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REGULAR ARTICLE

Developmental changes in the protective effect of exogenous brain-derived neurotrophic factor and neurotrophin-3 against ototoxic drugs in cultured rat vestibular ganglion neurons

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Abstract We examine developmental changes in the responsiveness of rat vestibular ganglion neurons (VGNs) to two neurotrophic factors (NTFs), brain-derived neurotrophic factor (BDNF) and neurotrophin-3 (NT-3) and investigate the protective effects of these NTFs against ototoxic drugs during postnatal development in dissociated cultures. VGNs were obtained from rats on postnatal days (P) 1, 3, 7 and 14. BDNF facilitated neuronal survival as well as neurite sprouting of VGNs obtained from younger rats (P1 and P3), whereas these effects were not observed in older rats (P7 and P14). BDNF was also effective in facilitating neurite extension in VGNs at each of the postnatal ages. NT-3 also facilitated neuronal survival and neurite extension of VGNs from younger rats but these effects were significantly smaller than those of BDNF ($p<0.05$). The protective effects of BDNF and NT-3 against ototoxic drugs, gentamicin and cisplatin, were also age-dependent: they were effective for neuronal survival, neurite sprouting and neurite extension in VGNs from younger rats, whereas these effects tended to disappear in VGNs from older rats. Analysis of the changes in the expression of the receptors of NTFs revealed that expression of TrkB and TrkC proteins and their mRNA did not change during the developmental period, whereas expression of $p75^{NTR}$ protein was down-regulated together with that of $p75^{NTR}$ mRNA during the developmental period. Developmental changes in the responsiveness to exogenous NTFs in VGNs, which is not caused by the changes of their receptors but probably caused by changes in the intracellular signaling pathways, should be taken into consideration in the prevention of neuronal degeneration caused by ototoxic drugs.

Keywords Vestibular ganglion · BDNF · NT-3 · Ototoxicity · Gentamicin

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Introduction

Brain-derived neurotrophic factor (BDNF) and neurotrophin-3 (NT-3) are two neurotrophic factors (NTFs) involved in regulating the development and maintenance of numerous populations of neurons in both the peripheral and the central nervous system (Kaplan and Miller 2000; Reichardt 2006). Both BDNF and NT-3 play important roles in regulating cell fate decisions, axon growth, dendrite growth, the pruning of neurons during embryonic stages (Fritzsch et al. 2004; Lessmann et al. 2003) and sustaining neuronal cell survival and morphology, as well as cell differentiation after birth (Skaper 2008). Signal transduction by these NTFs is initiated by activation of the high-affinity tropomyosin receptor kinase (Trk) family of receptor tyrosine kinases with some contribution from the low-affinity pan-neurotrophin receptors ($p75^{NTR}$). In vitro studies of both neuronal and non-neuronal cells have shown that BDNF is the specific ligand of TrkB whereas NT-3 preferentially interacts with TrkC. Both BDNF and NT-3 also bind to $p75^{NTR}$ (Kaplan and Miller 2000; Reichardt 2006).

Vestibular systems help to maintain posture, equilibrium and ocular stability during head movements through the vestibulo-ocular and vestibulo-spinal reflexes (Baloh and Honrubia 2001). Vestibular ganglion neurons (VGNs) are primary neurons whose neurites link the vestibular end-organs to the vestibular nuclei of the brainstem. It has been clearly shown as a result of studies on mutant mice lacking each gene that survival of VGNs and their innervations are dependent on BDNF and NT-3 (Ernfors et al. 1994, 1995). Other studies have shown that the mRNA of BDNF and NT-3 are present in the vestibular sensory epithelia, while mRNAs encoding their respective receptors are present in VGNs during the prenatal stages and that both BDNF and NT-3 have promoting effects on neuronal survival and neurite outgrowth in inner ear sensory neurons obtained from prenatal rats (Pirvola et al. 1992, 1994). Although BDNF, NT-3 and their receptors are still present after birth (Ylikoski et al. 1993), their role during the postnatal period remains unclear. We have previously examined the effects of exogenous NTFs on cultured VGNs obtained from developing rats and shown that the responsiveness to BDNF and NT-3 is age-dependent in rat VGNs (Chihara et al. 2011).

Since NTFs have effects on sustaining neuronal cell survival, morphology and differentiation after birth (Skaper 2008), they have been a major target for therapeutic agents in neural disorders (Nagahara and Tuszynski 2011). In the audiovestibular systems, BDNF and NT-3 have been reported to be effective in promoting neuronal survival and protecting cells from ototoxic damage caused by cisplatin (Cis) or gentamicin (GM) in spiral ganglion neurons from adult mice (Vieira et al. 2007) and in VGNs from postnatal day (P) 5 rats (Zheng et al. 1995). However, it remains unclear whether the protective effect of exogenous NTFs against damage caused by ototoxic drugs undergoes developmental changes in VGNs.

In the present study, we investigate in vitro the effects of BDNF and NT-3 in the presence of ototoxic drugs on rat VGNs at different postnatal days. Furthermore, to investigate the mechanism underlying the developmental changes in the responsiveness to the NTFs, we examine the expression of TrkB, TrkC and $p75^{NTR}$ by immunohistochemisty, western blotting and quantitative real-time reverse transcriptase-polymerase chain reaction (RT-PCR). Our results showed that the protective effects of NTFs against ototoxic damage change during the early postnatal period while the expression of their specific receptors did not change during the same period.

Materials and methods

Dissociated cell cultures of VGNs

Wistar rats were deeply anesthetized with etherization and sacrificed by decapitation in accordance with the Institutional Animal Care and Use Committee of the University of Tokyo. Superior vestibular ganglia were isolated from rats at P1, 3, 7 and 14. The dissected vestibular ganglia were incubated in Hank's solution (Gibco, Gaithersburg, MD, USA) with papain (20U/ml; Worthington Biochemical, Freehold, NJ, USA) at 37 °C for 20 min. Cells were dissociated by trituration using a sterile 500-μl pipette. The plating medium contained Leibovitz's L-15 solution (Gibco BRL, Grand Island, NY, USA), 10 % fetal calf serum, 26 mM $NaHCO_3$, 30 mM glucose and 50 U/ml penicillin. To determine the initial yield, the cells in the combined supernatants were counted with a hemocytometer and adjusted to 5.0×10^5 cells/ml in the above culture medium. The cells were plated onto poly-L-lysine-pretreated 13-mm-diameter cover glasses. When plating VGNs, each well received 300 μl of culture medium (containing 15.0×10^4 cells) with either: 50 ng/ml BDNF (Upstage, Lake Palcid, NY), or 50 ng/ml NT-3 (Calbiochem, Lajolla, CA, USA), or 0.5 mg/ml GM (Sigma, St. Louis, MO), or 2 μM Cis (Sigma), or both 50 ng/ml BDNF and 0.5 mg/ml GM, or both 50 ng/ml BDNF and 2 μM Cis, or both 50 ng/ml NT-3 and 0.5 mg/ml GM, or both 50 ng/ml NT-3 and 2 μM Cis. The control group had nothing added to their wells. Cells were maintained in an humidified atmosphere of 95 % air–5 % CO_2 at 37 °C for 48 h.

