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# Complementary Roles of BDNF and NT-3 in Vestibular and Auditory Development

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## Summary

**The physiological role of BDNF and NT-3 in the development of the vestibular and auditory systems was investigated in mice that carry a deleted *BDNF* and/or *NT-3* gene. BDNF was the major survival factor for vestibular ganglion neurons, and NT-3, for spiral ganglion neurons. Lack of BDNF and NT-3 did not affect ingrowth of nerve fibers into the vestibular epithelium, but *BDNF* mutants failed to maintain afferent and efferent innervation. In the cochlea, *BDNF* mutants lost type 2 spiral neurons, causing an absence of outer hair cell innervation. *NT-3* mutants showed a paucity of afferents and lost 87% of spiral neurons, presumably corresponding to type 1 neurons, which innervate inner hair cells. Double mutants had an additive loss, lacking all vestibular and spiral neurons. These results show that BDNF and NT-3 are crucial for inner ear development and, although largely coexpressed, have distinct and nonoverlapping roles in the vestibular and auditory systems.**

## Introduction

Balance, posture, and equilibrium are mediated by information conveyed from the vestibular sensory receptors to the brain, and hearing is conveyed from the cochlear sensory receptor. The sense organs of the vestibular inner ear include the hair cells in the sensory epithelia of the saccule and utricle and of the cristae of the three semicircular ducts, which are innervated by afferents from vestibular ganglion neurons. The auditory receptor of the cochlea includes the inner and outer hair cells of Corti's organ, which are innervated by afferents from spiral ganglion neurons.

The peripheral target of the cochleovestibular ganglion contains trophic activity that supports the survival of these neurons in culture (Ard et al., 1985; Hauger et al., 1989; Hemond and Morest, 1992; Lefebvre et al., 1992; Zhou and Van De Water, 1987). The best characterized gene family of trophic factors, the neurotrophins, include nerve growth factor (NGF; Levi-Montalcini, 1987), brain-derived neurotrophic factor (BDNF; Barde et al., 1982; Leibrock et al., 1989), neurotrophin-3 (NT-3; Ernfors et al., 1990; Hohn et al., 1990; Jones and Reichardt, 1990; Kaisho et al., 1990; Maisonpierre et al., 1990; Rosenthal et al., 1990) and neurotrophin-4 (NT-4; Berkemeier et al., 1991; Hallböök et al., 1991; Ip et al., 1992). The initial finding that the common low affinity neurotrophin receptor, p75NGFR, is expressed in the inner ear (Bernd and Represa, 1989; Despres et al., 1991; Hallböök et al., 1990; Raivich et al., 1987; von Bartheld et al., 1991) suggested a role for members of the neurotrophin family of neurotrophic factors in inner ear development.

The significance of neurotrophin interactions for inner ear development was apparent from the findings that vestibular and spiral ganglion neurons express mRNAs for the BDNF signal transducing receptor, trkB, and the NT-3 signal transducing receptor, trkC (Ernfors et al., 1992; Ylikoski, et al., 1993), and that cells of the inner ear sensory epithelia express mRNAs for BDNF and NT-3 in a distinct, partially overlapping pattern (Ernfors et al., 1992; Pirvola et al., 1992; Ylikoski et al., 1993; Schecterson and Bothwell, 1994). The lack of expression of *NGF* and *NT-4* mRNAs suggested a requirement only for BDNF and NT-3 in inner ear development. In the embryonic and neonatal rat, *NT-3* mRNA is localized to both the differentiating hair cells and surrounding supporting cells of the cochlear and vestibular sensory epithelia, whereas *BDNF* mRNA is localized exclusively to the differentiating hair cells (Pirvola et al., 1992). In addition, *BDNF*, but not *NT-3*, mRNA is expressed in the sensory epithelia of the crista ampullaris (Ernfors et al., 1992; Pirvola et al., 1992). The expression of BDNF and NT-3 at postnatal stages is more restricted. *NT-3* mRNA is present in inner hair cells of the cochlea and *BDNF* mRNA in hair cells of the vestibular maculae (Ylikoski et al., 1993). In culture, BDNF and NT-3 have mitogenic effects on cochleovestibular neurons (Represa et al., 1993) and, at later developmental stages, act on both vestibular and spiral ganglion neurons to promote the survival of these neurons (Avila et al., 1993; Davies et al., 1986; Pirvola et al., 1994; Vazquez et al., 1994). Both spiral and vestibular ganglion neurons respond to BDNF and NT-3 at the time of target encounter and innervation; however, BDNF is more potent in promoting the survival at later embryonic stages (Avila et al., 1993). In agreement with these studies, more than 80% of the vestibular ganglion neurons are lost in mice that genetically carry a mutation of the *BDNF* gene, and innervation of the saccular and utricular sensory epithelia and the cristae of the semicircular ducts is compromised (Ernfors et al., 1994a), apparently causing the deficiency in balance and coordination of movement manifested at 1 week after birth. *NT-3* mice display no overt phenotype related to inner ear deficiency but display severe defects in movements of the limbs, correlated with the absence of the proprioceptive sense organs (Ernfors et al., 1994b).

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Table 1. Number of Vestibular and Spiral Ganglion Neurons in *BDNF* (+ −), (− / −), *NT-3* (− / −), and *BDNF* (− / −)/*NT-3* (− / −) Mutant Mice

| | Vestibular Ganglion Neurons | | Spiral Ganglion Neurons | |
|---|---|---|---|---|
| | Number of Neurons ± SEM | Percent Loss | Number of Neurons ± SEM | Percent Loss |
| Control (+/+) mice | 2301 ± 76 (n = 3) | NA | 6008 ± 253 (n = 3) | NA |
| *BDNF* (+/−) mice | 1534 ± 53 (n = 3) | 33[a] | | |
| *BDNF* (−/−) mice | 408 ± 27 (n = 3) | 79[c] | 5616 ± 59 (n = 4) | 7 |
| *NT-3* (−/−) mice | 1702 ± 150 (n = 3) | 34[a] | 753 ± 46 (n = 3) | 87[b] |
| *BDNF* (−/−) mice | 32 ± 24 (n = 3) | 99[c] | 0 ± 0 (n = 4) | 100[c] |

All cell counts were performed on mice at postnatal day 10–15, except the double mutant mice, which were embryonic day 18. Sections were stained with cresyl violet and neurons counted by light microscopy. Only neurons with a clear nucleus and nucleoli were counted. Counts were not corrected for split nucleoli.

[a] $p < 0.05$, Student's t test.

[b] $p < 0.01$, Student's t test.

[c] $p < 0.001$, Student's t test.

Because BDNF and NT-3 are the only neurotrophins that are expressed in inner ear, the derivation of mice carrying a deleted *BDNF* (Ernfors et al., 1994a; Jones et al., 1994) and *NT-3* (Ernfors et al., 1994b; Farinas et al., 1994) gene provides a unique opportunity to understand physiological requirements for these neurotrophic factors in inner ear development. We have used ultrastructural, histological, immunohistochemical, and neuronal tracing methods to examine the importance of BDNF and NT-3 during fetal and postnatal life for the survival of vestibular and spiral ganglion neurons and their requirement for the establishment and maintenance of afferent and efferent innervation of vestibular and auditory hair cells.

## Results

Vestibular and cochlear innervation and the survival of afferent neurons in mice lacking BDNF and/or NT-3 was analyzed. Mice homozygous for a deleted *BDNF* (Ernfors et al., 1994a) or *NT-3* (Ernfors et al., 1994b) gene were generated by crossing animals heterozygous for a deleted *BDNF* or *NT-3* gene. To investigate the phenotype of mice deficient for both neurotrophins, mice heterozygous for both *BDNF* and *NT-3* were crossed, and the offspring analyzed. The results will be described in three sections. Analysis of *BDNF* mutant mice is described first, followed by the description of *NT-3* mutant mice. Finally, we will summarize the phenotype of mice lacking both neurotrophins. The principal findings of postnatal *BDNF* and *NT-3* mutant mice have been summarized in Table 2 and in the schematic drawings of Figures 6A and 6B.

