Published in final edited form as:

Brain Res. 2012 January 9; 1430: 25–34. doi:10.1016/j.brainres.2011.10.054.

Ras/p38 and PI3K/Akt but not Mek/Erk signaling mediate BDNF-induced neurite formation on neonatal cochlear spiral ganglion explants

Lina M. Mullen^b, Kwang K. Pak^a, Eduardo Chavez^b, Kenji Kondo^b, Yves Brand^{b,d,*}, and Allen F. Ryan^{a,b,c,*}

^aSan Diego VA Medical Center, 3350 La Jolla Village Drive, San Diego, CA 92161 ^bDepartment of Surgery/Otolaryngology, UCSD School of Medicine, 9500 Gilman Drive MC0666, La Jolla, CA 92093 ^cDepartment of Neurosciences, UCSD School of Medicine, 9500 Gilman Drive MC0666, La Jolla, CA 92093 ^dDepartment of Biomedicine and Clinic of Otolaryngology, Head and Neck Surgery, University Hospital Basel, Hebelstrasse 20, 4031 Basel, Switzerland

Abstract

Neurotrophins participate in regulating the survival, differentiation, and target innervation of many neurons, mediated by high-affinity Trk and low-affinity p75 receptors. In the cochlea, spiral ganglion (SG) neuron survival is strongly dependent upon neurotrophic input, including brain-derived neurotrophic factor (BDNF), which increases the number of neurite outgrowth in neonatal rat SG *in vitro*. Less is known about signal transduction pathways linking the activation of neurotrophin receptors to SG neuron nuclei. In particular, the p38 and cJUN Kinase (JNK), mitogen-activated protein kinase (MAPK) pathways, which participate in JNK signaling in other neurons, have not been studied. We found that inhibition of Ras, p38, phosphatidylinositol 3 kinase (PI3K) or Akt signaling reduced or eliminated BDNF mediated increase in number of neurite outgrowth, while inhibition of Mek/Erk had no influence. Inhibition of Rac/cdc42, which lies upstream of JNK, modestly enhanced BDNF induced formation of neurites. Western blotting implicated p38 and Akt signaling, but not Mek/Erk. The results suggest that the Ras/p38 and PI3K/Akt are the primary pathways by which BDNF promotes its effects. Activation of Rac/cdc42/JNK signaling by BDNF may reduce the formation of neurites. This is in contrast to our previous results on NT-3, in which Mek/Erk signaling was the primary mediator of SG neurite outgrowth *in vitro*. Our data on BDNF agree with prior results from others that have implicated PI3K/Akt involvement in mediating the effects of BDNF on SG neurons *in vitro*, including neuronal survival and neurite extension. However, the identification of p38 and JNK involvement is entirely novel. The results suggest that neurotrophins can exert opposing effects on SG neurons, the balance of competing signals influencing the generation of neurites. This competition could provide a potential mechanism for the control of neurite number during development.

Keywords

BDNF; Neuritogenesis; Ras/P38; Spiral ganglion; Signal transduction; TrkB receptor

Corresponding author: Allen F. Ryan, Ph.D., Tel.: +1 (858) 534-4594, Fax: +1 (858) 534-5319, afryan@ucsd.edu.

*Drs. Ryan and Brand contributed equally to the supervision of this work.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

1. Introduction

Neurotrophins play a critical role in neural development, regulating differentiation, neurite extension, target innervation and survival (Bibel and Barde, 2000). Brain-derived neurotrophic factor (BDNF) and neurotrophin-3 (NT-3) are well known to influence neurons in the inner ear.

In particular, mice deficient in BDNF exhibit reduced cochlear neuronal populations, especially in the apical turn (Ernfors et al., 1994 and 1995; Bianchi et al., 1996; Fritzsche et al., 1997; Fariñas et al., 2001). We, and others, have noted a dramatic effect of BDNF on developing spiral ganglion (SG) neurons in culture. BDNF treatment enhances survival of dissociated SG neurons (Malgrange et al., 1996), dramatically increases neurite number on SG explants (Pirvola et al., 1994) and promotes SG neurons survival *in vivo* (Leake et al., 2011).

Recently, Leake et al. (2011) demonstrated in neonatally deafened kittens and Landry et al. (2011) in adult deafened guinea pigs that chronic BDNF delivery from a miniosmotic pump improved electrically evoked auditory brainstem response thresholds. The authors therefore concluded that BDNF may have potential therapeutic value for the use with cochlear implants in the future. Furthermore, increasing reports are available on the potential therapeutic role of BDNF in a range of central nervous system (CNS) disorders such as amyotrophic lateral sclerosis, Parkinson's disease, peripheral neuropathy, Alzheimer's disease, Huntington's disease and stroke (reviewed by Nagahara and Tuszynski, 2011).

Neurotrophins signal primarily via high-affinity tyrosine kinase receptors in the cochlea, TrkB and TrkC (Pirvola et al., 1994), with some contribution from the low-affinity p75 receptor (Schedterson & Bothwell, 1994). BDNF signaling is mainly mediated via TrkB receptors and TrkB and p75 receptors are expressed by SG neurons throughout the inner ear (Pirvola et al., 1994; Knipper et al., 1996; Sano et al., 2001). Mice null for TrkB are reported to lose 15–20 % of SG neurons (Fritzsche et al., 2001). BDNF increases neurite number on SG explants *in vitro* throughout the entire length of the cochlea with no difference in the responses from different cochlear turns (our own unpublished data).

