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連絡先 〒951-8510 新潟市中央区旭町通1番町757

新潟大学医学部分子生理学分野 日比野 浩

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

Yulian Jin · Kenji Kondo · Munetaka Ushio ·  
Kimitaka Kaga · Allen F. Ryan · Tatsuya Yamasoba

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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 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.

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Y. Jin · K. Kondo (✉) · M. Ushio · K. Kaga · T. Yamasoba  
Department of Otolaryngology and Head and Neck Surgery,  
Graduate School of Medicine, The University of Tokyo,  
7-3-1 Hongo, Bunkyo-ku,  
Tokyo 113-8655, Japan  
e-mail: kondok-ky@umin.ac.jp

Y. Jin  
Department of Otolaryngology, Yanbian University Hospital,  
Yanji, China

K. Kaga  
National Institute of Sensory Organs,  
Tokyo, Japan

A. F. Ryan  
Department of Surgery, Division of Otolaryngology  
and Neurosciences, University of California, San Diego  
School of Medicine,  
La Jolla, Calif., USA

A. F. Ryan  
Veterans Administration Medical Center,  
La Jolla, Calif., USA

**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 (Fritzsche et al. 1997b, 2004; Rubel and Fritzsche 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; Fritzsche 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 (Orlke 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 subpopulation 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-sagittally. 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% collagenase 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 (Oriike 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  $\mu$ l per well. The cultures were maintained at 37°C in a humidified atmosphere containing 8% CO<sub>2</sub> for 2 h to promote the attachment of the neurons to the bottom of the coverslips. The cultures were then incubated in 300  $\mu$ l 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  $\mu$ l/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; Whilton 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% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) for 30 min at RT. After several PBS (pH 7.4) washes, endogenous 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.

**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

## 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  $\pm$  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 Dunnett'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  $\geq 2$  neurite group) and a chi-square test was used. Differences associated with *P* values of  $<0.05$  were considered as statistically significant.

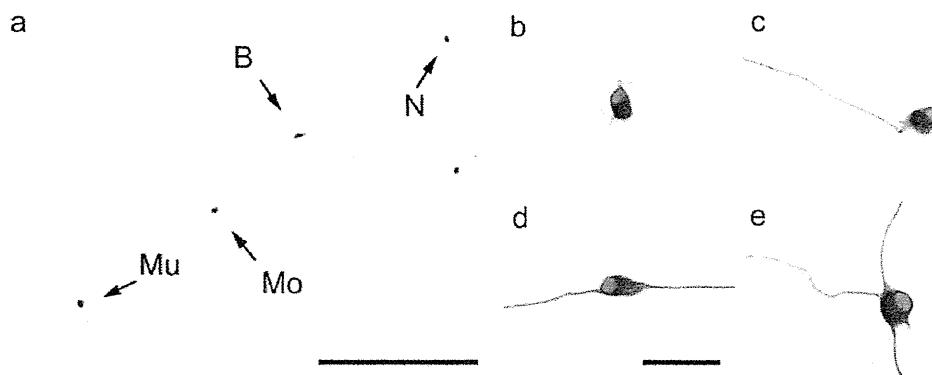
## Results

### 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  $2387 \pm 88$  in the P0 group,  $889 \pm 65$  in the P5 group and  $164 \pm 18$  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; Whitton 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-neuronal cells in P20 cultures (Fig. 2c).



**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  $\mu$ m (b–e)

#### 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 ( $8.7 \pm 0.7$ /well), followed by P20 cultures ( $7.0 \pm 1.5$ /well) and then by P0 cultures ( $0.6 \pm 0.2$ /well). Moreover, the greatest sensitivity to neurotrophic treatment was observed for P5 SGNs.

