Degeneration of vestibular neurons in late embryogenesis of both heterozygous and homozygous *BDNF* null mutant mice

Lynne M. Bianchi[1], Joanne C. Conover[1], Bernd Fritzsch[2], Thomas DeChiara[1], Ronald M. Lindsay[1],* and George D. Yancopoulos[1]

[1]Regeneron Pharmaceuticals Inc., 777 Old Saw Mill River Road, Tarrytown, NY 10591, USA
[2]Department of Biomedical Sciences, Creighton University, Omaha, NE 68178, USA

*Author for correspondence

SUMMARY

The generation of mice lacking specific neurotrophins permits evaluation of the trophic requirements of particular neuronal populations throughout development. In the present study, we examined the developing vestibulo-cochlear system to determine the time course of neurotrophin dependence and to determine whether competition occurred among developing cochlear or vestibular neurons for available amounts of either brain-derived neurotrophic factor (BDNF) or neurotrophin-4/5 (NT-4/5). Both cochlear and vestibular neurons were present in mice lacking NT-4/5. In contrast, vestibular neurons decreased in number beginning at mid-stages of inner ear development, in mice lacking BDNF. Early in development (E12.5-13), the size of the vestibular ganglion was normal in $bdnf^{-/-}$ mice. Decreased innervation to vestibular sensory epithelia was detected at E13.5-15, when progressive loss of all afferent innervation to the semicircular canals and reduced innervation to the utricle and saccule were observed. At E16.5-17, there was a reduction in the number of vestibular neurons in $bdnf^{-/-}$ mice. A further decrease in vestibular neurons was observed at P1 and P15. Compared to $bdnf^{-/-}$ mice, mice heterozygous for the *BDNF* null mutation ($bdnf^{+/-}$) showed an intermediate decrease in the number of vestibular neurons from E16.5-P15. These data indicate a late developmental requirement of vestibular neurons for BDNF and suggest competition among these neurons for limited supplies of this factor.

Key words: vestibulocochlear, neurotrophin, inner ear, neuron, BDNF, mouse

INTRODUCTION

During nervous system development, a period of extensive neuronal cell death is thought to result in part from competition for limited amounts of target-derived neurotrophic factors (Levi-Montalcini, 1987; Davies, 1988). This was first suggested from studies that demonstrated that manipulating the size of the target tissue directly influenced the number of neurons able to survive (Hollyday and Hamburger, 1976; Levi-Montalcini and Booker, 1960; Levi-Montalcini and Angeletti, 1966; Levi-Montalcini, 1987). At the molecular level, nerve growth factor (NGF) has served as the prototypical neurotrophic factor, with extensive evidence supporting a target-derived role of this molecule in the development of sympathetic and neural-crest-derived sensory neurons. Members of the recently discovered NGF-related neurotrophin family (reviewed by Lindsay et al., 1994), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3) and neurotrophin-4/5 (NT-4/5) have also been proposed to function as target-derived factors, but evidence for this is less well established.

The recent generation of mice lacking expression of specific neurotrophins (Crowley et al., 1994; Ernfors et al., 1994a,b; Farinas et al., 1994; Jones et al., 1994; Conover et al., 1995) is helping to refine details of the neuronal specificities of these factors and, in particular, is allowing the growth factor requirements of specific neuronal populations to be examined throughout development. By examining the status of neuronal populations in mice which lack neurotrophins, it is now possible to determine in vivo whether individual neuronal populations rely on specific neurotrophic factors during early neurogenesis, periods of initial neurite outgrowth, or at the stage of target encounter and subsequent innervation. Additionally, mice that are heterozygous (+/−) for a targeted null mutation of a neurotrophin provide a means to assess 'gene-dosage' effects by relating changes in neurotrophin production to the number of surviving neurons in a particular neuronal population.

Recent histological analyses of mice lacking one or another of the neurotrophins has provided insights into temporal aspects of their roles. For example, in mice lacking NGF, dorsal root ganglion (DRG) neurons were greatly decreased in number at birth, whereas sympathetic neuron numbers were not decreased until P10. Because sympathetic neurons become dependent on NGF later in development than DRG neurons, it was suggested that NGF regulated neuronal survival during the periods of programmed cell death (Crowley et al., 1994). Recent reports have also provided support for the hypothesis that there are reciprocal trophic interactions between neurons

and their target tissues. Muscle spindles, which depend on large sensory fiber afferent innervation for formation, were found to be completely absent at birth in mice lacking NT-3 (nt-$3^{-/-}$). In contrast, muscle spindle number was reduced by 50% in *NT-3* heterozygous null mutants (nt-$3^{+/-}$). These results also suggested that levels of NT-3 were reduced in nt-$3^{+/-}$ mice (Ernfors et al., 1994b). However, detailed evaluation of the age-dependent and the dose-dependent effects of neurotrophins have not yet been examined in detail in peripheral neuronal populations of neurotrophin-deficient mice.