### *BDNF* Mutant Mice

#### *Vestibular and Spiral Ganglion Neurons*

To determine the physiological role of BDNF in vivo for the survival of the sensory neurons that innervate the inner ear sense organs, the number of neurons in the vestibular and spiral ganglia of mutant mice was examined at postnatal days 10–15 (P10–15; Table 1). A significant loss of vestibular ganglion neurons was detected in the *BDNF* (−/−) mutant mice (79%; Table 1) similar to previous results (Ernfors et al., 1994a). Mice heterozygous for the mutation displayed a 33% loss of the vestibular ganglion neurons as compared with control ganglia counts (Table 1A). Spiral ganglion neurons were decreased by 7% in *BDNF* (−/−) mice (Table 1).

To determine the developmental period for neuronal loss of the vestibular ganglion in *BDNF* (−/−) mice, embryonic day 13 (E13), E16, and P0 ganglia were counted. A near normal number of vestibular neurons was present at E13; however, by E16, approximately 80% of the neurons had been lost. Because no significant further loss of vestibular ganglion neurons was evident at P0 or P14 (Figure 1), these results show that neuronal loss caused by the lack of BDNF occurs between E13 and E16.

#### *Peripheral Vestibular Afferent Innervation*

Pioneering nerve fibers penetrate the vestibular sensory epithelium at early E12, and a significant increasing number have accumulated by E16 (Galinovic-Schwartz et al., 1991). To study the afferent innervation of the vestibular compartments of the *BDNF* (−/−) mice, we employed immunocytochemistry, neuronal tracing, and electronmicroscopy. Polyclonal antiserum 9651 directed against extracellular domains of the p75NGFR and 9993 antiserum against intracellular domains of the p75NGFR were used for immunocytochemical detection of afferent innervation (Huber and Chao, 1994). Identical staining patterns were obtained with the two antisera, and immunostained tissue sections using the 9651 antiserum are displayed in the figures.

Vestibular ganglion neurons of control mice were not immunoreactive at E13, weakly at E16, and strongly at P0, but afferent nerve fibers exiting the ganglion were already stained at E13 (data not shown). Only rarely had immunoreactive fibers penetrated the saccular sensory epithelium of controls at E13, but nerve fibers were more often detected in the utricular epithelium at this stage (Figure 2A, small arrows). At E16, P0, and P24, numerous p75NGFR-positive fibers had penetrated both maculae epithelia, with immunoreactive nerve terminals ensheathing the hair cells in the shape of chalice formations (Figure 2C, P0). In E13 *BDNF* (−/−) mice, a few fibers were detected in the saccular and more in the utricular sensory epithelium (Figure 2B, small arrows), similar to control. However, at E16, P0, and P6, the vestibular sensory epithelia of *BDNF* (−/−) mice were devoid of immunoreactive material, and

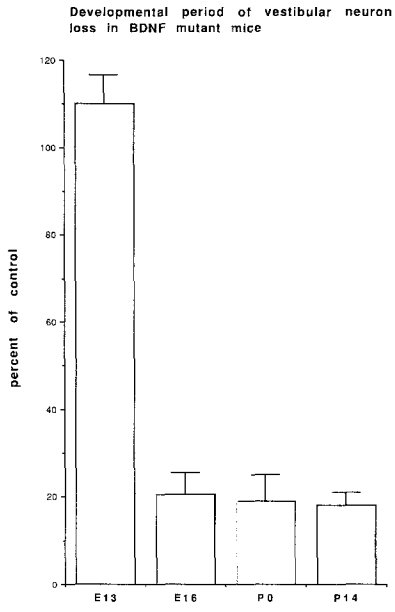


Figure 1. Developmental Time of Vestibular Ganglion Neuron Loss in *BDNF* (−/−) Mice

Vestibular ganglion neurons were counted in E13, E16, P0, and P14 *BDNF* (−/−) and control mice. The percentage of remaining vestibular ganglion neurons in *BDNF* (−/−) mice (n = 3 per stage) is plotted as a percentage of the controls (n = 3). Note the reduction of neurons in the *BDNF* (−/−) mice between E13 and E16, as compared with controls.

fibers were only detected in the underlying connective tissue layer (Figure 2D, P0), presumably corresponding to afferents from the surviving (i.e., 20%) vestibular ganglion neurons in the E16, P0, and P24 *BDNF* (−/−) mice (compare Table 1).

### *Central Vestibular Afferents*

The loss of peripheral vestibular afferents and most vestibular ganglion neurons suggested a deficiency also of the central vestibular afferents. Primary vestibular nerve afferents terminate in four major vestibular nuclei (the superior, lateral, medial, and spinal vestibular nuclei; Paxinos, 1985). The lipophilic carbocyanine drug 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (DiI) was applied to the eighth cranial nerve attached to the brain stem of P6 control and *BDNF* (−/−) mice to trace the central vestibular afferents. Vibratome sections prepared at the level of the vestibular nuclei of control mice revealed a dense network of afferents terminating in areas corresponding to the vestibular nuclei (Figure 3A). In addition, numerous vestibulocerebellar fibers (Figure 3A, curved arrow) terminated in the Purkinje cell layer of the uvula, nodulus, and flocculus (data not shown). In contrast to the control mice, afferents were almost completely absent in the vestibular nuclei of the *BDNF* (−/−) mice (Figure 3B), and a reduced number of vestibulocerebellar afferents was detected (data not shown). In addition to the vestibular nuclei and cerebellum, fibers were seen terminating in the nucleus of the solitary tract of both control and *BDNF* (−/−) mice, which likely represent a nonspecific crossing of the

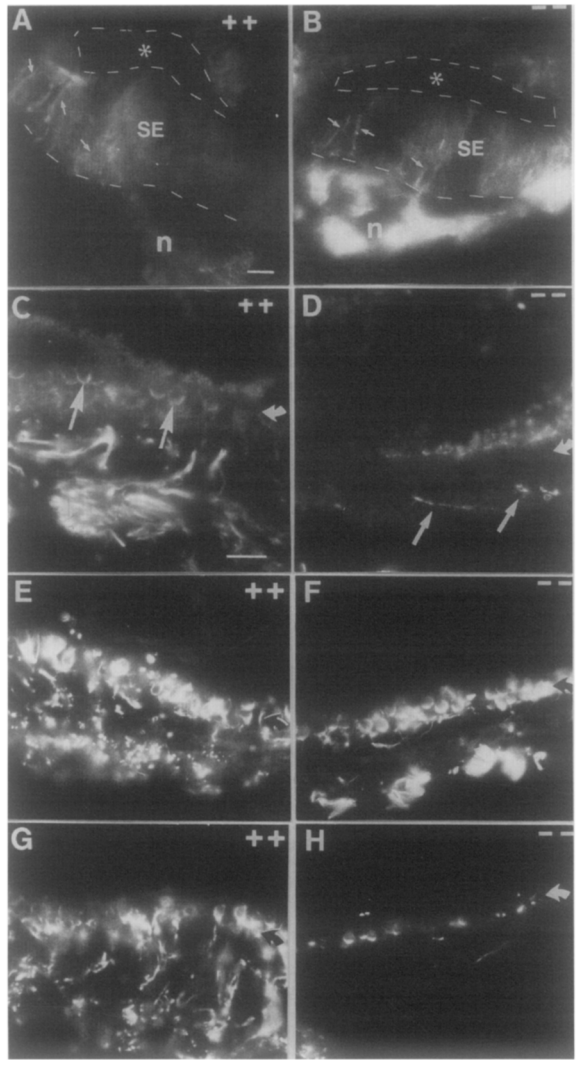


Figure 2. Afferent and Efferent Innervation of the Saccular and Utricular Sensory Epithelia in Control and *BDNF* (−/−) Mice during Development

(A, C, E, and G) control mice; (B, D, F, and H) *BDNF* (−/−) mice.