Immunocytochemistry

Seeded VGNs were fixed in 4 % paraformaldehyde in phosphate buffer saline (PBS; pH 7.4) for 30 min and immunostaining was performed using the avidin-biotin complex (ABC) method (Vectastain Elite kit; Vector Laboratories, Burlingame, CA, USA). Culture dishes were treated with 3 % hydrogen peroxide in methanol for 15 min at room temperature. After blocking with normal 4 % normal goat serum in PBS containing 0.3 M NaCl, 0.1 % Triton X-100 and 0.1 % NaN_3 for 30 min, the cultures were incubated overnight with the primary antibody, mouse monoclonal anti-neurofilament antibody (NF-200, clone NR52; Sigma) at dilutions of 1:1,000. After being washed with PBS three times, the cultures were incubated for 30 min in the secondary antibody, goat anti-rabbit biotinylated IgG (Vector Laboratories) and subjected to diaminobenzidine (DAB) –H_2O_2-$NiSO_4$ (Vector Laboratories) intensification for 2–3 min. After washing in distilled water, the cultures were dehydrated through a graded ethanol series and xylene, then mounted on glass slides for permanent preparation. Antibody specificity was confirmed by omission of the primary antibody and by pre-adsorption of the primary antibody with the target peptide. At least nine wells were analyzed for each condition.

Tissue preparation and immunohistochemistry

The superior vestibular ganglia on both sides were embedded in paraffin. Serial sections (4 μm) were made longitudinally.

The ABC method was used on the serial sections to compare the distribution of neurotrophin receptors. Mouse monoclonal antibodies against TrkB (BD Bioscience, Tokyo, Japan) were diluted 1/100, rabbit monoclonal antibody against TrkC (Cell Signaling Technology, Danverse, MA, USA) were diluted 1/1,000 and rabbit monoclonal antibody against $p75^{NTR}$ (Cell Signaling Technology) were diluted 1/1,000 in 0.1 M PBS containing 1 % goat serum albumin and 0.1 % Triton X-100. After blocking for avidin and biotin (Avidin/Biotin Blocking kit; Vector Laboratories), serial sections were incubated with the antibodies overnight, rinsed in PBS and incubated for 1 h with goat anti-mouse IgG (Vector Laboratories) diluted 1/500 for TrkB and with goat anti-rabbit IgG (Vector Laboratories) diluted 1/500 for TrkC and $p75^{NTR}$. They were then incubated with ABC-alkaline phosphatase (ABC-AP kit; Vector Laboratories). After being washed with distilled water, sections were counterstained with hematoxylin, dehydrated and mounted. For negative controls, the primary antibody was replaced with mouse IgG (Vector Laboratories) diluted 1/100 or rabbit IgG (Vector Laboratories) diluted 1/1,000. No obvious labeling corresponding to immunostaining by the primary antibodies was identified.

Western blotting

Proteins were separated on a 7.5 % polyacrylamide gel for 70 min at 200 V and then transferred to Amersham Hybond-P (GE Healthcare UK, Buckinghamshire, UK) for 75 min at 72 mA. The membrane was blocked with 3 % skim milk in PBS with 0.1 % Tween 20 (PBS-T) for 1 h. It was probed overnight at 4 °C with rabbit monoclonal antibodies against TrkB (Cell Signaling Technology) diluted to 1/500, TrkC (Cell Signaling Technology) diluted to 1/1,000 and $p75^{NTR}$ (Cell Signaling Technology) diluted to 1/500 and a mouse monoclonal antibody against β-actin (Sigma Aldrich) diluted to 1/500,000, then washed three times for 15 min in PBS-T and subsequently incubated with anti-rabbit IgG linked to peroxidase (Santa Cruz, Dallas, TX, USA) and anti-goat IgG linked to peroxidase (Santa Cruz) diluted to 1/1,000 with blocking buffer for 1 h at room temperature. After three additional washes, bound antibodies were detected by an Amersham ECL-plus (GE Healthcare UK) and analyzed with a LAS-3000 mini image analyzer (Fuji-Film, Tokyo, Japan). The expression level of β-actin was analyzed and used to normalize the value of the expression level of TrkB, TrkC and $p75^{NTR}$ proteins. For negative controls, we confirmed that no obvious band was identified when the primary antibody was replaced with rabbit IgG (Vector Laboratories) diluted 1/500. For positive controls, we confirmed the presence of immunoreactive bands corresponding to the molecular mass of each antibody in rat neocortex.

Real-time quantitative RT-PCR

Total cellular RNA was extracted from the vestibular ganglia using Trizol® (Invitrogen Life Technology, Carlsbad, CA, USA). For RT-PCR, complementary DNA (cDNA) was synthesized from 1 μg of total RNA with reverse transcriptase with random primers (Toyobo, Osaka, Japan). The reaction mixture was then subjected to PCR amplification with specific forward and reverse oligonucleotide primers for 35 cycles consisting in heat denaturation, annealing and extension. PCR products were size-fractionated on 2 % agarose gels and visualized under UV light. The primers chosen based on the sequence of rat TrkB, TrkC and $p75^{NTR}$ are shown in Table 1. Total RNA from adult mouse brain was used as a positive control.

Real-time quantitative RT-PCR was performed with the use of real-time Taq-Man technology and a sequence detector (ABI PRISM 7000; Applied Biosystems, Foster City, CA, USA). Gene-specific primers and Taq-Man probes were used to analyze transcript abundance. The 18S ribosomal RNA level was analyzed as an internal control and used to normalize the values for transcript abundance of each gene.

The probes used in this study were purchased as a part of a TaqMan Gene Expression Assay from Applied Biosystems: Rn01441719_m1 for TrkB, Rn00570389_m1 for TrkC and Rn00561634_m1 for $p75^{NTR}$. We performed six independent experiments.

Data analysis

Photomicrographs of immunostained cultures on glass slides were captured on an AxioVison digital camera (Zeiss) with a ×4 or ×10 objectives. Digital slide images were processed using Micro Analyzer analysis software, which enabled us to count the number of cells manually and measure the length of arbitrary lines, such as neurite outgrowth trajectory (Nihon

Table 1 PCR primers used for amplification of neurotrophin receptors

	Size (bp)		Sequence (′5-′3)
TrkB	486	Sense	GGC CAA GAA TGA ATA TGG GAA
		Antisense	TTG AGC TGG CTG TTG GTG AT
TrkC	527	Sense	AGC TGC TCA CTA ACC TGC AGC ATG
		Antisense	GGT AAA GAT CTC CCA AAG AAT AAC
$p75^{NTR}$	553	Sense	TGC TGC TGC TGC TGA TTC TA
		Antisense	GAC CTT GGG ATC CAT CGA C
GAPDH	162	Sense	TGA GGA CCA GGT TGT CTC CT
		Antisense	ATG GTA GGC CAT GAG TCC AC

Poladigital, Tokyo. Japan). The number of surviving VGNs, the ratio of VGNs with neuritis to total number of surviving VGNs and the longest neurite length of each VGN were measured. We counted all surviving VGNs in each well. Our criteria for identifying surviving neurons were based on their immunostained morphology: (1) positivity for NF200 and (2) a visible nucleus. Next, we divided VGNs into two groups: VGNs with neurite sprouting and VGNs without neurite sprouting. For VGNs with neurite outgrowth, we measured the length of the longest neurite of each VGN. When the number of VGNs with neurite outgrowth was over 30 neurons in a well, we selected 30 neurons at random to measure their neurite length. Randomization was performed using a computer program (Microsoft Excel 2003; Redmond, WA, USA). When measuring neurites length, only those VGNs whose processes did not overlap and could be traced clearly in the images were scored.