Unlike other more abundant or more accessible populations of primary afferent neurons, the growth factor requirements of vestibular and cochlear neurons are less well defined. The mature inner ear consists of both vestibular structures that regulate balance and cochlear structures responsible for hearing. The inner ear forms initially as a thickened patch of ectoderm that invaginates to form the otic vesicle or otocyst (Knowlton, 1967; Alvarez and Navascues, 1990; Li et al., 1978). The relatively simple, spherical-shaped otocyst then undergoes a series of morphological changes to give rise to the vestibular and cochlear regions of the inner ear. The stato-acoustic ganglia (SAG or vestibulocochlear ganglia) arises from the ventromedial wall of the otocyst initially as one complex that subsequently separates into distinct vestibular and cochlear segments (Li et al., 1978).

Detection of *BDNF* and *NT-3* mRNA in the early stage otocyst has led to speculation that these factors may be required for early formation and perhaps guidance of statoa-coustic (SAG) neurons to their target tissues (Hallböök et al., 1993; Schecterson and Bothwell, 1992; Pirvola et al., 1992, 1994). Consistent with this possibility, in situ hybridization analyses of SAG neurons have identified transcripts for neurotrophin receptors, TrkA, TrkB and TrkC (Pirvola et al., 1992, 1994; Dechant et al., 1993; Schecterson and Bothwell, 1992; Bernd et al., 1994). However, several in vitro studies have suggested that neurotrophins are unable to directly effect the survival or outgrowth of SAG at the early stages of inner ear development (Davies et al., 1986; Represa and Bernd, 1989; Bianchi and Cohan, 1993). Other in vitro studies have provided mixed results on whether neurotrophins influence auditory and vestibular neurons at various stages of development (Lefebvre et al., 1991; Avila et al., 1993; Vogel and Davies, 1991). Thus, in vitro models have not clearly established the developmental time course for neurotrophin-dependence in the vestibulo-cochlear system.

Initial reports describing neurotrophin null mutant mice provided evidence that BDNF and NT-3, but not NT-4/5, are ultimately required for maintenance of the vestibulocochlear system (Ernfors et al., 1994, 1995; Jones et al., 1994; Conover et al., 1995; Liu et al., 1995). The present study provides a detailed analysis of the development of vestibular and cochlear structures in mice heterozygous or homozygous for the *BDNF* and/or *NT-4/5* null mutations. Our results indicate that cochlear neurons are largely intact throughout development in mice lacking BDNF, NT-4/5, or both these neurotrophins. In contrast, beginning at mid-stages of inner ear development, vestibular ganglia demonstrate a developmental decrease in the number of vestibular neurons in the absence of BDNF. The absence of both BDNF and NT-4/5 does not result in any greater decrease in vestibular neurons. Compared to $bdnf^{-/-}$ mice, $bdnf^{+/-}$ mice show an intermediate decrease in vestibu-

lar neurons from E16.5-P15, suggesting that BDNF is present in 'intermediate' quantities in mice heterozygous for the null mutation. Support for this idea was obtained by comparing BDNF protein levels from neural tissue of $bdnf^{+/+}$, $bdnf^{+/-}$ and $bdnf^{-/-}$ mice. Consistent with the classical definition of the neurotrophic hypothesis, the results of this study provide further in vivo evidence that developing peripheral neurons compete for available levels of neurotrophic factor.

Following submission of the current manuscript, a paper by Ernfors et al. (1995) describing development of the vestibulo-cochlear system in mice lacking BDNF and NT-3 was published. Similarities and differences to their findings are described below.

MATERIALS AND METHODS

Generation of *BDNF*, *NT-4/5* and *BDNF-NT-4/5* null mutant mice

The generation of mice with targeted disruption of either the *BDNF* or *NT-4/5* genes has been described previously (Conover et al., 1995). Mice lacking in expression of both *BDNF* and *NT-4/5* were generated from mice heterozygous for the *BDNF* and *NT-4/5* null mutations. Southern blot analysis of genomic DNA obtained from mouse tails was performed to determine the genotype of each animal analyzed (Conover et al., 1995). Wild-type (+/+), heterozygous (+/−) and homozygous (−/−) mice were examined at embryonic (E12.5-13, E13.5-14, E14.5-15, E16.5-17) and postnatal (P1, P7 and P15) stages. All experimental groups contained 2-8 animals. All animal procedures were conducted under protocols reviewed and approved by an Institutional Animal Use and Care Committee in compliance with NIH guidelines

