BDNF, and by the repellent factor, MAG [45]. In this study, we confirmed that proBDNF participates in growth-cone morphology and motility by reducing the length and number of filopodia. It is also likely that during development, proBDNF may function to...
repulse axons and balance the effect of mature BDNF to guide axons to their right destination. This is consistent with our previous work demonstrating that proBDNF repulses the migration of cerebellar granule neurons and their neurites, whereas mature BDNF or proBDNF antibodies attract them [11].

Growth cone protrusion and retraction of neurons is due to the dynamic assembly and disassembly of tubulin and actin cytoskeletal proteins. RhoA, Cdc42, Rac1 GTPases provide the necessary integration sites for the complex regulation of growth cone motility, cell division, and actin dynamics in neuronal growth [4,46]. Over expression of dominant active forms of Rho initiate

**Figure 5. ProBDNF treatment increases RhoA activity and filopodial length on DRG growth cones.** A–H, Immunoreactivity of RhoA or Cdc42 activity in DRG growth cones after proBDNF (0, 5, 10, 20 min) treatment. Scale bar, 10 μm. I, Quantification of RhoA activity. There were significant inductions after exposure to proBDNF 10, 20 min. *p<0.05, **p<0.01, compared to untreated neurons; Students t test. J, Quantification of Cdc42 activity. There was no change during the whole 20 min exposure of proBDNF. K–N, F-actin of DRG growth cones after proBDNF (0, 5, 10, 20 min) treatment. Scale bar, 5 μm. O, ProBDNF decreased filopodial length on DRG growth cones in a time-dependent manner. P, ProBDNF decreased filopodia number on DRG growth cones in a time-dependent manner. n = 35 neurites/treatment from at least three independent experiments. **p<0.01, compared to untreated neurons; Students t test. doi:10.1371/journal.pone.0035883.g005
Axon outgrowth and control of growth cone filopodial dynamics and promote axonal regeneration [4,47]. Rho proteins, like other members of the Ras superfamily, have been shown to control the actin cytoskeleton by the active cycle changes between active GTP-bound and inactive GDP-bound states. p75NTR interacts with Rho-GDI which is stimulated by MAG, initiating the activation of RhoA to inhibit the growth [48,49]. We found that RhoA-GTPase is immediately activated in response to proBDNF, which was dependent on p75NTR. It is likely that the binding of proBDNF to p75NTR directly stimulates the interaction with Rho-GDI and promotes the generation of RhoA-GTPase which causes the subsequent collapse growth cones and filopodia.

The activation of RhoA and its downstream effector ROCK leads to growth cone collapse and neurite growth arrest, a mechanism that has been extensively studied in experiments on the myelin-associated inhibitors MAG, Nogo, and OMgp [50,51]. To directly link the proBDNF-induced activation of RhoA and ROCK with inhibition of neurite outgrowth, pharmacological approaches were used to prevent the activation to abrogate the deleterious effects of the proBDNF. The reversal of inhibition with C3-transferase was less than that of Y27632, and this may be attributable to relatively lower cell permeability of C3-transferase [52]. Our studies show that the inhibition induced by proBDNF is via engagement of activation of RhoA and its downstream effector ROCK.

In the present study, using p75NTR−/− neurons and mice we could show that p75NTR is the proBDNF downstream signal molecule which is essential for the neurite collapse. We found that proBDNF neurite collapsing effect in vitro and in vivo are lost in p75NTR−/− neurons and in p75NTR−/− mice. Furthermore, we found that the RhoA activation by proBDNF was also abolished in p75NTR−/− neurons. These data indicate that p75NTR is responsible for the action of proBDNF. Our data raise a question of how to reconcile the contradictory roles of p75NTR in neurite growth/collapse. Yamashita et al. showed that mature neurotrophins inhibit RhoA in the absence of Trk receptors, promoting neurite growth [48]. Recently, Yamashita’s group discovered another pathway demonstrating that p75NTR may activate paired immunoglobulin-like receptor B (PIR-B) which recruits Src homology 2-containing protein tyrosine phosphatase (SHP)-1 and SHP-2, that dephosphorylates Trk receptors and reduces neurite growth [53]. The activation by mature neurotrophins of p75NTR in the presence or absence of Trk receptors would promote neurite growth via inactivation of RhoA and/or activation of the phosphatidylinositide 3 kinase (PI3K) and the mitogen-activated protein kinase (MAPK) pathway [48,54,55,56]. On the other hand, the activation of p75NTR by proneurotrophins and/or myelin inhibitory factors in the absence of Trk or low levels Trk receptors in neurons would activate RhoA and suppress the signal transduction of Trk/MAPK, leading to the neurite collapse and degeneration. Thus, activation or inactivation of RhoA signaling pathway via the p75NTR is ligand- and coreceptor-dependent. The double-faced actions of p75NTR on neurite growth explains why no enhanced nerve regeneration is seen in p75NTR−/− mice after spinal cord injury [57].

