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.

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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 (Bianchi et al., 1996; Ernfors et al., 1994, 1995; Farinas et al., 2001; Fritzsch et al., 1997a, 1997b). 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 BNDF 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 (Schecterson and Bothwell, 1994). BDNF signaling is mainly mediated via TrkB receptors and TrkB and p75 receptors are expressed by SG neurons throughout the inner ear (Knipper et al., 1996; Pirvola et al., 1994; Sano et al., 2001). Mice null for TrkB are reported to lose 15–20% of SG neurons (Fritzsch et al., 1997a, 1997b). 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 phosphatidyl inositol 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 and 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 and 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 and 2. When BDNF treatment occurred in the presence of the pan-G-protein inhibitor GDPβS, there was no significant influence ($p>0.06$). In contrast, the specific Ras inhibitor FTI-277 virtually eliminated the BDNF-induced increase in SG neurite number at all inhibitor doses ($p<0.03$). While the MEK/Erk inhibitor UO126 had no effect ($p>0.08$), the p38 inhibitor SB203580 reduced the BDNF response at all doses ($p<0.02$). Interestingly, the Rac/cdc42 inhibitor C *difficile* toxin B significantly increased the BDNF effect on neurite number, but only at the lowest dose employed ($p<0.04$). The PI_3 kinase inhibitor Wortmannin reduced the BDNF effect, but only at the highest dose employed ($p<0.0001$). Akt inhibitor II significantly attenuated the BDNF effect at 100 nM ($p<0.0001$) and 1 nM ($p<0.01$), but not at 0.1 ($p<0.08$). 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 ($p<0.04$). The PI3K inhibitor Wortmannin decreased length at the highest dose ($p<0.04$). The PKA inhibitor KT5720 increased neurite length at all doses ($p<0.04$). When explants were

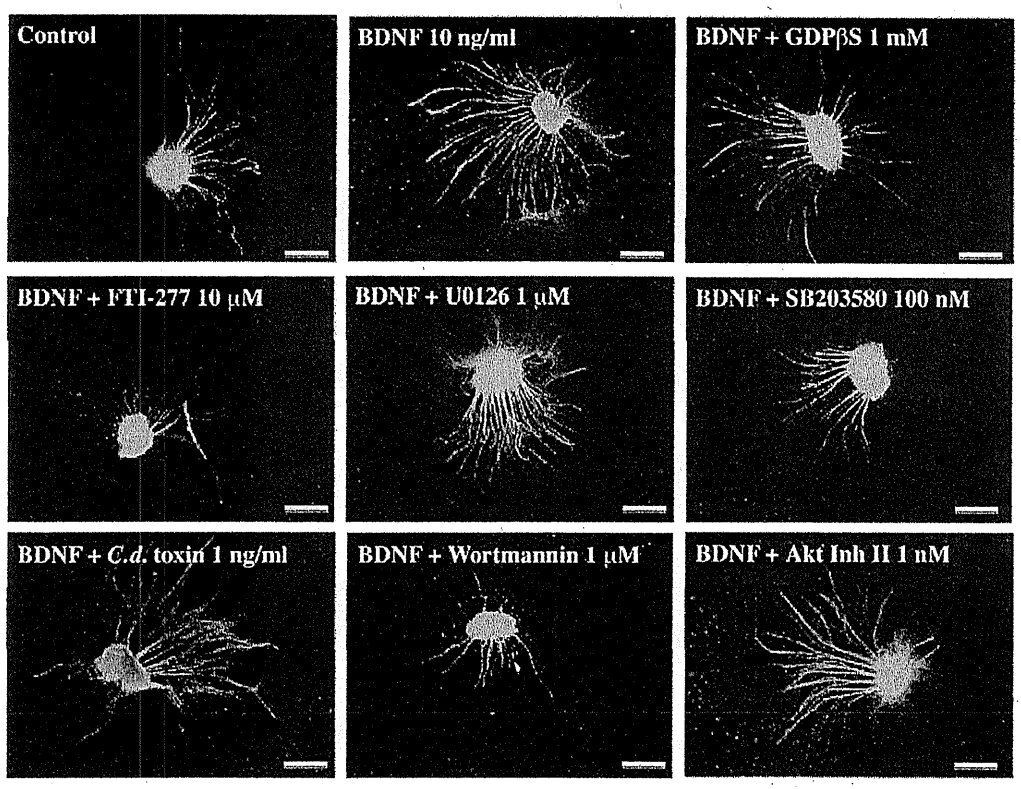

Fig. 1 – Representative SG explants stained with anti-200 kDa neurofilament antibody, for each experimental condition. Scale bar 300 μm.

exposed to the inhibitors alone, neurite numbers were increased by UO126 at 1000 nM ($p<0.05$), and decreased by Akt inhibitor II at 1 nM ($p<0.02$). 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/μm of ganglion. Control samples cultured without BDNF for 72 hours showed 0.050 (±.010) neurons/μm, while explants cultured with BDNF showed 0.131 (±.014) neurons/μm. 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 (± .006) neurites/μm. 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 phosho-p38 and phosho-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

