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Developmental changes in the responsiveness of rat spiral ganglion neurons to neurotrophic factors in dissociated culture: differential responses for survival, neuritogenesis and neuronal morphology

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Abstract

The way that the development of the inner ear innervation is regulated by various neurotrophic factors and/ or their combinations at different postnatal developmental stages remains largely unclear. Moreover, survival and neuritogenesis in deafferented adult neurons is important for cochlear implant function. To address these issues, developmental changes in the responsiveness of postnatal rat spiral ganglion neurons (SGNs) to neurotrophin-3 (NT-3), brain-derived neurotrophic factor (BDNF) and leukemia inhibitory factor (LIF) were examined by using a dissociated cell culture system. SGNs at postnatal day (P) 0, P5 and P20 (young adult) were cultured with the addition of NT-3, BDNF, or LIF or of a combination of NT-3 and BDNF (N + B) or of NT-3, BDNF and LIF (ALL factors). SGNs were analyzed for three parameters: survival, longest neurite length (LNL) and neuronal morphology. At P0, SGNs required exposure to N + B or ALL factors for enhanced survival and the ALL factors combination showed a synergistic

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effect much greater than the sum of the individual factors. At P5, SGNs responded to a wider range of treatment conditions for enhanced survival and combinations showed only an additive improvement over individual factors. The survival percentage of untreated SGNs was highest at P20 but combinations of neurotrophic factors were no more effective than individual factors. LNL of each SGN was enhanced by LIF alone or ALL factors at P0 and P5 but was suppressed by NT-3, BDNF and N + B at P5 in a dose-dependent manner. The LNL at P20 was enhanced by ALL factors and suppressed by N + B. Treatment with ALL factors increased the proportion of SGNs that had two or more primary neurites in all age groups. These findings suggest that NT-3, BDNF, LIF and their combinations predominantly support different ontogenetic events at different developmental stages in the innervation of the inner ear.

Keywords

NT-3; BDNF; LIF; Age-related changes; Inner ear; Rat (Sprague Dawley)

Introduction

In the auditory system, various growth factors are known to play roles in the development of the innervation of the inner ear (Fritzsch et al. 1997b, 2004; Rubel and Fritzsch 2002). For example, studies of transgenic mice have shown that two members of the nerve growth factor (NGF) family of proteins, namely brain-derived neurotrophic factor (BDNF) and neurotrophin-3 (NT-3), play essential roles in the normal development of innervation (Agerman et al. 2003; Ernfors et al. 1995; Farinas et al. 2001; Fritzsch et al. 1997a, 2004; Tessarollo et al. 2004). In vitro studies of spiral ganglion neurons (SGNs) have revealed that both NT-3 and BDNF can promote SGN survival and neuritogenesis in organotypic SG explants (Aletsee et al. 2001; Hartnick et al. 1996; Mou et al. 1997; Mullen et al. 2012; Pirvola et al. 1992) and in dissociated cultures (Gillespie et al. 2001; Hartnick et al. 1996; Hegarty et al. 1997; Malgrange et al. 1996; Marzella et al. 1999; Pirvola et al. 1994; Vieira et al. 2007; Wei et al. 2007; Whitlon et al. 2006; Zheng et al. 1995). Recent studies have shown that neurotrophins also regulate the physiological properties of SGNs (Adamson et al. 2002; Davis and Liu 2011). Other peptide growth factors, such as ciliary-derived neurotrophic factor and leukemia inhibitory factor (LIF), also promote survival and neuritogenesis in SGNs in vitro (Gillespie et al. 2001; Hartnick et al. 1996; Marzella et al. 1997; Vieira et al. 2007; Whitlon et al. 2006).

In various peripheral nervous systems, the magnitude and manner of the neuron’s response to neurotrophic factors is known to be age-dependent. For example, sympathetic neurons require NGF for their survival in early development. After they become mature, they are less dependent on NGF for survival (Easton et al. 1997; Putcha et al. 2000) but still respond to NGF with increased neurite growth (Orike et al. 2001b). Moreover, the survival dependence of trigeminal ganglion neurons has been shown to switch from BDNF and NT-3 to NGF during the early stages of target field innervation (Buchman and Davies 1993). Similarly, a sub-population of dorsal root ganglion (DRG) neurons depends on NGF during embryonic development but switches its dependence to glial-cell-derived neurotrophic factor in early postnatal life (Molliver et al. 1997). These data suggest that various neurotrophic factors can exert their effects on the same neuronal population, either simultaneously or sequentially, at different phases of their development and further, that each factor might regulate different ontogenetic events at different developmental stages. Although a few studies have addressed this issue in the vestibular system (Chihara et al. 2011; Hashino et al. 1999), the degree to which similar age-dependent regulation of SGNs by neurotrophic factors occurs remains unclear.

Accordingly, we have performed a systematic developmental study of rat SGNs by culturing dissociated SGNs harvested at various postnatal stages from birth to the age at which the hearing function is mature. We have tested the responsiveness of these SGNs to NT-3, BDNF and LIF, alone or in various combinations, focusing mainly on SGN survival, neuritogenesis and the neuronal morphology of individual SGNs.

Materials and methods

All animal procedures were approved by the relevant local animal subjects committees (Graduate School of Medicine, The University of Tokyo, #P08-029) and performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Preparation of tissue culture plates

Circular coverslips (13 mm; Matsunami glass, Tokyo, Japan) were incubated with a mixed solution of fibronectin (Sigma Aldrich Japan, Tokyo, Japan; 10 μg/ml in phosphate-buffered saline [PBS; pH 7.4]) and laminin (Sigma Aldrich Japan; 10 μg/ml in PBS) at 4°C overnight and then washed three times in PBS (pH 7.4). The coverslips were then incubated with poly-L-lysine (Sigma Aldrich Japan; 20 μg/ ml in Dulbecco's modified Eagle's medium [DMEM; Invitrogen life technologies, Tokyo Japan]) at 37°C for 1 h, washed three times in PBS (pH 7.4), air-dried and placed in each well of 24-well plates (BD Biosciences, Tokyo, Japan).

Animal dissection and dissociated cell culture of SGNs

Cultures of dissociated SGNs were prepared from Sprague Dawley rats (Saitama experimental animals, Saitama, Japan) at postnatal day 0 (P0), day 5 (P5) and day 20 (P20). We chose these developmental ages for the following reason: the period of P0 culture (equivalent to P0–P4 in vivo) corresponds to the time of naturally occurring SGN death (Echteler et al. 2005; Rueda et al. 1987); P5 culture (equivalent to P5–P9 in vivo) corresponds to the terminal stage of the remodeling of afferent projections within the sensory epithelium (Echteler 1992; Wiechers et al. 1999); and P20 culture (equivalent to P20–P24 in vivo) corresponds to the stage in which all of the major developmental events for SGNs are complete and the hearing function has achieved maturity (Pujol et al. 1998).

For the dissociation of SGNs, rats were deeply anesthetized with an intraperitoneal injection of ketamine hydrochloride (100 mg/kg) and xylazine hydrochloride (9 mg/kg) and then decapitated. The mandible was removed and skulls were opened mid-sagitally. Under a dissecting microscope, the brain was removed and the temporal bones were harvested and transferred to Petri dishes containing sterile PBS (pH 7.4). The membranous labyrinth was exposed by removal of the bony or cartilaginous cochlear capsule. After the removal of the spiral ligament, stria vascularis and organ of Corti, the SG was carefully dissected from the modiolus by using fine forceps and placed in calcium/magnesium-free Hank's balanced salt solution (HBSS, Invitrogen life technologies). Pooled ganglia (n=10–16) were then enzymatically digested in HBSS with 0.1% collagenese and then in HBSS with 0.25% trypsin (both from Invitrogen life technologies) in a gently shaking 37°C water bath. Incubation times varied depending on the age of the tissue (see Table 1). Enzymatic digestion was terminated by the addition of fetal bovine serum (Invitrogen life technologies) to a final concentration of 10% and the digested ganglia were centrifuged at 1,500 rpm for 5 min at room temperature (RT). The supernatant was discarded and fresh primary growth medium consisting in DMEM (Invitrogen life technologies), fetal bovine serum (10%; Invitrogen life technologies), HEPES buffer (10 mM; Invitrogen life technologies) and penicillin (300 U/ ml; Sigma Aldrich Japan) was then added, following which the ganglia

were dissociated by mechanical trituration through a plastic pipette tip. In this procedure, a maximum of four triturations were carried out before the cells were removed by decanting the supernatant medium. Fresh medium was added, a further four triturations were carried out and the cells were again removed. These sequential triturations were repeated until the majority of the neurons were removed, with the number of repeats depending on the age of the donor. This method of avoiding excess mechanical damage to the dissociated SGNs enhanced the survival of SGNs several fold, especially in P20 SGNs, as demonstrated in the dissociation of superior cervical ganglion neurons (Orike et al. 2001a).