We previously found that Ras or Mek/Erk inhibition blocked NT-3 effects on SG neurites, while p38 inhibition had no effect (Aletsee et al., 2001). Mice with mutations in the docking site for the Shc adaptor protein on the TrkB receptor, which would be expected to reduce both Ras/MAPK and phosphatidylinositol 3 kinase (PI3K) signaling, showed modest reduction in SG neuron survival (Postigo et al., 2002).

To explore BDNF signal transduction in SG neurons, SG explants were treated with BDNF in the presence of specific inhibitors of intracellular signaling pathways involved in TrkB signaling in the inner ear and other neuronal systems, and activation of signaling proteins was assessed by Western blotting.

2. Results

2.1 BDNF increases SG neurite number but not length

Consistent with previous studies (Hartnick et al., 1996; Hegarty et al., 1997), treatment of neonatal SG explants with BDNF resulted in a significant increase ($p < 0.05$) in the number of SG neurites present on each explant (Figs. 1 & 2). In contrast, and also consistent with prior results (Malgrange et al., 1996), there was no effect of BDNF treatment on the length of SG neurites (Figs. 1 & 3).

2.2 Inhibitors of several signal transduction pathways alter BDNF-induced increases in SG neurite number

The influence of signaling inhibitors on the BDNF-induced increase in neurites on SG explants is illustrated in Figs. 1 & 2. When BDNF treatment occurred in the presence of the pan-G-protein inhibitor GDP β S, there was no significant influence ($p > .06$). In contrast, the specific Ras inhibitor FTI-277 virtually eliminated the BDNF-induced increase in SG neurite number at all inhibitor doses ($p < .03$). While the MEK/Erk inhibitor UO126 had no effect ($p > .08$), the p38 inhibitor SB203580 reduced the BDNF response at all doses ($p < .02$). Interestingly, the Rac/cdc42 inhibitor *C difficile* toxin B significantly increased the BDNF effect on neurite number, but only at the lowest dose employed ($p < .04$). The PI₃ kinase inhibitor Wortmannin reduced the BDNF effect, but only at the highest dose employed ($p < .0001$). Akt inhibitor II significantly attenuated the BDNF effect at 100 nM ($p < .0001$) and 1 nM ($p < .01$), but not at 0.1 ($p < .08$). The PKA inhibitor KT5720 did not alter BDNF effects on SG neurites. When applied alone at the effective dose, or at the highest dose used when no effect was observed, none of the inhibitors influenced SG neurite number.

2.3 Signal transduction inhibitors influence SG neurite length

As discussed above, BDNF alone did not affect SG neurite length. Nevertheless, some signaling inhibitors in the presence of BDNF significantly altered neurite length (Fig. 3). The Rac/cdc42, G protein, Mek/Erk and Akt inhibitors each decreased SG neurite length at all doses employed ($p < .04$). The PI3K inhibitor Wortmannin decreased length at the highest dose ($p < .04$). The PKA inhibitor KT5720 increased neurite length at all doses ($p < .04$). When explants were exposed to the inhibitors alone, neurite numbers were increased by UO126 at 1000 nM ($p < .05$), and decreased by Akt inhibitor II at 1 nM ($p < .02$). None of the other inhibitors used affected neurite length when applied alone.

2.4 BDNF increases both SG neuron survival and neurites/neuron

The methods used above could not distinguish whether BDNF-induced increases in the number of neurites on SG explants were due to increased SG neuron survival, neurite branching within the explant, or both. We therefore explored alternate methods, and found that a different fixation and staining regimen combined with clearing allowed visualization of SG somata in explants larger than those used for the studies above. The results of culture and BDNF treatment on SG neuron survival in this model are illustrated in Fig. 4. Freshly dissected SG explants contained an average of 0.466 SG neurons/ μ m of ganglion. Control samples cultured without BDNF for 72 hours showed 0.050 ($\pm .010$) neurons/ μ m, while explants cultured with BDNF showed 0.131 ($\pm .014$) neurons/ μ m. Thus, BDNF resulted in a 162% increase in SG neuron survival compared to untreated explants. Of course, no neurites were observed on freshly dissected explants. However, control explants cultured without BDNF for 72 hours showed 0.020 ($\pm .006$) neurites/ μ m. Thus, neurites extending from the explants represented only 40% of surviving neurons. BDNF resulted in a 520% increase in the number of neurites that extended from the explant when compared to control explants, representing both increased survival and increased neurites/neuron.

2.5 BDNF activates p38 and Akt in SG

Western blotting revealed specific activation of cell signaling in SGNs by BDNF. Using Actin as an internal control, normalized phospho-38, phospho-Akt and phospho-Erk levels were expressed as % of control. In three replicates, the relative intensity of phospho-p38 and phospho-Akt was increased in BDNF treated tissue compared to tissue in culture media only. In contrast, only a modest not statistically significant increase in activated Erk MAPK was noted (Fig. 5).

3. Discussion

In the current study, we show that Ras/P38 and PI3K/Akt but not Mek/Erk signaling mediate BDNF-induced neurite formation on neonatal cochlear SG explants. In order to assess the signaling pathways mentioned above, we first evaluated the effects of BDNF alone on SG neurites *in vitro*. Then, SG explants were treated with BDNF in the presence of specific inhibitors of the intracellular signaling pathways involved downstream from TrkB signaling. Finally, we confirmed activation of signaling proteins by Western blotting.

The observation that BDNF treatment results in substantially more neurites on SG explants is consistent with increases in neuronal survival that have been observed with dissociated SG neurons (e.g. Hartnick et al., 1996). However, when survival and neurite number were compared directly, we noted an even greater increase in the number of neurites/neuron following BDNF treatment. This was not associated with an obvious branching of the fibers, nor did the number of neurites exceed one per neuron, indicating that BDNF also increased the production of individual, unbranched neurites on SG neurons. Thus, BDNF appears to be both a survival promoting and neurotogenic factor for SG neurons. The lack of effect of BDNF on neurite length also agrees with several previous studies (Hartnick et al., 1996; Malgrange et al., 1996).