(A–D) p75NGFR-immunoreactive afferents nerve fibers in the vestibular sensory epithelia. Sections from E13 embryos (A and B) and P0 mice (C and D) were cut and stained for p75NGFR immunoreactivity. Note stained nerve fibers (small arrows) in both control and *BDNF* (−/−) E13 utricular sensory epithelia (SE). The sensory epithelia are outlined by dashed lines and the utricular duct by asterisks. At E16 and P0, numerous nerve fibers and nerve chalices (large arrows) were present in the sensory epithelium (curved arrows) of control mice (C; P0). At E16 and P0, nerve fibers also were seen in the connective tissue of *BDNF* (−/−) mice (D; P0, straight arrow), but not in the epithelium (D; curved arrow).

(E–H) NF 150 kDa-immunoreactive nerve fibers of the saccular macula of *BDNF* (−/−) and control mice. (E and F) P6; (G and H) P24. Note the reduction of stained efferents in the sensory epithelium (curved arrows) between P6 (F) and P24 (H) in the *BDNF* (−/−) mice.

++, control mice; −−, *BDNF* (−/−) mice; n, nerves in the connective tissue; SE, sensory eptihelium of the utricle. All images are at same magnification. Bars, 20 μm.

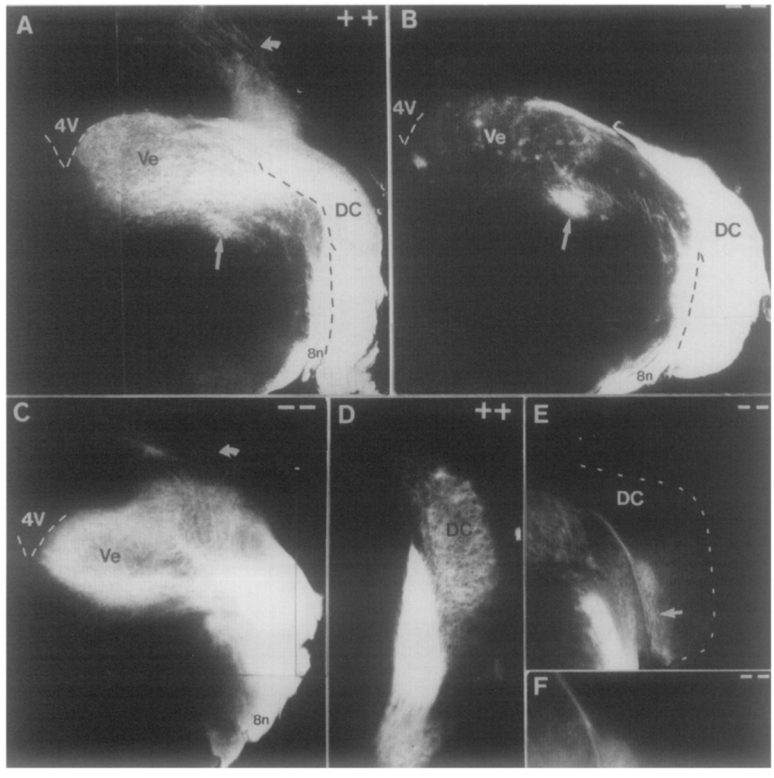


Figure 3. DiI Tracing of Central Vestibular and Cochlear Afferents in *BDNF* (−/−), *NT-3* (−/−), and Control Mice

(A–C) Tracing of central vestibular afferents. DiI was applied to the eighth cranial nerve to trace the vestibular afferents of P6 control (A), *BDNF* (−/−) (B), and *NT-3* (−/−) (C) mice, and coronal vibratome sections were cut at the level of the vestibular nuclei. Note the near complete absence of vestibular afferents terminating in the vestibular nuclei (Ve) of the *BDNF* (−/−) mouse (B), but apparent normal complement in the *NT-3* (−/−) mouse (C). Curved arrows in (A) and (C) indicate vestibulocerebellar afferents. Arrow indicates the nucleus of the solitary tract.

(D–F) Tracing of central cochlear afferents. DiI was applied to the eighth cranial nerve of control (D) and P6 *NT-3* (−/−) mice (E and F), and coronal sections were cut at the level of the cochlear nuclei. Note paucity of nerve fibers in the dorsal cochlear nucleus (DC) of *NT-3* (−/−) mice (E and F; [F] is higher magnification of part of [E]), as compared with control (D). Arrow in (E) indicates a few remaining afferents in the *NT-3* (−/−) mouse.

++, control mice; −−, *BDNF* (−/−) mice; 4V, fourth ventricle; 8n, eighth cranial nerve; DC, dorsal cochlear nucleus; Ve, vestibular nuclei.

dye to central trigeminal or facial afferents terminating in this nucleus (Paxinos, 1985). The results in Figures 3A and 3B show that the lack of BDNF leads to a paucity of central vestibular afferents.

### *Vestibular Efferent Innervation*

In addition to afferent innervation, vestibular sense organs receive efferent innervation. Efferents penetrate the vestibular sensory epithelium close to the embryonic period of afferent innervation (Fritzsch and Nichols, 1993). Efferent hair cell contacts are established neonatally but continue to mature up to 3 weeks after birth (Emmerling et al., 1990; Shnerson et al., 1981; Sobkowicz and Emmerling, 1989). An anti-neurofilament (NF) 150 kDa antisera and an anti-acetylcholine esterase (AChE) antibody were used for detection of efferents. Whereas NF 150 kDa is associated with most or all afferents and efferents, only some efferents have been described as containing AChE (McLamb and Park, 1992; Sobkowicz and Emmerling, 1989).

Control mice displayed strongly reactive NF 150 kDa-positive fibers innervating the hair cells of the saccular and utricular epithelia at P0, P6 (see Figure 2E), and P24 (see Figure 2G). Similar to control mice, a large number of NF 150 kDa-positive fibers were detected at P0 and P6 (see Figure 2F) in the *BDNF* (−/−) mice. However, at P24, only a few NF 150 kDa-positive fibers were detected in the sensory epithelia of the saccular (see Figure 2H) and utricular maculae. The loss of efferents was also evident using AChE immunohistochemistry. In P24 control mice, AChE immunoreactivity was detected along the basal lamina and underneath some of the hair cells. In the *BDNF* (−/−) mice, AChE-immunoreactive material was completely absent in the vestibular sensory epithelia (data not shown).

Because afferents in the vestibular sensory epithelia of *BDNF* (−/−) mice are completely lost between E13 and E16, only efferents are assumed to be detected with the NF 150 kDa antisera in the postnatal *BDNF* (−/−) mice. Thus, the above results indicate that efferents innervate hair cells in the vestibular sensory epithelia of *BDNF* (−/−) mice, similar to control, but are lost between P6 and P24.