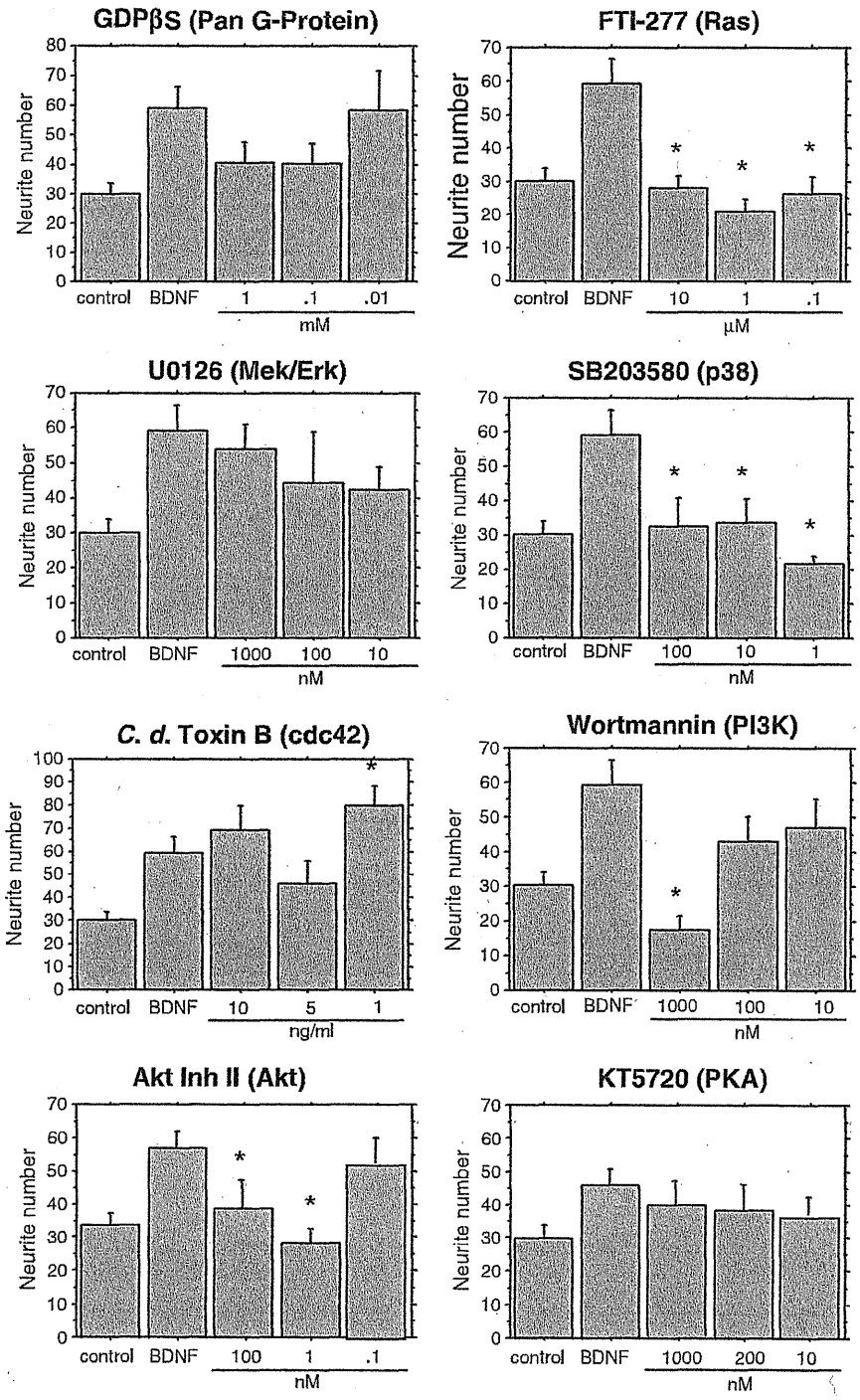

Fig. 2 – Average number of SG neurites observed on SG explants. The number of neurites observed on control and BDNF-treated explants are compared to that seen with three different levels of each signaling inhibitor in addition to BDNF. Lines represent one SEM. BDNF was significantly different from control 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 $n=18$.

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 neuritogenic 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).

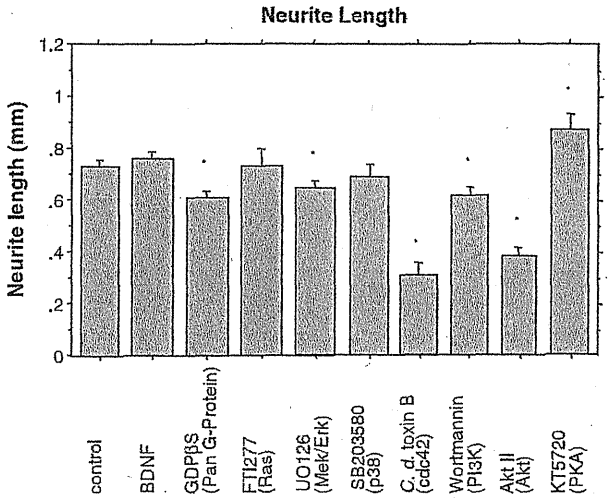

Fig. 3 – The average length of SG neurites observed on SG explants. The length of neurites observed on control and BDNF-treated explants is compared to that seen with signaling inhibitors in addition to BDNF. Lines represent one SEM. Asterisks denote statistical difference of inhibitor plus BDNF groups 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.

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. Weichers et al. (1999) 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

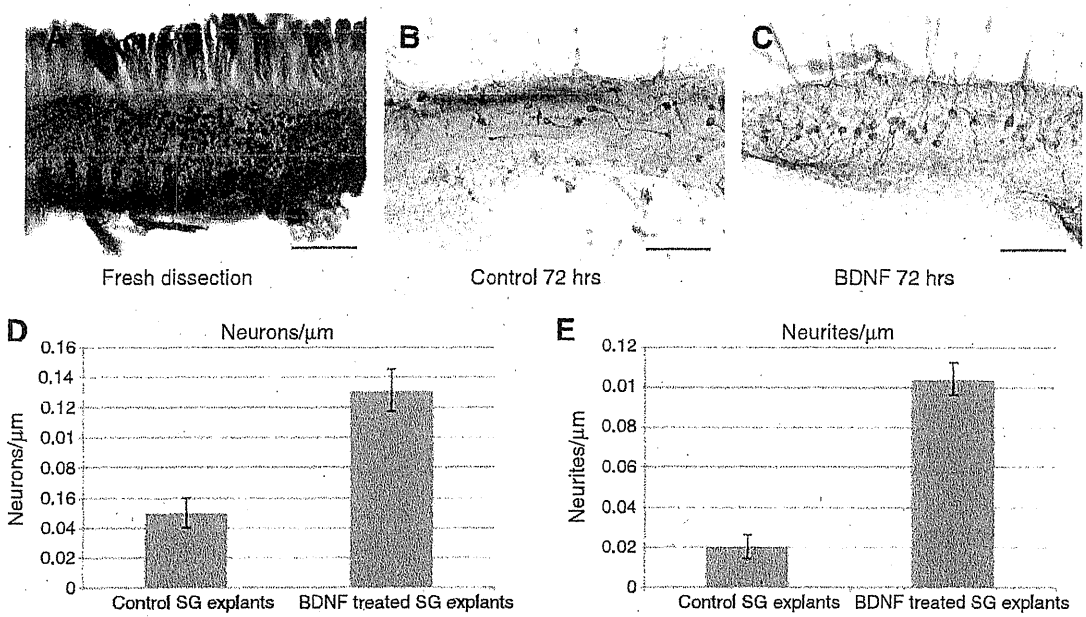

Fig. 4 – Effects of BDNF treatment on neuronal survival and extension of neurites from half-turn SG explants. (A) Explant from a fresh dissection. (B) Explant after 72 hours in culture without BDNF treatment. (C) Explant after 72 hours in culture with BDNF treatment. (D) BDNF resulted in an increased 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.

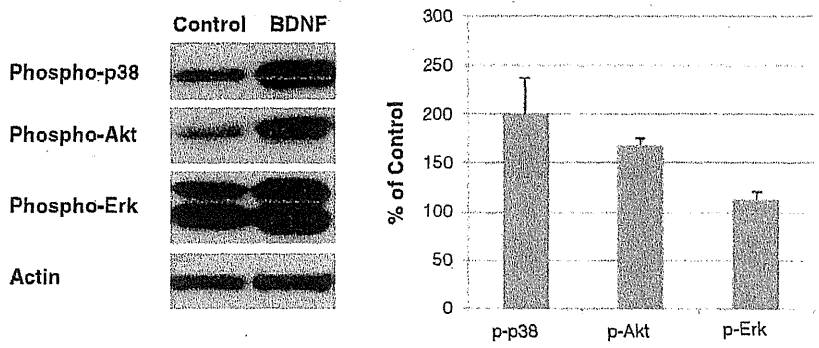

Fig. 5 – Representative Western blots of phosphorylated p38, phosphorylated Akt and phosphorylated Erk. SG explants were exposed either to culture media alone or with 25 ng/ml BDNF. Six whole SG were used per individual blot. Actin was used as an internal control. Phospho-protein levels were determined 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.

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 μM). 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 μm, 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 and Herdegen, 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 (Bredensen and Rabizadeh, 1997).