The function of p75NTR in neurite growth does not only depend on neuronal expression of p75NTR, but also on the glial expression of p75NTR [58]. This interrelationship between different cell types expressing p75NTR is even more complicated.
in the peripheral nervous system. For example, macrophages express p75NTR and have been reported to affect peripheral nerve regeneration [59], p75NTR is highly expressed by peripheral neurons and also Schwann cells after Wallerian degeneration. We and others showed that Schwann cell p75NTR positively regulates nerve regeneration. The depletion of p75NTR causes retardation of peripheral nerve regeneration after nerve injury [57,60,61] and reduces the nerve elongation during development [32,62].

In summary, proBDNF is a powerful neurite outgrowth inhibitor which activates RhoA via p75NTR. ProBDNF also decreases filopodial length and number of growth cones and triggers a polarized distribution of activated RhoA in the tips of growth cones. Neutralization of endogenous proBDNF enhances the innervation. Endogenous proBDNF may play an opposing role to that of mature BDNF regulating neurite growth.

**Materials and Methods**

**Animals and Reagents**

All experimental procedures were under the guidelines of the National Health and Medical Research Council of Australia and
approved by the Animal Welfare Committee of Flinders University. Adult 9–10 weeks old Sprague-Dawley (SD) rats (n = 12), neonatal 129 sv wild-type (p75NTR+/+), n = 24), postnatal day 5 (P5, p75NTR++/+, n = 15), p75NTR deficient mice (p75NTR−/−, n = 12) were used.

Recombinant proBDNF with a RR-AA mutation of the cleavage site and the neutralizing proBDNF antiserum were produced in our lab and their biological activities have been characterized [63]. The antiserum directed against proBDNF for immunohistochemistry have been characterized [27]. The neutralizing antiserum specifically recognize proBDNF but not mature BDNF and other neurotrophins [11,12,25,26,63]. The antisera directed against proBDNF for Western Blot of proBDNF, p75NTR and Sortilin in Cortex were obtained from Invitrogen and other reagents were obtained from Sigma (St. Louis, MO) unless specified otherwise.

Primary Neuron Culture

Lumbar dorsal root ganglia (DRG) were dissected from SD rats (~250 g, n = 6) and cut into eight pieces, digested at Collagenase II Ca²⁺ free Hank’s solution as described previously [64]. The tissues were dissociated using a fire-polished Pasteur pipette; the cell suspension was layered on top of a 15% fatty acid-free albumin bovine serum (BSA, Sigma) solution. After a 400 g centrifugation for 20 min the pellet was resuspended in Neurobasal medium (Invitrogen) containing 0.25% fetal calf serum (FCS, Invitrogen), penicillin–streptomycin (100 U), L-glutamine (2 mM) and B27 (2%) serum-free supplement (Invitrogen). The number of viable cells was determined by trypan blue (Sigma) exclusion and 2 × 10⁴ DRG cells were plated onto poly-D-lysine-coated cell culture dishes and cultured at 37°C in 95% O₂ / 5% CO₂ incubator. Cultured DRG neurons were treated with proBDNF (0, 3, 10, 30, 100 ng/ml), anti-proBDNF (10 C in 95% O₂ /5% CO₂ incubator, neurons were incubated followed by incubation with Alexa Fluor 488 anti-rabbit secondary antibody (Jackson Laboratories, USA). Images were acquired from randomly selected fields (n = 2–12) under Fluorescence Microscope (Olympus BX50). The length of the longest neurite of 80–120 neurons per condition was determined using ImageJ software [66]. Each experimental condition was done in duplicate wells, and at least three independent experiments were conducted to acquire the final results.

Indirect Immunofluorescence and Neurite Outgrowth Assay

For outgrowth assays, primary cultured neurons were fixed with 4% paraformaldehyde (PFA), then incubated overnight with 1:1000 Alexa Fluor 488 anti-RhoA secondary antibody (Jackson Laboratories, USA). For outgrowth assays, primary cultured neurons were fixed with 4% paraformaldehyde (PFA), then incubated overnight with 1:1000 Alexa Fluor 488 anti-RhoA secondary antibody (Jackson Laboratories, USA). Images were acquired from randomly selected fields (n = 2–12) under Fluorescence Microscope (Olympus BX50). The length of the longest neurite of 80–120 neurons per condition was determined using ImageJ software [66]. Each experimental condition was done in duplicate wells, and at least three independent experiments were conducted to acquire the final results.