Each cell suspension was again spun at 1,500 rpm for 5 min and the resultant pellet was resuspended in fresh primary growth medium. Cell counts were made by using a hemocytometer and dissociated cells were plated onto the center of the glass coverslips at a density of 20,000 cells/100 μl per well. The cultures were maintained at 37°C in a humidified atmosphere containing 8% CO_2 for 2 h to promote the attachment of the neurons to the bottom of the coverslips. The cultures were then incubated in 300 μl primary growth medium for 10 h for the complete attachment of the SGNs, followed by incubation in a serum-free maintenance medium consisting in DMEM, N2 supplement (10 μl/ml; Invitrogen life technologies), HEPES buffer (10 mM), penicillin (300 U/ml), glucose (at a final concentration of 6 g/l; Sigma Aldrich Japan) for an additional 72 h. To compare the effects of neurotrophic factors, cultures in each age group were further divided into six subgroups and supplemented with the following factors: (1) recombinant human NT-3 (catalog number 480875; EMD biosciences, La Jolla, Calif., USA; 50 ng/ml), (2) recombinant human BDNF (catalog number 01-194; Upstate Biotechnology, Lake Placid, N.Y., USA; 50 ng/ml), (3) LIF (catalog number LIF3005; Chemicon, Temecula, Calif., USA; 50 ng/ ml), (4) a combination of NT-3 and BDNF (50 ng/ml each; referred to as the N + B group), (5) a combination of NT-3, BDNF and LIF (50 ng/ml each; referred to as the ALL factor group). One group was cultured without addition of any of the factors to serve as an untreated control. The concentration of each factor was determined based on previous studies that had examined the responses of SGNs from postnatal mice and rats in vitro (Gillespie et al. 2001; Hegarty et al. 1997; Malgrange et al. 1996; Marzella et al. 1999; Vieira et al. 2007; Wei et al. 2007; Whitlon et al. 2006). To assess the effects of concentration on SGN neurite extension (see Results), additional P5 cultures were treated with NT-3 (10 ng/ml), BDNF (10 ng/ml), or N + B (10 ng/ml each).

To determine the initial yields of plated SGNs, cultures (n=4 in each age group) were maintained in primary growth medium for 4 h after plating, followed by fixation for immunostaining.

Immunohistochemistry

Cultured cells were fixed by immersion in 4% paraformal-dehyde in 0.1 M phosphate buffer (pH 7.4) for 30 min at RT. After several PBS (pH7.4) washes, endogeneous peroxidase activity was blocked by treatment with 0.3% hydrogen peroxide in methanol for 30 min at RT. The cells were then incubated with blocking solution (PBS, pH 7.4, containing 4% fetal bovine serum [Invitrogen life technologies], 0.2% Triton X-100 and 0.1% sodium azide) for 30 min each at RT to reduce non-specific antibody binding, followed by incubation with anti-neurofilament (NF) 200 mouse monoclonal antibody (clone NR52, Sigma; 1:500 in blocking solution) at 4°C overnight. After three washes in PBS (pH 7.4), the tissues were incubated with biotinylated horse anti-mouse IgG secondary antibody (Vector Laboratories Japan, Tokyo, Japan) for 1 h at RT. Following three washes in PBS (pH 7.4), the tissues were then reacted with ABC solution (Vectastain Elite kit; Vector Laboratories Japan) for 30 min at RT, according to the manufacturer's instructions. After further washing steps in PBS (pH 7.4), immunoreactivity was visualized by using the diaminobenzidine (Vector Laboratories Japan) reaction. After additional washes in distilled water, the cells were

dehydrated through graded ethanol and xylene and then mounted on glass slides. The primary antibody was omitted from the procedure as a negative control; this gave no labeling corresponding to the immunostaining of the primary antibody.

Image presentation

Photomicrographic images of the immunostained SGNs were taken on a Nikon E800 microscope (Nikon, Tokyo, Japan) under brightfield illumination and phase-contrast optics with a digital microscope camera (AxioCam, Carl Zeiss Japan, Tokyo, Japan). Digital images were edited with Adobe Photoshop CS1 software (Adobe Systems Incorporated, Tokyo, Japan). The images were not modified except for minor adjustments of size, orientation, brightness, contrast and conversion to grayscale consistent with analysis.

Data analysis

We used the following indices for evaluation of the effects of neurotrophic factors on SGNs: the number of surviving neurons in each culture, the number of surviving SGNs relative to the initial seed number, the length of the longest neurite extending from each SGN and neuronal morphology. Data were collected from at least four independent experiments with at least duplicate wells for each of the experimental conditions. Quantitative analyses described below were performed by independent observers in a blinded manner.

To evaluate SGN survival, we counted all neurons present on each coverslip. Every cell that was labeled with the anti-NF200 antibody and also had a visible nucleus was counted as a surviving neuron, without regard to morphology or the number of processes.

To evaluate the length of the longest neurite on SGNs, neurons were sampled from the center field of view (4× objective) within each coverslip. Two additional sampling fields adjacent to the center field were employed in P20 cultures, because of the small number of SGNs in each field. The neurons within each 4× microscopic field were individually photographed and every SGN inside each field was included for analysis. All processes that emanated from the cell body of the SGNs were traced and their lengths were measured by using image analysis software (Microanalyzer, Polaroid Japan). The length of the longest neurite from each SGN was defined as the longest neurite length (LNL). The length of the neurite in SGNs without a neurite was listed as 0. When measuring neurite length, only those neurons whose processes could be clearly traced were included. This might have biased our results toward cells with shorter neurites, as some neurites exited the microscopic field. A total of 1247, 1355 and 953 neurons were analyzed from P0, P5 and P20 cultures, respectively.

Neuronal morphology was assessed for the same SGNs evaluated for the neurite extension. Each neuron analyzed was categorized into monopolar, bipolar, multipolar, or no processes. Neurons extending only neurite(s) shorter than the diameter of their cell body were categorized as having no processes. The percentage of the various morphologies was calculated from the total number of neurons analyzed.

Statistical analysis

Results were presented as the mean ± SE of samples in each experimental group. Statistical analyses for the number of surviving neurons and LNL were performed by using one-way analysis of variance (ANOVA) followed by Dunnet’s post hoc test with GraphPad Prism 5 (GraphPad Software, La Jolla, Calif., USA) to compare each neurotrophin subgroup with the untreated control group. For the statistical analysis of the effect of neurotrophic factors on neuronal morphology, neurons were divided into two categories, i.e., SGN without or one neurite (referred to as the 0–1 neurite group) and SGN with two or more neurites (referred to

as the ≥2 neurite group) and a chi-square test was used. Differences associated with *P* valuesof <0.05 were considered as statistically significant.

Results

General appearance of cultures

Immunostaining of the cultured cells at 4 h after plating revealed that the mean seed number of SGNs per well was 2387±88 in the P0 group, 889±65 in the P5 group and 164±18 in the P20 group.

Figure 1a illustrates an example of cultured SGNs after 12 h in primary growth medium containing serum and a further 72 h in serum-free maintenance medium. The surviving SGNs were identified as NF200-positive cells. As described in previous studies of murine dissociated SGN cultures (Vieira et al. 2007; Whitlon et al. 2007), cultured neurons either had no neurite (Fig. 1b) or showed monopolar (with one neurite emanating from the cell body; Fig. 1c), bipolar (with two neurites emanating from the cell body; Fig. 1d), or multipolar (with three or more neurites emanating from the cell body; Fig. 1e) morphologies.

We also examined the cultures under phase-contrast optics to evaluate the appearance of SGNs and non-neuronal cells (possibly consisting in glial cells and fibroblasts that were unlabeled). As shown in Fig. 2, non-neuronal cells in each culture exhibited two morphologies, i.e., flat-shaped cells with large nuclei and spindle-shaped cells with small nuclei and processes that extended from the cell body. This difference was most readily observed in P20 cultures (Fig. 2c). The density of non-neuronal cells decreased with increasing age of the donor animals; in P0 and P5 cultures, the non-neuronal cells formed a confluent layer on the glass surface (Fig. 2a, b), whereas the glass surface was partially free of non-neural cells in P20 cultures (Fig. 2c).

Effects of NT-3, BDNF and LIF on SGN survival

Figure 3 shows representative photomicrographs of cultured SGNs in P0, P5 and P20 groups, with or without neurotrophic factor supplementation. As illustrated, increases in SGN numbers as a result of neurotrophic support are apparent for SGNs harvested at younger ages.

Figure 4 provides a quantitative analysis of SGN numbers across ages and treatment conditions. Figure 4a shows the average number of SGNs/well, uncorrected for initial seed number. In the absence of any neurotrophic factor treatment, P5 cultures obviously showed the greatest yield of surviving neurons (8.7±0.7/well), followed by P20 cultures (7.0±1.5/well) and then by P0 cultures (0.6 ±0.2/well). Moreover, the greatest sensitivity to neurotrophic treatment was observed for P5 SGNs.

Because of the difference in the initial yield of SGNs among age groups, we normalized the number of surviving SGNs/well by the initial seeding level. These data are presented in Fig. 4b, which demonstrate that the survival rate of untreated SGNs in culture increases dramatically with increasing age. In control cultures, surviving SGNs at 72 h corresponded to 0.027%, 0.98%, or 4.2% of those seeded initially in the P0, P5 and P20 groups, respectively. The normalized survival rate of SGNs in each treatment group also tended to increase for older SGNs (Fig. 4b).