It should be noted that we could not distinguish between the dendrites and axons of SG neurons, since we have not found markers that distinguish between the two in explants. Similarly, we could not distinguish between type I and type II SG neuron neurites, since peripherin labeling does not distinguish these two classes of neurons in the rat in culture, due to up-regulation of peripherin in type I neurons *in vitro* (Lallemend et al., 2007). However, since 95% of SG neurons are type I cells, it seems likely that this class of neuron dominates our results.

Our *in vitro* data on neuronal survival can also be related to *in vivo* observations of the SG. The endogenous expression of BDNF in the cochlea appears to vary during the period under study. At birth, BDNF is seen in rat inner and outer hair cells (HC) and along the length of the cochlea (Pirvola et al., 1992) and is present in the supporting cells (SCs) of the mouse organ of Corti only in the apical turn (Farinas et al., 2001). Wheeler et al. (1994) and Wiechers et al. (1999) reported that BDNF mRNA in HCs declined to background levels by P3–P4. Wiechers et al. (1999) observed BDNF mRNA in SCs and outer HCs at P6–P8, while Ylikoski et al. (1993) noted BDNF mRNA in both inner HCs and outer HCs at P7. Wiechers et al. (1993) evaluated the expression of BDNF at the protein level during the first two postnatal weeks in mice, using immunohistochemistry. They found that BDNF is present in inner HCs and outer HCs at P1, and then disappears at P3. However, at P3 BDNF is found in some SG neurons. BDNF then reappears in HCs and SCs at P6, and is observed at high levels in SG neurons. At P10, BDNF is only present in some SCs and in scattered SG neurons. These results suggest that HCs produce BDNF during the first few days after birth, with a decline around P3–P4, but recovery by P6–P7. SG neurons also transiently express BDNF, beginning around P6. Rüttiger et al. (2007) showed that BDNF is not expressed in the organ of Corti, but in the SG in adult gerbils. While there was no change in BDNF expression in the apical turn, a moderate decrease in expression was seen in midbasal turns during aging. In contrast, a recent study by Liu et al. (2011) on adult surgical human cochlear specimens showed no expression of BDNF protein either in the organ of Corti or in the SG.

Our data indicate that SG neurons and neurites are highly sensitive to BDNF during the period in which declines in production are observed, around P3–P5. This is in line with electrophysiological experiments on P3–P8 neonatal mouse SG. Adamson et al. (2002)

demonstrated that BDNF alters the endogenous membrane properties and channel types in such a way as to generate faster accommodation and kinetics. It can be speculated that Akt and/or p38 signaling may contribute to these effects.

It is possible that early postnatal production of BDNF in the organ of Corti maintains SG neurons and neurites during the period of reorganization of innervation. The decline in production may then induce apoptosis, with those neurons that ultimately survive having successfully innervated HCs, while neurons that fail to synapse on HCs die from lack of trophic support. SG neurons are reported to undergo substantial apoptosis during the first postnatal week in rodents (Echteler and Nofsinger, 2000).

Our signaling results suggest that a number of pathways participate in transmitting the effects of TrkB receptor activation to the nucleus. Our conclusions are summarized in Fig. 6. The strong effects of FTI-277 on neurite number suggest a major role for Ras in mediating the survival- and neuritogenesis-promoting effects of BDNF. The reduction in neurite number was observed at all FTI-277 dosages employed, including the lowest (0.1 μ M). This implies that the effect is mediated at least in part by H-Ras, since other isoforms of Ras such as N- or K-Ras are only inhibited at higher levels (5 and 10 μ M, respectively; Lerner et al., 1995). Also, a combination of p38 MAPK and PI3K-Akt signaling appears to stimulate SG neurites, while the UO126 data suggest that the promotion of SG neurite number by BDNF does not involve the canonical Ras-Mek-Erk MAPK survival pathway. This conclusion is supported by our Western blotting data, which demonstrated strong activation of p38 and Akt, but not Erk, in SG neurons after BDNF treatment. Similarly, in sympathetic neurons, NGF promotes survival via a Ras-PI3K-Akt pathway rather than Mek-Erk (Vaillant et al., 1999). Other studies have also shown BDNF mediated activation of PI3K-Akt signaling in SG *in vitro* (Lallemend et al., 2005; Hansen et al., 2001). However, our observation that BDNF does not involve the canonical Ras-Mek-Erk MAPK survival pathway is in contrast to a report by Lallemend et al. (2005) who found that BDNF enhancement of dissociated SG neuron survival was decreased by UO126. Since they used rat SG neurons of a similar age, the difference may be related to dissociation of the ganglion.

The p38 and cJUN kinase (JNK) mitogen-activated protein kinase (MAPK) families have not yet been investigated in BDNF signal transduction in the SG. Our findings that Ras/p38 promotes BDNF mediated effects on SNG while Rac/cdc42/JNK signaling reduces the BDNF mediated formation of neurites are novel.