Data are presented as means±SEM. Statistical significance was examined using a Kruskal–Wallis test with a post hoc Steel-Dwass test. Differences were considered significant when $p<0.05$.

Results

Developmental changes in the effectiveness of NTFs on cell survival, neurite sprouting and neurite extension

VGNs were obtained from rats at P1, 3, 7 and 14. After 48 h in culture, the effect of exogenous 50 ng/ml BDNF or 50 ng/ml NT-3 on cell survival, neurite sprouting and neurite extension was examined.

In the absence of exogenous NTFs, the number of surviving neurons, the ratio of neurons with neurites and the length of the longest neurites were significantly dependent on their postnatal ages ($p<0.01$; Figs. 1 and 2). The number of surviving neurons and the ratio of neurons with neurites obtained from P1, 3 and 7 rats were significantly greater than those from P14 rats ($p<0.01$). On the other hand, the length of the longest neurites in control conditions was greatest at P3 and significantly greater than those from P1, 7 and 14 rats ($p<0.01$).

The effects of BDNF and NT-3 on cell survival, neurite sprouting and neurite outgrowth were also dependent on their postnatal ages (Fig. 2). Both BDNF and NT-3 significantly increased the number of surviving neurons obtained from younger rats (P1 and P3) when compared to control condition ($p<0.01$: Fig. 2a, b), whereas neither of BDNF nor NT-3 had significant effects on the number of surviving neurons from older rats (P7 and P14; $p>0.1$). BDNF also significantly increased the ratio of VGNs with neurites from younger rats (P1 and P3; $p<0.05$ for P1 and $p<0.01$ for P3; Fig. 2c, d) and the length of the longest neurites from each of the postnatal ages ($p<0.01$; Fig. 2e, f). On the other hand, NT-3 increased the length of neurites of VGNs only from P1 rats ($p<0.01$; Fig. 2e, f), while it did not increase the ratio of VGNs with neurites from any of the postnatal ages (Fig. 2c, d).

To investigate the possibility that different concentrations of BDNF and NT-3 might have some effects on VGNs from older rats, we also tested 1, 50 and 200 ng/ml of these two NTFs in VGNs from P14 rats. But none of them had any significant differences on cell survival, neurite sprouting and neurite outgrowth in VGNs from P14 rats ($p>0.8$; Supplementary Fig. 1).

Developmental changes in the protective effect of neurotrophins against gentamicin and cisplatin

Exogenous application of GM (0.5 mg/ml) or Cis (2 μM) significantly decreased the number of surviving neurons, the

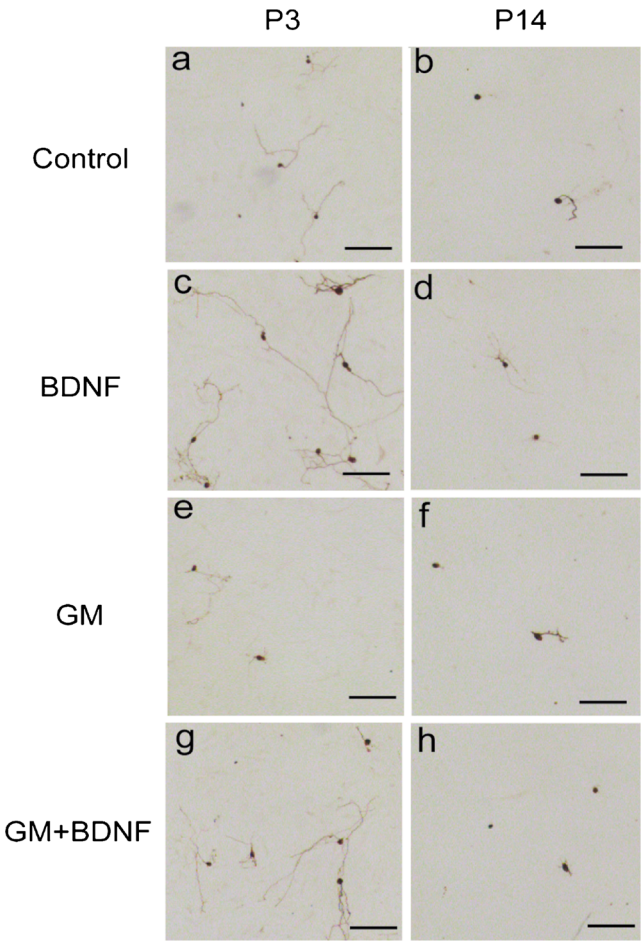

Fig. 1 Representative images of vestbular ganglion neurons (VGNs) obtained from postnatal days (P) 3 (**a**, **c**, **e**, **g**) and 14 rats (**b**, **d**, **f**, **h**) after 48 h in dissociated culture. Viable VGNs were stained by a neurofilament monoclonal antibody (NF-200) in the control condition (**a**, **b**) and in the presence of 50 ng/ml brain-derived neurotrophic factor (*BDNF*; **c**, **d**), 0.5 mg/ml gentamicin (*GM*; **e**, **f**), or both GM and BDNF (*GM+ BDNF*; **g**, **h**). *Bars* 50 μm