### *Ultrastructural Characterization of Vestibular Innervation*

Ultrastructural analysis was used to confirm the absence of afferent and paucity of efferent innervation of the macula utriculus in *BDNF* (−/−) mice. Toluidine blue–stained semithin sections of utricles from P24 control mice revealed axonal bundles (Figure 4A, small arrows) and the presence of nerves projecting through the connective tissue that underlies the macula (Figure 4A, curved arrows). The hair cells of the control mice were enveloped by afferent nerve chalices, characterized by the light staining of this material (Figure 4A, large arrows). Hair cells of the *BDNF* (−/−) utricular epithelium were completely devoid of afferents contacting their soma (Figure 4B), and nerve fibers were absent in the sensory epithelium, but a few small-caliber axons were present in the connective tissue that underlies the utricular macula (Figure 4B, small arrows). Control and *BDNF* (−/−) maculae utriculus were also studied by electron microscopy. Afferents in the control maculae were characterized by electron translucent nerve chalices enveloping the type 1 hair cells (Figure 4C, Ch). The chalices received efferent synapses on their outer membrane

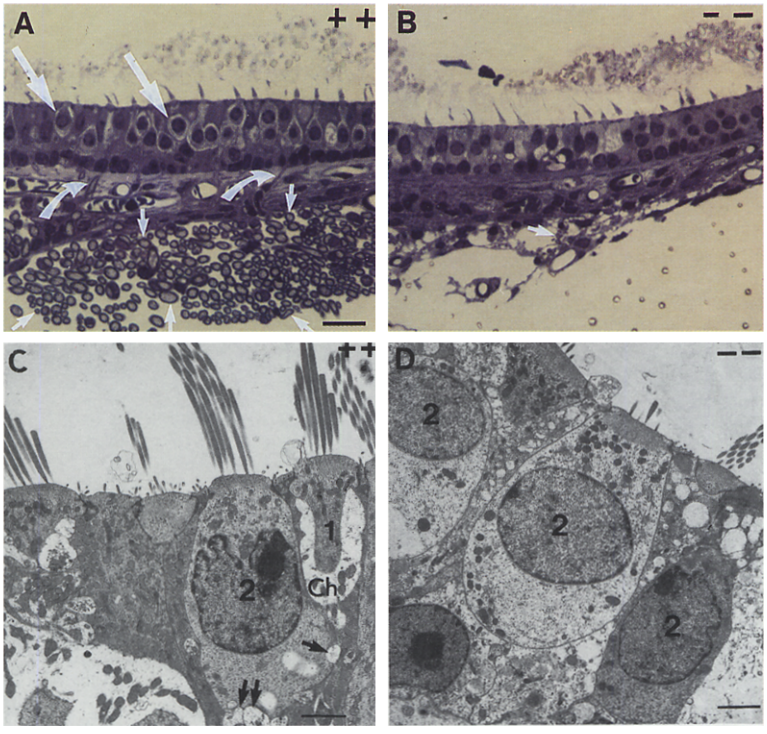


Figure 4. Semithin and Thin Plastic Sections of Maculae of Sensory Epithelia from Utricles of P24 Control and *BDNF* (–/–) Mice

(A and C) control mice; (B and D) *BDNF* (–/–) mice.

(A and B) Toluidine blue–stained semithin sections.

(A) The predominant sensory cell type of this sensory receptor in control mice was the type 1 hair cell characterized by its envelopment by the afferent nerve chalice (large arrows). Many myelinated afferent nerve fibers were present below the basement membrane (curved arrows) and the vascular bed (small arrows) of this utricle and were seen penetrating the basement membrane.

(B) Few of the sensory cells present in the *BDNF* (–/–) utricle had a type 1–like soma, with the predominant hair cell type being more closely related to the soma of a type 2 hair cell. The hair cells of the *BDNF* (–/–) macula utriculus possessed no nerve chalices. No myelinated or unmyelinated nerve fibers were seen in relationship to the basement membrane, but a few nerve fibers were seen directly below the vascular bed of the *BDNF* (–/–) utricle (small arrow). Many of the supporting cells stained lightly and appeared almost translucent in the *BDNF* (–/–) mice.

(C and D) Ultrastructure of the sensory epithelium of macula. Note type 2 (2) and neck of type 1 (1) hair cells in control (C). Note numerous efferent endings on the type 2 hair cell (arrows) and the electrontranslucent nerve chalice (Ch) surrounding the type 1 hair cell. The vestibular hair cells present in the *BDNF* (–/–) utricles were predominantly of a type 2–like character (2) but without any neuronal contacts (D).

++, control mice; ––, *BDNF* (–/–) mice; 1, type 1 hair cell; 2, type 2 hair cell; Ch, nerve chalice. Bars, 25 μm (A and B); 0.6 μm (C and D).

(Figure 4C, arrows). *BDNF* (–/–) mice displayed a complete absence of afferents and a paucity of efferents (Figure 4D). The remaining few efferents contacted some of the hair cells of type 2 characteristics (data not shown). Thus, these results show that ultrastructural elements characteristic of afferents are absent in the *BDNF* (–/–) mice and that there is a paucity of efferent innervation, with few remaining efferent endings contacting the somas of type 2–like hair cells.

### *Mutant Vestibular Hair Cell Morphology*

Preceded at earlier developmental stages (e.g., E14–15) by an immature hair cell precursor, two morphologically distinct types of hair cells have been described for mature vestibular sensory receptors of mammals: the type 1 hair cell (pear-shaped soma enveloped by a chalice-like afferent nerve ending with efferent terminals contacting only the nerve chalice), and the type 2 hair cell (cylindrical soma with numerous small bouton-like nerve endings of both afferent and efferent character contacting its base). Analysis of utricular epithelium of P24 control mice revealed the presence of both of these hair cell types, with the type 1 hair cells being the most numerous type present in the sensory macula (Figures 4A and 4C). In comparison, the utricular sensory epithelium of the P24 *BDNF* (–/–) mice contained many vestibular hair cells that could not be clearly classified as either type 1 or type 2, but were closer in general appearance to the type 2 vestibular hair cell soma. These hair cells appeared immature, with a type 2–like morphology, and lacked both afferent and efferent nerve contacts (Figure 4D). Counts on the number of type 1 and type 2 hair cells in semithin sections prepared from the center of each utricle revealed no difference in the total number of vestibular hair cells present in *BDNF* mutant mice, as compared with controls. However, these results quantitatively confirmed a clear change in the predominant morphology from type 1 to type 2–like hair cells in *BDNF* mutant mice. Thus, most vestibular macula hair cells of *BDNF* (–/–) mice appear immature and lack afferent and efferent innervation.

### *Cochlear Afferent and Efferent Innervation*

The polyclonal antisera 9651 and 9993 against p75NGFR were used for immunocytochemical detection of cochlear afferent innervation. The 9651 and 9993 antisera stained only spiral ganglion neurons and afferents, with no immunoreactive material in the intraganglionic spiral bundle. The 9651 and 9993 antisera may therefore be staining predominantly afferents in the cochlea. The same staining pattern was obtained with these two antisera, and immunostaining of neural elements using the 9651 antisera is displayed in the figures.