While signal transduction pathways that mediate BDNF effects have received little attention in the inner ear, several pathways have been implicated in other neuronal systems. Results from pharmacological studies suggest that both MAPK and PI3K pathways mediate BDNF-induced neurite outgrowth from retinal ganglia (Bonnet et al., 2004), while Erk5 activation is critical to BDNF-promoted survival of developing cortical neurons (Liu et al., 2003). Activation of the PI3K target Akt (also known as protein kinase B), mediates BDNF effects on hippocampal neurons (Lee et al., 2002). It has been shown that p38 and JNK MAPK pathways can also be activated by Trk receptors in the nervous system. While in general they promote apoptosis (Mielke et al., 2000; Ishikawa et al., 2003), several examples of survival enhancement by these pathways have been documented (Nishina et al., 1997; Du et al., 2004). The p75 receptor can also be involved in BDNF signaling. As a dependence receptor (Mehlen and Bredesen, 2004), p75 requires neurotrophin binding to prevent cleavage of its intracellular domain and release of an apoptosis-promoting fragment. Alternatively, neurotrophin binding to p75 can induce apoptosis. This is thought to be Trk-dependent when a neurotrophin binds to a mismatched Trk in association with p75 (Bredesen and Rabizadeh, 1997).

It is intriguing that Rac/cdc42 inhibition enhanced the neurite-promoting effects of BDNF. This observation suggests that BDNF may have a complex effect on SG neurons, with neurite number being promoted by p38 and Akt signaling, while being opposed by a Rac/cdc42/JNK pathway. However, the neurite-promoting effects of BDNF were only enhanced at the lowest concentration of the Rac/cdc42 inhibitor applied. A BDNF-independent effect seems unlikely, since Brors et al. (2003a) showed that Rac/cdc42 inhibition led to a dose-dependent decrease of SG neurite number cultured on laminin. The idea that BDNF may activate competing survival and death signals is consistent with current theories of apoptosis regulation in which it is the balance of such competing signals that determine a cell's fate (Salvesen, 2002).

The general G protein inhibitor GDP β S did not influence BDNF effects at any dosage. However, specific inhibition of the G protein Ras reduced BDNF effects, while inhibition of the Rho family G protein Rac/cdc-42 enhanced BDNF. The simplest explanation for the lack of effect of GDP β S is that inhibition of Ras and Rac/cdc42 signaling cancelled each other, resulting in no net effect. While this may well be the case, the very large number of G proteins that might potentially be involved in SG neurons suggests that there may well be a more complex explanation.

Agerman et al. (2003) replaced the coding sequence of the BDNF gene in mice with that of NT3, to analyze the selective roles of BDNF and NT3 during inner ear development. They found that NT3 largely replaced the actions of BDNF in the cochlea, indicating that these two neurotrophins have common and redundant functions. Interestingly, our data indicate that despite the fact that NT3 can largely replace the effects of BDNF in the cochlea, the signaling pathways activated by these neurotrophins are quite different. Aletsee et al. (2001) demonstrated that Ras/Mek but not p38 signaling mediates NT3-induced effects on SG neurons *in vitro*. This implies that the different signaling pathways activated by BDNF versus NT3 nevertheless converge on similar cell functions. The reason for the utilization of different signaling cascades is unclear. However, this might relate to the evolutionary history of the two receptors involved. It might also be speculated that different opportunities for regulation are provided by the two patterns of intracellular signaling.

In the current study, BDNF treatment alone did not affect neurite length. Therefore, the effects of signaling inhibitors on neurite extension without BDNF presumably reflect an influence independent of this neurotrophin. One candidate for the mediation of length effects is alteration of extracellular matrix signaling via integrins. We have previously shown that extracellular matrix molecules enhance neurite outgrowth at the level used to coat the culture wells in the present experiment (Aletsee et al., 2001; Evans et al., 2007). It should be noted that integrin signaling is unlikely to mediate the effects of BDNF on SG neuron survival of neuritogenesis as discussed above, as we have not found in past experiments that ECM molecules influence SG neurite number (Aletsee, et al., 2001). In the case of inhibitors that only influenced length in the presence of BDNF, it is possible that BDNF has both positive and negative influences upon neurite length, that on balance result in no effect. Inhibitors may upset this balance. While this hypothesis is perhaps too complex to be attractive without additional supporting data, it is at least consistent with our observations.

4. Experimental Procedures

4.1 Culture of Spiral Ganglion Neurons

Surgical procedures were approved by the animal subject committee of the San Diego VA Medical Center in accordance with the guidelines laid down by NIH regarding the care and use of animals for experimental procedures. Three to five day old Sprague-Dawley rat pups (P3–P5) were decapitated and the skulls were opened midsagittally under sterile conditions.

The membranous labyrinth was exposed by peeling off the cartilaginous cochlear capsule under a dissecting microscope. The stria vascularis and the organ of Corti were removed to expose the SG. The ganglion was excised from the entire length of the cochlea and divided into explants that were approximately $300 \times 300 \mu\text{m}$. These individual explants were cultured in 24-well plates previously coated with fibronectin (Sigma-Aldrich, St. Louis, MO) and poly-L-lysine (Sigma-Aldrich). The tissue was incubated in $170 \mu\text{l}$ of an attachment media consisting of DMEM (Invitrogen/Gibco, Grand Island, NY, USA), 10% FCS (Invitrogen/Gibco), 5% HEPES (Invitrogen/Gibco) and 30 units/ml penicillin (Sigma-Aldrich) for 24 hours at 37°C , 5% CO_2 . After 24 hours, the culture medium was changed to $200 \mu\text{l}$ of a maintenance media consisting of DMEM supplemented with 1X N2 and 5g/L glucose (Invitrogen/Gibco). For neurotrophin stimulation, the maintenance media contained BDNF (10ng/ml; Calbiochem, La Jolla, CA, USA). BDNF control cultures received maintenance media alone. It should be noted that hearing in the rat cochlea begins on about postnatal day 10 (Henley et al., 1989; Rybak et al., 1992). Prehearing neurons were studied since older neurons are more difficult to culture and neurite development is ongoing at this age (Ernfors et al., 1995; Echteler et al., 2000).