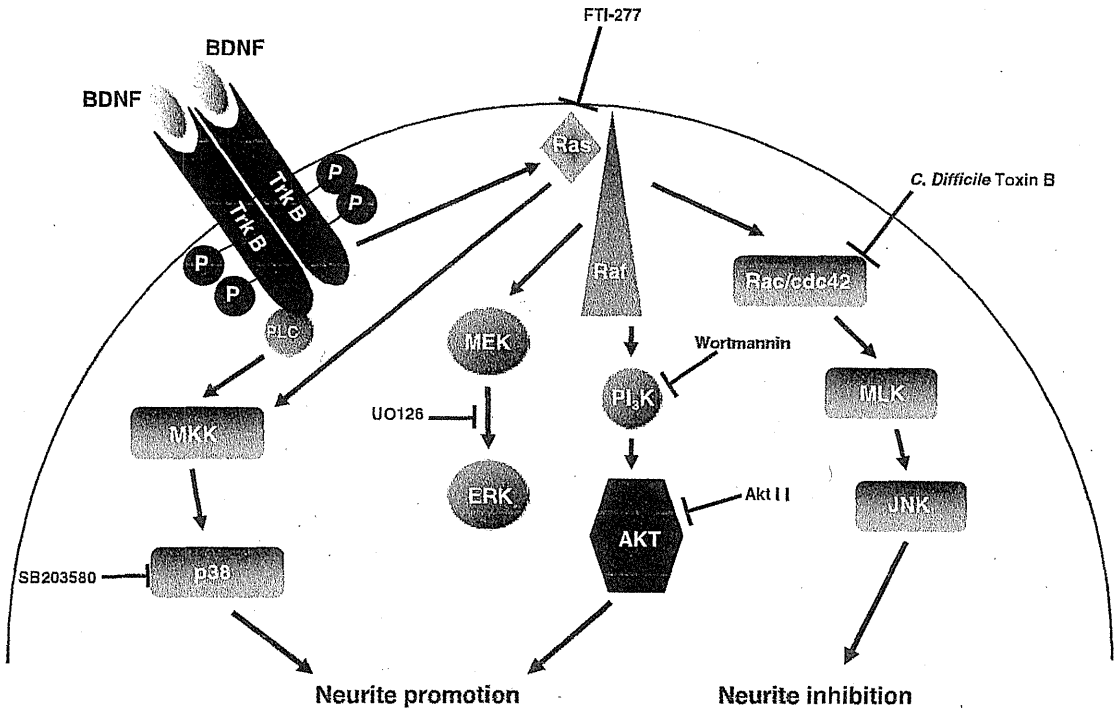

Fig. 6 – Schematic representation of possible signal transduction pathways involved in BDNF effects on SG neuritis, and the inhibitor used in the present study.

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 GDPβS 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 GDPβS 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 midsagitally 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 300×300 μm. 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 μl 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 μl of a maintenance media consisting of DMEM supplemented with 1X N2 and 5 g/L glucose (Invitrogen/Gibco). For neurotrophin stimulation, the maintenance media contained BDNF (10 ng/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 and Nofsinger, 2000).

Experimental cultures contained BDNF with different concentrations of signaling inhibitors: 0.01, 0.1 or 1 mM of the general G-protein inhibitor GDPβS (Sigma-Aldrich); 0.1, 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 1000 nM 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 min and then washed with PBS. The samples were blocked with 1% donkey serum (Sigma-Aldrich) for 10 min 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 25 ng/ml BDNF for 5 min. Explants were collected from media, and lysed with 100 μl 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 10,000×*g* for 10 min 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 [50 mM 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).

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Original Article

Changes in responsiveness of rat spiral ganglion neurons to neurotrophins across age: differential regulation of survival and neuritogenesis

Kenji Kondo,[1,2] Kwang Pak,[1] Eduardo Chavez,[1] Lina Mullen,[1] Sara Euteneuer,[1,3] and Allen F. Ryan[1]

[1]*Division of Otolaryngology and Neurosciences, Department of Surgery and Veterans Administration Medical Center, University of California, San Diego School of Medicine, La Jolla, CA, USA,* [2]*Department of Otorhinolaryngology and Head and Neck Surgery, Graduate School of Medicine, University of Tokyo, Tokyo, Japan, and* [3]*Department of Otorhinolaryngology, Head and Neck Surgery, School of Medicine, Ruhr-University Lubeck, Lubeck, Germany*

Developmental changes in responsiveness of rat spiral ganglion neurons (SGNs) to neurotrophin-3 (NT-3) and brain-derived neurotrophic factor (BDNF) were examined using an explant culture system. Spiral ganglion (SG) explants at embryonic Day 18 (E18), postnatal Day 0 (P0), P5, P10 and P20 were cultured with the addition of either NT-3 or BDNF at various concentrations (0.1–100 ng/ml) and analyzed the dose-response characteristics of three parameters: SGN survival, the number of neurites emanating from the explants and the length of neurite extension. In E18 cultures, SGN survival and neurite number were enhanced more strongly by NT-3 than by the BDNF. As the explants became more mature, the effects of NT-3 decreased, whereas those of BDNF increased, peaking at P0. Although the intrinsic capacity of SGNs to produce and extend neurites declined considerably by P20, they still retained the capacity to respond to both NT-3 and BDNF. These temporal patterns in responsiveness of SGNs to neurotrophins correspond well to the expression pattern of the two neurotrophins in cochlear sensory epithelium *in vivo* and also correlate with the time course of developmental events in SGNs such as cell death and the establishment of mature hair cell innervation patterns.

KEYWORDS: NT-3, BDNF, age-related, neurite growth, neurite extension

Introduction

Spiral ganglion neurons (SGNs) are primary afferent bipolar neurons, with a peripheral dendrite receiving synaptic input from hair cells in organ of Corti, and a central axon projecting to the cochlear nucleus. During development, immature SGNs derived from the otic placode extend neurites toward the presumptive sensory epithelium and brainstem, then establish functional connections [1,2]. Initially, overproduced SGNs are then eliminated by programmed cell death [3–5]. The surviving neurons undergo further maturational processes including remodeling of the dendritic projection to hair cells [6–10].

Studies of the developing auditory system have shown that the neurotrophic factors neurotrophin-3 (NT-3) and brain-derived neurotrophic factor (BDNF) are essential for the development of innervation in the inner ear [see 2,11,12 for reviews], including the response properties of SGNs [e.g. 13,14]. In various peripheral nervous systems, it is known that neuronal responses to neurotrophins can be age dependent. For example, trigeminal neuron survival dependence switches from BDNF and NT-3 to nerve growth factor (NGF) during the early stages of target field innervation [15]. Similarly, some dorsal root ganglion (DRG) neurons depend on NGF in the embryonic stage and switch to glial cell line-derived neurotrophic factor (GDNF) in early postnatal life [16]. These data indicate that neurotrophic factors can support different events in different developmental stages. Although some studies have addressed this issue in avian auditory and vestibular end organs [17,18], the extent to which neurotrophin effects in the mammalian inner ear vary across age remains unclear.

To address this, we performed a systematic study of the effects of NT-3 and BDNF on rat SGNs, from the late embryonic stage through maturation, in explant cultures.

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Correspondence: Allen F. Ryan, Ph.D., UCSD School of Medicine, 9500 Gilman Drive #0666, La Jolla, CA 92093, USA. Tel: 858-534-4594. Fax: 858-534-5319. E-mail: afryan@ucsd.edu

Materials and methods

Animal dissection and explant culture

All animal procedures were approved by the local animal subjects committee (San Diego VA Medical Center) in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals.

Explant cultures of SGNs were prepared from 95 Sprague-Dawley rats (Harlan, Indianapolis, IN, USA) at embryonic Day 18 (E18; the day the positive vaginal plug was considered as E0), postnatal Day 0 (P0, the day of the birth), P5, P10 and P20. The embryonic animals were obtained from timed pregnant rats anesthetized with an intraperitoneal injection of rodent cocktail (ketamine 12.5 mg/ml, xylazine 1.26 mg/ml, acepromazine 0.25 mg/ml; 0.4 ml/100g body weight). After decapitation of the pups and embryos, the mandible was removed and skulls were opened midsagitally. The brain was removed and the temporal bones were harvested and transferred to Petri dishes containing sterile phosphate buffered saline (PBS; pH 7.4). The membranous labyrinth was exposed by peeling off the bony or cartilaginous cochlear capsule. The spiral ligament, stria vascularis and organ of Corti were removed and then the spiral ganglion (SG) was separated from the modiolus using fine forceps. The SG of P10 and P20 were covered with a thin bony layer. Since the bone inhibits the SG from efficient attachment to the bottom of the well, special attention was paid to removing bony fragments as completely as possible in this procedure. The procedure used for dissection allowed for harvesting of the entire SG, from base to apex. The ganglion was then divided into eight explants of approximately equal size, which ensured that here was no sampling bias toward any part of the ganglion.