In control mice, most or all spiral ganglion neurons stained for p75NGFR from E16 to P24 (Figure 5A). A few p75NGFR-positive afferents had penetrated the cochlear epithelium in E16 *BDNF* (–/–) mice, similar to the immunostaining pattern for control cochlear tissue (data not shown). In P0 and P24 control mice, immunoreactive material was detected close to the outer hair cells (Figure 5A, small arrows). In P0 and P24 *BDNF* (–/–) mice, no

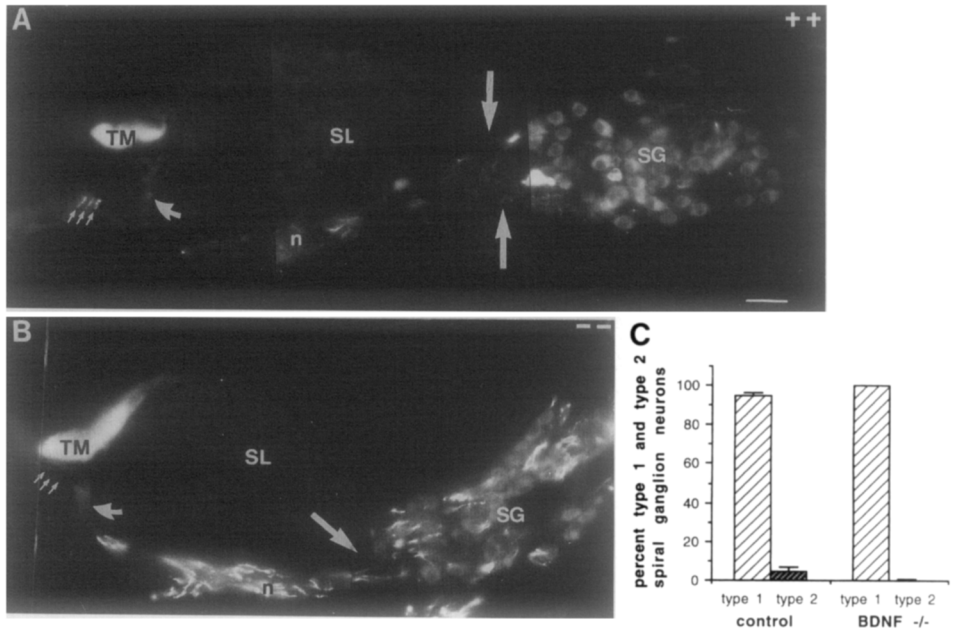


Figure 5. Selective Loss of Type 2 Spiral Ganglion Neurons and Afferent Innervation of Outer Hair Cells in *BDNF* (−/−) Mice

p75NGFR immunoreactivity in the cochlea of P24 control (A) and *BDNF* (−/−) (B) mice. Note immunoreactive material at the base of outer hair cells in the control (small arrows in [A]). Immunoreactivity at the level of outer hair cells (small arrows in [B]) is absent in the *BDNF* (−/−) mice. The arrows denote the intraganglionic spiral bundle. Nonspecific staining of the tectorial membrane (TM) was detected in both control and *BDNF* (−/−) mice. The percentage of type 1 and type 2 neurons in the spiral ganglion was plotted in control and *BDNF* (−/−) mice (C). Whereas the spiral ganglion of control mice contained approximately 5% of type 2 neurons, these were almost completely absent in the *BDNF* (−/−) mice. ++, control mice; −−, *BDNF* (−/−) mice; n, nerves projecting through the spiral limbus to the hair cells; SG, spiral ganglion; SL, spiral limbus; TM, tectorial membrane. Bar, 20 μm. Both panels are displayed at the same magnification.

p75NGFR immunoreactivity was present at the level of the outer hair cells (Figure 5B, small arrows). To obtain additional evidence for a deficiency of afferent innervation in outer hair cells in *BDNF* (−/−) mice, the number of type 1 and type 2 spiral ganglion neurons was determined in semithin plastic sections of P24 control and *BDNF* (−/−) mice. Type 1 neurons have been shown to innervate inner hair cells, and type 2, only outer hair cells (Berglund and Ryugo, 1987). The results in Figure 5C demonstrate that lack of BNDF resulted in an almost total loss of type 2 neurons but did not affect the number of type 1 neurons. Our results show that BDNF is crucial for outer hair cell innervation but appears not to affect significantly inner hair cell innervation. The absence of afferents at the level of the outer hair cells already at PO suggests a failure of outer hair cell innervation in *BDNF* (−/−) mice. Both P24 control and *BDNF* (−/−) mice displayed NF 150 kDa and AChE-positive fibers in approximation to both inner and outer hair cells (data not shown). These findings show that postnatal *BDNF* (−/−) mice lack afferent, but not efferent, innervation of the outer hair cells.

## NT-3 Mutant Mice

### *Vestibular and Spiral Ganglion Neurons*

At P10–15, *NT-3* (−/−) mice displayed a 34% decrease in the number of vestibular ganglion neurons as compared with control specimens (Table 1). The lack of NT-3 also affected the survival of spiral ganglion neurons. *NT-3* (−/−) mice showed an 87% loss of spiral ganglion neurons in the cochlea as compared with control mice (Table 1).

### *Vestibular Afferent and Efferent Innervation*

Despite the loss of 34% of the vestibular ganglion neurons in the *NT-3* (−/−) mice, no overt deficiencies could be detected in peripheral afferent innervation of the maculae of the saccule, utricule, or the cristae of the semicircular ducts at P6, as revealed by p75NGFR immunohistochemistry. Central vestibular afferents were traced with DiI in *NT-3* (−/−) mice. Vestibular afferents of P6 *NT-3* (−/−) mice were detected in the vestibular nuclei (Figure 3C), similar to controls (see Figure 3A). No obvious deficiencies were detected in NF 150 kDa-positive fibers of the vestibular sensory epithelium (data not shown). Thus, a subpopulation of vestibular neurons is lost in the *NT-3* (−/−) mice; however, this loss did not lead to any detectable deficiencies of innervation in central and peripheral targets.

### *Cochlear Afferent and Efferent Innervation*

The loss of most spiral ganglion neurons in *NT-3* (−/−) mice suggested a deficiency also of cochlear afferents. The central processes of spiral ganglion neurons terminate in the cochlear nuclei of the brain stem. Similar to the tracing of *BDNF* (−/−) mice, DiI was applied to the eighth cranial nerve attached to the brain stem of P6 control and *NT-3* (−/−) mice. Vibratome sections prepared at the level of the cochlear nuclei of control mice revealed a dense network of afferents terminating in areas corresponding to the cochlear nuclei (see Figure 3D). In contrast to control mice, *NT-3* (−/−) mice displayed an almost complete absence of cochlear afferents, and only occasionally were a few fibers identified (see Figure 3E, arrow; Figure