Experimental cultures contained BDNF with different concentrations of signaling inhibitors: .01, .1 or 1 mM of the general G-protein inhibitor GDP β S (Sigma-Aldrich); .1, 1 or 10 μM of the Ras inhibitor FTI-277 (Calbiochem); 10, 100 or 1000 nM of the MEK/Erk inhibitor UO126 (Calbiochem); 1, 10 or 100 nM of the p38 inhibitor SB 203580 (Calbiochem); 1, 5, or 10 ng/ml of the Rac/cdc42 inhibitor *C. difficile* toxin B (an upstream activator of JNK; Calbiochem); 10, 100 or 1000 nM of the PI3K inhibitor Wortmannin (Calbiochem); 0.1, 1.0, or 100 nM of the Akt inhibitor Akt inhibitor II (Calbiochem: 124008); 10, 200 or 1000nM of the PKA inhibitor KT5720 (Cell Signaling Technology, Beverly, MA). Inhibitor control media contained the lowest effective dosage of the inhibitor alone. For each condition, 12 explants were studied, except Rac/cdc42 inhibitor *C. difficile* toxin B 18 explants were studied.

4.2 Fixation and Immunohistochemistry

After 3 days of incubation, cultures were fixed with 4% paraformaldehyde for 20 minutes and then washed with PBS. The samples were blocked with 1% donkey serum (Sigma-Aldrich) for 10 minutes at room temperature to reduce nonspecific binding. Specimens were incubated with rabbit polyclonal anti-200 kDa neurofilament antibody (Sigma-Aldrich) diluted 1:500 at 4°C overnight. Explants were then incubated in FITC-conjugated donkey anti-rabbit secondary antibody (Jackson ImmunoResearch, West Grove, PA) diluted 1:100 in PBS. Immunolabeling controls in which rabbit serum was substituted for the primary antibody exhibited no labeling.

The explants were digitally imaged on a fluorescence inverted microscope (Olympus, IX 70) and the number and length of neurites were determined by image analysis software (Spot) as previously described (Brors et al., 2003b). Briefly neurites were traced from the edge of the explant to the tip. All neurites on all explants were measured.

4.3 Quantitation of Neuronal Survival

To assess BDNF effects on neuronal survival, half-turn SG explants were cultured as above with and without 25 ng/ml BDNF for 72 hours, except that the explants were grown on glass cover slips. In order to provide higher penetration and potential for effects on the ganglion body, we used 25 ng/ml in our Western Blot and neuronal studies. The explants were fixed as above, treated with 0.5% peroxide in methanol to block endogenous peroxidases, reacted with a mouse monoclonal antibody IgG against rat neurofilament 200 (Sigma-Aldrich), followed by a biotinylated secondary anti-mouse IgG and developed by an avidin and DAB

procedure (Vector Laboratories, Burlingame, CA). The tissue was cleared with citrosol (Fischer Scientific, Waltham, MA, USA) to allow visualization of the cell soma and mounted for evaluation of neuronal survival and neurite number. Soma survival results from cultured explants were compared to those from freshly dissected explants.

4.4 Assessment of Signaling Protein Activation

To assess the activation of signaling pathways, intact SG were harvested and placed in attachment media for 24 hours. They were then placed in maintenance media, with or without 25ng/ml BDNF for 5 minutes. Explants were collected from media, and lysed with 100 μ l T-Per Tissue Protein Extraction Reagent (Thermo Scientific, Rockford, IL) in 1X phosphatase/proteases inhibitors (Roche, Indianapolis, IN) and sonicated for 10 min to shear chromosomal DNA. Samples were centrifuged at 10,000G for 10 minutes to separate the cytosolic from the membranous components. Equal quantities of these lysates were separated by Bis-Tris Mini Gels 4–12% gels, and electrotransferred to polyvinylidene difluoride (PVDF) membranes (Bio-Rad, Hercules, CA). The membranes were blocked with 5.5% nonfat dried milk in TBS-Tween [50mM Tris-HCL(pH 7.4), 150 mM NaCl, 0.05% Tween 20] for 60 min at room temperature. Blots were incubated with primary antibodies in blocking buffer overnight at 4°C and then incubated with horseradish peroxidase-linked secondary antibodies (Jackson ImmunoResearch) followed by chemiluminescent detection (GE Healthcare, Piscataway, NJ). Blots were evaluated with antibodies against phosphorylated Akt (Cell Signaling Technology), phosphorylated p38 (Cell Signaling Technology), phosphorylated Erk (Santa Cruz Biotechnology, Santa Cruz, CA) and to an internal control protein actin (BD Transduction Laboratories, San Diego, CA). After chemiluminescent exposure each membrane was placed inside a dark chamber, an autoradiography film 5×7 was laid over the membrane to capture light emission and scanned with an Agfa Arcus II scanner. The intensity of the bands corresponding to phosphorylated-p38, phosphorylated-Akt and phosphorylated-Erk were quantified using Image J software. Band intensity for the phosphoproteins was corrected for intensity of our internal control protein (actin) and then expressed as the percentage increase, compared with non-treated tissue. Western blotting was replicated three times with independent biological replicate. With each biological replicate, Western blotting was performed twice. Six whole SG were used per individual blot. Ratio data were analyzed using the Mann-Whitney nonparametric statistical test.

4.5 Quantitation of neurite outgrowth

Statistical analysis, using a one-way analysis of variance (ANOVA) followed by a Tukey least significant difference post hoc test was performed, including a correction for the use of multiple post hoc tests (Statview 5.0).