In P0 cultures, neurotrophic factors had a significant effect on the survival of SGNs ($P<0.0001$; ANOVA) and this effect was additive/synergistic. When the culture was treated with N + B, the number of surviving neurons per culture (10.5±0.8) increased to 17-fold that

of untreated cultures (0.6±0.2). This number almost corresponded to that of the sum of the number of surviving neurons in NT-3 (0.9±0.2) and BDNF cultures (5.3±0.5). When the culture was treated with ALL factors, the number of surviving neurons further increased to 74-fold that of untreated cultures (74.0±6.7), which is approximately 9.1-fold that of the sum of the number of surviving SGNs in NT-3-treated (0.9±0.2), BDNF-treated (5.3±0.5) and LIF-treated (1.9±0.4) cultures. The post-hoc test revealed that the treatment with N + B and ALL factors had statistically significant survival-promoting effects compared with the untreated control ($P<0.05$, $P<0.001$, respectively).

In P5 cultures, SGN survival responded to the widest range of treatments amongst the age groups; ANOVA revealed significant effect on survival ($P<0.0001$) and the post-hoc test showed that each of NT-3, BDNF, N + B and ALL factors significantly enhanced the survival of SGNs ($P<0.001$ for each; Fig. 3). In this age group, the additive/synergistic effect seen in P0 cultures was decreased under our culture conditions. When the culture was treated with N + B, the number of surviving neurons per culture (63.4±7.1), which was 6.3-fold that of untreated cultures (10.0±1.1), almost corresponded to the number of surviving neurons in BDNF cultures (5.9-fold, 59.0±4.7). When the culture was treated with ALL factors, the number of surviving neurons (nine-fold, 90.3±6.0) was slightly less than the sum of the number of SGNs treated with the individual factors: BDNF, NT-3 (3.2-fold, 31.5±2.0) and LIF(1.9-fold, 19.4±1.6).

In P20 cultures, the survival-promoting effect of each treatment was the smallest of any of the age groups ($P=0.0097$; ANOVA). The post-hoc test revealed that only treatment with NT-3 or with BDNF significantly promoted the survival of SGNs compared with the untreated control ($P<0.01$, $P<0.001$, respectively). Moreover, cultures treated with N + B or ALL factors exhibited survival no greater than that observed in controls.

Effects of NT-3, BDNF and LIF on neurite extension

The LNL of untreated SGNs was shortest for neurons harvested at P0, slightly longer for P5 neurons and longest for P20 neurons.

In P0 cultures, the overall effect of treatment on the LNL was significant (Fig. 5a; $P<0.0001$; ANOVA). However, when factors were delivered alone, only LIF elicited an enhancement of LNL compared with the control group ($P<0.001$; Dunnet post hoc test). ALL factors also showed a significant increase in LNL ($P<0.01$) but this effect was lower than that observed with LIF alone. LNL was unaffected by the presence of NT-3, BDNF, or both factors combined ($P>0.05$).

LNL of P5 SGNs responded to the widest range of treatment with neurotrophic factors among the age groups (Fig. 5b; $P<0.0001$; ANOVA). Again, LIF and ALL factors showed significantly increased LNL ($P<0.001$), although ALL factors once more produced a lower increase than LIF alone. In contrast, treatment with NT-3, BDNF, or N + B inhibited neurite extension compared with that in the control group ($P<0.05$, $P<0.001$, $P<0.001$, respectively). To test whether this inhibitory effect depended on the concentration of neurotrophic factors, we also treated P5 SGNs with BDNF and/or NT-3 at 10 ng/ml. Although a survival-promoting effect was observed at this lower concentration, as seen at 50 ng/ml (Fig. 6a), LNL in the treated groups was not significantly different from that in the untreated controls (Fig. 6b). However, the addition of LIF (ALL factors) produced a significant enhancement of both survival and LNL at this age.

In P20 cultures, the effect of neurotrophic factors on LNL was the smallest among the age groups, although it remained significant (Fig. 5c; $P=0.0008$) by ANOVA. The post-hoc test

revealed that treatment with ALL factors weakly enhanced LNL ($P<0.05$), whereas N + B had a strong inhibitory effect ($P<0.001$).

Effects of NT-3, BDNF and LIF on neuronal morphology

For all age groups, monopolar neurons were the most prevalent morphological type in untreated cultures (Fig. 7). In general, neurotrophic factor treatment had modest effects on neuronal morphology. An exception was ALL factors, which enhanced the proportion of bipolar and multipolar neurons, while decreasing the monopolar and especially the “no” neurites phenotypes.

To assess these effects statistically, neurons in each age group were divided into two categories, i.e., SGN without neurites or with one neurite versus SGN with two or more neurites. In all age groups, ALL factors significantly increased the proportion of ≥2 neurite SGNs compared with the control group (Table 2; $P<0.05$ in P0, $P<0.001$ in P5 and P20; chi-square test). In the P20 group, BDNF alone and LIF alone also more modestly enhanced the proportion of ≥2 neurite SGNs compared with the control group (Table 2; $P<0.05$; chi-square test).

Discussion

The present study was designed to extend our knowledge regarding age-dependent changes in the responsiveness of SGNs to NT-3, BDNF and LIF by using dissociated cultures. Our study appears to be the first systematic in vitro study to compare the regulation of survival, neurite extension and neuronal morphology of rat SGNs among different age groups under the same culture conditions. The results demonstrate that each of these indices of SGN maturation are differentially regulated by NT-3, BDNF and/or LIF in an age-dependent manner. Our data further suggest that each of these neurotrophic factors predominantly support different ontogenetic events at different developmental stages in the innervation of the inner ear.

Effects of neurotrophic factors on SGN survival

A striking feature of our results was the dramatic age-dependent increase in the proportion of neurons that survived in culture in the absence of neurotrophic factor treatment. This finding suggests that SGNs are highly dependent for their survival on exogenous neurotrophic factors at early developmental stages but become more neurotrophic-factor-independent as they approach adulthood. A similar tendency has been reported for the trigeminal ganglion (Scott and Davies 1993) and sympathetic neurons (Easton et al. 1997; Orike et al. 2001b; Putcha et al. 2000). Because the period of our P0 culture (equivalent to P0–P4 in vivo) corresponds to a period of naturally occurring SGN cell death (Echteler et al. 2005; Rueda et al. 1987), we can speculate that the higher trophic factor dependence of P0 and P5 SGNs contributes to the elimination of SGNs that fail to establish connection with a factor-producing target, as has been suggested for gerbil SGN (Mou et al. 1998).

Arguing against this interpretation is the limited survival response of P0 SGN to neurotrophin treatment, since none of the factors tested were effective in promoting the survival of SGN when applied individually. However, combinations of factors were highly effective, especially when LIF was added to BDNF plus NT-3. BDNF or NT-3 alone only became effective at older ages, whereas the synergistic effects observed at P0 declined at P5 and disappeared at P20. These data suggest that more than one factor is required to rescue SGN from apoptosis during early developmental target cell interactions, thereby serving to promote the survival of neurons that make contacts with separate sources of trophic factors, such as the peripheral and central contacts of SGN. This possibility is supported by the

finding that both NT-3 and BDNF are expressed in the developing cochlea and cochlear nucleus (Sugawara et al. 2007; Tierney et al. 2001; Wiechers et al. 1999). The elements of the LIF receptor-gp130 heterodimer (Auernhammer and Melmed 2000) are expressed in the P1 mouse spiral ganglion (Oshima et al. 2007) and LIF is strongly expressed in the adult cochlear sensory epithelium after noise injury (Cho et al. 2004). Although we could locate no published information regarding LIF expression in developing cochlear nucleus, LIF is expressed by many neurons in the brain (Lemke et al. 1996).

Notably, the initial seeding number of SGNs/well decreased dramatically with increasing age. Since the number of cells seeded/well was constant, this means that a greater proportion of the culture consisted in non-neuronal cochlear cells in older cultures. Therefore, we cannot exclude the possibility that the neurotrophic factor independence of older SGN reflects trophic support from these non-neuronal cells. Although the density of non-neuronal cells decreased with the increasing age of the animals at the endpoint of culture period, probably reflecting the high proliferation rate of younger non-neuronal cells, the possibility that the initial support of non-neuronal cells at the early culture periods might have been associated with the higher survivial rate of SGNs at P20 cannot be excluded. The mature glial cells in P20 cultures might also have provided stronger trophic support than those in younger animals.

At P5 and P20, BDNF was more potent than NT-3 or LIF for survival. This observation is in good agreement with previous studies of dissociated early postnatal rat SGNs (Hegarty et al. 1997; Marzella et al. 1999; Zheng et al. 1995) and with the enhancement of the survival of adult SGNs by BDNF in vivo (Leake et al. 2011; Miller et al. 2007; Wise et al. 2005) and in vitro (Vieira et al. 2007; Wei et al. 2007). Although BDNF expression is almost absent in the peripheral target field of the SGNs at the later stages of development (Wheeler et al. 1994; Wiechers et al. 1999; Ylikoski et al. 1993), it is expressed by neurons in the ventral cochlear nucleus beginning on P3 (Tierney et al. 2001). BDNF is also observed in SGNs themselves until the adult stage (Ruttiger et al. 2007; Schimmang et al. 2003; Singer et al. 2008), raising the possibility of an autocrine mechanism (Schimmang et al. 2003).