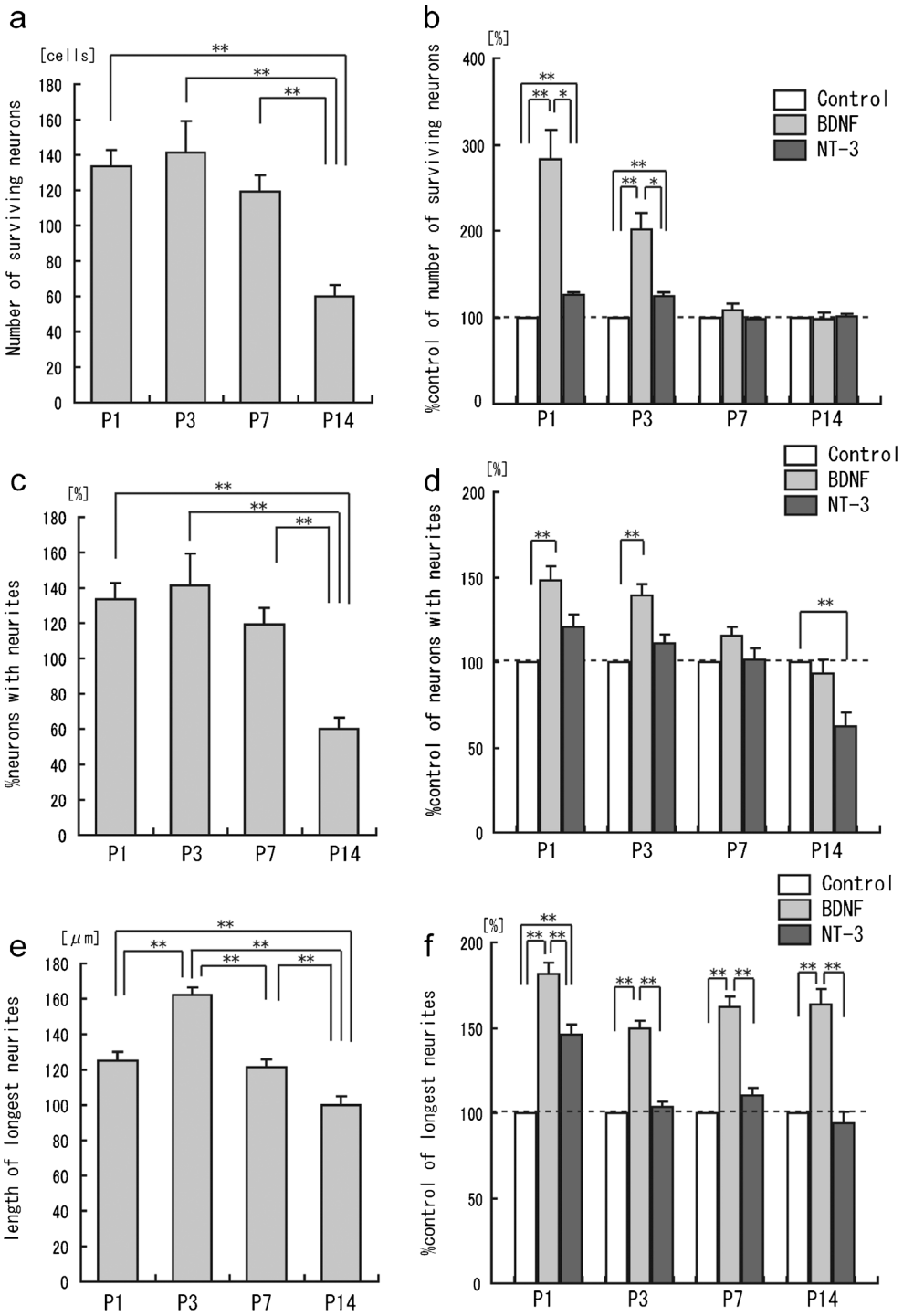

Fig. 2 The effects of BDNF and neurotrophin-3 (*NT-3*) on neuronal survival, neurite sprouting and neurite extension in VGNs obtained from developing rats. **a**. Number of surviving VGNs in the control condition. **b**. The ratio (%) of the number of surviving VGNs in the presence of 50 ng/ml BDNF or 50 ng/ml NT-3 relative to the control condition. Number of surviving neurons in control condition was expressed as 100 % (*dashed line*) in the *righthand* graph. **c**. The ratio (%) of VGNs with neurites to total surviving VGNs in the control condition **d**. The % control of the ratio of VGNs with neurites in the presence of BDNF and NT-3. The ratio of VGNs with neurites in the control condition was expressed as 100 % (*dashed line*) in the *righthand* graph. **e**. Length of the longest neurites in the control condition. **f**. The % control of the length of VGNs in the presence of BDNF and NT-3. The length of the longest neurites in the control condition was expressed as 100 % (*dashed line*) in the *righthand* graph. Each *bar* represents a mean for 70–120 VGNs. *$p<0.05$, **$p<0.01$

ratio of neurons with neurites and the length of neurites in VGNs from most of the postnatal ages ($p<0.05$; Fig. 2e, f).

Application of BDNF together with GM or Cis significantly increased the number of surviving neurons compared to the condition without NTFs in VGNs obtained from younger rats (P1 and P3: $p<0.01$; Fig. 3a), whereas these effects disappeared in VGNs from older rats (P7 and P14: $p>0.3$) except against Cis at P7 ($p<0.05$). BDNF also significantly increased the ratio of neurons with neurites from younger rats (P1 and P3) against GM or Cis ($p<0.01$ at P3 for GM and at P1 for Cis; $p<0.05$ at P1 for GM, and at P3 and P7 for Cis; Fig. 3b) and significantly increased the length of neurites from each of the postnatal ages against GM or Cis ($p<0.01$; Fig. 3c) except the length of neurites from P14 rats against GM ($p>0.05$). The