Each SG explant was placed on the center of one well of a 48-well tissue culture plate that had been precoated overnight at 4 °C with 10 μg/ml fibronectin from human plasma (Sigma, St. Louis, MO, USA) and with 20 μg/ml poly-L-lysine (Sigma, St. Louis, MO, USA) for 1 hour at 37 °C. To promote attachment of the tissue to the bottom of the wells, the explants were incubated overnight at 37 °C with 5% CO_2, in 80 μl of primary growth media consisting of Dulbecco's modified Eagle's medium (DMEM; Sigma, St. Louis, MO, USA), 10% fetal bovine serum (FBS) (Invitrogen Life Technologies, Grand Island, NY, USA), 10-mM HEPES buffer (Invitrogen Life Technologies, Grand Island, NY, USA) and 300 U/ml penicillin (Sigma, St. Louis, MO, USA). The explants were then cultured in 200 μl of maintenance medium consisting of DMEM, 10 μl/ml N2 supplement (Invitrogen Life Technologies, Grand Island, NY, USA), 10-mM HEPES buffer, 300 U/ml penicillin and glucose (to achieve a final concentration of 6 g/l; Sigma, St. Louis, MO, USA) for an additional 72 hours. To compare the effects of neurotrophins, the cultures of each age were divided into 15 groups consisting of 8–12 explants each, and supplemented with either recombinant human NT-3 (EMD Biosciences, La Jolla, CA, USA) or recombinant human BDNF (Upstate Biotechnology, Lake Placid, NY, USA) at concentrations ranging from 0.1 to 100 ng/ml, with one group cultured without neurotrophin to serve as an untreated control, for a total of 75 groups across ages.

Immunohistochemistry

Immunostaining was performed using the avidin–biotin complex (ABC) method (Vectastain Elite kit; Vector Laboratories, Burlingame, CA, USA). SG explants were fixed by immersion in 4% paraformaldehyde in 0.1-M phosphate buffer for 30 minutes at room temperature (RT). After washes with PBS, endogeneous peroxidase activity was blocked by treatment with 0.3% hydrogen peroxide in methanol for 30 minutes at RT. The tissues were then incubated with PBS containing 1% glycine and blocking solution (PBS containing 4% FBS and 0.2% Triton X-100) for 30 minutes each at RT to reduce nonspecific antibody binding. The explants were then incubated with anti-neurofilament (NF) 200 mouse monoclonal antibody (1:500 in blocking solution; Sigma, St. Louis, MO, USA) or anti-active caspase-3 rabbit polyclonal antibody (1:1000 in blocking solution; Abcam, Cambridge, MA, USA) at 4 °C overnight. After three washes in PBS, the tissues were then incubated with biotinylated horse anti-mouse IgG or goat anti-rabbit IgG secondary antibody (Vector Laboratories, Burlingame, CA, USA) corresponding to the primary antibody for 1 hour at RT. After three washes in PBS, the tissues were then reacted with ABC solution (Vector Laboratories, Burlingame, CA, USA) for 30 minutes at RT, following the manufacturers' instruction. Immunoreactivity was visualized by diaminobenzidine (DAB; Vector Laboratories, Burlingame, CA, USA) reaction. After washing in distilled water, the tissues were coated with crystal mount (Fisher Scientific, Tustin, CA, USA) and air dried for several days. The primary antibody was omitted from the procedure as a negative control, which showed no labeling.

RNA isolation and polymerase chain reaction (PCR)

The SG tissue from E18, P0, P5, P10 and P20 animals were harvested in the same manner, as described above for explant culture, and immediately immersed and kept in RNA later (Qiagen, Valencia, CA, USA). Total RNA was isolated using a guanidine thiocyanate/phenol/ chloroform-based extraction procedure (TRIzol Reagent; Invitrogen Life Technologies, Grand Island, NY, USA) according to the company's instructions. Reverse transcription was performed using Superscript First-Strand cDNA synthesis system for reverse transcription-polymerase chain reaction

(RT-PCR) (Invitrogen Life Technologies, Grand Island, NY, USA) using oligo (dT) primers. Subsequent PCR amplification was performed using the Platinum Taq DNA Polymerase kit (Invitrogen Life Technologies, Grand Island, NY, USA) in a 50-μl solution, with 3 μl of the reverse-transcribed complementary DNA (cDNA), 1 × PCR buffer, 1.0-U Taq DNA polymerase, 200-nM dNTPs, 1.5-mM $MgCl_2$, 4% DMSO and 200-nM primers. Primer sequences for TrkB were sense: 5′-AGT CCA GAC ACT CAG GAT TTG TAC-3′; antisense: 5′-CTC CGT GTG ATT GGT AAC ATG-3′. Primer sequences for TrkC were sense: 5′-CAA CCA TGG CAT CAC TAC ACC-3′; antisense: 5′-TAA GAG GCT TGG AAT GTC CG-3′. Primer sequences for p75 were sense: 5′-GCA TAA GCC TGA AGC CAA CAC G-3′; antisense: 5′-CCC ACT CAT TCC AAC AGC AAG C-3′. Primer sequences for beta-actin were sense: 5′-GCT CGT CGT CGA CAA CGG CTC-3′; antisense: 5′-CAA ACA TGA TCT GGG TCA TCT TCT C-3′. The predicted fragment lengths of the PCR products were 522 base pairs (bp) for TrkB, 365 bp for TrkC, 583 bp for p75 and 353 bp for actin, respectively. The PCR reaction was performed on cDNA for 35 cycles at 94 °C for 15 seconds, 56 °C for 30 seconds and 72 °C for 1 minute with a final extension at 72 °C for 5 minutes. The omission of reverse transcriptase during reverse transcription served as a negative control to confirm the absence of genomic DNA contamination. Eight microliters of the reaction product were analyzed by agarose gel electrophoresis with ethidium bromide staining.

Image presentation and data analysis

Images of the immunostained explants were captured by a Spot digital camera (Diagnostic Instruments, Sterling Heights, MI, USA) attached to an inverted microscope (Olympus IX70) under brightfield illumination. Digital images were then assembled into panels using Adobe Photoshop 6.0 software (Adobe Systems Incorporated, San Jose, CA, USA). The images were not modified except for the minor adjustment of the brightness and contrast.

We used three parameters for evaluation of the effects of neurotrophins on SGNs: number of surviving neurons in each explant, number of neurites emanating from each explant and the length of neurite extension. We also evaluated the neurites visually for differences in other aspects of growth behavior.

To evaluate the SGN survival and the neurite number, each explant after immunostaining was examined under an inverted microscope. The number of surviving neurons and the number of neurites were counted manually by adjusting the focus, if necessary, to assess the full depth of the explant. Our criteria of identifying surviving neurons were based on their immunostained morphology: (1) positivity for NF200 and (2) visible nucleus. The immunostained E18, P0 and P5 explants were sufficiently transparent that individual neurons could easily be identified and counted (Figure 1). For P10 and P20 explants, however, the tissue after immunostaining was not as transparent, and it was more difficult to count the surviving SGNs reliably. Therefore, we excluded these developmental ages from the analysis of SGN survival. Neurite number was determined by counting neurites emanating from each explant at its edge. Therefore, branching in more peripheral areas was not taken into account. Since we could not distinguish central axons from peripheral dendrites, we refer to them simply as neurites.

The magnitude of apoptosis in the culture was evaluated similarly by manually counting immunolabeled cells for active caspase-3 in each explant. This evaluation was performed only for E18, P0 and P5 cultures and could not be performed on P10 and P20 cultures because of the intransparency of older explants.

For the evaluation of neurite length extending from the explant, we used the five longest neurites from each explant for analysis. Using Spot advanced software (Diagnostic Instruments, Sterling Heights, MI, USA), each neurite was traced by drawing a line along the neurite, resulting in a series of short, linear segments. The lengths of these segments were then summed and calculated by the program. The selected neurites from all cultures in the same condition were pooled for analysis. When the explant had fewer than five neurites, all of the neurites were included in the analysis.