With regard to the survival-promoting effects of LIF, previous in vitro studies have documented that LIF has survival-promoting effects on early postnatal rat and mouse SGNs (Gillespie et al. 2001; Whitlon et al. 2006) and adult mouse SGNs (Vieira et al. 2007). Our results suggest that the survival-promoting effects of LIF alone are not as strong as those of BDNF and NT-3. It is more potent as a synergistic enhancer of neurotrophins for the survival of P0 and, to a lesser extent, P5 rat SGNs.

Effects of neurotrophic factors on neurite extension

The effect of neurotrophic factors on neurite extension in SGNs in vitro has been less extensively studied compared with neuronal survival or neurite number from explants and remains a relatively controversial issue. We have demonstrated that, although LNL in untreated SGNs is maximal at P20, the effect of neurotrophic factors on neurite length is greatest at P5. The period of our P5 culture (equivalent to P5–P9 in vivo) corresponds to a stage of late remodeling of afferent projections to the sensory epithelium (Echteler 1992; Wiechers et al. 1999) and cochlear nucleus (Limb and Ryugo 2000). The strong regulation of neurite extension by neurotrophic factors at P5 might be necessary to achieve the rearrangement of afferent innervation mediated by target-derived neurotrophic factors in the organ of Corti and/or brainstem.

Our study has revealed that LIF has a strong effect on neurite extension on P0 and P5 SGNs. At P0 and P5, treatment with LIF alone or with ALL factors enhanced neurite extension, whereas treatment with NT-3 and/or BDNF showed no significant effect at P0 and an

inhibitory effect at P5. We can reasonably conclude that the effect of treatment with ALL factors is primarily attributable to the effect of LIF at these ages. LIF has been reported to enhance neurite extension in a variety of other neuronal types (Cafferty et al. 2001; Leibinger et al. 2009). In particular, recent studies have demonstrated that LIF mediates the enhanced intrinsic growth status after a conditioning lesion (Cafferty et al. 2001; Hyatt Sachs et al. 2010) suggesting that LIF plays a role in the regeneration of injured neurites. Therefore, our results, together with those of previous reports (Gillespie et al. 2001; Vieira et al. 2007; Whitlon et al. 2007) indicate that the application of exogenous LIF, alone or together with neurotrophins, should be clinically valuable as a treatment for central axon injury from trauma or the surgical removal of acoustic tumors and for peripheral dendrites to improve the efficacy of cochlear implants.

In contrast to LIF, NT-3 and BDNF at 50 ng/ml showed inhibitory effects for neurite extension on P5 SGNs. NT-3 and BDNF showed a survival-promoting effect on the same neuronal population at this concentration, whereas NT-3 and BDNF at 10 ng/ml did not show this inhibitory effect, suggesting that this is not a toxic effect but a specific suppression of neuritogenesis by high concentrations of NT-3 and BDNF. This finding contrasts with the extension-promoting effects of NT-3 and BDNF during in vivo and in vitro studies (Brors et al. 2008; Leake et al. 2011; Miller et al. 2007; Wise et al. 2005). The reason underlying this discrepancy is unclear but several possibilities can be proposed.

One possibility is that the production of other growth factors such as LIF by SGNs themselves or by adjacent tissue is higher in explants or in vivo situations. Alternatively, the discrepancy could be attributable to the difference in the mode of exposure of SGNs to the factors: in vivo administration of neurotrophic factors by osmotic pump through the scala tym-pani (Leake et al. 2011; Miller et al. 2007; Wise et al. 2005) might primarily expose the SGN neurites. In contrast, in our dissociated culture system, the entire surface of the SGNs is presumably exposed to the factors and so the factors might not drive the neurites to extend. Indeed, a study of rat DRG neurons in compartmented cultures has revealed that NGF promotes neurite extension when applied to the neurite alone but suppresses neurite elongation when the cell body is exposed (Kimpinski et al. 1997). Another study of rat DRG neurons has demonstrated a dose-dependent inhibitory effect on neurite extention for NGF (≥50 ng/ml; Conti et al. 1997, 2004). The authors (Conti et al. 2004) speculate that this might be necessary to terminate axon growth when the growth cone reaches its target. Therefore, the finding that 50 ng/ml NT-3 and BDNF show greater inhibitory effects on P5 SGNs than other age groups might be associated with the observation that the period of P5 culture corresponds to the stage of afferent rearrangement. The molecular mechanisms underlying this inhibition are unknown but a higher concentration of neurotrophins might change the balance of signaling mediated through Trks and p75, the latter of which has been shown to suppress neurite extension in SGNs (Brors et al. 2008).

Effects of neurotrophic factors on neuronal morphology

SGNs are bipolar neurons possessing two neuronal processes (an axon and a dendrite), which are anatomically and functionally distinct. Previous studies have indicated that not all SGNs show this in vivo morphology in culture (Vieira et al. 2007; Whitlon et al. 2007) suggesting that the initiation of neuritogenesis is regulated differentially for SGN axons and dendrites.

In the current study, the fraction of monopolar neurons was highest for P0 SGNs, whereas the fraction showing bipolar morphology was highest at P20, especially after treatment with ALL factors. These findings suggest that the capacity of SGNs to initiate regrowth of neurites changes in an age-dependent manner and that the SGNs retain the capacity to

respond to exogenous neurotrophic factors for neurite regrowth, even when the neurons are functionally mature.

A few studies have addressed the regulation of neurite morphology as modulated by growth factors. When dissociated DRG neurons are cultured in the absence of NGF, most of the neurons exhibit unipolar morphology, whereas NGF or NT-3 treatment dramatically increases the percentage of bipolar neurons (Lentz et al. 1999). NGF and NT-3 have also been demsonstrated to produce differential effects on central and peripheral neurite growth patterns of trigeminal ganglion neurons in whole-mount explant cultures (Ulupinar et al. 2000). Neonatal mouse SGNs tend to show increased bipolar morphology after LIF, ciliary neurotrophic factor, or oncostatin treatment (Whitlon et al. 2007). These observations clearly show that neurotrophic factors are involved in the initiation of neuritogenesis, although molecular mechanisms regulating this morphology remain largely unknown. The findings in our study suggest that our cocktail of neurotrophic factors should be useful in studies of the transplantation of neural stem cells into spiral ganglia, in which the development of neurons with bipolar morphology is necessary to restore the functional connection between the organ of Corti and the brainstem.

Potential limitations of the study

Several limitations need to be acknowledged with regard to the present study. First, we harvested and dissociated SGNs from along the entire length of the cochlea. Therefore, our results reflect the average response of SGNs from all cochlear turns at each developmental age. Recent studies have demonstrated that SGNs present at different cochlear positions can vary in their physiological properties in response to neurotrophins (Adamson et al. 2002; Davis and Liu 2011). SGNs located in the apex show NT-3-specific patterns in terms of their electrophysiological properties and their molecular expression in response to the higher expression of NT-3 in the apical region, whereas SGNs in the base show BDNF-specific patterns in response to the higher expression of BDNF in the basal region. This finding raises the possibility that the SGNs of different turn origin might respond to neurotrophins differently with respect to survival and neuritogenesis.

A second limitation is that our study did not differentiate the responses of type I versus type II SGNs, since the reliable differentiation of these two neuronal types is difficult based purely on their morphology in culture. Moreover, rat type I SGNs unfortunately up-regulate the type II marker peripherin in culture. Although the majority of SGNs harvested should have been type I, a recent study has demonstrated that the proportion of type II/type I in mice decreases with age (Barclay et al. 2011). In addition, BDNF more strongly supports the survival and neuritogenesis of type II SGNs in explants (Barclay et al. 2011). Therefore, similar neuronal-type-specific differences in trophic dependence might have influenced the data of our study.

A third limitation concerns the possible influence of other experimental factors that could affect the survival and neuritogenesis of SGNs in dissociated cell culture. Our data could have been influenced by mechanical and chemical stresses during dissociation, the specific coating of the glass culture surface and the type of culture media, in addition to the supplemention of neurotrophic factors. The response of SGNs to these factors might also be age-dependent and might have influenced our results. These issues need to be addressed in further investigations.

Concluding remarks

The present study demonstrates that the responsiveness of rat postnatal SGNs to neurotrophic factors with regard to several indices of growth changes in an age-dependent

manner, that various factors can exert influences that are quite distinct from one another and that strong synergistic effects are observable between factors. Improved knowledge of the processes that occur during the development of SGNs should be valuable to facilitate the maintenance of SGNs and their dendrites and also the development of regenerative therapies to improve the efficacy of cochlear implants.