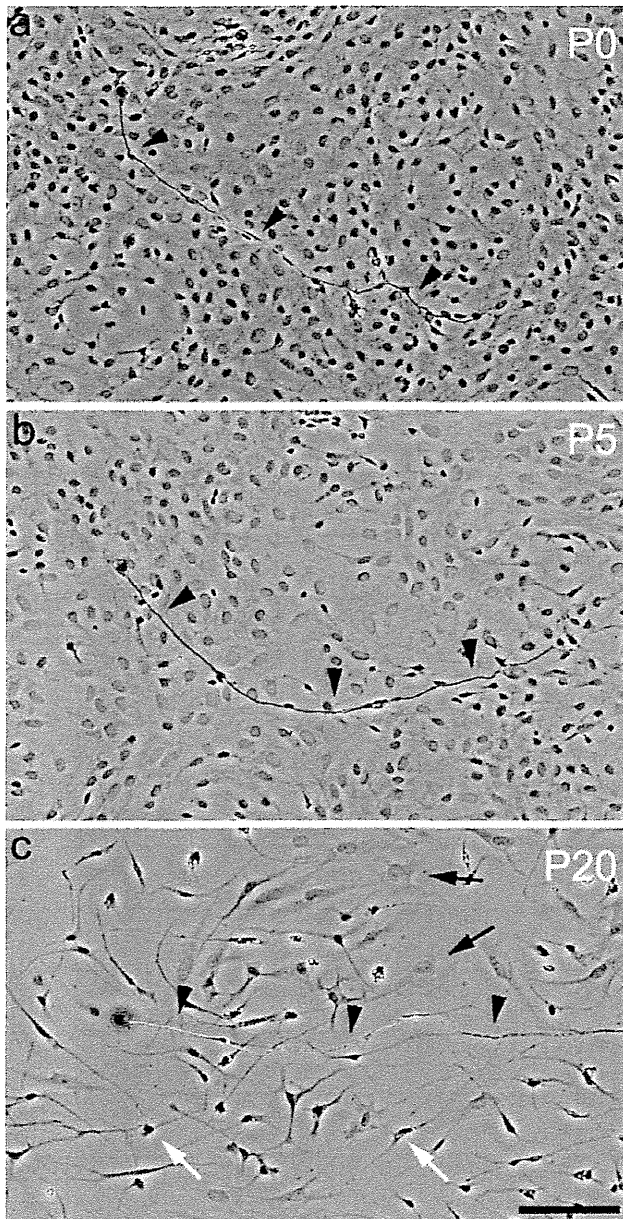
Because of the difference in the initial yield of SGNs among age groups, we normalized the number of surviving SGNs/well by the initial seeding level. These data are presented in Fig. 4b, which demonstrate that the survival rate of untreated SGNs in culture increases dramatically with increasing age. 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 ( $10.5 \pm 0.8$ ) increased to 17-fold that of untreated cultures ( $0.6 \pm 0.2$ ). This number almost corresponded to that of the sum of the number of surviving neurons in

NT-3 ( $0.9 \pm 0.2$ ) and BDNF cultures ( $5.3 \pm 0.5$ ). When the culture was treated with ALL factors, the number of surviving neurons further increased to 74-fold that of untreated cultures ( $74.0 \pm 6.7$ ), which is approximately 9.1-fold that of the sum of the number of surviving SGNs in NT-3-treated ( $0.9 \pm 0.2$ ), BDNF-treated ( $5.3 \pm 0.5$ ) and LIF-treated ( $1.9 \pm 0.4$ ) 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 ( $63.4 \pm 7.1$ ), which was 6.3-fold that of untreated cultures ( $10.0 \pm 1.1$ ), almost corresponded to the number of surviving neurons in BDNF cultures (5.9-fold,  $59.0 \pm 4.7$ ). When the culture was treated with ALL factors, the number of surviving neurons (nine-fold,  $90.3 \pm 6.0$ ) was slightly less than the sum of the number of SGNs treated with the individual factors: BDNF, NT-3 (3.2-fold,  $31.5 \pm 2.0$ ) and LIF (1.9-fold,  $19.4 \pm 1.6$ ).

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



**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

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 ( $P < 0.001$ ; Dunnett 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 ( $P < 0.001$ ).