Histological analysis
Serial sections

Embryonic mice were immersed in 4% paraformaldehyde for 2-4 days, then processed for paraffin embedding. Postnatal mice were perfused intracardially with 4% paraformaldehyde. Inner ear tissues from P15 mice were decalcified prior to processing for paraffin embedding. Serial sections (7-10 µm) from embryonic and postnatal mice were stained with hematoxylin and eosin. Each section was examined to identify the number of sections containing neuronal somata. Measures of the area occupied by neuronal cell bodies in 10 randomly selected, but evenly spaced sections throughout the regions containing neurons were obtained (Jandel Image Analysis System, Zeiss Axioplan). Areas containing nerve fibers were excluded. The volume of each vestibular ganglion ($\mu m^3 \times 10^6$) was then calculated from the cross-sectional area, section thickness and number of sections containing neurons. Comparisons of neuronal numbers were made by counting the number of neurons with a clear nucleus in the plane of section in every second or third section (18-21 µm intervals). At P15, neuronal counts were also corrected based on the nuclear diameter and section thickness using the formula of Abercrombie (1946). Similar reductions of neuronal number in heterozygous and homozygous mice were obtained with both counting methods. Data shown represent uncorrected counts. To compare neuronal soma size, the diameter of 100 randomly selected neurons throughout each ganglia of P15 mice was measured. In the figures, values represent the means and standard errors.

Immunohistochemistry for neurofilament detection

Neurofilaments were detected with the RT97 antibody. Sections through the cochlear ganglia were deparaffinized, cleared, blocked with serum then incubated in the primary RT97 antibody (1:500, room

temperature 1-2 hours). Sections were then processed using the avidin-biotin-peroxidase technique (Vectastain Elite, Burlingame, CA). Adjacent sections lacking the primary antibody were used as controls.

Whole-mount surface preparations

An analysis of afferent connections to the inner ear was performed as previously described (Fritzsch and Nichols, 1993). Briefly, DiI (1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine percholate; Molecular Probes) soaked filter strips were applied to the alar plate rostral to the statoacoustic (octaval) nerve root in E13.5-14, E14.5-15 and P7 *bdnf*⁺/⁺ and *bdnf*⁻/⁻ mice. After a diffusion time of 3-8 days at 36°C, the ears, with otic ganglion and facial nerve attached, were dissected free from adjacent periotic mesenchyme and viewed and photographed as whole mounts. To examine the integrity of cochlear sensory hair cells and myelinated nerve fibers, aldehyde-fixed cochleae from postnatal mice were perfused through the round window with 1.5% osmium tetroxide. The organ of Corti was then dissected and viewed as whole-mount surface preparations.

Analysis of BDNF protein levels

BDNF protein levels were obtained from neural tissues using an enzyme-linked immunoabsorbant assay (ELISA) as previously described (Radke et al., 1995). Briefly, equal numbers of dorsal root ganglia were dissected from *bdnf*⁺/⁺, *bdnf*⁺/⁻ and *bdnf*⁻/⁻ mice and homogenized in lysis buffer (50 mM Tris HCl, 0.6 M NaCl, 0.2% Triton X-100, 1% BSA, 0.1 mM benzethonium chloride, 1 mM ben-zamidine, and 0.1 mM PMSF at pH 7.4). Tissues were homogenized and centrifuged at 4°C for 30 minutes at 10,000 *g*. Supernatants (50 μl/sample) were then assayed using a BDNF-specific monoclonal antibody as a capture antibody and a biotinylated, affinity-purified rabbit anti-BDNF polyclonal antibody as a reporter system (Regeneron Pharmaceuticals). An HRP-avidin conjugate was detected using TMB as the substrate for colormetric analysis. The optical density was read at 450 nm with a 570 nm correction for the plastic interference (Molecular Devices). The ELISA detected BDNF at concentrations of 10 pg/ml-2.5 ng/ml, but did not recognize NGF, NT-3, or NT-4/5 at concentrations up to 100 ng/ml.

RESULTS

Formation of SAG in the absence of BDNF

Previous studies of mice lacking BDNF (*bdnf*⁻/⁻) had revealed a significant deficit in the normal complement of neurons of the vestibular ganglion by the second postnatal week (Ernfors et al., 1994a; Jones at al., 1994; Conover et al., 1995). To determine more accurately at what stage of development BDNF is required for survival of vestibular neurons, we examined the ganglia at stages corresponding to the periods of initial neurite outgrowth through late-synaptogenesis (Desmadryl and Sans, 1990, Desmadryl et al., 1992).

Examination of serial cross-sections indicated that the SAG at E12.5-E13 was similar in size and appearance in both wild-type mice (*bdnf*⁺/⁺) and *bdnf*⁻/⁻ mice (Fig. 1). At this early stage of development, corresponding to the period of initial neurite outgrowth, the ganglia is not yet separated into distinct vestibular and cochlear regions. However, the volumes of the entire SAG complex were similar in both *bdnf*⁺/⁺ ($12.4\pm1.6\times10^6$ μm³) and *bdnf*⁻/⁻ mice ($11.6\pm1.0\times10^6$ μm³), suggesting that at early stages of development, the development of the vestibular component of the SAG is not dependent on BDNF.