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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-Leffler J, Blanchard MP, Scarfone E, Canlon B, Nosrat C, Ernfors 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, Beros A, Mullen L, Palacios S, Pak K, Dazert S, Ryan AF. Ras/MEK but not p38 signaling mediates NT-3-induced neurite extension from spiral ganglion neurons. J Assoc Res Otolaryngol. 2001; 2:377–387. [PubMed: 11833610]

Auernhammer CJ, Melmed S. Leukemia-inhibitory factor-neuroimmune modulator of endocrine function. Endocr Rev. 2000; 21:313–345. [PubMed: 10857556]

Barclay M, Ryan AF, Housley GD. Type I vs type II spiral ganglion neurons exhibit differential survival and neuritogenesis during cochlear development. Neural Dev. 2011; 6:33. [PubMed: 21989106]

Brors D, Hansen S, Mlynski R, Volkenstein S, Aletsee C, Sendtner M, Ryan AF, Dazert S. Spiral ganglion outgrowth and hearing development in p75-deficient mice. Audiol Neurootol. 2008; 13:388–395. [PubMed: 18663291]

Buchman VL, Davies AM. Different neurotrophins are expressed and act in a developmental sequence to promote the survival of embryonic sensory neurons. Development. 1993; 118:989–1001. [PubMed: 8076530]

Cafferty WB, Gardiner NJ, Gavazzi I, Powell J, McMahon SB, Heath JK, Munson J, Cohen J, Thompson SW. Leukemia inhibitory factor determines the growth status of injured adult sensory neurons. J Neurosci. 2001; 21:7161–7170. [PubMed: 11549727]

Chihara Y, Iwasaki S, Kondo K, Yamasoba T. Responsiveness of rat vestibular ganglion neurons to exogenous neurotrophic factors during postnatal development in dissociated cultures. Brain Res. 2011; 1408:1–7. [PubMed: 21774920]

Cho Y, Gong TW, Kanicki A, Altschuler RA, Lomax MI. Noise overstimulation induces immediate early genes in the rat cochlea. Brain Res Mol Brain Res. 2004; 130:134–148. [PubMed: 15519684]

Conti AM, Fischer SJ, Windebank AJ. Inhibition of axonal growth from sensory neurons by excess nerve growth factor. Ann Neurol. 1997; 42:838–846. [PubMed: 9403475]

Conti AM, Brimijoin S, Miller LJ, Windebank AJ. Suppression of neurite outgrowth by high-dose nerve growth factor is independent of functional p75NTR receptors. Neurobiol Dis. 2004; 15:106–114. [PubMed: 14751775]

Davis RL, Liu Q. Complex primary afferents: what the distribution of electrophysiologically-relevant phenotypes within the spiral ganglion tells us about peripheral neural coding. Hear Res. 2011; 276:34–43. [PubMed: 21276843]

Easton RM, Deckwerth TL, Parsadanian AS, Johnson EM Jr. Analysis of the mechanism of loss of trophic factor dependence associated with neuronal maturation: a phenotype indistinguishable from Bax deletion. J Neurosci. 1997; 17:9656–9666. [PubMed: 9391020]

Echteler SM. Developmental segregation in the afferent projections to mammalian auditory hair cells. Proc Natl Acad Sci USA. 1992; 89:6324–6327. [PubMed: 1631126]

Echteler SM, Magardino T, Rontal M. Spatiotemporal patterns of neuronal programmed cell death during postnatal development of the gerbil cochlea. Brain Res Dev Brain Res. 2005; 157:192–200.

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. [erratum appears in Neuron 15:739]. [PubMed: 7605630]

Farinas I, Jones KR, Tessarollo L, Vigers AJ, Huang E, Kirstein M, de Caprona DC, Coppola V, Backus C, Reichardt LF, Fritzsch B. Spatial shaping of cochlear innervation by temporally regulated neurotrophin expression. J Neurosci. 2001; 21:6170–6180. [PubMed: 11487640]

Fritzsch B, Farinas I, Reichardt LF. Lack of neurotrophin 3 causes losses of both classes of spiral ganglion neurons in the cochlea in a region-specific fashion. J Neurosci. 1997a; 17:6213–6225. [PubMed: 9236232]

Fritzsch B, Silos-Santiago I, Bianchi LM, Farinas I. The role of neurotrophic factors in regulating the development of inner ear innervation. Trends Neurosci. 1997b; 20:159–164. [PubMed: 9106356]

Fritzsch B, Tessarollo L, Coppola E, Reichardt LF. Neurotrophins in the ear: their roles in sensory neuron survival and fiber guidance. Prog Brain Res. 2004; 146:265–278. [PubMed: 14699969]

Gillespie LN, Clark GM, Bartlett PF, Marzella PL. LIF is more potent than BDNF in promoting neurite outgrowth of mammalian auditory neurons in vitro. Neuroreport. 2001; 12:275–279. [PubMed: 11209934]

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]

Hashino E, Dolnick RY, Cohan CS. Developing vestibular ganglion neurons switch trophic sensitivity from BDNF to GDNF after target innervation. J Neurobiol. 1999; 38:414–427. [PubMed: 10022582]

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 $[Ca^{2+}]_i$ within a set range. J Neurosci. 1997; 17:1959–1970. [PubMed: 9045725]

Hyatt Sachs H, Rohrer H, Zigmond RE. The conditioning lesion effect on sympathetic neurite outgrowth is dependent on gp130 cytokines. Exp Neurol. 2010; 223:516–522. [PubMed: 20144891]

Kimpinski K, Campenot RB, Mearow K. Effects of the neurotrophins nerve growth factor, neurotrophin-3, and brain-derived neurotrophic factor (BDNF) on neurite growth from adult sensory neurons in compartmented cultures. J Neurobiol. 1997; 33:395–410. [PubMed: 9322157]

Leake PA, Hradek GT, Hetherington AM, Stakhovskaya O. Brain-derived neurotrophic factor promotes cochlear spiral ganglion cell survival and function in deafened, developing cats. J Comp Neurol. 2011; 519:1526–1545. [PubMed: 21452221]

Leibinger M, Muller A, Andreadaki A, Hauk TG, Kirsch M, Fischer D. Neuroprotective and axon growth-promoting effects following inflammatory stimulation on mature retinal ganglion cells in mice depend on ciliary neurotrophic factor and leukemia inhibitory factor. J Neurosci. 2009; 29:14334–14341. [PubMed: 19906980]

Lemke R, Gadient RA, Schliebs R, Bigl V, Patterson PH. Neuronal expression of leukemia inhibitory factor (LIF) in the rat brain. Neurosci Lett. 1996; 215:205–208. [PubMed: 8899749]

Lentz SI, Knudson CM, Korsmeyer SJ, Snider WD. Neurotrophins support the development of diverse sensory axon morphologies. J Neurosci. 1999; 19:1038–1048. [PubMed: 9920667]

Limb CJ, Ryugo DK. Development of primary axosomatic endings in the anteroventral cochlear nucleus of mice. J Assoc Res Otolaryngol. 2000; 1:103–119. [PubMed: 11545139]

Malgrange B, Lefebvre P, Van de Water TR, Staecker H, Moonen G. Effects of neurotrophins on early auditory neurones in cell culture. Neuroreport. 1996; 7:913–917. [PubMed: 8724672]

Marzella PL, Clark GM, Shepherd RK, Bartlett PF, Kilpatrick TJ. LIF potentiates the NT-3-mediated survival of spiral ganglia neurones in vitro. Neuroreport. 1997; 8:1641–1644. [PubMed: 9189906]

Marzella PL, Gillespie LN, Clark GM, Bartlett PF, Kilpatrick TJ. The neurotrophins act synergistically with LIF and members of the TGF-beta superfamily to promote the survival of spiral ganglia neurons in vitro. Hear Res. 1999; 138:73–80. [PubMed: 10575116]

Miller JM, Le Prell CG, Prieskorn DM, Wys NL, Altschuler RA. Delayed neurotrophin treatment following deafness rescues spiral ganglion cells from death and promotes regrowth of auditory nerve peripheral processes: effects of brain-derived neurotrophic factor and fibroblast growth factor. J Neurosci Res. 2007; 85:1959–1969. [PubMed: 17492794]

Molliver DC, Wright DE, Leitner ML, Parsadanian AS, Doster K, Wen D, Yan Q, Snider WD. IB4-binding DRG neurons switch from NGF to GDNF dependence in early postnatal life. Neuron. 1997; 19:849–861. [PubMed: 9354331]

Mou K, Hunsberger CL, Cleary JM, Davis RL. Synergistic effects of BDNF and NT-3 on postnatal spiral ganglion neurons. J Comp Neurol. 1997; 386:529–539. [PubMed: 9378849]

Mou K, Adamson CL, Davis RL. Time-dependence and cell-type specificity of synergistic neurotrophin actions on spiral ganglion neurons. J Comp Neurol. 1998; 402:129–139. [PubMed: 9831050]

Mullen LM, Pak KK, Chavez E, Kondo K, Brand Y, Ryan AF. Ras/p38 and PI3K/Akt but not Mek/Erk signaling mediate BDNF-induced neurite formation on neonatal cochlear spiral ganglion explants. Brain Res. 2012; 1430:25–34. [PubMed: 22119396]

Orike N, Thrasivoulou C, Cowen T. Serum-free culture of dissociated, purified adult and aged sympathetic neurons and quantitative assays of growth and survival. J Neurosci Methods. 2001a; 106:153–160. [PubMed: 11325435]

Orike N, Thrasivoulou C, Wrigley A, Cowen T. Differential regulation of survival and growth in adult sympathetic neurons: an in vitro study of neurotrophin responsiveness. J Neurobiol. 2001b; 47:295–305. [PubMed: 11351340]

Oshima K, Teo DT, Senn P, Starlinger V, Heller S. LIF promotes neurogenesis and maintains neural precursors in cell populations derived from spiral ganglion stem cells. BMC Dev Biol. 2007; 7:112. [PubMed: 17935626]

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 USA. 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]

Pujol, R.; Lavigne-Rebillard, M.; Lenoir, M. Development of sensory and neural structures in the mammalian cochlea. In: Rubel, EW.; Popper, AN.; Fay, RR., editors. Development of the auditory system. Springer; New York: 1998. p. 146-192.