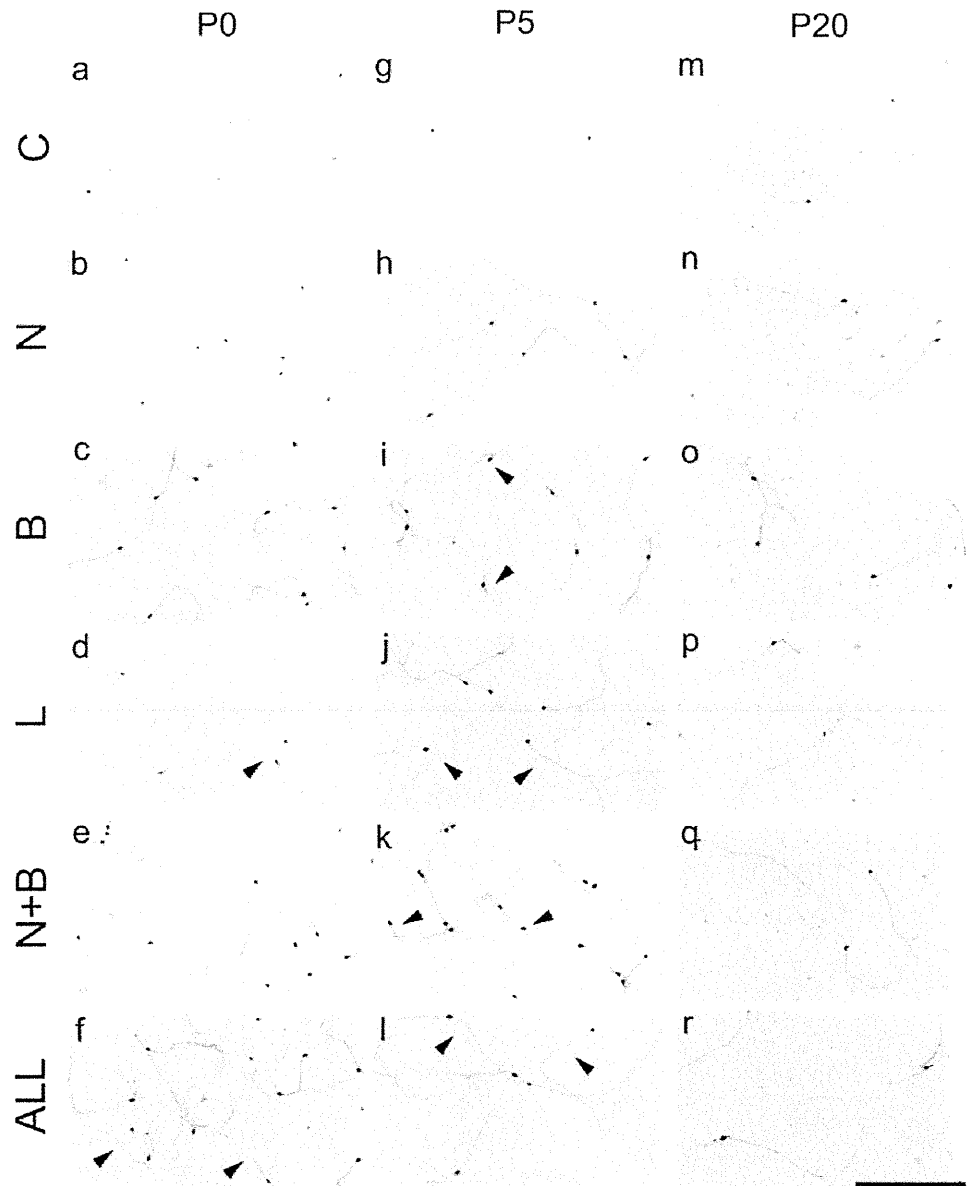
#### Effects of NT-3, BDNF and LIF on neuronal morphology

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  $\geq 2$  neurite SGNs compared with the control group (Table 2;  $P < 0.05$  in P0,



**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 supplementation with BDNF or N+B in P5 cultures (arrowheads in i, k). Bar 0.5 mm



$P < 0.001$  in P5 and P20; chi-square test). In the P20 group, BDNF alone and LIF alone also more modestly enhanced the proportion of  $\geq 2$  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