Vestibular innervation in the absence of BDNF

By E13.5-14, innervation to all vestibular sensory epithelia of the inner ear was established in *bdnf*⁺/⁺ mice (Fig. 2A,C; *n*=8). However, the innervation to vestibular sensory epithelia was reduced in *bdnf*⁻/⁻ mice at this stage (*n*=8). Among the *bdnf*⁻/⁻ mice, afferent innervation to the posterior vertical canal was absent (Fig. 2B). In addition, the innervation to the anterior vertical and horizontal canals, if present at all (Fig. 2B,D), was reduced compared to the *bdnf*⁺/⁺ littermates. By E14.5-15, all innervation to the semicircular canals was lost in *bdnf*⁻/⁻ mice (Fig. 3, *n*=5). In contrast, the utricle and saccule showed only reduced innervation at both E13.5 and E14.5. Although the
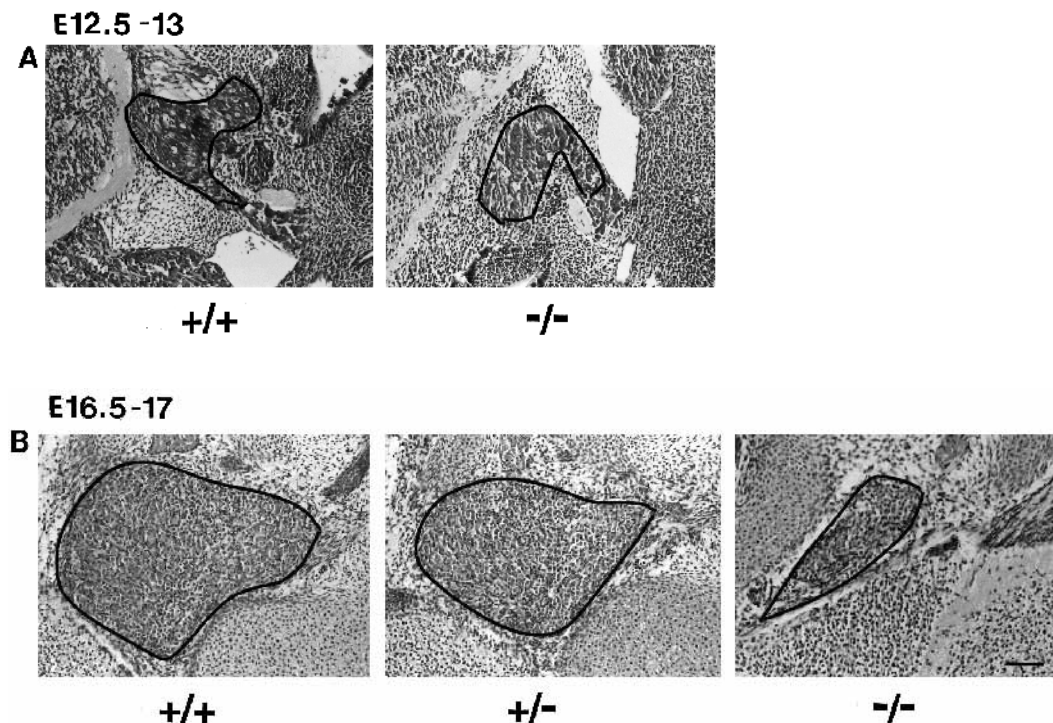

E12.5 -13

A   **+/+**   **-/-**

E16.5 -17

B   **+/+**   **+/-**   **-/-**

Fig. 1. Cross sections of embryonic SAG and vestibular ganglia. (A) At E12.5-13 the SAG similar is in size in both wild-type (+/+) and *bdnf*⁻/⁻ mice. (B) At E16.5-17 the vestibular ganglia is dramatically reduced in size in *bdnf*⁻/⁻ mice. The *bdnf*⁺/⁻ mice show an intermediate reduction in the size of vestibular ganglia at this stage. Area of ganglia is outlined in black. Scale bar, 100 μm.