Putcha GV, Deshmukh M, Johnson EM Jr. Inhibition of apoptotic signaling cascades causes loss of trophic factor dependence during neuronal maturation. J Cell Biol. 2000; 149:1011–1018. [PubMed: 10831605]

Rubel EW, Fritzsch B. Auditory system development: primary auditory neurons and their targets. Annu Rev Neurosci. 2002; 25:51–101. [PubMed: 12052904]

Rueda J, de la Sen C, Juiz JM, Merchan JA. Neuronal loss in the spiral ganglion of young rats. Acta Otolaryngol. 1987; 104:417–421. [PubMed: 3434262]

Ruttiger L, Panford-Walsh R, Schimmang T, Tan J, Zimmermann U, Rohbock K, Kopschall I, Limberger A, Muller M, Fraenzer JT, Cimerman J, Knipper M. BDNF mRNA expression and protein localization are changed in age-related hearing loss. Neurobiol Aging. 2007; 28:586–601. [PubMed: 16580094]

Schimmang T, Tan J, Muller M, Zimmermann U, Rohbock K, Kopschall I, Limberger A, Minichiello L, Knipper M. Lack of Bdnf and TrkB signalling in the postnatal cochlea leads to a spatial reshaping of innervation along the tonotopic axis and hearing loss. Development. 2003; 130:4741–4750. [PubMed: 12925599]

Scott SA, Davies AM. Age-related effects of nerve growth factor on the morphology of embryonic sensory neurons in vitro. J Comp Neurol. 1993; 337:277–285. [PubMed: 8277001]

Singer W, Panford-Walsh R, Watermann D, Hendrich O, Zimmermann U, Kopschall I, Rohbock K, Knipper M. Salicylate alters the expression of calcium response transcription factor 1 in the cochlea: implications for brain-derived neurotrophic factor transcriptional regulation. Mol Pharmacol. 2008; 73:1085–1091. [PubMed: 18198284]

Sugawara M, Murtie JC, Stankovic KM, Liberman MC, Corfas G. Dynamic patterns of neurotrophin 3 expression in the postnatal mouse inner ear. J Comp Neurol. 2007; 501:30–37. [PubMed: 17206617]

Tessarollo L, Coppola V, Fritzsch B. NT-3 replacement with brain-derived neurotrophic factor redirects vestibular nerve fibers to the cochlea. J Neurosci. 2004; 24:2575–2584. [PubMed: 15014133]

Tierney TS, Doubell PT, Xia G, Moore DR. Development of brain-derived neurotrophic factor and neurotrophin-3 immunoreactivity in the lower auditory brainstem of the postnatal gerbil. Eur J Neurosci. 2001; 14:785–793. [PubMed: 11576182]

Ulupinar E, Jacquin MF, Erzurumlu RS. Differential effects of NGF and NT-3 on embryonic trigeminal axon growth patterns. J Comp Neurol. 2000; 425:202–218. [PubMed: 10954840]

Vieira M, Christensen BL, Wheeler BC, Feng AS, Kollmar R. Survival and stimulation of neurite outgrowth in a serum-free culture of spiral ganglion neurons from adult mice. Hear Res. 2007; 230:17–23. [PubMed: 17521837]

Wei D, Jin Z, Jarlebark L, Scarfone E, Ulfendahl M. Survival, synaptogenesis, and regeneration of adult mouse spiral ganglion neurons in vitro. Dev Neurobiol. 2007; 67:108–122. [PubMed: 17443776]

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]

Whitlon DS, Ketels KV, Coulson MT, Williams T, Grover M, Edpao W, Richter CP. Survival and morphology of auditory neurons in dissociated cultures of newborn mouse spiral ganglion. Neuroscience. 2006; 138:653–662. [PubMed: 16413120]

Whitlon DS, Grover M, Tristano J, Williams T, Coulson MT. Culture conditions determine the prevalence of bipolar and monopolar neurons in cultures of dissociated spiral ganglion. Neuroscience. 2007; 146:833–840. [PubMed: 17331652]

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]

Wise AK, Richardson R, Hardman J, Clark G, O'Leary S. Resprouting and survival of guinea pig cochlear neurons in response to the administration of the neurotrophins brain-derived neurotrophic factor and neurotrophin-3. J Comp Neurol. 2005; 487:147–165. [PubMed: 15880560]

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]

Zheng JL, Stewart RR, Gao WQ. Neurotrophin-4/5 enhances survival of cultured spiral ganglion neurons and protects them from cisplatin neurotoxicity. J Neurosci. 1995; 15:5079–5087. [PubMed: 7623136]

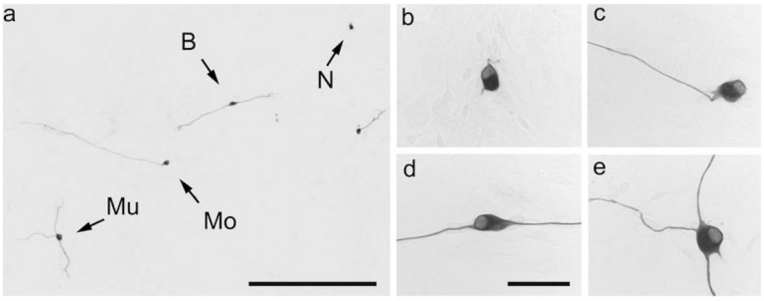

Fig. 1.

a Example of cultured spiral ganglion neurons (SGNs) after 12 h in primary growth medium containing serum and a further 72 h in serum-free maintenance medium. SGNs were then fixed and immunostained with anti-NF200 antibody. Surviving SGNs are identified as NF200-positive cells. Cultured neurons either have no neurite (*N*) or show monopolar (*Mo*; with one neurite emanating from the cell body), bipolar (*B*; with two neurites emanating from the cell body), or multipolar (*Mu*; with three or more neurites emanating from the cell body) morphologies. High magnification views of the *N*, *Mo*, *B* and *Mu* SGNs shown in **a** are presented in **b**–**e**, respectively. *Bars* 0.5 mm (**a**), 50 μm (**b**–**e**)

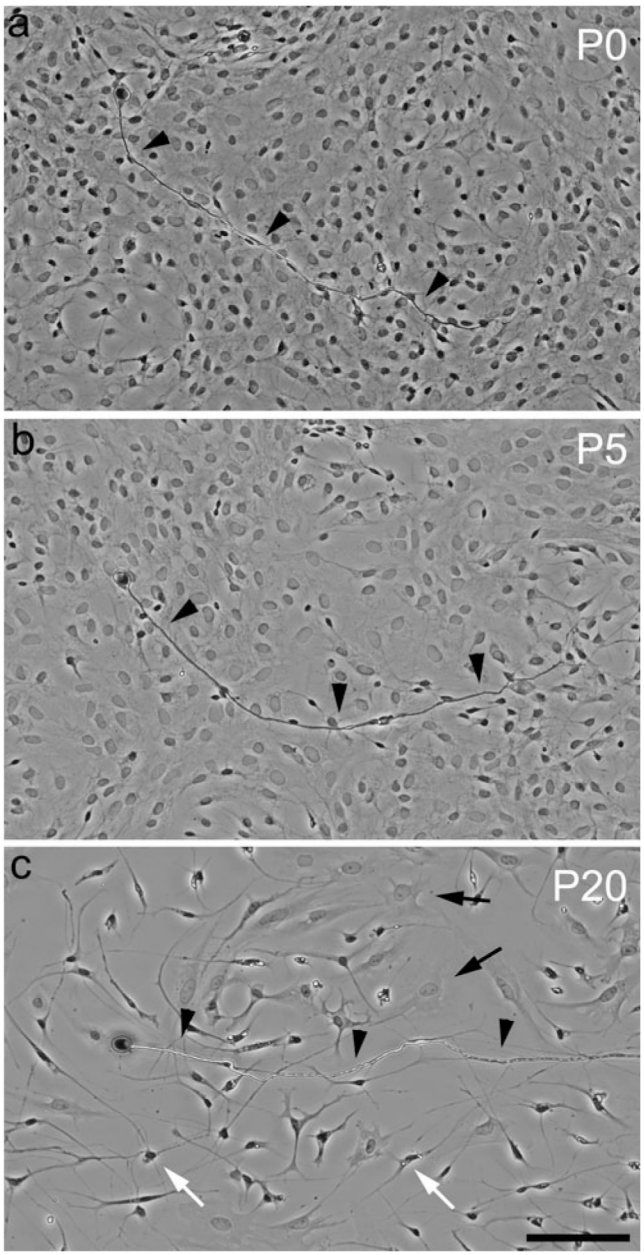

Fig. 2.