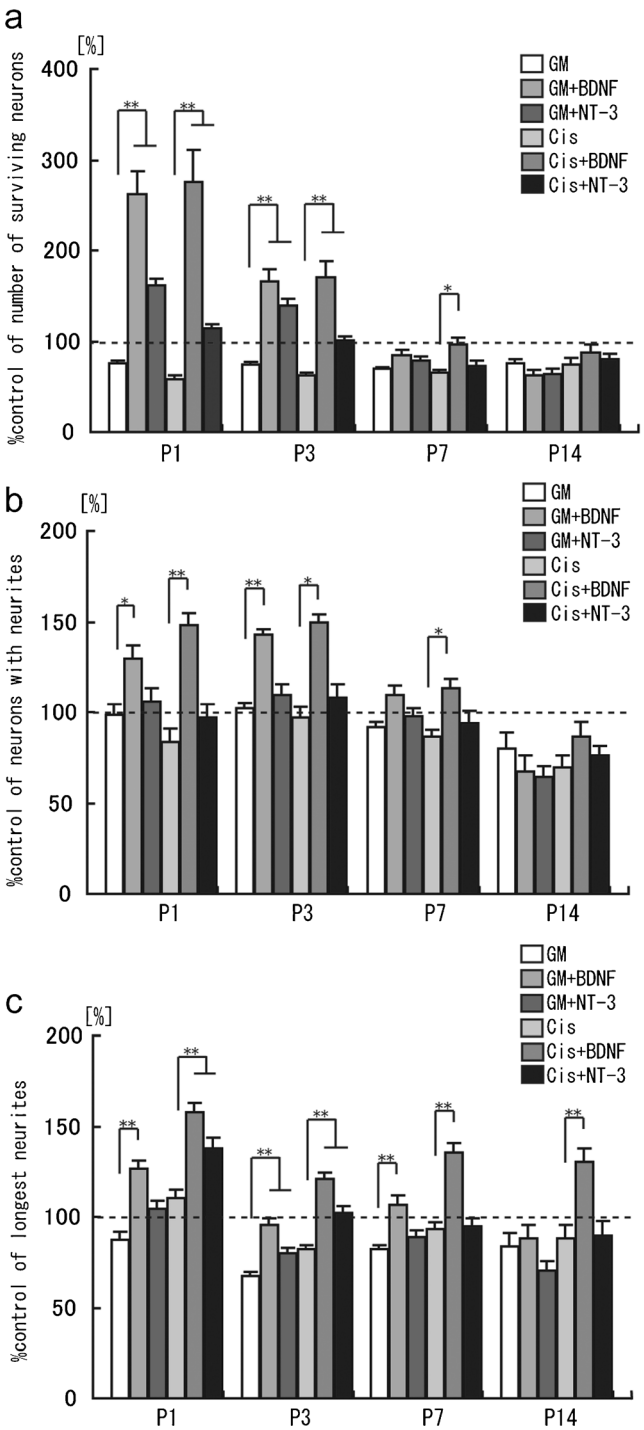

Fig. 3 Protective effect of BDNF and NT-3 against gentamicin (*GM*) and cisplatin (*Cis*) in VGNs from developing rats. **a**. The % control of the number of surviving VGNs in the presence of 0.5 mg/ml GM, both GM and BDNF (*GM+BDNF*), both GM and NT-3 (*GM+NT-3*), 2 μM Cis, both Cis and BDNF (*Cis+BDNF*) and both Cis and NT-3 (*Cis+NT-3*). Number of surviving neurons in the control condition was expressed as 100 % (*dashed line*). **b**. The % control of the ratio of the VGNs with neurites in the conditions, GM, GM+BDNF, GM+NT-3, Cis, Cis+BDNF and Cis+NT-3. The ratio of neurons with neurites in the control condition was expressed as 100 % (*dashed line*). **c**. The % control of the length of the longest neurites in the conditions, GM, GM+BDNF, GM+NT-3, Cis, Cis+BDNF and Cis+NT-3. The length of the longest neurites in the control condition was expressed as 100 % (*dashed line*)

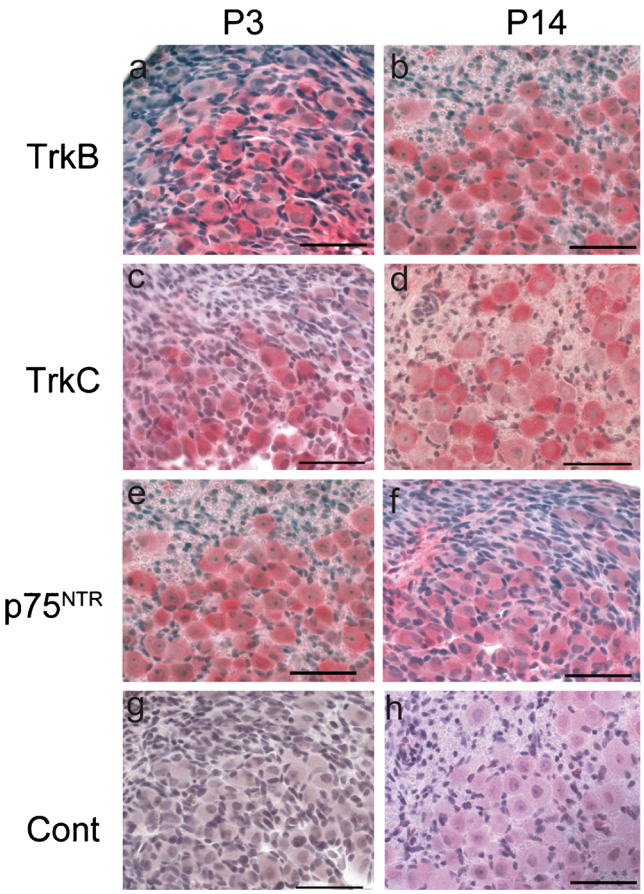

Fig. 4 Representative images for TrkB (**a**, **b**), TrkC (**c**, **d**) and P75^{NTR} (**e**, **f**) protein immunoreactivity in vestibular ganglia from P3 (**a**, **c**, **e**, **g**) and P14 rats (**b**, **d**, **f**, **h**). Cell nuclei (*blue*) were counterstained with hematoxylin. No obvious labeling corresponding to immunoreactivity was identified by substitution of the antibody with similarly diluted IgG from rabbit (**g**, **h**). *Bars* 50 μm

application of NT-3 together with GM or Cis also significantly increased the number of surviving neurons from younger rats (P1 and P3: $p<0.01$) but the effects were smaller than those seen with BDNF. NT-3 increased the length of neurites from younger rats (P1 and P3) against GM or Cis ($p<0.01$ at P1 for Cis and at P3 for GM and Cis; Fig. 3c) whereas it did not have a significant effect on the ratio of VGNs with neurites from any of the postnatal ages under GM or Cis ($p>0.1$; Fig. 3b). Different concentrations of BDNF and NT-3 (1 ng/ml, 50 ng/ml and 200 ng/ml) did not any have significant differences on cell survival, neurite sprouting and neurite outgrowth against GM or Cis in VGNs from P14 rats ($p>0.5$; Supplementary Fig. 2).

Developmental changes in the expression of TrkB, TrkC and p75NTR in VGNs

To examine the mechanism underlying the developmental changes in responsiveness of VGNs to BDNF and NT-3, we examined changes in the expression of proteins and mRNAs

of TrkB, TrkC and p75^{NTR} using immunohistochemisty, western blotting and quantitative real-time RT-PCR.

Figure 4 shows typical immunohistochemical images for TrkB, TrkC and p75^{NTR} of the vestibular ganglia obtained from P3 and P14 rats. At P3, VGNs were positively stained for TkB, TrkC and p75^{NTR}, while at P14 there was reduced immunopositivity for P75^{NTR} although TrkB and TrkC immunopositivity remained the same. No obvious labeling corresponding to the immunostaining was identified by substitution of the antibody with similarly diluted IgG in VGNs either at P3 and P14.

To quantitatively confirm the developmental changes in the expression of NTF receptors observed by immunohistochemistry, we compared the expression of NTF receptor proteins using western blotting analysis (Fig. 5). The analysis showed that the expression of TrkB and TrkC proteins did not show significant changes during development ($p>0.05$). On the other hand, expression of the p75^{NTR} protein showed a significant decrease during the period between P1 and P14 ($p<0.01$).

We also examined the developmental changes in the expression mRNA encoding NTF receptors in rat VGNs. Definite expression of TrkB, TrkC and p75NTR was confirmed by RT-PCR (Fig. 6a). Real-time quantitative RT-PCR revealed that the expression of TrkB and TrkC mRNAs did not change during the developmental period, whereas expression of the p75NTR mRNA significantly decreased during the period between P3 and P14 ($p<0.01$; Fig. 6b-d).