utricle was clearly innervated throughout the entire sensory epithelia, a reduction in afferent fiber density was detected (compare Figs 2C,D, 3). The fibers that were present appeared to branch more profusely within the sensory epithelium and formed growth cones surrounding most, if not all, sensory hair cells. Within the saccule, a reduction in innervation was observed in the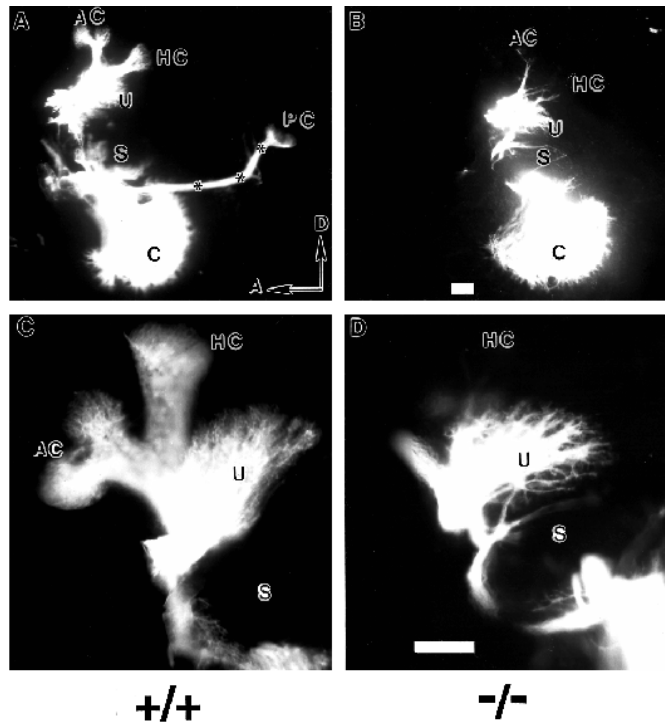 dorsal region (supplied by fibers running with the vestibular root), but not ventral region (supplied by fibers from the cochlear nerve root).

Loss of vestibular neurons during mid-stages of inner ear development in the absence of BDNF

In the mouse inner ear, the vestibular component of the SAG is clearly distinguishable from the cochlear portion by E16.5-17. By this stage, the vestibular fibers have begun to make synaptic contact with their sensory hair cells. Examination of E16.5-17 *bdnf*^{-/-} mice revealed a decrease in vestibular ganglion volume and neuronal number compared to *bdnf*^{+/+} mice (Table 1; Fig. 1). By P1 there was a further reduction in the volume and number of neurons in the vestibular ganglia of *bdnf*^{-/-} mice (Table 1). At P15, *bdnf*^{-/-} mice had very few neurons remaining in vestibular ganglia (Fig. 4). The volume of the vestibular ganglia represented only 4% of the volume measured in *bdnf*^{+/+} mice (Table 1), whereas the number of vestibular neurons in *bdnf*^{-/-} mice was only 2% of the number found in *bdnf*^{+/+} mice (Table 1).

Mice heterozygous for the *BDNF* null mutation display a gene dosage decrease in vestibular ganglia and reduction in BDNF protein levels

Compared to wild-type or *bdnf*^{-/-} mice, mice heterozygous for the *BDNF* null mutation (*bdnf*^{+/-}) had an intermediate decrease
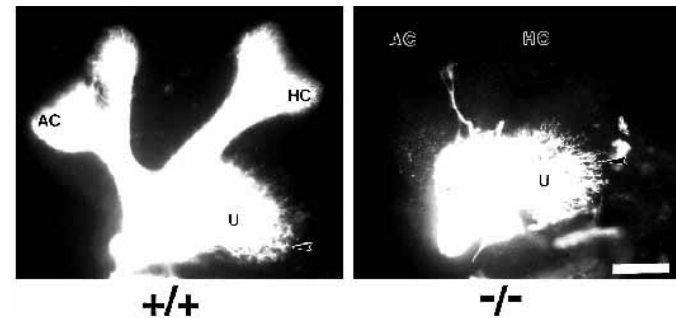

Fig. 2. Comparison of DiI-labeled flat mounts of E13.5-14 *bdnf*^{+/+} (A,C) and *bdnf*^{-/-} (B,D). Innervation to sensory epithelia is present in *bdnf*^{+/+} mice, however innervation is reduced to the vestibular epithelia in *bdnf*^{-/-} mice. In *bdnf*^{-/-} mice, fibers innervating the posterior canal (PC, *) are absent whereas fibers to the anterior vertical (AC) and horizontal canals (HC) are greatly reduced. In contrast to the semicircular canals, the utricle (U) and saccule (S) reveal a more modest reduction in nerve fiber density. Innervation to the cochlea (C) was similar in both *bdnf*^{+/+} and *bdnf*^{-/-} mice. Scale bar, 100 µm, Arrows indicate anterior, A and dorsal, D.

Fig. 3. Comparison of DiI-labeled flat mounts of E14.5-15 *bdnf*^{+/+} and *bdnf*^{-/-} mice. In contrast to *bdnf*^{+/+} mice, *bdnf*^{-/-} mice have nearly complete loss of innervation to the semicircular canals. A few fibers extend toward, but do not reach, the anterior vertical canal (AC). Fibers are not detected in the region of the horizontal canal (HC). The utricular innervation (U) extends throughout the area of sensory hair cells with many processes ending in boutons at the level of the hair cells (arrowhead). Scale bar, 100 µm.