Highlights

1. BDNF increases neonatal spiral ganglion survival and neurite number but not length in vitro.
2. Inhibition of p38, Ras, PI₃ kinase and Akt II signal transduction reduced BDNF-induced responses, while inhibition of Mek/Erk had no effect.
3. Western blotting revealed specific activation of Akt and p38, but not Erk cell signaling in spiral ganglion neurons by BDNF.
4. Neurotrophins can exert opposing effects on spiral ganglion neurons, competing signals influence cell survival and the generation of neurites.

Abbreviations

ANOVA	one-way analysis of variance
BDNF	brain-derived neurotrophic factor
CNS	central nervous system
HC	hair cells
JNK	cJUN Kinase
MAPK	mitogen-activated protein kinase
NT-3	neurotrophin-3
PI3K	phosphatidyl inositol 3 kinase
PVDF	polyvinylidene difluoride
SCs	supporting cells
SG	spiral ganglion

Acknowledgments

Supported by the Research Service of the VA (AFR), NIH/NIDCD grant R01-DC000139 (AFR) and by Swiss National Science Foundation grant PBBSP3-13097 (YB).

REFERENCES

- Adamson CL, Reid MA, Davis RL. Opposite actions of brain-derived neurotrophic factor and neurotrophin-3 on firing features and ion channel composition of murine spiral ganglion neurons. *J Neurosci.* 2002; 22:1385–1396. [PubMed: 11850465]
- Agerman K, Hjerling-Leffer J, Blanchard MP, Scarfone E, Canlon B, Nosrat C, Enfors P. BDNF gene replacement reveals multiple mechanisms for establishing neurotrophin specificity during sensory nervous system development. *Development.* 2003; 130:1479–1491. [PubMed: 12620975]
- Aletsee C, Bros A, Mullen L, Palacios C, Pak K, Dazert S, Ryan AF. Ras/MEK but not p38 signaling mediates neurite extension from spiral ganglion neurons. *JARO.* 2001; 2:377–387. [PubMed: 11833610]
- Bianchi LM, Conover JC, Fritzsche B, DeChiara T, Lindsay RM, Yancopoulos GD. Degeneration of vestibular neurons in late embryogenesis of both heterozygous and homozygous BDNF null mutant mice. *Development.* 1996; 122:1965–1973. [PubMed: 8674435]
- Bibel M, Barde YA. Neurotrophins: key regulators of cell fate and cell shape in the vertebrate nervous system. *Genes Dev.* 2000; 14:2919–2937. [PubMed: 11114882]
- Bonnet D, Garcia M, Vecino E, Lorentz JG, Sahel J, Hicks D. Brain-derived neurotrophic factor signalling in adult pig retinal ganglion cell neurite regeneration in vitro. *Brain Res.* 2004; 1007:142–151. [PubMed: 15064145]
- Bredensen DE, Rabizadeh S. p75NTR and apoptosis: Trk-dependent and Trk-independent effects. *Trends Neurosci.* 1997; 120:287–290.
- Brors D, Aletsee C, Dazert S, Huverstuhl J, Ryan AF, Bodmer D. Clostridium difficile toxin B, an inhibitor of the small GTPases Rho, Rac, and Cdc42, influences spiral ganglion neurite outgrowth. *Acta Otolaryngol.* 2003a; 123:20–25. [PubMed: 12625568]
- Brors D, Bodmer D, Pak K, Aletsee C, Schafers M, Dazert S, Ryan AF. EphA4 provides repulsive signals to developing cochlear ganglion neurites mediated through ephrin- B2 and -B3. *J Comp Neurol.* 2003b; 462:90–100. [PubMed: 12761826]
- Du L, Lyle CS, Obey TB, Gaarde WA, Muir JA, Bennett BL, Chambers TC. Inhibition of cell proliferation and cell cycle progression by specific inhibition of basal JNK activity: evidence that mitotic Bcl-2 phosphorylation is JNK-independent. *J. Biol. Chem.* 2004; 279:11957–11966. [PubMed: 14704147]