Discussion

Developmental changes in the responsiveness to NTFs have been reported in several kinds of neurons, such as motoneurons, trigeminal neurons and VGNs (Becker et al. 1998; Chihara Iwasaki et al. 2011; Enokido et al. 1999; Hashino et al. 1999; Nikolaou et al. 2006). In mouse trigeminal ganglion neurons, BDNF supports the survival of the neurons obtained from mice in the early embryonic period but the response decreases across the developmental period. On the other hand, neurons obtained from older mice become responsive to nerve growth factors (Enokido et al. 1999). In chick VGNs, BDNF and NT-3 facilitate neurite outgrowth during the early postnatal period but the effect declined thereafter. Instead, glial cell line-derived neurotrophic factor (GDNF) appears after completion of synaptogenesis on their peripheral and central targets (Hashino et al. 1999). In the present study, we have shown that the effects of BDNF and NT-3 on neuronal survival, neurite sprouting and neurite extension in rat VGNs change during the developmental period and that the effect of BDNF on VGNs is more potent than that of NT-3. These results are consistent with those of Chihara et al. (2011),

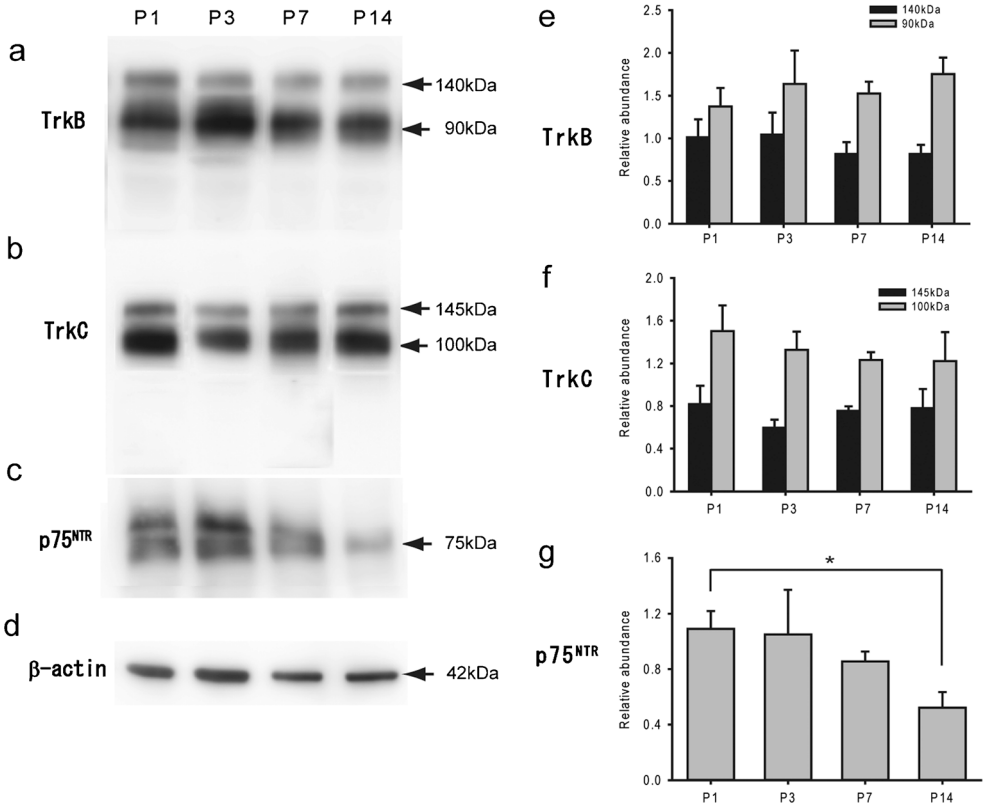

Fig. 5 Developmental changes in the expression of TrkB, TrkC and p75^{NTR} proteins in rat VGNs. **a-d** Results of the expression TrkB (**a**), TrkC (**b**), p75^{NTR} (**c**) and β-actin (**d**) proteins in P1–14 rat VGNs by western blot analysis. The results show a developmental decrease of p75^{NTR} protein between P1 and P14, whereas TrkB and TrkC proteins did not decrease during the same period. **e–g** Developmental changes in the relative abundance of TrkB (**e**), TrkC (**f**) and p75^{NTR} (**g**). The expression level of each protein was measured in bands at 90 kDa and 140 kDa for TrkB, 100 kDa and 145 kDa for TrkC and 75 kDa for p75^{NTR}. They were normalized to the expression level of β-actin. Five independent experiments were performed. In each experiment, 10 ganglia were used from each postnatal stage

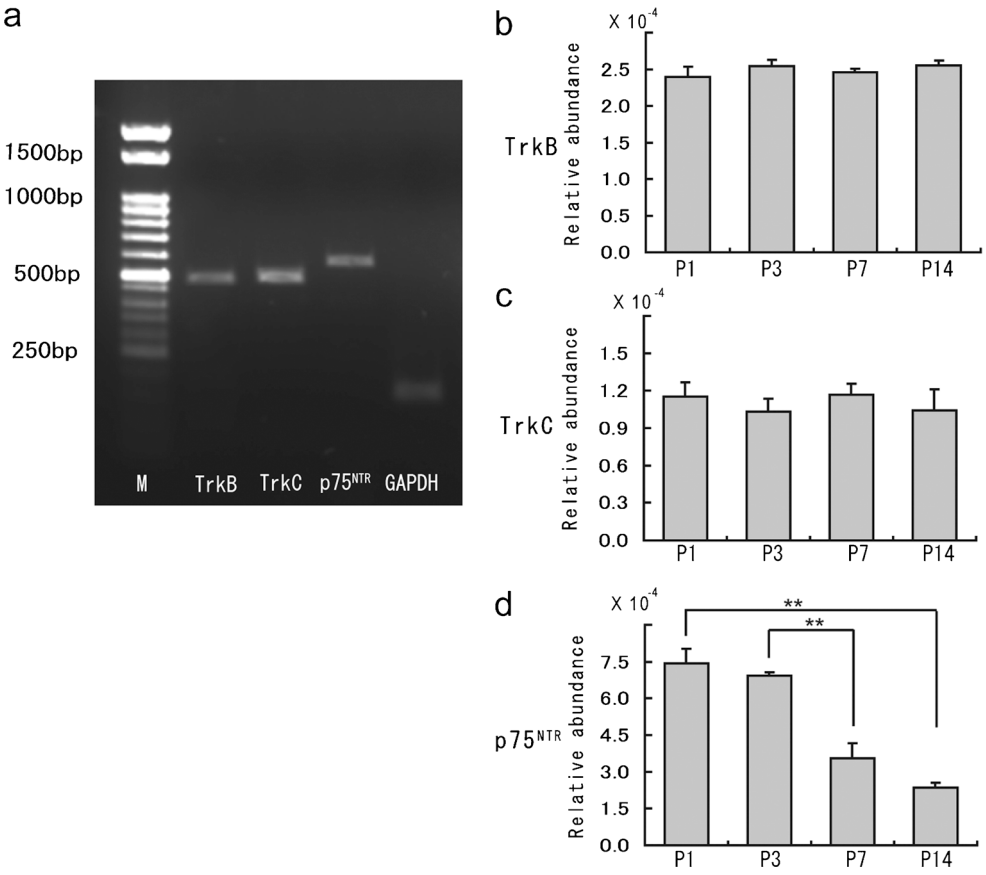

Fig. 6 Developmental changes in the expression of TrkB, TrkC and $p75^{NTR}$ mRNAs in rat VGNs. **a** Expression analysis of TrkB, TrkC and $p75^{NTR}$ mRNAs in VGNs obtained from P3 rats by RT-PCR. Ten ganglia were used from each developmental stage. Expected sizes were: 486 bp for TrkB, 527 bp for TrkC, 553 bp for $p75^{NTR}$ and 162 bp for GAPDH. *M* marker. **b**–**d** Developmental changes in the expression of TrkB (**b**), TrkC (**c**) and $p75^{NTR}$ (**d**) quantified by real-time RT-PCR. The expression levels of these receptor genes were normalized to those of the 18S ribosomal RNA levels. Five independent experiments were performed. In each experiment, 10 ganglia were used from each postnatal stage

who also showed that GDNF did not have any effects on rat VGNs during the postnatal period.