Table 1. Comparison of vestibular ganglia volume and neuronal number

Developmental stage	*bdnf*^{+/+}			*bdnf*^{+/-}			*bdnf*^{-/-}		
E16.5-17									
Volume ($\times 10^6$ µm^3)	11.9±0.17	(*n*=3)	100%	7.3±0.6	(*n*=3)	61%	3.4±0.3	(*n*=3)	28%
Number of neurons	1597±140	(*n*=3)	100%	921±56	(*n*=3)	57%	593±89	(*n*=3)	37%
P1									
Volume ($\times 10^6$ µm^3)	11.4±0.5	(*n*=5)	100%	8.4±0.4	(*n*=2)	73%	1.3±0.3	(*n*=6)	11%
Number of neurons	1417±54	(*n*=5)	100%	972±120	(*n*=2)	69%	297±18	(*n*=6)	21%
P15									
Volume ($\times 10^6$ µm^3)	25.0±4.2	(*n*=2)	100%	11.3±0.3	(*n*=3)	45%	1.0±0.4	(*n*=3)	4%
Number of neurons	1248±139	(*n*=3)	100%	739±48	(*n*=3)	59%	29±8	(*n*=3)	2%

Comparison of the reduction of vestibular ganglion volume and neuronal number in *bdnf*^{+/+}, *bdnf*^{+/-} and *bdnf*^{-/-} mice. Mean volumes of ganglia ($\times 10^6$ µm^3, ±s.e.m.) represent regions occupied by neuronal cell bodies and exclude areas containing nerve fibers. The number of neurons (± s.e.m.) in the vestibular ganglia of P15 mice was estimated by counting the number of neurons with a nucleus in the plane of section at 18-21 µm intervals throughout the ganglia. Due to the small number of neurons present in P15 *bdnf*^{-/-} mice, all neurons were counted within the ganglia. For each stage of development examined the volume of the vestibular ganglia and number of neurons in wild-type animals is set at 100%. The percentages of ganglion volume or neuronal numbers given for heterozygous (+/−) and homozygous (−/−) mice are relative only to wild-type mice at the same stage of development.
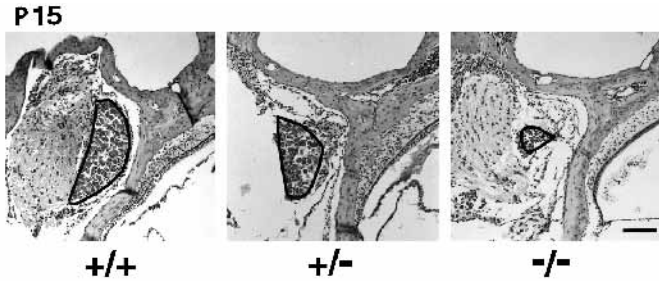
Fig. 4. Cross sections of P15 vestibular ganglia. Very few neurons remain in *bdnf*⁻/⁻ mice. Compared to *bdnf*⁻/⁻ mice, *bdnf*⁺/⁻ mice reveal an intermediate reduction in the size of the vestibular ganglia. Area of ganglia is outlined in black. Scale bar, 100 μm.

Fig. 5. Comparison of BDNF protein levels in DRG from *bdnf*⁺/⁺, *bdnf*⁺/⁻ and *bdnf*⁻/⁻ mice. Mice heterozygous from the *BDNF* null mutation show a 50% reduction in BDNF protein compared to *bdnf*⁺/⁺ littermates.

Fig. 6. Cross sections through peripheral vestibular targets. Hair cells are present in the sacculus of both wild-type (+/+) and *bdnf*⁻/⁻ mice at P15. Scale bar, 100 μm.

in vestibular ganglion size and neuronal number at E16.5-17. Similarly, P1 *bdnf*⁺/⁻ mice also displayed an intermediate decrease in ganglion size and number of vestibular neurons (Table 1). By P15, the volume of vestibular ganglia in *bdnf*⁺/⁻ mice was 45% of the volume of *bdnf*⁺/⁺ mice. The number of vestibular neurons in *bdnf*⁺/⁻ mice was 59% of the number counted in *bdnf*⁺/⁺ mice (Table 1). The average diameter of vestibular neurons was slightly decreased in *bdnf*⁺/⁻ mice (13.4±0.06 μm; n=100) compared to *bdnf*⁺/⁺ mice (15.3±1.4 μm; n=100). Thus, both the volume of the vestibular ganglia and the number of neurons present were reduced by approximately half in mice heterozygous for the *BDNF* null mutation. The intermediate decrease in the number of vestibular neurons in *bdnf*⁺/⁻ mice, compared to *bdnf*⁺/⁺ and *bdnf*⁻/⁻ mice, is highly suggestive of a dose-dependent effect of target-derived BDNF on the survival of developing vestibular neurons.