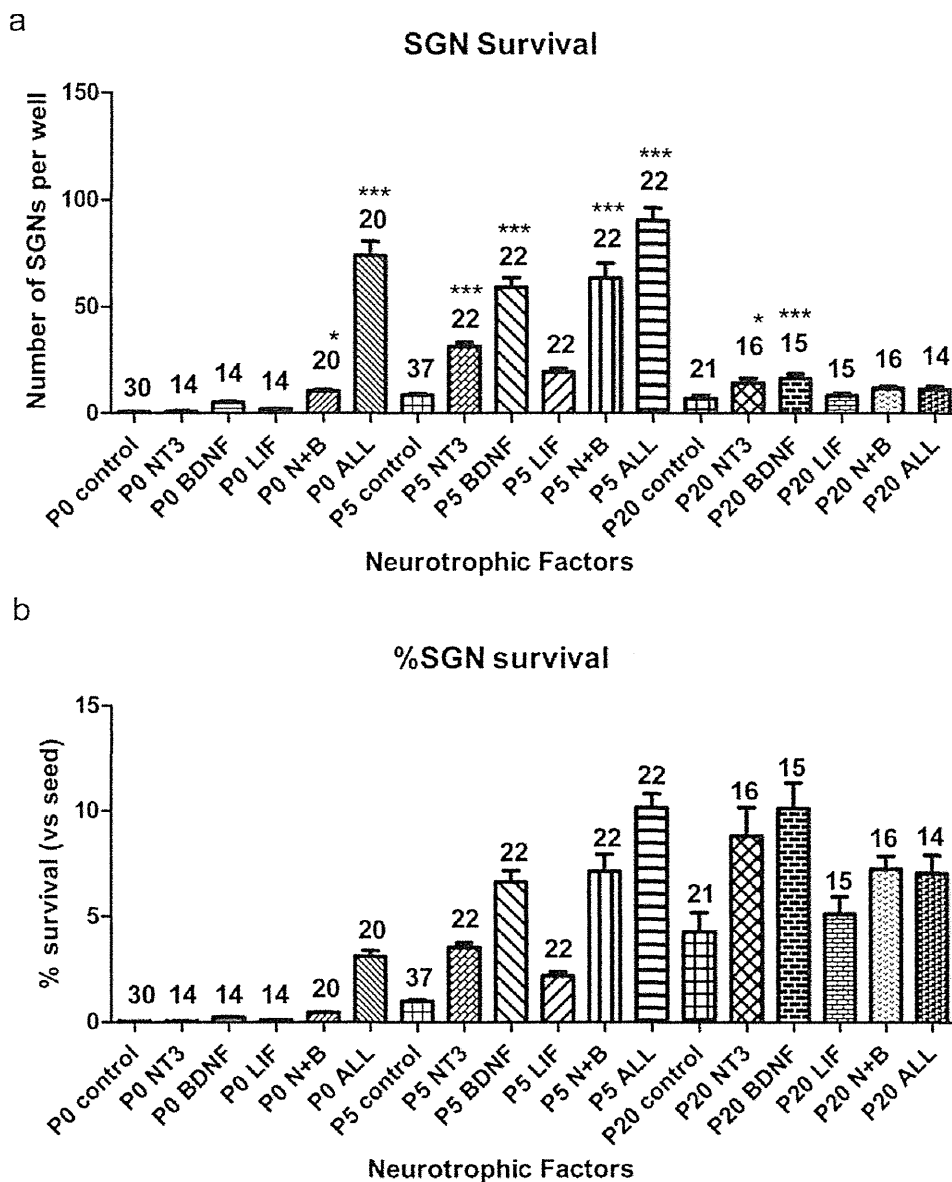
demonstrate that each of these indices of SGN maturation are differentially regulated by NT-3, BDNF and/or LIF in an age-dependent manner. Our data further suggest that each of these neurotrophic factors predominantly support different ontogenetic events at different developmental stages in the innervation 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-