Statistical analysis

The data for each experimental condition were compiled from at least four independent experiments, each of which contained two to three explant cultures for each given age and concentration of neurotrophin. Results were presented as the mean ± standard error of all samples. For neurite length, the average value of all the neurite length selected for analysis in each condition was designated as the maximum neurite length (MNL). Statistical analyses for survival, neurite number and MNL were performed using one-way analysis of variance (ANOVA), followed by Dunnet *post hoc* test with correction for multiple tests, using Statview 5.0 software to compare each neurotrophin subgroup with the corresponding untreated control group. Differences associated with p values < 0.05 were considered as statistically significant.

Results

General morphology of the rat SGN explants *in vitro*

Figure 1 illustrates the effects of NT-3 or BDNF on the appearance of SG explants at various ages. Generally,

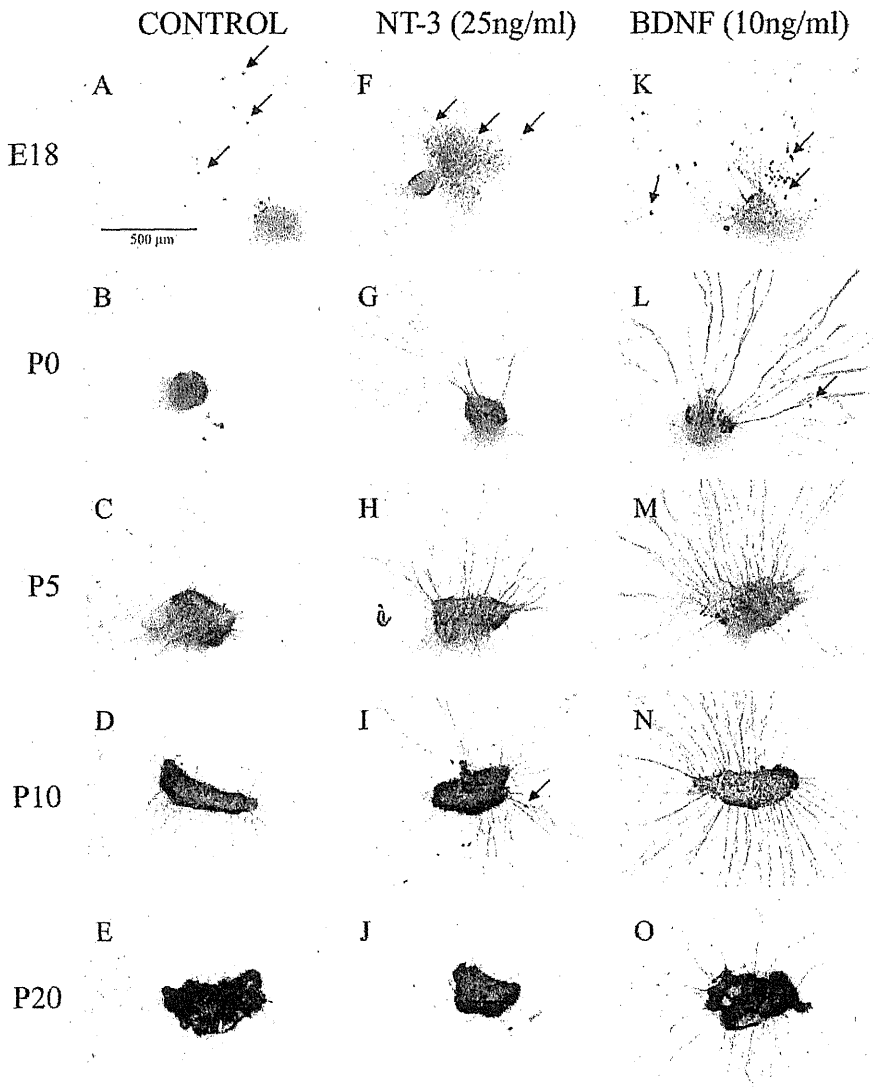

Figure 1. Representative photomicrographs of SG explants from E18 to P20 cochlea. Each explant was cultured for 3 days in media without neurotrophin (A–E) or supplemented with either 25 ng/ml of NT-3 (F–J) or 10 ng/ml of BDNF (K–O), the lowest maximally effective dose of each factor. Explants were then fixed and immunostained with anti-NF200 antibody. In E18 cultures, large number of neurons migrated outside of the explants (arrows in A, F and K), whereas only a few such migrating neurons were observed at other ages (arrows in I and L). Neurites were thinnest at E18, thickest from P0–P10. Thickness was variable at P20. Neurite number and length declined significantly at P20. Responses to NT-3 and BDNF varied depending upon the ages of the culture, with NT-3 responses greatest at E18 (F) and BDNF dominant at later ages (L–O). Scale bar = 0.5 mm.

neurites extended from SG explants in a radial direction, as previously described [19,20]. In E18 explants, some neurons migrated from the explants (Figure 1A, F, K). Older SGNs exhibited little migratory capacity and remained inside the original explant (Figure 1B–E, G–J, L–O). We also observed an age-dependent difference in neurite thickness. E18 neurites appeared consistently thinner upon visualization (Figure 1A, F, K) when compared with P0, P5 and P10 neurites (Figure 1B–D, G–I, L–N). In contrast, P20 explants showed considerable variability in apparent neurite thickness (Figure 1E, J, O). These age-related morphological differences in thickness did not appear to be affected by either neurotrophin.

An unusually large number of neurons showed morphological signs of apoptosis in P0 explants (Figure 2A). That is they showed anti-NF200 immunoreactivity, but the cell bodies were smaller and exhibited pyknotic

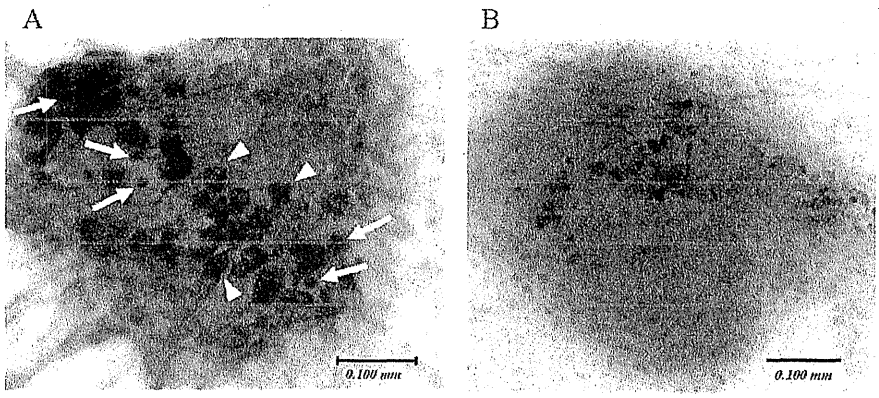

Figure 2. Degenerating neuronal cells in a P0 explant culture (A). The explant was maintained for 3 days in maintenance medium supplemented with BDNF (10 ng/ml), then fixed and immunostained with anti-NF200 antibody. Degenerating cells (white arrows) are small, exhibit condensed immunolabeling for NF200, lack neurites and do not show a clear contour of nucleus. Surviving SGNs (white arrowheads) show a clear, unstained nucleus and typically form neurites. Apoptosis in a P0 explant culture (B). The explant was immunostained with anti-active caspase-3 antibody. Scale bar = 0.1 mm.

nuclei. The average number of such cells per explant in P0 control explants (22.9 ± 7.7) was significantly greater than in E18 (11.7 ± 4.4, $p < 0.02$) or P5 (2.3 ± 0.6, $p < 0.01$) explants.

Effect of neurotrophins on neuronal survival

Survival of the SGN somata could readily be evaluated in E18, P0 and P5 explants. However, at older ages, explant density precluded reliable visualization of cell bodies. A dose-response analysis of SGN survival, assessed by normalized SGN cell body counts (Figure 3), revealed that NT-3 exhibited a strong effect on neuronal survival at only E18. In contrast, BDNF supported survival at all the ages.