In order to test whether BDNF protein levels were decreased in *bdnf*⁺/⁻ and *bdnf*⁻/⁻ mice, ELISA measurements were conducted. Due to the small size of the vestibular target tissues, it was not possible to generate enough tissue for ELISA analysis. Therefore, BDNF protein levels were measured from the more abundant dorsal root ganglia (DRG). Results from these experiments revealed a gene dose-dependent decrease in BDNF protein levels in *bdnf*⁺/⁻ and *bdnf*⁻/⁻ mice (Fig. 5). In DRG, the levels of BDNF protein in *bdnf*⁺/⁻ mice were reduced by 50% of that observed in *bdnf*⁺/⁺ mice. BDNF protein levels were not above background in *bdnf*⁻/⁻ mice. These results indicate that BDNF protein levels in *bdnf*⁺/⁻ mice are essentially half of that found in *bdnf*⁺/⁺ littermates.

Formation of peripheral and central vestibular targets in mice lacking BDNF

The morphological appearance of target hair cells appeared normal in both *bdnf*⁻/⁻ and *bdnf*⁺/⁻ mice. Sensory hair cells were present (Fig. 6) in all vestibular regions peripherally (saccule, utricle, cristae ampullaris) and central vestibular nuclei were observed (not shown). Thus, the degeneration of vestibular ganglion neurons was not a consequence of missing target cells, nor did neuronal degeneration result in a notable loss of target cells.

Development of cochlear structures in the absence of BDNF or NT-4/5

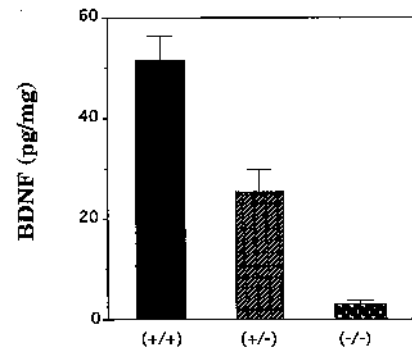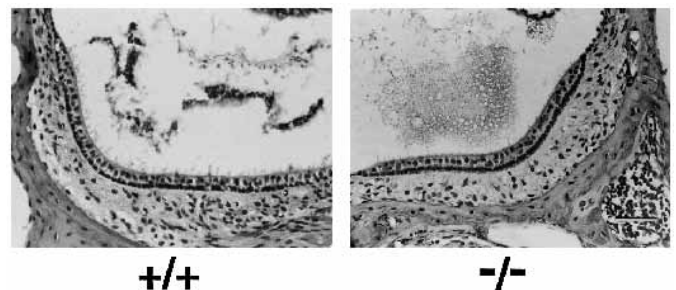*BDNF* mRNA has been detected in developing and adult cochlear sensory epithelium and mRNA for Trk B has been

detected on cochlear neurons (Pirvola et al., 1992; Wheeler et al., 1994). Although previous reports have indicated that cochlear ganglia are largely intact in *bdnf*⁻/⁻ mice (Ernfors et al., 1994a; Conover et al., 1995), there may be a subpopulation of neurons that is disrupted in these mice. The cochlear (spiral) ganglion contains two types of neurons (type I and type II) that relay acoustic information from inner and outer sensory hair cells, respectively.

DiI labeling of cochlear fibers at E13.5-14 and E14.5-15 did not reveal any morphological differences between *bdnf*⁺/⁺ and *bdnf*⁻/⁻ mice (Figs 2, 3). Evaluation of whole-mount surface preparations of the postnatal (P15) organ of Corti from *bdnf*⁻/⁻ mice revealed that sensory hair cells and support cells were present. However, an apparent decrease in the myelinated nerve fiber density at the very apical region of the organ of Corti in *bdnf*⁻/⁻ mice was observed (Fig. 7B). All other cochlear regions were similar in *bdnf*⁺/⁺ (n=4) and *bdnf*⁻/⁻ mice (n=4). Nerve fiber density in the apical region increased in *bdnf*⁻/⁻ mice from P11 to P15. However, because these mice typically die by the third postnatal week, it is not known whether normal innervation density is ever achieved in the absence of BDNF. Nerve fiber density in the organ of Corti of mice lacking NT-4/5 appeared normal.