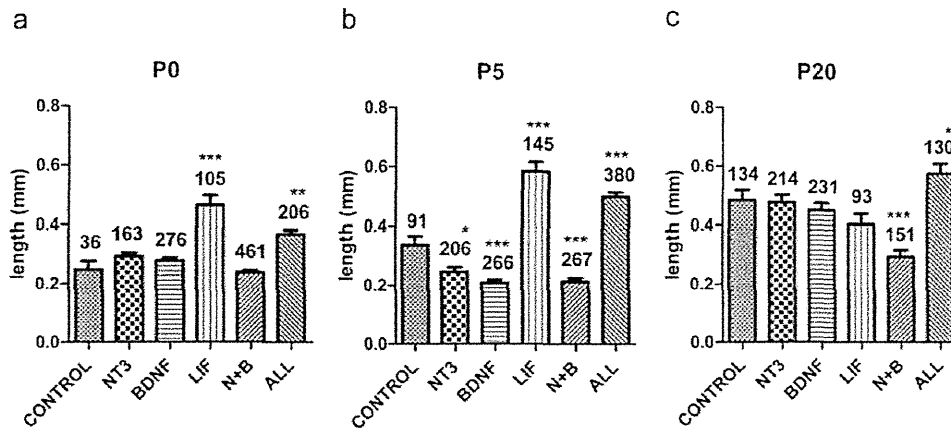
**Fig. 4 a** Effects of neurotrophic factors on SGN survival. Dissociated SG cells at P0, P5 and P20 were plated at 20,000 cells/culture well and cultured for 12 h in serum-containing primary growth medium and for a further 72 h in serum-free maintenance medium without any neurotrophic factors (*control*) 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  $\pm$  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  $\pm$  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



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).



**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  $\pm$  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

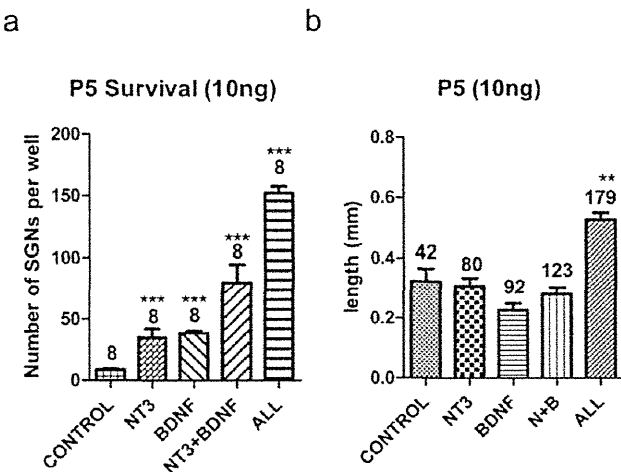
Although we could locate no published information regarding LIF expression in developing cochlear nucleus, LIF is expressed by many neurons in the brain (Lemke et al. 1996).

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 survival 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.

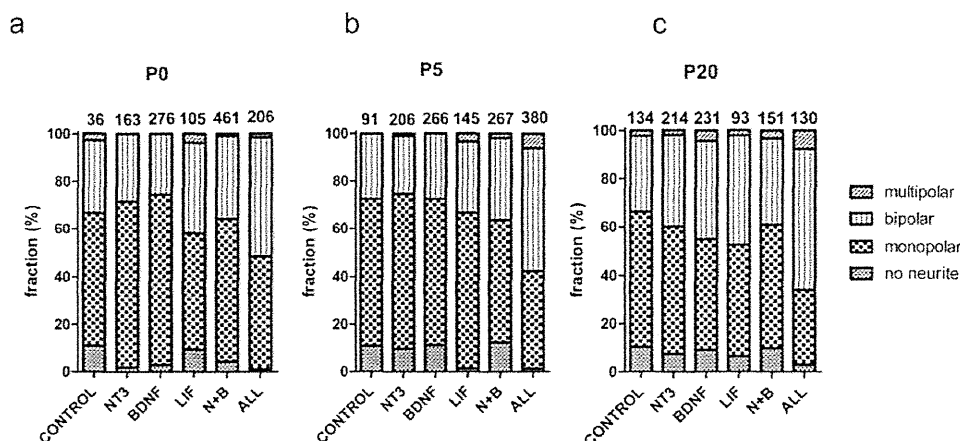
At P5 and P20, BDNF was more potent than NT-3 or LIF for survival. This observation is in good agreement with previous studies of dissociated early postnatal rat SGNs (Hegarty et al. 1997; Marzella et al. 1999; Zheng et al. 1995) and with the enhancement of the survival of adult SGNs by BDNF in vivo (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; Whitton 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.