Activation Assay of RhoA and Cdc42

The proBDNF-dependent activation of RhoA and Cdc42 was assessed using a pull-down assay kit according to manufacturer instructions (NewEast Biosciences, USA). Due to the limited number of DRG neurons we used cortical neurons in this assay. 10⁴ cortical cells from p75NTR++/+ and p75NTR−/− mice were cultured and treated with 30 ng/ml proBDNF for 0, 5, 10, 20 min, and subsequently homogenized and lysed with RIPA buffer containing protease inhibitors (Roche Applied Science). One aliquot was used for immunoblot (IB) analysis, the second aliquot was incubated with GTP-RhoA or GTP-Cdc42 antibody, bound with protein A/G agarose beads for 1 h at 4°C, precipitated and subjected to SDS-PAGE, detected by anti-RhoA or anti-Cdc42 antibodies. GTP-activated levels were normalized to the corresponding total RhoA levels using densitometric analysis using ImageJ software. The data were expressed as a percentage of the signals obtained at 5, 10 and 20 min in comparison with the time point 0.

Immunohistochemical Detection of Activated RhoA and Cdc42

Primary cultured rat DRG neurons (n = 6) were exposed to proBDNF for 0, 5, 10, 20 min, fixed with 4% PFA and incubated with antiserum directed against GTP-RhoA or GTP-Cdc42 (NewEast Biosciences, USA) respectively, followed by incubation with Alexa Fluoro 594 anti-mouse secondary antibody [Jackson Laboratories, USA]. The presence of GTP-activated RhoA or Cdc42 in growth cones was quantified by measuring signal intensity in each growth cone using ImageJ. For F-actin staining, primary cultured DRG neurons were fixed with 0.25% glutaraldehyde, quenched with 1 mg/ml sodium borohydride for 15 min, followed by incubation rhodamine-conjugated phalloidin (Molecular Probes, USA). The staining of growth cones were captured using a confocal microscope (Leica SP5, Germany).
Neurite Outgrowth Assay Detects Activation of RhoA and ROCK Pathway

The chemical approach included treatment of cultured DRG neurons with the RhoA inhibitor, C3-transferase (25 μg/ml; Cytoskeleton), and/or the ROCK inhibitor, Y27632 (10 μM; Calbiochem), before adding proBDNF (30 ng/ml). 2×10⁴ cultured DRG neurons were treated after 24 h later, neurite length was measured as above mentioned. Cell lysates were assayed for activated RhoA and for phosphorylated CRMP-2 (rabbit polyclonal anti phosphorylated CRMP-2 affinity purified antibody was a gift from Dr S. Petrotas, Monash University, Clayton, Australia).

In vivo Delivery of Exogenous ProBDNF and its Neutralizing Antiserum into the Footpad of Pups

10 μl of proBDNF (1 μg), anti-proBDNF (5 μg) or IgG (5 μg) were injected into the footpad of P5 p75NTR+/- and p75NTR-/- mice (n = 5/treatment) on three consecutive days. On day ten, the pups were anesthetized and immobilized perfused through the heart with 30 ml saline followed by 30 ml Zamboni’s fixative as described previously [11]. After perfusion, the injected ventral trunk was dissected and post-fixed overnight in the same fixative. The next day, the trunk was cryopreserved in 0.1 M phosphate-buffered saline 18% sucrose. Sagittal cryostat sections (30 μm thickness) were cut into five series using a freezing microtome (Leica, Germany) and mounted on gelatin-coated glass slides as described previously [11]. The series in every fifth mid-sagittal sections were used for nerve fiber density quantification. These sections were blocked using 5% donkey serum for 1 h at room temperature then incubated with anti-neurofilament antibody (1:1000, RT97, Abcam, UK) overnight at 4 °C. The next day, the sections were incubated with Alexa Fluor 594 anti-mouse secondary antibody. Images were taken from 8 sections per animal under Fluorescence Microscope (Olympus BX50). The nerve fiber density was determined by determining the area occupied by nerve fibers at 150 μm intervals using ImageJ program. Five animals per treatment were used in this study. The data were expressed as a normalized nerve fiber density using normal sheep IgG group as 1.

Statistical Analysis

The in vivo experiments of footpad injection were carried out in a double blinded manner. The data are expressed as mean ± standard error of the mean (SEM). Experiments with intra or inter group were analyzed by using Student’s t test or one way ANOVA with Least-significant Difference (LSD) Post Hoc Test. p values <0.05 were considered statistically significant. All statistical analyses of the experimental data were performed using 13.0 SPSS.

Supporting Information

Figure S1 ProBDNF treatment decreases neurite length on cultured DRG neurons.

Figure S2 BDNF treatment increases neurite length on cultured DRG neurons.

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Author Contributions

Conceived and designed the experiments: XZF. Performed the experiments: YS YL FL JHZ. Analyzed the data: YS RH SL JJL. Contributed reagents/materials/analysis tools: YS YL JHZ. Wrote the paper: YS RH XZF.

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