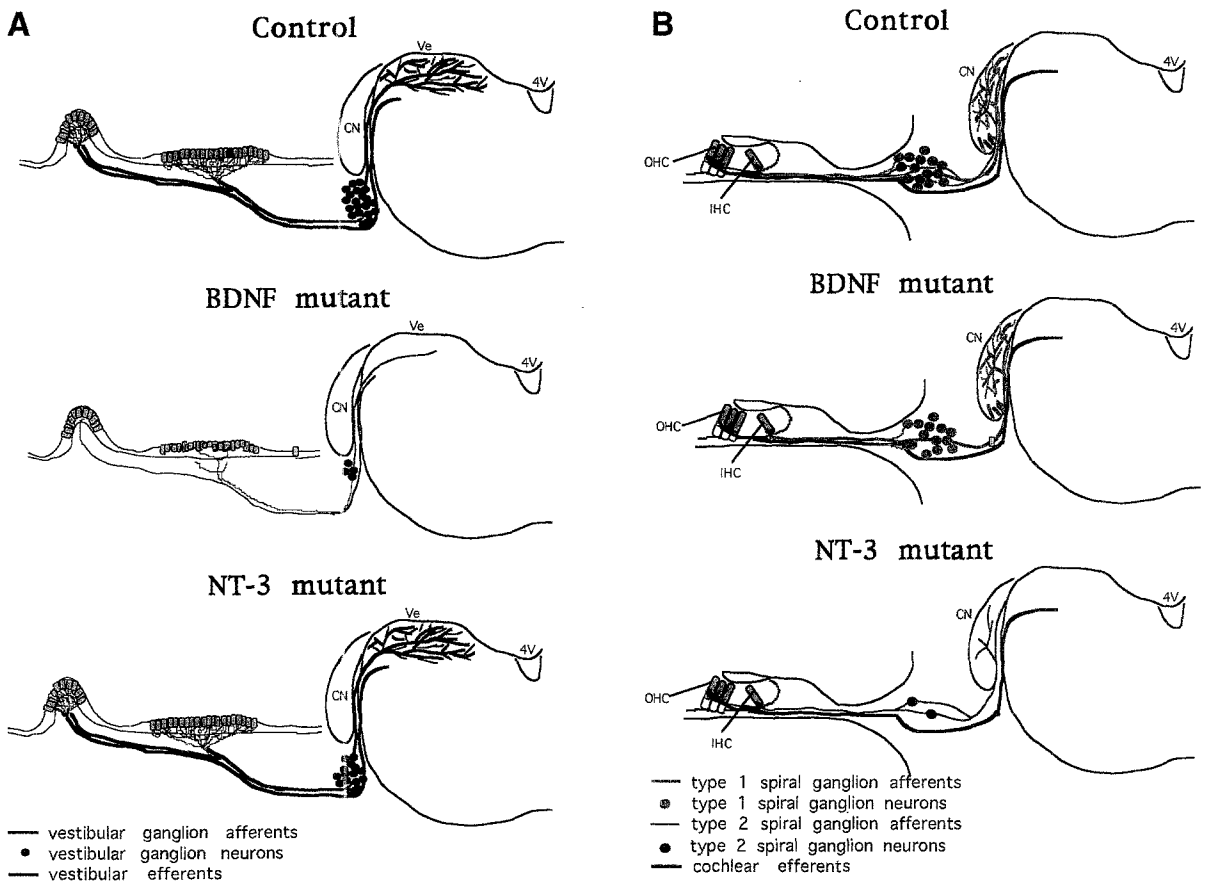


Figure 6. Schematic Representation of Vestibular and Auditory Deficits in Postnatal *BDNF* and *NT-3* Mutant Mice

Where stated, a deficit was suggested by indirect evidence. The structures are not drawn proportionally.

(A) Vestibular deficits. Red represents the vestibular ganglion neurons, central afferents terminating in the vestibular nuclei (Ve) and peripheral afferents terminating in the maculae of the utricule and saccule containing hair cells (grey). Efferents are represented by blue. *BDNF* (−/−) mice displayed a loss of approximately 80% of the vestibular ganglion neurons and a complete loss of afferent innervation of the vestibular sensory epithelia. Some afferents were detected in the connective tissue adjacent to the utricular and saccular sensory epithelia. *BDNF* (−/−) mice also showed a near complete absence of central afferents terminating in the vestibular nuclei. Most efferents in all vestibular epithelia were absent in the *BDNF* (−/−) mice. Vestibular hair cells of *BDNF* (−/−) mice appeared immature. *NT-3* (−/−) mice showed a 34% loss of vestibular ganglion neurons, but no visible loss of central and peripheral afferents and peripheral efferents as detected using immunohistochemistry and DiI tracing.

(B) Auditory deficits. Green represents the type 1 spiral ganglion neurons and their afferents, red the type 2 spiral ganglion neurons and their afferents. Type 1 afferents innervate the inner hair cells (IHC) and type 2 afferents the three rows of outer hair cells (OHC). Both type 1 and type 2 central afferents terminate in the cochlear nuclei (CN). *BDNF* (−/−) mice displayed a loss of 7% of the spiral ganglion neurons and innervation of OHC was absent (red), but the innervation of the IHC (green) appeared normal. An absence of OHC innervation correlated to a loss in the spiral ganglion of the type 2 neurons, which innervate the OHC cells. Efferent innervation of IHC and OHC appeared not to be compromised. *NT-3* (−/−) mice displayed an 87% loss of spiral ganglion neurons and had a near complete loss of central afferents in the cochlear nuclei. Deficits of peripheral afferent innervation were suggested by the lack of central afferents and a loss of only type 1 spiral ganglion neurons in the *NT-3* (−/−) mice was suggested by a complete absence of spiral ganglion neurons in the cochlea of the double mutant mice, which shows that BDNF and NT-3 act on distinct populations of spiral ganglion neurons. Efferent cochlear innervation appeared not to be compromised in *NT-3* (−/−) mice. Note that this table does not describe the developmental events leading to these deficits, some of which are described in Results.

4V, fourth ventricle; CN, cochlear nuclei; Ve, vestibular nuclei; IHC, inner hair cells; OHC, outer hair cells.

3F). Peripheral efferent cochlear innervation of *NT-3* (−/−) mice appeared normal, displaying NF 150 kDa-positive nerve fibers and AChE-positive material in approximation to both inner and outer hair cells of the cochlea, similar to control (data not shown). These results show that most spiral ganglion afferents are lost in the *NT-3* (−/−) mice, but AChE-positive efferents appear not to be compromised.

### *BDNF* and *NT-3* Double Mutant Mice

The observation that the deficiency in *BDNF* (−/−) mice primarily reflected a loss of vestibular ganglion neurons, and the deficiency in *NT-3* mice, primarily a loss of spiral ganglion neurons suggested a nonoverlapping role for neurotrophins in vestibular and auditory development. To confirm this, mice deficient in both BDNF and NT-3 were generated. *BDNF* (+/−) and *NT-3* (+/−) mice were crossed, and double heterozygous mice were generated at an expected frequency of approximately 25%. These mice were crossed and embryos were collected at E18. Homozygous double mutant mice were found at an expected frequency of 1/16, indicating that the lack of both BDNF and NT-3 did not compromise the survival of these embryos to late stages of gestation. In contrast to the single neurotrophin

Table 2. Summary of Innervation Deficiencies in the Postnatal Vestibular and Auditory Systems of *BDNF* and *NT-3* Mutant Mice

| | Control | *BDNF* (−/−) | *NT-3* (−/−) |
|---|---|---|---|
| Vestibule | | | |
| Vestibular ganglion neurons | +++ | + | ++ |
| Saccule + utricle | | | |
| Afferents | +++ | − | +++ |
| Efferents | +++ | + | +++[a] |
| Crista | | | |
| Afferents | +++ | − | +++ |
| Efferents | +++ | + | +++[a] |
| Central vestibular afferents | +++ | + | +++ |
| Cochlea | | | |
| Spiral ganglion neurons | +++ | ++ | + |
| IHC | | | |
| Afferents | +++ | +++ | ND |
| Efferents | +++ | +++ | +++ |
| OHC | | | |
| Afferents | +++ | − | ND |
| Efferents | +++ | +++ | +++ |
| Central cochlear afferents | +++ | +++ | + |

+++, normal complement; ++, reduced; +, severely reduced; −, none detected; ND, not determined. Note that this table does not describe the developmental events leading to these deficits, some of which are described in the Results section. IHC, inner hair cells; OHC, outer hair cells.

[a] Based only on NF 150 kDa immunohistochemistry.

homozygous mutant mice, the homozygous double mutant mouse embryos displayed an almost complete loss of vestibular as well as spiral ganglion neurons (99% and 100% loss, respectively; Table 1). These results show that virtually all vestibular and spiral ganglion neurons require either BDNF or NT-3 for their survival. They also indicate that distinct populations of neurons within the vestibular and spiral ganglion require either BDNF or NT-3, confirming the phenotypic analyses of the single mutant mice.