**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



**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; Whitton 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.

**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). 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)

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

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 tympani (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 extension for NGF ( $\geq 50$  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 demonstrated to produce differential effects on central and peripheral neurite growth patterns of trigeminal ganglion neurons in whole-mount explant cultures (Ulu-pinar 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 supplementation 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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# Changes in responsiveness of rat spiral ganglion neurons to neurotrophins across age: differential regulation of survival and neuritogenesis

Kenji Kondo,<sup>1,2</sup> Kwang Pak,<sup>1</sup> Eduardo Chavez,<sup>1</sup> Lina Mullen,<sup>1</sup> Sara Euteneuer,<sup>1,3</sup> and Allen F. Ryan<sup>1</sup>

<sup>1</sup>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; <sup>2</sup>Department of Otorhinolaryngology and Head and Neck Surgery, Graduate School of Medicine, University of Tokyo, Tokyo, Japan; <sup>3</sup>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.

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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



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.

## 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 midsagittally. 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 there 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<sub>2</sub>, 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 sup-

plement (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, endogenous 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

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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- $\mu$ l solution, with 3  $\mu$ l of the reverse-transcribed complementary DNA (cDNA), 1  $\times$  PCR buffer, 1.0-U Taq DNA polymerase, 200-nM dNTPs, 1.5-mM MgCl<sub>2</sub>, 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  $\pm$  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 Dunnett *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.

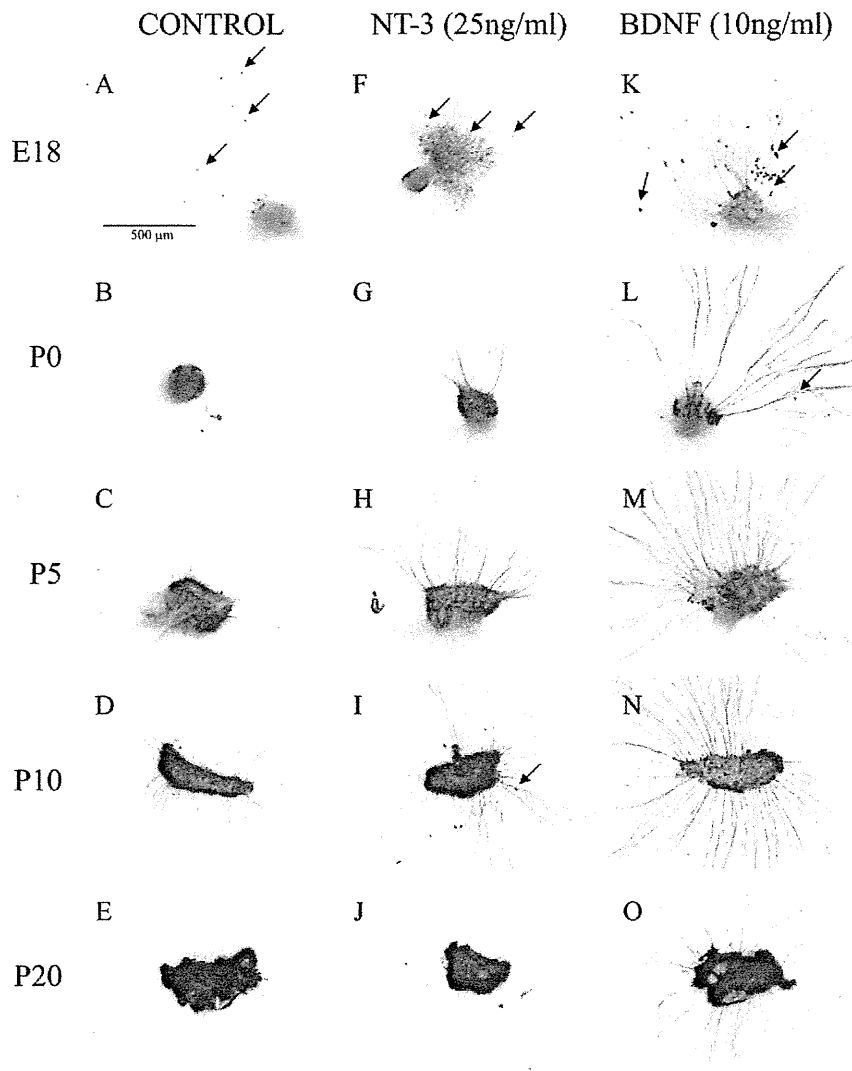


Figure 1. Representative photomicrographs of SG explants from E18 to P20 cochlea. Each explant was cultured for 3 days in media without neurotrophin (A–E) or supplemented with either 25 ng/ml of NT-3 (F–J) or 10 ng/ml of BDNF (K–O), the lowest maximally effective dose of each factor. Explants were then 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.