- Echteler SM, Nofsinger YC. Development of ganglion cell topography in the postnatal cochlea. *J Comp Neurol.* 2000; 425:436–446. [PubMed: 10972943]
- Ernfors P, Lee KF, Jaenisch R. Mice lacking brain-derived neurotrophic factor develop with sensory deficits. *Nature.* 1994; 368:147–150. [PubMed: 8139657]
- Ernfors P, Van De Water T, Loring J, Jaenisch R. Complementary roles of BDNF and NT-3 in vestibular and auditory development. *Neuron.* 1995; 14:1153–1164. [PubMed: 7605630]
- Evans AR, Euteneuer S, Chavez E, Mullen LM, Hui EE, Bhatia SN, Ryan AF. Laminin and fibronectin modulate inner ear spiral ganglion neurite outgrowth in an in vitro alternate choice assay. *Dev Neurobiol.* 2007; 67:1721–1730. [PubMed: 17600813]
- Farinas I, Jones KR, Tessarollo L, Vigers AJ, Huang E, Kirstein M, de Caprona DC, Coppola V, Backus C, Reichardt LF, Fritzsche B. Spatial shaping of cochlear innervation by temporally regulated neurotrophin expression. *J Neurosci.* 2001; 21:6170–6180. [PubMed: 11487640]
- Fritzsche B, Silos-Santiago I, Bianchi LM, Farinas I. The role of neurotrophic factors in regulating the development of inner ear innervation. *Trends Neurosci.* 1997; 20:159–164. [PubMed: 9106356]
- Fritzsche B, Silos-Santiago I, Bianchi LM, Farinas I. Effects of neurotrophin and neurotrophin receptor disruption on the afferent inner ear innervation. *Semin Cell Dev Biol.* 1997; 8:277–284. Review. PMID 11542690.
- Hansen MR, Zha XM, Bok J, Green SH. Multiple distinct signal pathways, including an autocrine neurotrophic mechanism, contribute to the survival-promoting effect of depolarization on spiral ganglion neurons in vitro. *J Neurosci.* 2001; 21:2256–2267. [PubMed: 11264301]
- Hartnick CJ, Staecker H, Malgrange B, Lefebvre PP, Liu W, Moonen G, Van de Water TR. Neurotrophic effects of BDNF and CNTF, alone and in combination, on postnatal day 5 rat acoustic ganglion neurons. *J Neurobiol.* 1996; 30:246–254. [PubMed: 8738753]
- Hegarty JL, Kay AR, Green SH. Trophic support of cultured spiral ganglion neurons by depolarization exceeds and is additive with that by neurotrophins or cAMP and requires elevation of Ca^{2+} within a set range. *J Neurosci.* 1997; 17:1958–1970.
- Henley CM, Owings MH, Stagner BB, Martin GK, Lonsbury-Martin BL. Postnatal development of 2f₁-f₂ otoacoustic emissions in pigmented rat. *Hear Res.* 1989; 43:141–148. [PubMed: 2312410]
- Ishikawa Y, Kusaka E, Enokido Y, Ikeuchi T, Hatanaka H. Regulation of Bax translocation through phosphorylation at Ser-70 of Bcl-2 by MAP kinase in NO-induced neuronal apoptosis. *Mol Cell Neurosci.* 2003; 24:451–459. [PubMed: 14572466]
- Knipper M, Zimmermann U, Rohbock K, Kopschall I, Zenner HP. Expression of neurotrophin receptor trkB in rat cochlear hair cells at time of rearrangement of innervation. *Cell Tissue Res.* 1996; 283:339–353. [PubMed: 8593664]
- Lallemend K, Hadjab S, Hans G, Moonen G, Lefebvre PP, Malgrange B. Activation of protein kinaseC β constitutes a new neurotrophic pathway for deafferented spiral ganglion neurons. *J Cell Sci.* 2005; 118:4511–4525. [PubMed: 16179609]
- Lallemend F, Vandenbosch R, Hadjab S, Bodson M, Breuskin I, Moonen G, Lefebvre PP, Malgrange B. New insights into peripherin expression in cochlear. *Neurons. Neurosci.* 2007; 150:212–222.
- Landry TG, Wise AK, Fallon JB, Shephers RK. Spiral ganglion neuron survival and function in the deafened cochlea following chronic neurotrophic treatment. *Hear Res.* 2011 Epub ahead of print.
- Leake PA, Hradek GT, Hetherington AM, Stakhovskaya O. Brain-derived neurotrophic factor (BDNF) promotes cochlear spiral ganglion survival and function in deafened, developing cats. *J Comp Neurol.* 2011; 519:1526–1545. [PubMed: 21452221]
- Lee FS, Rajagopal R, Kim AH, Chang PC, Chao MV. Activation of Trk neurotrophin receptor signaling by pituitary adenylate cyclase-activating polypeptides. *J Biol Chem.* 2002; 277:9096–9102. [PubMed: 11784714]
- Lerner EC, Qian Y, Blaskovich MA, Fossum RD, Vogt A, Sun J, Cox AD, Der CJ, Hamilton AD, Sefti SM. Ras CAAX peptidomimetic FTI-277 selectively blocks oncogenic Ras signaling by inducing cytoplasmic accumulation of inactive Ras-Raf complexes. *J Biol Chem.* 1995; 270:26802–26806. [PubMed: 7592920]
- Liu L, Cavanaugh JE, Wang Y, Sakagami H, Mao Z, Xia Z. ERK5 activation of MEF2-mediated gene expression plays a critical role in BDNF-promoted survival of developing but not mature cortical neurons. *Proc Natl Acad Sci U S A.* 2003; 100:8532–8537. [PubMed: 12826611]