## Discussion

Our results are consistent with the notion that BDNF and NT-3 play crucial and specific roles in development of the vestibular and auditory systems (schematically depicted in Figure 6 and Table 2). The deficiency in the *BDNF* (−/−) mice is reflected in the loss of vestibular ganglion neurons, central and peripheral afferents, peripheral efferents, and hair cell differentiation. Also, the partial loss of neurons in heterozygous *BDNF* mutant mice suggests that the factor is limiting in vivo, similar to NT-3 for the proprioceptive system (Ernfors at al., 1994b). Whereas the vestibular ganglion neurons and vestibular afferents were affected embryonically, efferent innervation declined postnatally in *BDNF* (−/−) mice. The deficits of *NT-3* (−/−) mice reflected a loss of a subpopulation of vestibular ganglion neurons. Both BDNF and NT-3 influenced survival of spiral ganglion neurons and affected cochlear innervation. Lack of BDNF led to the loss of only a few spiral ganglion neurons and an absence of outer hair cell innervation, whereas *NT-3* (−/−) mice displayed a loss of most spiral ganglion neurons and afferents. Neurons of both vestibular and spiral ganglion were almost completely absent in double mutant mice deficient for BDNF and NT-3. Both BDNF and NT-3 are expressed by hair cells of the inner ear, and the BDNF and NT-3 receptor mRNAs (trkB and trkC) are expressed in vestibular and spiral ganglion neurons (Ernfors et al., 1992; Pirvola et al., 1992; Ylikoski et al., 1993). Furthermore, BDNF and NT-3 support the survival and neurite outgrowth from embryonic vestibular ganglion neurons (Avila et al., 1993; Davies et al., 1986; Pirvola et al., 1992; Vazquez et al., 1994). Together with our results, it therefore appears likely that BDNF and NT-3 represent the major, physiologically important neurotrophic factors required for normal development of the vestibular and auditory systems of the inner ear. Furthermore, comparison of *BDNF* and *NT-3* single mutant mice with double mutant mice suggests that these neurotrophins have distinct complementary and nonoverlapping roles in vestibular and cochlear development; i.e., one neurotrophin cannot functionally replace the other.

### Crucial Role of BDNF for Survival of Vestibular Neurons and Maintenance of Afferent and Efferent Vestibular Innervation

At concentrations suggestive of a high affinity receptor interaction, BDNF and NT-3 have the ability to induce volume increases of explanted chick vestibuloacoustic ganglia in culture and have thus been implicated in promoting proliferation of these neurons (Represa et al., 1993). Vestibular ganglion neurons are born between E10 and E12 in the mouse, with a peak in proliferation at E11 (Ruben, 1967). The attainment of a neuronal population equivalent to that of controls at E13 and the loss of vestibular ganglion neurons in the *BDNF* (−/−) mice between E13 and E16 suggests that BDNF is required for the survival of neurons after terminal mitosis, rather than as a stimulus for the proliferation of neuroblasts to form this ganglion. Pioneering afferent nerve fibers penetrate the vestibular sensory epithelia at E12, and a significant increase in nerve fibers has occurred by E16. Thus, the loss of vestibular ganglion neurons in the *BDNF* (−/−) mice occurs during the developmental period of target innervation. This is consistent with the classical neurotrophic factor hypothesis that target-derived neurotrophic factors regulate the survival of neurons at the time of programmed cell death.

Afferents penetrate the vestibular sensory epithelia of control mice at E13, and hair cell–nerve contact was suggested at E16 by the presence of immunoreactive material at the base of the hair cells forming nerve chalices. This corresponds well with the previous reported period of fiber ingrowth (Galinovic-Schwartz et al., 1991) and the ultrastructural detection of afferent nerve endings contacting hair cells (Anniko et al., 1979a; Mbiene et al., 1988; Van De Water et al., 1978). Local chemoattractant fields have been proposed for the establishment of inner ear sensory innervation (Van De Water et al., 1989; Van De Water and Ruben, 1983). The presence of nerve fibers in the *BDNF* (−/−) utricular sensory epithelia at E13 suggests that target tissue recognition and ingrowth of nerve fibers occur independently of BDNF.

Only a subpopulation of vestibular neurons was absent

in the *NT-3* (−/−) mice. Peripheral and central afferent vestibular innervation in these mice appeared qualitatively normal. It is possible that the remaining vestibular neurons compensate for the loss by sprouting, leading to a normal complement of nerve fibers in the targets. However, we cannot exclude the possibility that these mice have a quantitative deficiency of nerve fibers corresponding to the loss of vestibular neurons.

BDNF may be required either directly or indirectly for vestibular efferent innervation. The efferents invade the sensory epithelia embryonically (Fritzsch and Nichols, 1993) but continue to mature biochemically and morphologically for several weeks after birth (Emmerling et al., 1990; Shnerson et al., 1981; Sobkowicz and Emmerling, 1989). *BDNF* mRNA is expressed throughout development and in the mature hair cells of the vestibular sensory epithelia (Ylikoski et al., 1993). Thus, it is possible that BDNF is acting directly on the efferent innervation and perhaps as a survival factor for the vestibular efferent neurons. Because the efferent endings form synaptic contact with afferent nerve chalices of the type 1 hair cells (Ohno et al., 1993a, 1993b), it is also possible that the loss of efferents is indirect, caused by the absence of afferents in the *BDNF* (−/−) mice. The finding of few efferent contacts in *BDNF* (−/−) utricles terminating directly on the hair cell soma (i.e., on the type 2–like hair cells), but no efferents on any type 1–like hair cells, which would normally receive efferent endings on their afferent nerve chalices, suggests that the loss of efferents is indirect and caused by a lack of afferents in the *BDNF* (−/−) mice.

### Role of BDNF for Inner Hair Cell Innervation and NT-3 for Outer Hair Cell Innervation in the Cochlea

Our results strongly suggest that BDNF and NT-3 also exert different functions in the cochlea. Whereas lack of *NT-3* eliminated most spiral ganglion neurons (87%), only approximately 7% were absent in *BDNF* (−/−) mice. Mouse spiral ganglion neurons consist of two distinct populations of neurons, the type 1 afferent cell (92%-94% of the neuronal population), which are myelinated, have numerous cytoplasmic organelles, and a more lightly colored nucleus with a prominent nucleolus, and the type 2 afferent cell (approximately 6%-8% of the neurons), which are unmyelinated, have few cytoplasmic organelles, and an irregular nucleus including a small nucleolus (Romand and Romand, 1987). The myelinated type 1 neurons have been shown to innervate exclusively inner hair cells, and the unmyelinated type 2 neurons, only outer hair cells in the mouse (Berglund and Ryugo, 1987). A loss of 7% of the spiral ganglion neurons in *BDNF* (−/−) mice and loss of the morphological population of type 2 spiral ganglion neurons in these mice suggest that BDNF acts exclusively as a survival factor for type 2 spiral ganglion neurons. This is also consistent with the absence of afferent outer hair cell innervation in the *BDNF* (−/−) mice, which is achieved by the 6%–8% type 2 neurons in the spiral ganglion. The observation that all spiral ganglion neurons are lost in the double mutant mice is entirely consistent with the notion that BDNF and NT-3 act on distinct neuronal populations within the spiral ganglion. It therefore appears likely that BDNF is a survival factor for the type 2 spiral ganglion neurons affecting outer hair cell innervation and NT-3 for the type 1 neurons of this ganglion affecting the innervation of the inner hair cells.