## 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, 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

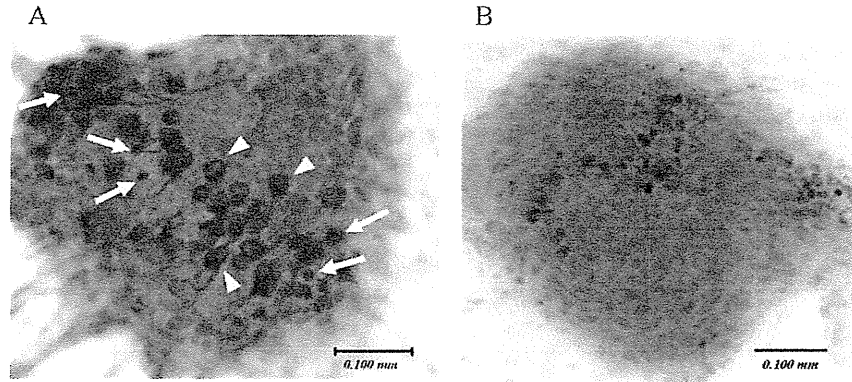


Figure 2. Degenerating neuronal cells in a P0 explant culture (A). The explant was maintained for 3 days in maintenance medium supplemented with BDNF (10 ng/ml), then fixed and immunostained with anti-NF200 antibody. Degenerating cells (white arrows) are small, exhibit condensed immunolabeling for NF200, 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.

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 nuclei. The average number of such cells per explant in P0 control explants ( $22.9 \pm 7.7$ ) was significantly greater than in E18 ( $11.7 \pm 4.4$ ,  $p < 0.02$ ) or P5 ( $2.3 \pm 0.6$ ,  $p < 0.01$ ) 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  $15.7 \pm 1.6$  at E18,  $58.2 \pm 13.0$  at P0 and  $38.7 \pm 10.2$  at P5. The number of labeled cells at P0

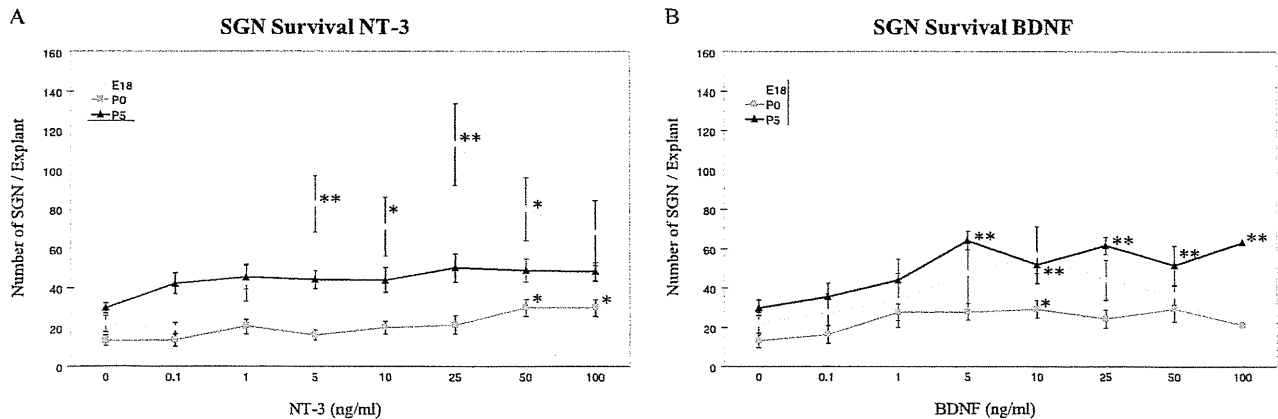


Figure 3. Dose-response effects of neurotrophins on SGN survival in E18, P0 and P5 explant cultures. Graphs show the average number of surviving SGNs observed after 3 days in maintenance medium with various concentrations of NT-3 (A) or BDNF (B). Error bars represent the standard error of the mean. Asterisks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