- Liu W, Kinnfors A, Boström M, Rask-Andersen H. Expression of TrkB and BDNF in human cochlea-an immunohistochemical study. *Cell Tissue Res.* 2011; 345:213–221. [PubMed: 21739244]
- Malgrange B, Lefebvre PP, Martin D, Staecker H, Van de Water TR, Moonen G. NT-3 has a tropic effect on process outgrowth by postnatal auditory neurones in vitro. *Neuroreport.* 1996; 7:2495–2499. [PubMed: 8981411]
- Mehlen P, Bredesen DE. The dependence receptor hypothesis. *Apoptosis.* 2004; 9:37–49. [PubMed: 14739597]
- Mielke K, Herdegen T. JNK and p38 stress kinases degenerative effectors of signal transduction-cascades in the nervous system. *Prog. Neurobiol.* 2000; 61:45–60. [PubMed: 10759064]
- Nagahara AH, Tuszynski MH. Potential therapeutic uses of BDNF in neurological and psychiatric disorders. *Nat. Rev. Drug. Discov.* 2011; 10:209–219. [PubMed: 21358740]
- Nishina H, Fischer KD, Radvanyi L, Shahinian A, Hakem R, Rubie EA, Bernstein A, Mak TW, Woodgett JR, Penninger JM. Stress-signalling kinase Sek1 protects thymocytes from apoptosis mediated by CD95 and CD3. *Nature.* 1997; 385:350–353. [PubMed: 9002521]
- Pirvola U, Ylikoski J, Palgi J, Lehtonen E, Arumae U, Saarma M. Brain-derived neurotrophic factor and neurotrophin 3 mRNAs in the peripheral target fields of developing inner ear ganglia. *Proc Natl Acad Sci U S A.* 1992; 89:9915–9919. [PubMed: 1409719]
- Pirvola U, Arumae U, Moshnyakov M, Palgi J, Saarma M, Ylikoski J. Coordinated expression and function of neurotrophins and their receptors in the rat inner ear during target innervation. *Hear Res.* 1994; 75:131–144. [PubMed: 8071140]
- Postigo A, Calella AM, Fritsch B, Knipper M, Katz D, Eilers A, Schimmang T, Lewin GR, Klein R, Minichiello L. Distinct requirements for TrkB and TrkC signaling in target innervation by sensory neurons. *Genes Dev.* 2002; 16:633–645. [PubMed: 11877382]
- Rüttiger L, Panford-Walsh R, Schimmang T, Tan J, Zimmermann U, Rohbock K, Köpschall I, Limberger A, Müller M, Fraenzer JT, Cimerman J, Knipper M. BDNF mRNA expression and protein localization are changed in the age-related hearing loss. *Neurobiol Aging.* 2007; 28:586–601. [PubMed: 16580094]
- Rybak LP, Whitworth C, Scott V. Development of endocochlear potential and compound action potential in the rat. *Hear Res.* 1992; 59:189–194. [PubMed: 1319988]
- Salvesen GS. Caspases and apoptosis. *Essays Biochem.* 2002; 38:9–19. Review. [PubMed: 12463158]
- Sano H, Mukai J, Monoo K, Close LG, Sato TA. Expression of p75NTR and its associated protein NADE in the rat cochlea. *Laryngoscope.* 2001; 111:535–538. [PubMed: 11224788]
- Schecterson LC, Bothwell M. Neurotrophin and neurotrophin receptor mRNA expression in developing inner ear. *Hear Res.* 1994; 73:92–100. [PubMed: 8157510]
- Vaillant AR, Mazzoni I, Tudan C, Boudreau M, Kaplan DR, Miller FD. Depolarization and neurotrophins converge on the phosphatidylinositol 3-kinase-Akt pathway to synergistically regulate neuronal survival. *J Cell Biol.* 1999; 146:955–966. [PubMed: 10477751]
- Wheeler EF, Bothwell M, Schecterson LC, von Bartheld CS. Expression of BDNF and NT-3 mRNA in hair cells of the organ of Corti: quantitative analysis in developing rats. *Hear Res.* 1994; 73:46–56. [PubMed: 8157505]
- Wiechers B, Gestwa G, Mack A, Carroll P, Zenner HP, Knipper M. A changing pattern of brain-derived neurotrophic factor expression correlates with the rearrangement of fibers during cochlear development of rats and mice. *J Neurosci.* 1999; 19:3033–3042. [PubMed: 10191320]
- Ylikoski J, Pirvola U, Moshnyakov M, Palgi J, Arumae U, Saarma M. Expression patterns of neurotrophin and their receptor mRNAs in the rat inner ear. *Hear Res.* 1993; 65:69–78. [PubMed: 8080462]

Fig. 1.
Representative SG explants stained with anti-200kDa neurofilament antibody, for each experimental condition. Scale bar 300 μ m.

Fig. 2.

Average number of SG neurites observed on SG explants. The number of neurites observed on control and BDNF-treated explants are compared to that seen with three different levels of each signaling inhibitor in addition to BDNF. Lines represent one SEM. BDNF was significantly different from control in all cases. Asterisks denote statistical difference of inhibitor plus BDNF groups from the BDNF-alone group. n=12 for each experimental condition, except Rac/cdc42 inhibitor *C. difficile* toxin B n=18.

Fig. 3.

The average length of SG neurites observed on SG explants. The length of neurites observed on control and BDNF-treated explants is compared to that seen with signaling inhibitors in addition to BDNF. Lines represent one SEM. Asterisks denote statistical difference of inhibitor plus BDNF groups from the BDNF-alone group. $n=12$ for each experimental condition, except *Rac/cdc42* inhibitor *C. difficile* toxin B $n=18$. Since BDNF did not alter neurite length, the inhibitors are presumably affecting alternative signaling, perhaps integrin activation by the fibronectin substrate.

Fig. 4.

Effects of BDNF treatment on neuronal survival and extension of neurites from half-turn SG explants. **(A)** Explant from a fresh dissection. **(B)** Explant after 72 hours in culture without BDNF treatment. **(C)** Explant after 72 hours in culture with BDNF treatment. **(D)** BDNF resulted in an increased SG neuron survival and **(E)** increased number of neurites that extended from the explant compared to untreated explants. Scale bar 100 μm . $n=12$ in each experimental condition. Lines represent one SEM.

Fig. 5.

Representative Western blots of phosphorylated p38, phosphorylated Akt and phosphorylated Erk. SG explants were exposed either to culture media alone or with 25 ng/ml BDNF. Six whole SG were used per individual blot. Actin was used as an internal control. Phospho-protein levels were determined by densitometry and were normalized against actin. BDNF treated levels are expressed as % of control values. Phospho-p38 and phospho-Akt were significantly increased by BDNF treatment ($p < 0.05$), whereas phospho-Erk levels were not. Bars show the mean + one SEM of 3 independent experiments.

Fig. 6. Schematic representation of possible signal transduction pathways involved in BDNF effects on SG neuritis, and the inhibitor used in the present study.