BDNF and NT-3 act on neurons through the high-affinity Trk family receptors, TrkB and TrkC, respectively, as well as the low-affinity $p75^{NTR}$ (Kaplan and Miller 2000; Reichardt 2006). The Trk receptors are activated by neurotrophin-mediated dimerization and transphosphorylation of activation loop tyrosines, which promote various intracellular signaling cascades and their downstream effectors (Huang and Reichardt 2003). There are three main intracellular pathways for Trk-mediated signaling: the Ras/mitogen activated protein kinase (MAPK) pathway, the phosphoinositide 3-kinase (PI3K) pathway activated through Shc (Src homology and collagen) and the calcium/calumodulin-dependent protein kinase II (CaMKII) pathway activated through phospholipase C-γ (PLCγ). A point mutation in the SHC binding site of TrkB resulted in reduced fiber growth and loss of target innervation with only mild effects on neuronal survival of VGNs, suggesting that the Shc binding and subsequent activation of the Ras/MAPK or PI3K pathway is important for target innervation of VGNs (Postigo et al. 2002). On the other hand, a point mutation in the PLCγ binding site of TrkB resulted in exuberant projections of VGNs to the basal turn of cochlea with limited effects on neuronal survival, suggesting that the PLCγ site-activated CaMKII pathway is essential for the proper navigation of vestibular nerve fibers (Tessarollo et al. 2004). Recently, Sciarretta et al. (2010) showed that a double point mutation in both the Shc and PLCγ docking sites resulted in a loss of vestibular neurons that is essentially indistinguishable from that of TrkB null mutant mice, indicating that the survival function of BDNF/TrkB is mediated predominantly through the cooperative action of these two binding sites (Sciarretta et al. 2010). In the present study, we examined the changes in the level of mRNA and protein of TrkB, TrkC and $p75^{NTR}$s by quantitative RT-PCR and western blotting in VGNs obtained from developing rats in order to investigate the mechanism underlying the developmental decline of the effect of NTFs in rat VGNs. Unexpectedly, the results showed that the expression of the TrkB and TrkC proteins, as well as their mRNA, did not show significant changes during development but that the expression of $p75^{NTR}$ proteins decreased after P3. These results suggest that developmental changes of these intracellular signaling pathways and their downstream effectors might underlie the developmental decline of the effects of NTFs in VGNs. It is also possible that developmental decline of $p75^{NTR}$ affected the responsiveness of VGNs to NTFs. The Trks and $p75^{NTR}$s have many associated proteins that are the starting points of their signaling cascades, which in turn modulate Trk receptor functions (Geetha et al. 2005; Harrison et al. 2000). Furthermore,

neurotrophin binding to $p75^{NTR}$ itself controls neuronal survival and neuronal apoptosis through activation of the NF-κB and Jun kinase pathways, respectively (Hamanoue et al. 1999; Harrington et al. 2002). Other possibilities include developmental changes in the quantity of Trk receptors expressed on the cell surface and changes in the activation of inhibitory molecules such as protein tyrosince phosphatases (Gerling et al. 2004; Yang et al. 2006) and Sprouty proteins (Mason et al. 2006).

Aminoglycoside and platinum-containing drugs have been shown to have ototoxic effects on the peripheral endorgans in the inner ear (Clerici et al. 1996; Song and Schacht 1996; Xie et al. 2011) as well as on their primary afferents (Jeong et al. 2010; Zheng et al. 1995). A principal mechanism underlying the toxic effects of these drugs is presumed to be the generation of reactive oxygen species (ROS). It has been shown that administration of aminoglycoside generates ROS and their reaction products in hair cells of the inner ear (Clerici et al. 1996; Song and Schacht 1996) and in their primary afferents (Jeong et al. 2010) and that co-application of antioxidants with aminoglycoside or the introduction of antioxidant enzymes via viral vectors can protect them against aminoglycoside-induced ototoxicity (Kawamoto et al. 2004; Song and Schacht 1996). Cis also generates ROS, by depletion of reduced glutathione and antioxidant enzymes and/or activation of ROS generating systems, such as NADPH oxidase (Clerici et al. 1996). It has been reported that BDNF can protect neurons from ototoxicity of Cis by reducing intracellular levels of ROS in rat spiral ganglion neurons (Gabaizadeh,et al. 1997). Zheng et al. (1995) previously showed the protective effect of BDNF and NT-3 against the neurotoxic effect of GM and Cis in VGNs obtained from P5 rats. In the present study, we investigated developmental changes in the protective effects of these NTFs against GM and Cis agents in VGNs from developing rats and have shown that the protective effects of NTFs against GM and Cis were limited during the early postnatal period as were the effects of NTFs in control conditions.

Attempts to develop clinical treatments using systemic application of NTFs against neural diseases have been disappointing, primarily owing to their short half-life in the blood and possible side effects in other organs (Jang et al. 2010; Thoenen and Sendtner 2002). However, direct infusion of exogenous NTFs into the inner ear has been shown to be effective for preventing loss of spiral ganglion neurons and/or hair cells in experimental animals (Nakaizumi et al. 2004; Noushi et al. 2005; Richardson et al. 2006). In the present study, we have shown that the protective effects of BDNF and NT-3 against ototoxic damage in cultured VGNs are limited in the early postnatal period but that expression of the TrkB and TrkC did not change during these periods. Our results suggest that simple administration of NTFs might be insufficient for treating peripheral vestibular damages by ototoxic drugs and that the developmental changes in their responsiveness to NTFs should be taken into consideration. Furthermore, in order to implement NTF treatment for maintaining VGNs after peripheral vestibular damage, it is necessary to clarify the developmental changes in the intracellular signaling pathways and their downstream effector following activation of Trk receptors and $p75^{NTR}$s.