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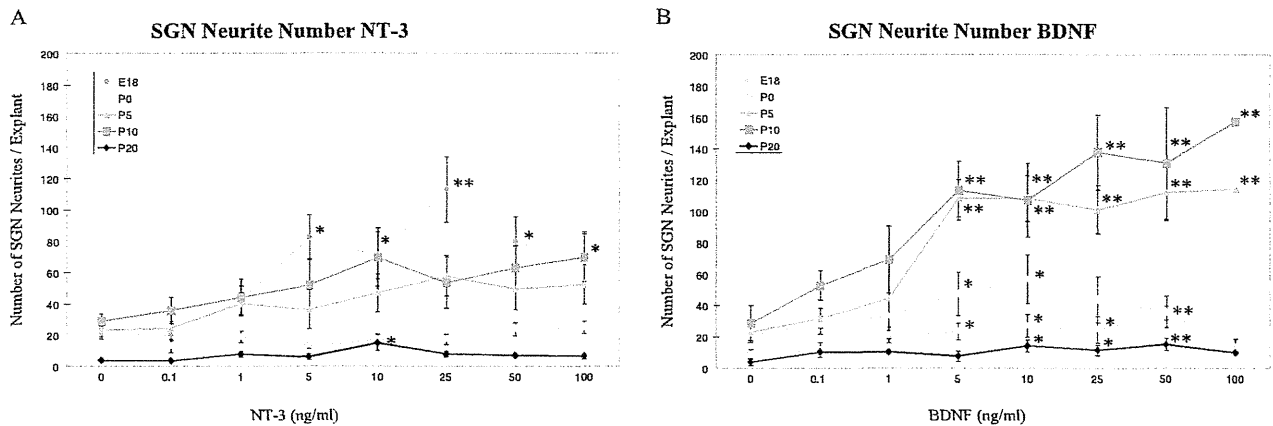


Figure 4. Dose-response effects of neurotrophins on the neurite number observed in SG explant cultures at E18, P0, P5, P10 and P20. Graphs show the average number of neurites emanating from each explant after 3 days in maintenance medium with various concentrations of NT-3 (A) or BDNF (B). Error bars 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).

was significantly larger than the other ages ( $p < 0.01$ ), representing  $\sim 10\%$  of all neurons present.

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

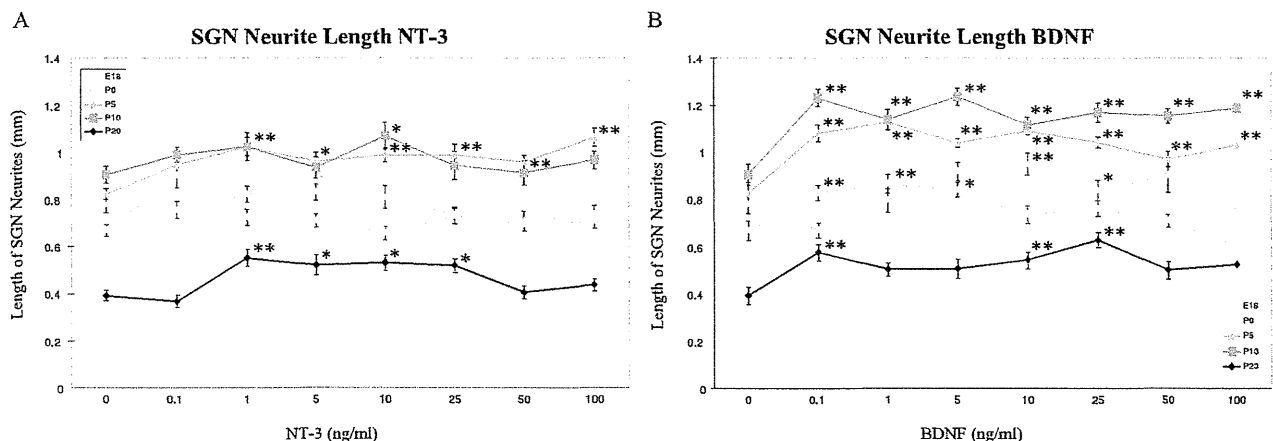


Figure 5. Dose-response effects of neurotrophins on the neurite extension observed in SG explant cultures at P0, P5, P10 and P20. Graphs show the average length of longest neurites selected from each explant (5 neurites/explant) and pooled in the same culture condition. Each explant was cultured for 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).