Caspase-3 activation in the explant cultures

Since morphology suggested some apoptosis, we immunostained E18, P0 and P5 control explants with anti-active caspase-3 antibody and counted the number of labeled cells in each explant (Figure 2B). The number of active caspase-3-positive cells/explant observed across all turns was 15.7 ± 1.6 at E18, 58.2 ± 13.0 at P0 and 38.7 ± 10.2 at P5. The number of labeled cells at P0 was significantly larger than the other ages ($p < 0.01$), representing ~10% of all neurons present.

When P0 explants were treated with NT-3, significantly fewer cells (33.7 ± 5.6) were caspase-positive ($p < 0.03$). However, although fewer cells were caspase-positive after BDNF treatment (38.8 ± 4.9), this difference was not statistically significant ($p = 0.073$).

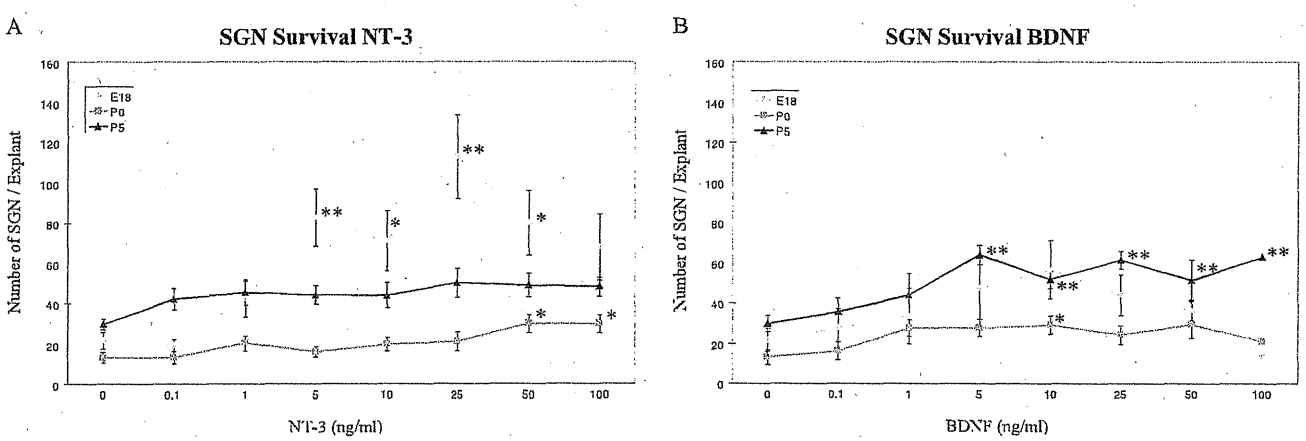

Figure 3. Dose-response effects of neurotrophins on SGN survival in E18, P0 and P5 explant cultures. Graphs show the average number of surviving SGNs observed after 3 days in maintenance medium with various concentrations of NT-3 (A) or BDNF (B). Error bars represent the standard error of the mean. Asterisks indicate a significant difference (*$p < 0.05$; **$p < 0.01$) compared with control culture (Dunnett *post hoc* test).

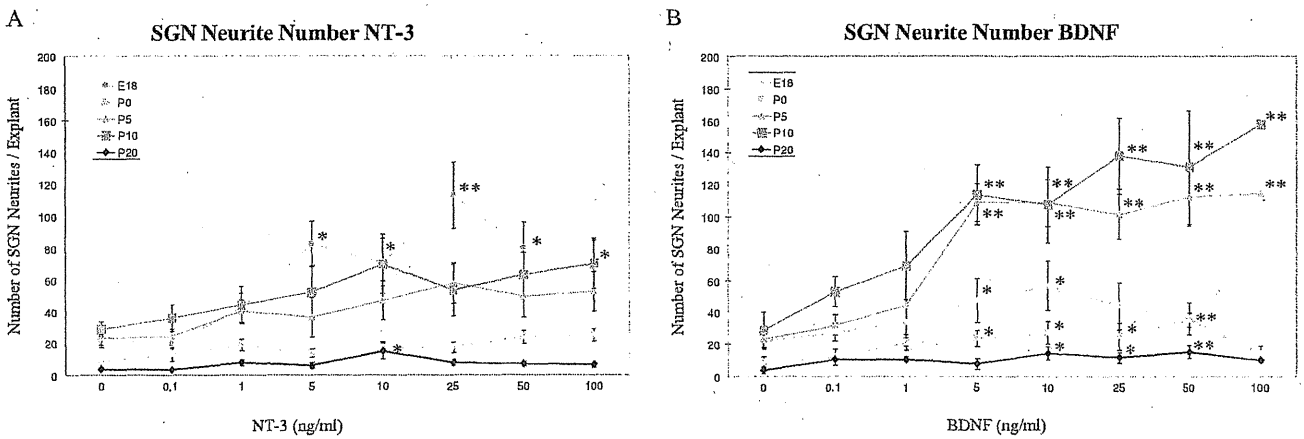

Figure 4. Dose-response effects of neurotrophins on the neurite number observed in SG explant cultures at E18, P0, P5, P10 and P20. Graphs show the average number of neurites emanating from each explant after 3 days in maintenance medium with various concentrations of NT-3 (A) or BDNF (B). Error bars represent the standard error of the mean. Asterisks indicate a significant difference ($^{*}p < 0.05$, $^{**}P < 0.01$) compared with the control culture (Dunnett *post hoc* test).

Neurotrophin treatment had no effect on caspase positivity for either E18 or P5 explants.

Effect of neurotrophins on neurite number

Quantitative analysis for the number of neurites extending from each explant also demonstrated clear age-dependent differences (Figure 4). In untreated explants (neurotrophin concentration 0), the fewest neurites were observed at P0 and P20, and the most at P5 and P10. NT-3 had a large and significant effect on neurite number only at E18, although a more modest increase was seen at one NT-3 dose at P20. In contrast, BDNF increased neurite number at all ages, although it exhibited an especially strong effect at P5 and P10.

Effect of neurotrophins on neurite length

As shown in Figure 5, there was also a strong effect of age on neurite length. The shortest neurites were observed on untreated explants from P20 rats, and the longest at P5 and P10. These age differences were generally maintained during neurotrophin treatment. However, NT-3 treatment produced a modest increase in neurite length at P5, P10 and P20, and no effect at E18 or P0. BDNF treatment produced moderate increases in

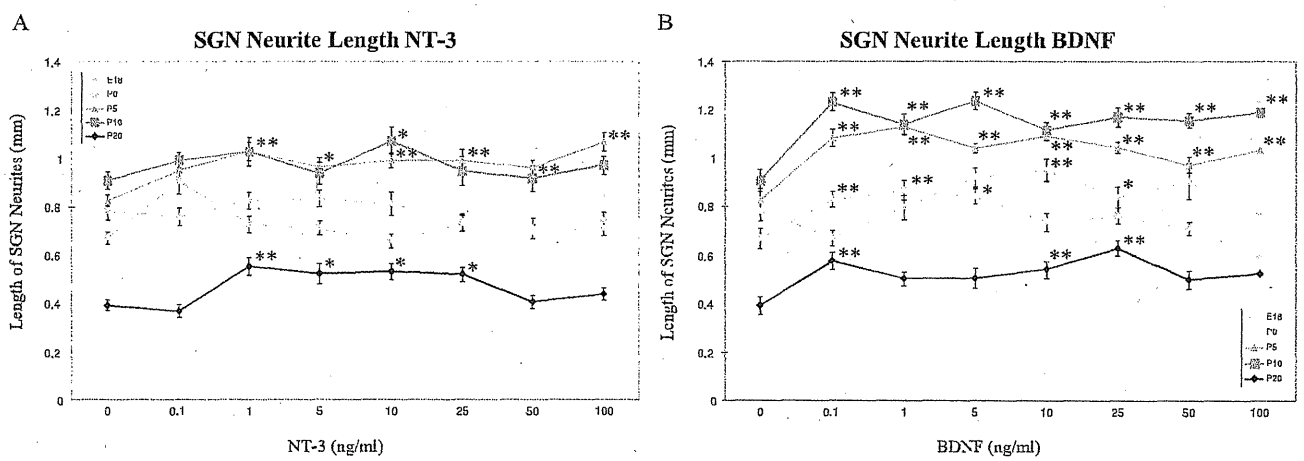

Figure 5. Dose-response effects of neurotrophins on the neurite extention observed in SG explant cultures at P0, P5, P10 and P20. Graphs show the average length of longest neurites selected from each explant (5 neurites/explant) and pooled in the same culture condition. Each explant was cultured for 3 days in maintenance medium with various concentrations of NT-3 (A) or BDNF (B). Error bars represent the standard error of the mean. Asterisks indicate a significant difference ($^{*}p < 0.05$, $^{**}p < 0.01$) compared with the control culture (Dunnett *post hoc* test).