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References

Baloh T, Honrubia V (2001) Clinical neurophysiology of the vestibular system. Oxford University Press, New York

Becker E, Soler RM, Yuste VJ, Gine E, Sanz-Rodriguez C, Egea J, Martin-Zanca D, Comella JX (1998) Development of survival responsiveness to brain-derived neurotrophic factor, neurotrophin 3 and neurotrophin 4/5, but not to nerve growth factor, in cultured motoneurons from chick embryo spinal cord. J Neurosci 18:7903–7911

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

Clerici WJ, Hensley K, DiMartino DL, Butterfield DA (1996) Direct detection of ototoxicant-induced reactive oxygen species generation in cochlear explants. Hear Res 98:116–124

Enokido Y, Wyatt S, Davies AM (1999) Developmental changes in the response of trigeminal neurons to neurotrophins: influence of birthdate and the ganglion environment. Development 126:4365–4373

Ernfors P, Van De Water T, Loring J, Jaenisch R (1995) Complementary roles of BDNF and NT-3 in vestibular and auditory development. Neuron 14:1153–1164

Ernfors P, Lee KF, Jaenisch R (1994) Mice lacking brain-derived neurotrophic factor develop with sensory deficits. Nature 368:147–150

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

Gabaizadeh R, Staecker H, Liu W, Van De Water TR (1997) BDNF protection of auditory neurons from cisplatin involves changes in intracellular levels of both reactive oxygen species and glutathione. Brain Res Mol Brain Res 50:71–78

Geetha T, Jiang J, Wooten MW (2005) Lysine 63 polyubiquitination of the nerve growth factor receptor TrkA directs internalization and signaling. Mol Cell 20:301–312

Gerling N, Culmsee C, Klumpp S, Krieglstein J (2004) The tyrosine phosphatase inhibitor orthovanadate mimics NGF-induced neuroprotective signaling in rat hippocampal neurons. Neurochem Int 44:505–520

Hamanoue M, Middleton G, Wyatt S, Jaffray E, Hay RT, Davies AM (1999) p75-mediated NF-kappaB activation enhances the survival response of developing sensory neurons to nerve growth factor. Mol Cell Neurosci 14:28–40

Harrington AW, Kim JY, Yoon SO (2002) Activation of Rac GTPase by p75 is necessary for c-jun N-terminal kinase-mediated apoptosis. J Neurosci 22:156–166

Harrison SM, Jones ME, Uecker S, Albers KM, Kudrycki KE, Davis BM (2000) Levels of nerve growth factor and neurotrophin-3 are affected differentially by the presence of p75 in sympathetic neurons in vivo. J Comp Neurol 424:99–110

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

Huang EJ, Reichardt LF (2003) Trk receptors: roles in neuronal signal transduction. Annu Rev Biochem 72:609–642

Jang SW, Liu X, Yepes M, Shepherd KR, Miller GW, Liu Y, Wilson WD, Xiao G, Blanchi B, Sun YE, Ye K (2010) A selective TrkB agonist with potent neurotrophic activities by 7,8-dihydroxyflavone. Proc Natl Acad Sci USA 107:2687–2692

Jeong SW, Kim LS, Hur D, Bae WY, Kim JR, Lee JH (2010) Gentamicin-induced spiral ganglion cell death: apoptosis mediated by ROS and the JNK signaling pathway. Acta Otolaryngol 130:670–678

Kaplan DR, Miller FD (2000) Neurotrophin signal transduction in the nervous system. Curr Opin Neurobiol 10:381–391

Kawamoto K, Sha SH, Minoda R, Izumikawa M, Kuriyama H, Schacht J, Raphael Y (2004) Antioxidant gene therapy can protect hearing and hair cells from ototoxicity. Mol Ther 9:173–181

Lessmann V, Gottmann K, Malcangio M (2003) Neurotrophin secretion: current facts and future prospects. Prog Neurobiol 69:341–374

Mason JM, Morrison DJ, Basson MA, Licht JD (2006) Sprouty proteins: multifaceted negative-feedback regulators of receptor tyrosine kinase signaling. Trends Cell Biol 16:45–54

Nagahara AH, Tuszynski MH (2011) Potential therapeutic uses of BDNF in neurological and psychiatric disorders. Nat Rev Drug Discov 10: 209–219

Nakaizumi T, Kawamoto K, Minoda R, Raphael Y (2004) Adenovirus-mediated expression of brain-derived neurotrophic factor protects spiral ganglion neurons from ototoxic damage. Audiol Neurootol 9: 135–143

Nikolaou KE, Malamitsi-Puchner A, Boutsikou T, Economou E, Boutsikou M, Puchner KP, Baka S, Hassiakos D (2006) The varying patterns of neurotrophin changes in the perinatal period. Ann NY Acad Sci 1092:426–433

Noushi F, Richardson RT, Hardman J, Clark G, O'Leary S (2005) Delivery of neurotrophin-3 to the cochlea using alginate beads. Otol Neurotol 26:528–533

Pirvola U, Ylikoski J, Palgi J, Lehtonen E, Arumae U, Saarma M (1992) Brain-derived neurotrophic factor and neurotrophin 3 mRNAs in the peripheral target fields of developing inner ear ganglia. Proc Natl Acad Sci USA 89:9915–9919

Pirvola U, Arumae U, Moshnyakov M, Palgi J, Saarma M, Ylikoski J (1994) Coordinated expression and function of neurotrophins and their receptors in the rat inner ear during target innervation. Hear Res 75:131–144

Postigo A, Calella AM, Fritzsch B, Knipper M, Katz D, Eilers A, Schimmang T, Lewin GR, Klein R, Minichiello L (2002) Distinct requirements for TrkB and TrkC signaling in target innervation by sensory neurons. Genes Dev 16:633–645

Reichardt LF (2006) Neurotrophin-regulated signalling pathways. Philos Trans R Soc Lond B 361:1545–1564

Richardson RT, Noushi F, O'Leary S (2006) Inner ear therapy for neural preservation. Audiol Neurootol 11:343–356

Sciarretta C, Fritzsch B, Beisel K, Rocha-Sanchez SM, Buniello A, Horn JM, Minichiello L (2010) PLCgamma-activated signalling is essential for TrkB mediated sensory neuron structural plasticity. BMC Dev Biol 10:103

Skaper SD (2008) The biology of neurotrophins, signalling pathways, and functional peptide mimetics of neurotrophins and their receptors. CNS Neurol Disord Drug Targets 7:46–62

Song BB, Schacht J (1996) Variable efficacy of radical scavengers and iron chelators to attenuate gentamicin ototoxicity in guinea pig in vivo. Hear Res 94:87–93

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

Thoenen H, Sendtner M (2002) Neurotrophins: from enthusiastic expectations through sobering experiences to rational therapeutic approaches. Nat Neurosci 5(Suppl):1046–1050

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

Xie J, Talaska AE, Schacht J (2011) New developments in aminoglycoside therapy and ototoxicity. Hear Res 281:28–37

Yang T, Massa SM, Longo FM (2006) LAR protein tyrosine phosphatase receptor associates with TrkB and modulates neurotrophic signaling pathways. J Neurobiol 66:1420–1436

Ylikoski J, Pirvola U, Moshnyakov M, Palgi J, Arumae U, Saarma M (1993) Expression patterns of neurotrophin and their receptor mRNAs in the rat inner ear. Hear Res 65:69–78

Zheng JL, Stewart RR, Gao WQ (1995) Neurotrophin-4/5, brain-derived neurotrophic factor, and neurotrophin-3 promote survival of cultured vestibular ganglion neurons and protect them against neurotoxicity of ototoxins. J Neurobiol 28:330–340