### Is BDNF Required for Vestibular Hair Cell Maturation?

Little is known of the signals that participate in inner ear hair cell differentiation. Most hair cells of the vestibular sensory epithelia appeared immature and un/dedifferentiated in P24 *BDNF* (−/−) mice. Because vestibular hair cells differentiate close to birth in the mouse (Anniko et al., 1979a, 1979b; Mbiene and Sans, 1986; Mbiene et al., 1988; Van De Water et al., 1978), these findings are consistent with either a direct or indirect effect of BDNF on hair cell maturation or maintenance of the differentiated state. A direct effect could be mediated by an autocrine loop, because BDNF is expressed in the embryonic and postnatal sensory epithelia (Ernfors et al., 1992; Pirvola et al., 1992; Ylikoski et al., 1993). However, in this case, BDNF would have to act through a noncatalytic form of the trkB receptor, which appears to be the only trkB receptor expressed in the vestibular sensory epithelia (Ylikoski et al., 1993). The vestibular sensory epithelia of *BDNF* (−/−) mice lacked innervation, and it is possible that the deficient hair cell differentiation is indirect, caused by the loss of afferent and/or efferent innervation. Embryonic maturation of deafferented hair cells in culture, after in vivo destruction of the afferent innervation, occurs normally, suggesting that short-term loss of afferents do not influence differentiation (Anniko et al., 1979a; Hirokawa, 1977; Van De Water, 1976). However, prolonged denervation of vestibular hair cells may lead to a dedifferentiation of hair cells (Favre and Sans, 1991). It is therefore possible that the immature appearance of the hair cells in the P24 *BDNF* (−/−) mice is caused by the loss of innervation instead of a direct action of BDNF on hair cell differentiation.

Hair cells of the mammalian sensory epithelia in the inner ear cease proliferation close to birth, followed by maturation (Anniko et al., 1979a, 1979b; Hirokawa, 1977; Ruben, 1967; Sans and Chat, 1982; Van De Water et al., 1978). However, trauma in the inner ear caused by aminoglycoside antibiotic-induced ototoxicity induces proliferation in the adult mammalian sensory epithelia and the reappearance of immature hair cells (Forge et al., 1993; Lefebvre et al., 1993; Warchol et al., 1993). The failure of regenerated hair cells to mature and their poor reinnervation (Forge et al., 1993) would be consistent with the permanent condition of hearing impairment or balance dysfunction in affected humans. Perhaps exogenously administered BDNF and/or NT-3 could enhance hair cell innervation and differentiation.

## Experimental Procedures

### Animals and Genotyping

Offspring from crosses of mice heterozygous for a deletion of the *BDNF* or *NT-3* genes were used in this study (Ernfors et al., 1994a, 1994b). The day of the vaginal plug was considered E0. In all experiments, the tails were cut and DNA was purified according to Laird et al. (1991). The mice were genotyped by the polymerase chain reaction. Double

mutant mice were generated by crossing mice heterozygous for both *BDNF* and *NT-3*.

### Tissue Processing

For immunohistochemistry on postnatal tissues, mice were anesthetized and perfused through the heart with 4% paraformaldehyde (PFA) and the tissues postfixed at 4°C for 2 hr. For immunocytochemistry on embryonic and neonatal tissues, the heads were cut and immersion fixed in 4% PFA overnight. All tissues were cryoprotected in 30% sucrose (in phosphate-buffered saline [pH 7.4]) after the fixation and frozen at −70°C. For immunohistochemistry on embryonic and P0, P4, and P6 mice, transversal frozen sections (10 μm) of the head were cut at the level of the inner ear. For mice older than 1 week, inner ears were dissected and decalcified prior to embedding for frozen sections. Two or three mice from each age and genotype were analyzed. The slides were incubated in dilution buffer (0.5 M NaCl, 0.01 M phosphate buffer [pH 7.3], 3% bovine serum albumin, and 0.3% Triton X-100) for 1 hr, followed by overnight incubation with the indicated concentration of antisera in dilution buffer. After four washes in phosphate-buffered saline, sections were incubated for 2 hr with the appropriate rhodamine-conjugated secondary antiserum, washed three times for 10 min, and covered with glycerol/phosphate-buffered saline (9:1) for viewing. The rabbit 9651 and 9993 antisera were diluted at 1:200; the rabbit anti-NF 150 kDa, 1:500; the mouse monoclonal anti-AChE, 1:200; the rabbit anti-synaptophysin, 1:50. Rhodamine-conjugated goat anti-mouse and goat anti-rabbit antisera (diluted 1:200) were used as secondary antisera. In addition to the above antibodies, an anti-phosphorylated NF 200 kDa antibody (RT 97) was used. This antibody was found unreliable for the detection of efferents because no staining was found in the sensory epithelia of neonatal control and *BDNF* (−/−) mice or older *BDNF* (−/−) mice despite the presence of efferents as revealed by NF 150 kDa immunoreactivity, bouton-like synaptophysin-positive endings on type 1 hair cells, and the ultrastructural identification of efferents.

For neuronal counts of postnatal vestibular ganglia, these were dissected from perfused mice, postfixed for 2 hr in 4% PFA, equilibrated in sucrose, frozen on dry ice, and sectioned at 7 μm thickness on a cryostat. The sections were stained with cresyl violet, and the number of neurons in every third section was counted. For neuronal counts of postnatal spiral ganglia, the inner ears were dissected, decalcified, paraffin embedded, and 5 μm sections were cut and stained with cresyl violet. Neurons of every fourth section were counted. For counts of embryonic and neonatal vestibular and spiral ganglia, heads were immersion fixed in 4% PFA overnight, paraffin embedded, and 5 μm sections were cut and stained with cresyl violet. Every fourth section was counted. Only the neurons with a clear nucleus and nucleoli were counted, and the counts were not corrected for split nucleoli.

### Specificity of the p75NGFR Antisera

The specificity of the 9651 and 9993 antisera has been established. These antisera detect only a single product on Western blots, corresponding to the p75NGFR protein (M. Chao, personal communication). In addition, similar staining patterns were obtained with the two different antisera against p75NGFR. Structures rich in glycoproteins, such as the tectorial membrane and the otolithic membrane, have previously been shown to absorb antibodies nonspecifically (Despres et al., 1991). The staining of this material was therefore considered to be nonspecific.

### Semithin Sections and Electron Microscopy

P24 *BDNF* (−/−) (n = 3) and control mice (n = 3) were anesthetized and perfused through the heart with a fixative containing 2.5% glutaraldehyde and 2% PFA in 0.1 M cacodylate buffer. Vestibular endorgans and the apical and basal turn of the cochlea were dissected bilaterally and immersion fixed for an additional 2 hr, postfixed for 1 hr in 1% $OsO_4$, and embedded in Eponate 12. Alternating semithin and thin sections were collected. The semithin (1 μm sections) were stained with toluidine blue for light microscopic examination. Thin sections (0.1 μm) were stained with uranyl acetate and lead citrate, carbon coated, and viewed with a Hitachi H-600 scanning transmission electron microscope operating at 75 kV.

The number of type 1 and type 2 spiral ganglion neurons were counted in the apical turn of P24 control and *BDNF* (−/−) cochlea. A total of 1000 spiral ganglion neurons were sampled for each mouse.

### DiI Tracing

P6 *BDNF* (−/−) (n = 3), *NT-3* (−/−) (n = 5), and control mice (n = 6) were perfused with 4% PFA, and the heads, with the brain exposed, were postfixed overnight in 4% PFA. The brains were carefully lifted dorsally and caudally, holding the olfactory bulbs with a pair of forceps. The intact fifth and seventh cranial nerves were identified and cut to expose the eighth cranial nerve. Crystals of DiI (Molecular Probes) were applied unilaterally to the intact nerve, and the nerve was cut distal to the DiI using fine iris scissors. The brains were dissected and pinned down on Sylgard gel petri dishes with insect pins and submerged in 4% PFA. To reduce nonspecific diffusion of the dye, insect pins were used to prevent distal parts of the nerve stump from coming into direct contact with the brain. For the tracing of vestibular afferents, *BDNF* (−/−), *NT-3* (−/−), and control brains were incubated at 50°C for 14 days. For tracing of cochlear afferents, *NT-3* (−/−) and control brains were incubated at 50°C for 6 days. The brains were rinsed several times in phosphate-buffered saline followed by embedding in 2% agar at 50°C. Cross-sections (70 μm) of the brain stems were cut on a vibratome and viewed with a rhodamine filter on a Zeiss microscope.

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