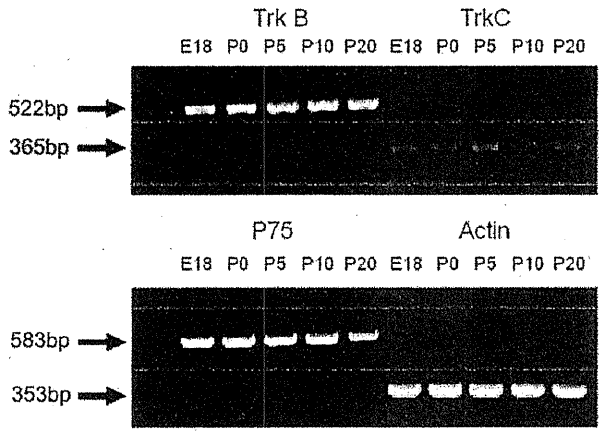

Figure 6. Expression of mRNA for TrkB, TrkC and p75 receptors in SG during rat development. Ethidium bromide-stained agarose gel demonstrating RT-PCR products for TrkB (522 bp), TrkC (365 bp) and p75 (583 bp) throughout the developmental period examined (E18–P20). DNA ladder and the expression of mRNA for actin (internal control) are also shown.

neurite length at all ages tested, with the greatest effect at P5, P10 and P20.

Expression of neurotrophin receptors in developing SGNs determined by RT-PCR

We assessed the expression of messenger RNA (mRNA) for TrkC, TrkB and p75, the receptor proteins for NT-3 and BDNF, in SG across age using RT-PCR. As shown in Figure 6, we observed robust PCR products for the mRNA of each receptor throughout the developmental period analyzed. Assuming that the mRNA is translated, these data suggest that changes in Trk or p75 receptors may be unlikely to mediate changes in the responsiveness of SG explants. However, it must be noted that the PCR products potentially represent expression by both neuronal and nonneuronal elements within the ganglion. Therefore, variation in expression between neurons and other cell types is certainly possible.

Discussion

The aim of the present study was to investigate the age-dependence of SGN survival, neurite outgrowth and neurite extension regulation by NT-3 and BDNF. Our results demonstrate that both NT-3 and BDNF support these aspects of SGN development in a dose- and stage-dependent manner and that the response pattern to neurotrophins varies between each of these parameters. The findings suggest that BDNF and NT-3 mediate separate ontogenetic events at different developmental stages.

A switch in neurotrophin dependence for SGN survival

We found that SGNs change their dependence for survival from NT-3 at E18 to BDNF at older ages. Although this report appears to be the first longitudinal study to show such a shift in mammals, it is consistent with previous reports from individual developmental stages. Pirvola et al. [21] observed greater survival-promoting effect of NT-3 over BDNF on rat embryonic (E13) cochleovestibular ganglion neurons, whereas others [22–24] reported greater BDNF dependence for postnatal SGNs. Developmental changes in neurotrophin dependence occur in other parts of the peripheral nervous system as well: trigeminal ganglion neurons switch from NT-3 and BDNF to NGF [15], whereas a subpopulation of DRG neurons switch from NGF to GDNF [16].

Our data are also similar to the developmental changes in the chick cochlea described by Avila et al. [17]. In their report, the chick cochlear neurons in culture predominantly depend on NT-3 for their survival in the early embryonic period. The response is maximum at E7 and decreased thereafter, being negligible from E13 to hatching. In contrast, the effect of BDNF for survival is more delayed and peaked at E9–E11 and although diminishing from then onward, remains in a significant range until hatching. This is roughly comparable with the timing of neurotrophin dependence in the rat, suggesting that age-dependent support of primary auditory neuron survival by NT-3 followed by BDNF may be a common molecular mechanism shared by birds and mammals.

Changes in survival response of SGNs to neurotrophins during development could be due to the selective death of a subset of neurons that respond preferentially to NT-3. For example, based on knockout mouse data, it has been suggested that developing type I SGNs depend preferentially upon NT-3 for their survival, whereas type II neurons depend upon BDNF [25]. Alternatively, our observation could be due to changes in the neurotrophin responsiveness of individual neurons. We do not have evidence to reach a definite conclusion regarding this point. Because rat SGNs upregulate peripherin in culture [26], we could not distinguish between type I and type II neurons. However, Mou et al. [27] found that the survival of dissociated postnatal (P1–P10) type I and type II mouse SGNs was preferentially enhanced by BDNF when compared with NT-3, in agreement with our results at P5 and P10. With respect to other potential subtypes of SGNs, previous immunohistochemical examinations showed that all SGNs in both embryonic and postnatal mammals uniformly express both TrkB and TrkC receptors and that there appear to be no distinct subsets of neurons based on Trk expression [22,28]. This suggests

that the overall population of SGNs may switch their dependence for survival from NT-3 to BDNF based on changes in the intracellular responses to TrkB and TrkC stimulation, rather than on receptor expression.

Effects of neurotrophin concentration

For each of the measures employed, a neurotrophin concentration effect was noted. This typically consisted of an increase in neuronal survival, neurite number or neurite length with increasing dose. In most cases, the response appeared to saturate at between 5 and 10 ng/ml. An exception was NT-3, where 25 ng/ml produced significantly greater SGN survival and neurite number than either lower or higher concentrations. The response saturation may reflect maximal utilization of all Trk receptors at relatively low concentrations. Another possibility is that neurotrophin receptors were downregulated at higher ligand concentrations, as has been observed in other systems (e.g. [29]). In this case, greater amounts of ligand may have been required to produce the same effect. In general, we did not observe systematic changes in threshold dose across age. When an effect was robust, it typically showed a similar minimal effective dose at all ages (e.g., Figure 4, BDNF; Figure 5, NT-3 and BDNF). Only when responses were minimal did we see variation in threshold dose. These data suggest that neurotrophin receptors are not expressed on SGNs gradually with age. Rather, age-dependent changes observed in some SGN responses may be related to changes in intracellular signaling in response to receptor activation.

A critical period of SGN death

Comparing the total number of rat SGNs/cochlea *in situ* (18 000–25 000; [4]) and the small numbers of surviving SGNs after 4 days *in vitro* in our culture system (20–120 neurons/explant in about 1/8 of the whole SG), considerable cell death occurred when SGNs were placed into explant cultures at any age. However, P0 cultures exhibited a significantly lower number of surviving SGNs, both in the presence or absence of neurotrophins than did either in E18 or P5 explants. P0 explants also exhibited significantly higher levels of caspase activity, indicating apoptosis. Interestingly, the dose response of SGNs to NT-3 for survival appeared to shift to higher concentrations, and the enhancement of survival at P0 was saturated at a lower magnitude (2.3-fold compared with control explants) than in E18 culture (more than fivefold compared with control explants; Figure 3A). At the same time, BDNF influence on survival remains low (Figure 3B). It is significant that the period of culture for P0 ends at the time equivalent to P4 *in vivo*. This corresponds to the peak of naturally occurring cell death in the rat SG *in vivo* [4]. Our findings in culture suggest that the *in vivo* increase in cell death may reflect a downregulation of SGN sensitivity to NT-3, without an increase in BDNF sensitivity, resulting in neuronal apoptosis.