Representative phase-contrast images of postnatal day 0 (P0; **a**), P5 (**b**) and P20 (**c**) control cultures maintained for 12 h in serum-containing primary growth medium and for a further 72 h in serum-free maintenance medium (*arrowheads* neurites of SGNs). Non-neuronal cells in each culture can be morphologically differentiated into two cell types, i.e., *flat-shaped* cells with large nuclei and *spindle-shaped* cells with small nuclei and prominent processes. This differentiation is most readily observed in P20 culture (*black arrows* flat-shaped cells, *white arrows* spindle-shaped cells). The density of these non-neuronal cells decreases with the increasing age of the animals. In P0 and P5 cultures, the non-neuronal cells form a confluent layer on the glass surface, whereas the glass surface is partially free of cellular covering in P20 culture. *Bar* 0.1 mm

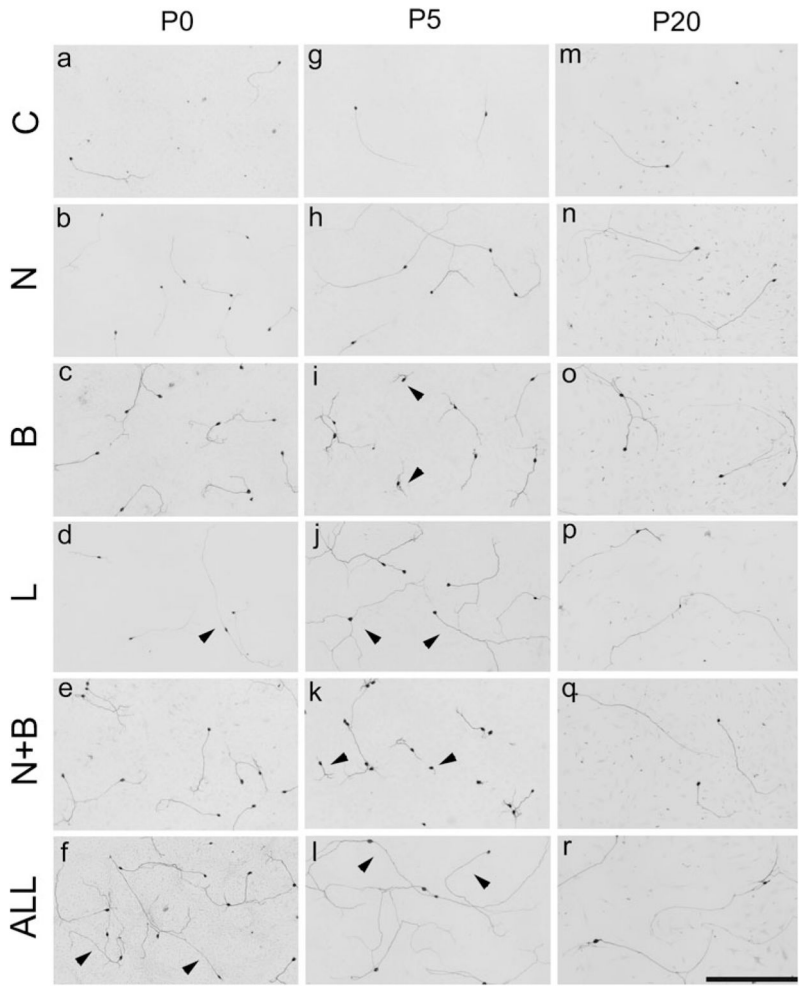

Fig. 3.

Representative photomicrographs of cultured SGNs in each age group. Neurons at P0 (**a**–**f**), P5 (**g**–**l**) and P20 (**m**–**r**) were cultured for 12 h in serum-containing primary growth medium and for a further 72 h in serum-free maintenance medium without any neurotrophic factors (**a**, **g**, **m**) or supplemented with 50 ng/ ml neurotrophin-3 (NT-3; *N*, **b**, **h**, **n**), brain-derived neurotrophic factor (BDNF; *B*, **c**, **i**, **o**), leukemia inhibitory factor (LIF; *L*, **d**, **j**, **p**), a combination of NT-3 and BDNF (50 ng/ml each; *N+B*, **e**, **k**, **q**), or a combination of NT-3, BDNF and LIF (50 ng/ ml each; *ALL*, **f**, **l**, **r**) and then fixed and immunostained with anti-NF200 antibody. Survival effects of NT-3, BDNF, LIF and their combinations compared with the untreated control varied depending upon the ages of SGNs. At P0, the additive/ synergistic effect of treatment in the N + B and ALL factors groups is clearly apparent, whereas this effect appears to be decreased in P5 and is not obvious in P20 cultures. The length of neurites seems to be increased by treatment with LIF or ALL factors in P0 and P5 cultures (*arrowheads* in **d**, **f**, **j**, **l**). In contrast, neurite length appears to be decreased by supplemention with BDNF or N + B in P5 cultures (*arrowheads* in **i**, **k**). *Bar* 0.5 mm

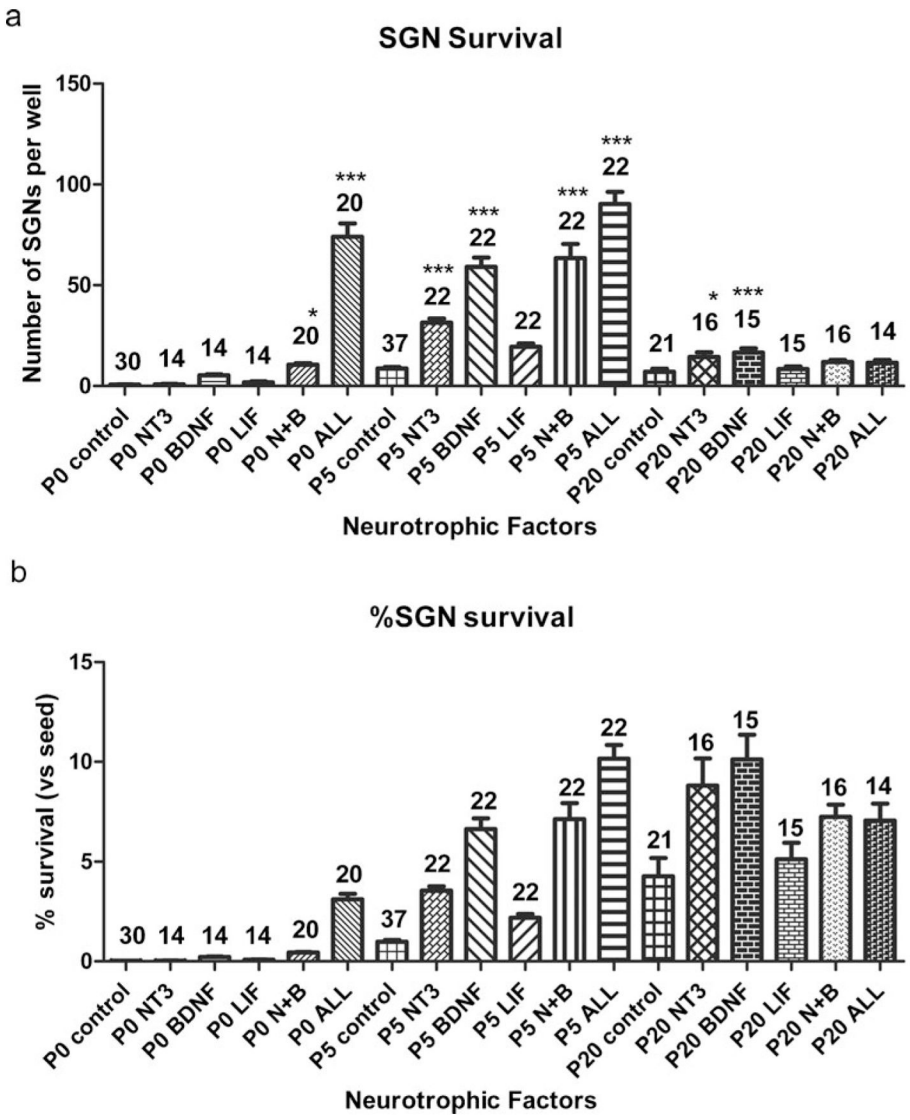

Fig. 4.