The expression of p75 by SG explants suggests another potential pathway for the regulation of cell death and survival. Stimulation of the p75 receptor by the proforms of neurotrophins is well known to mediate apoptosis, including in the SG [30,31] and has also been implicated in regulation of neurite length [32]. Since we applied mature neurotrophins to our cultures, this could not have resulted directly from our experimental manipulations. Although neurotrophins are not expressed in the SG of neonatal or adult rats *in vivo* [33], the potential for autocrine neurotrophic effects in SGNs [34] should be considered. The low level of survival of SGNs that we observed in untreated SGNs suggests that there is not extensive production of neurotrophins in our cultures. However, Zha et al. [35] have reported that neonatal SGNs can express neurotrophins, at least in culture. Thus neurotrophin genes expressed in their proforms could potentially mediate apoptosis *in vitro* via an autocrine process. Stimulation of p75 by mature neurotrophins is also well known to modulate the response of Trk receptors to mature neurotrophins [30]. Thus, changes in the interaction of p75 and Trk receptors across age could also contribute to altered responses to neurotrophins. On the basis of our PCR results, the expression of p75 mRNA did not appear to vary across age. Assuming that this mRNA was translated, any age-related changes would not be based on differential expression but could reflect downstream signaling changes.

The effects of neurotrophins on neurite number

Quantitative assessment of explants indicated a larger number of neurites in the presence of NT-3 than BDNF at E18 (Figure 1F and K, Figure 4). In contrast, at P0 neither neurotrophin had a strong effect. However, the number of neurites emanating from P5 and P10 SG explants was much more strongly enhanced by BDNF than NT-3. Thus, the effects of neurotrophins on neurite number resemble those observed for survival in E18, P0 and P5 cultures.

Our neurite outgrowth index (the ratio of neurite number/number of SGNs in each explant) demonstrated that none of the neurotrophin subgroups, either for NT-3 or for BDNF, had significantly different ratios compared with the untreated control in P0 culture. In contrast, the BDNF subgroups at higher concentrations (10 ng/ml and 50 ng/ml) had significantly greater index values compared with the control group in P5 culture. This finding suggests that the modest increase in neurite number induced at P0 by neurotrophins reflects primarily an increase in surviving SGNs, whereas the effects on

neurite number at P5 and P10 are mediated primarily by neuritogenesis.

Comparison of neurotrophic effects *in vitro* with the expression of neurotrophins and developmental events *in vivo*

The period of E18 culture (equivalent to E18–E21 *in vivo*), the earliest developmental stage we examined, corresponds to the period when the afferent fibers of SGNs reach the cochlear sensory epithelium [1,36]. By this stage, expression of both NT-3 and BDNF extends throughout the organ of Corti longitudinally [37], implying that both neurotrophins are available for all SGNs. NT-3 is more strongly expressed than BDNF [37–39] and distributed more widely in the sensory epithelium, since both hair cells and supporting cells express this neurotrophin, whereas BDNF is more restricted to hair cells [37,39]. This is consistent with our observation of greater enhancement of SGN survival by NT-3 than BDNF in E18 explants.

It should be noted, however, that there is a discrepancy between our result and the results of gene deletion studies [37]. Although mice null for the NT-3 gene show a considerably reduced number of SGNs in the basal cochlea (less than 20% of wild type) at birth [25,40], replacement of the NT-3-coding sequence with that for BDNF almost completely rescues the loss of basal turn SGNs (85%) by NT-3 absence [37,41]. Similar rescue in the number of SGNs has been demonstrated in mice for which the coding part of the BDNF gene was replaced with that of NT-3 [42]. These results suggest that NT-3 and BDNF can be functionally equivalent for the survival of SGNs prenatally [37,42]. The discrepancy between this finding and ours may be related to the mode of exposure of SGNs to neurotrophins: in the *in vivo* condition, neurotrophins are supplied to the SGNs basically through the targets of their neurites, and concentrations at these targets may be very high. In the *in vitro* condition, the entire SGN is exposed to the neurotrophins and concentration is uniform. The synergistic effects of neurotrophins with other survival factors *in vivo* must also be considered.

The period of P0 explant culture (equivalent to P0–P4 *in vivo*) corresponds not only to that of naturally occurring cell death [4,5] as mentioned above, but also to a relatively low level of responsiveness to NT-3 and BDNF with respect to survival, neurite number and neurite length. Interestingly, the expression of BDNF in the cochlear sensory epithelium, which disappears in the early postnatal period [10,39], reappears at P6–P7 in hair cells and supporting cells [10], which is temporally coincident with strong effects of BDNF, which we observed on survival, neurite number and neurite extension. SGNs may, therefore, tailor their responsiveness to coincide with developmental trends in neurotrophin availability.

P20 cultures, the oldest stage we examined, correspond to P20–P24 *in vivo*. Almost all of the major developmental events for SGNs are complete and hearing function has matured by this age [1]. Although the intrinsic capacity for neurite growth has considerably declined in this period, SGNs still retained the capacity to respond to both NT-3 and BDNF. This finding is consistent with the enhancement of survival and neurite regrowth of adult SGNs by neurotrophins *in vivo* [43–46]. NT-3 is highly expressed in inner hair cells and their supporting cells [36,39,47,48], so responsiveness to this neurotrophin is not surprising. However, BDNF expression is almost absent in the target field of adult SGNs [10,33,39]. Moreover, BDNF expression is observed in SGNs themselves [49] and may be acting in an autocrine manner [49].

Mechanisms for differential regulation by neurotrophins

Our RT-PCR results demonstrate that three kinds of neurotrophin receptors, TrkC, TrkB and p75, all of the neurotrophin receptors involved in the signaling of NT-3 and BDNF, are expressed in SG throughout the developmental period we examined. These data are in good agreement with previous immunohistochemical and *in situ* hybridization studies [9,22,28,33,37,47].

Our results appear in line with the results of transgenic mouse studies in which the coding part of the NT-3 gene is replaced by BDNF [37,50] and *vice versa* [42]. These studies suggest that although NT-3 and BDNF can be functionally equivalent for the survival of SGNs prenatally [37,42], they have distinct roles for the axon guidance and innervations in the cochlea [50]. These findings cannot be explained simply by the expression pattern of each neurotrophin receptor. As noted above, the biological responses to neurotrophins are presumably regulated by molecular cascades downstream of Trk/p75 signaling. It has been shown that multiple signal transduction pathways are involved in neurotrophin-mediated biological effects on neurons, which may contribute to age-dependent differential regulation of SGN biological responses to neurotrophins.

Conclusion

The present study demonstrates that neurotrophins regulate developing rat SGNs in an age-dependent manner. The temporal patterns of responsiveness of SGNs to NT-3 and BDNF presented here correspond well to the expression pattern of the two neurotrophins in cochlear sensory epithelium *in vivo* and also correlate with the time course of developmental events in the SG, such

as neuronal cell death and the remodeling of afferent innervation. Our data, therefore, suggest multiple, age-specific roles for NT-3 and BDNF in the ontogeny of cochlear innervation.

Declaration of interest

The authors report no conflict of interest. The authors alone are responsible for the content and writing of this paper. This work was supported by the Research Service of the VA Merit grant 1108966, the NIH/NIDCD grant DC000139 and the Japan Foundation for Aging and Health, Promoting Projects of Researches on Sensory and Communicative Disorders.

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