a Effects of neurotrophic factors on SGN survival. Dissociated SG cells at P0, P5 and P20 were plated at 20,000 cells/culture well and cultured for 12 h in serum-containing primary growth medium and for a further 72 h in serum-free maintenance medium without any neurotrophic factors (*control*) or supplemented with 50 ng/ml NT-3, BDNF, LIF, or N + B at 50 ng/ ml each or with ALL factors at 50 ng/ml each. The data are presented as an average number ± SE of surviving SGNs per culture well at each developmental age. Significant differences compared with control cultures are indicated: *P<0.05, **P<0.01, ***P<0.001 (ANOVA followed by Dunnett's post-hoc test). The *number above* each *bar* indicates the number of cultures analyzed. **b** Percentage of surviving SGNs divided by initial number of neurons seeded. Data are presented as an average percentage ± SE of surviving SGNs compared with the initial seed number of SGNs at each developmental stage. The *number above* each *bar* indicates the number of cultures analyzed. The survival rate of SGNs in each treatment group tended to increase with increasing age of the donor animals

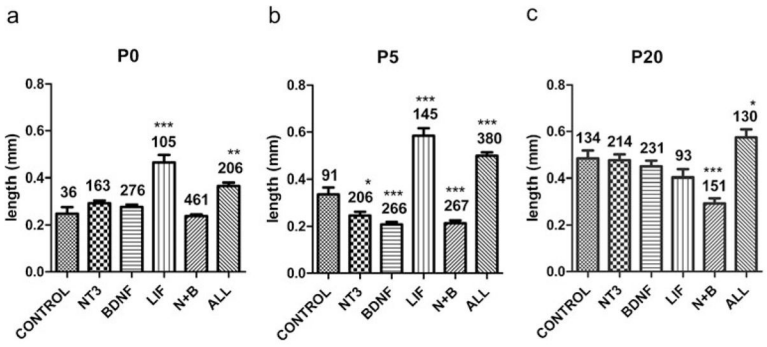

Fig. 5.

Effects of neurotrophic factors on the longest neurite length (LNL) in P0 (**a**), P5 (**b**) and P20 (**c**) cultures. Data are presented as an average length ± SE of the longest neurite of each neuron at each developmental age. Significant differences compared with control cultures (*CONTROL*) are indicated: **P*<0.05, ***P*<0.01, ****P*<0.001 (ANOVA followed by Dunnett's post-hoc test). The *number above* each *bar* indicates the number of neurons analyzed. LIF supplementation of P0 and P5 cultures and ALL factors added to P0, P5 and P20 cultures significantly increased LNL compared with control SGNs. In contrast, treatment with NT-3, BDNF, or N + B in P5 cultures and with N + B in P20 cultures inhibited neurite extension

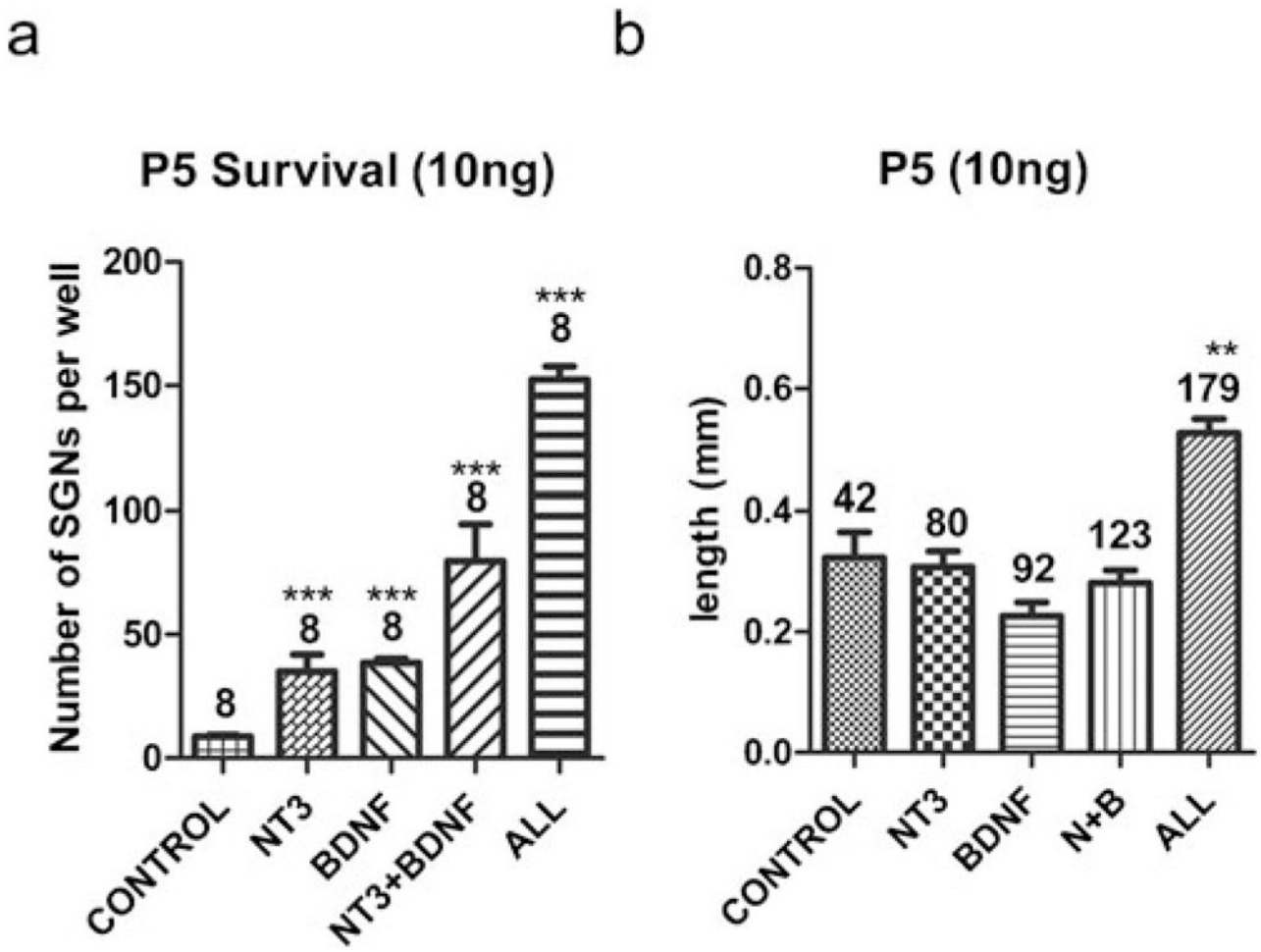

Fig. 6.

Effects of NT-3 and BDNF at 10 ng/ml on survival (**a**) and LNL (**b**) in P5 cultures. Significant differences compared with control cultures are indicated: **P*<0.05, ***P*<0.01, ****P*<0.001 (ANOVA followed by Dunnett's post-hoc test). The *number above* each *bar* indicates the number of cultures (**a**) and neurons (**b**) analyzed. At 10 ng/ml, NT-3, BDNF, or N + B enhanced SGN survival, as also seen at 50 ng/ml, whereas inhibitory effects on neurite extension were not observed at this concentration

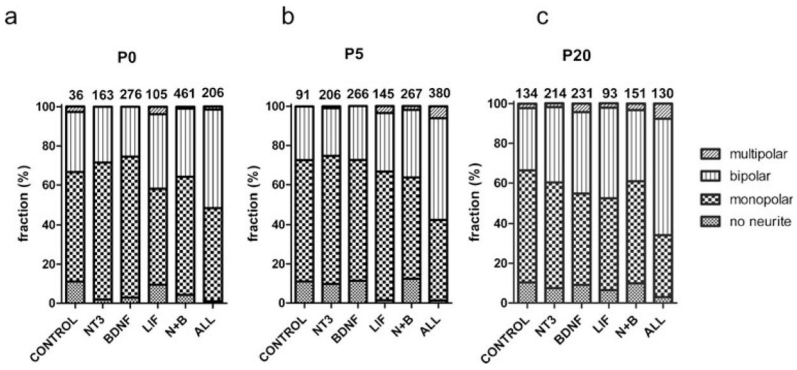

Fig. 7.

Effects of NT-3, BDNF, LIF, N + B, or ALL factors on neuronal morphology. The fraction of SGNs that were without a neurite or that were monopolar, bipolar and multipolar under each culture condition are indicated. ALL factors enhanced the proportion of bipolar neurons and reduced the number of neurons without neurites at all ages. The *number above* each *bar* indicates the number of neurons analyzed

Table 1

Conditions for dissociation of spiral ganglion neurons (SGNs) at postnatal day 0 (P0), P5 and P20

Treatment	P0	P5	P20
Duration of collagenase incubation (min)	15	20	30
Duration of trypsin incubation (min)	20	30	40

Table 2

Fraction of SGN without or with one neurite (referred to as the 0–1 neurite group) and SGN with two or more neuritis (referred to as the ≥2 neurite group).

Group	Control	NT-3	BDNF	LIF	NT-3 + BDNF	ALL
P0						
0–1 neurite group	24	115	205	61	296	100
≥2 neurite group	12	46	70	44	165	106*
P5						
0–1 neurite group	66	154	193	96	170	161
≥2 neurite group	25	52	73	48	97	219**
P20						
0–1 neurite group	89	129	127	49	92	45
≥2 neurite group	45	85	104*	44*	59	87***

Significant differences in the fraction of ≥2 neurite group compared with control culture are indicated:

* $P<0.05$,

** $P<0.01$,

*** $P<0.001$ (chi-square test)