Immunohistochemistry and DiI labeling were performed to test whether type II cochlear neurons, which represent 5% of the cochlear neuron population and innervate outer hair cells, were disrupted in *bdnf*⁻/⁻ mice. These neurons, as distinguished
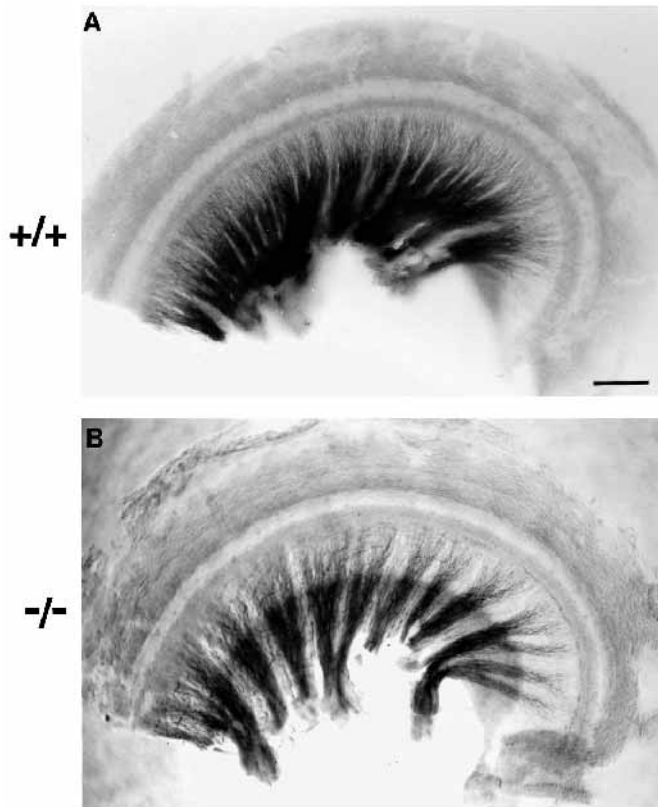
Fig. 7. Whole-mount surface preparations stained with osmium tetroxide reveal myelinated afferent fibers innervating the organ of Corti. (A) Wild-type mice (*bdnf*$^{+/+}$) have dense afferent nerve fiber innervation to the apical region of the organ of Corti at P15. (B) Mice lacking BDNF (−/−) show a reduction in the density of myelinated afferent fiber innervation to this region at P15. Innervation to all other regions of the cochlea appears similar in *bdnf*$^{+/+}$ and *bdnf*$^{−/−}$ mice. Scale bar, 100 μm.

Fig. 8. Comparison of type II cochlear neurons in *bdnf*$^{+/+}$ and *bdnf*$^{−/−}$ mice. (A) RT97-positive neurons are detected in cross sections of cochlear ganglia in mice lacking BDNF (−/−). The neurofilament antibody RT97 preferentially stains the neurofilament-rich type II cochlea neurons. (B,C) DiI-labeled, flat-mount preparations of P7 organ of Corti reveal cochlear afferent fibers extending from the cochlear ganglia through radial fibers (r) to the inner hair cell region (asterisks) and the three rows of outer hair cells (arrows). (B) Cochlear afferent fibers are observed innervating inner and outer hair cells in *bdnf*$^{+/+}$ mice at the middle-basal turn. All other regions of the cochlea receive similar innervation. (C) Afferent fibers extend to the inner and outer hair cells in the basal turn of *bdnf*$^{−/−}$ mice; however, compared to *bdnf*$^{+/+}$ mice, the fiber density to the outer hair cells is reduced. Innervation density to the outer hair cells in the apical and middle turns (not shown) was more severely reduced than in the basal turn. Scale bars, 100 μm.

by characteristic immunostaining with the neurofilament antibody RT97 (Berglund and Ryugo, 1986) were present in the cochlear ganglia of P15 *bdnf*$^{−/−}$ mice (Fig. 8A). DiI labeling of afferent fibers also revealed that the unmyelinated type II fibers innervating the outer hair cells of the organ of Corti, were present (Fig. 8C, arrows). However, compared to wild-type mice, P7 *bdnf*$^{−/−}$ mice revealed a reduction in nerve fiber innervation to the outer hair cells (compare arrows Fig. 8B and C). The reduction in the apical and middle turns of the cochlea (not shown) was even more prominent than the reduction observed in the basal regions (Fig. 8C). Thus, type II neurons were detected in *bdnf*$^{−/−}$ mice, but displayed location-specific reductions in innervation density.

Vestibular neurons in mice lacking BDNF and NT-4/5

Although both BDNF and NT-4/5 activate the Trk B receptor, which is present in both vestibular and cochlear neurons (Pirvola et al., 1992, 1994; Ernfors et al., 1992; Dechant et al., 1993; Schecterson and Bothwell, 1994), mice lacking NT-4/5 did not show any dramatic reduction in either cochlear or vestibular neurons at P15 (Conover et al., 1995). Because nearly all vestibular neurons were lost by P15 in *bdnf*$^{−/−}$ mice, it was not possible to accurately determine whether there was
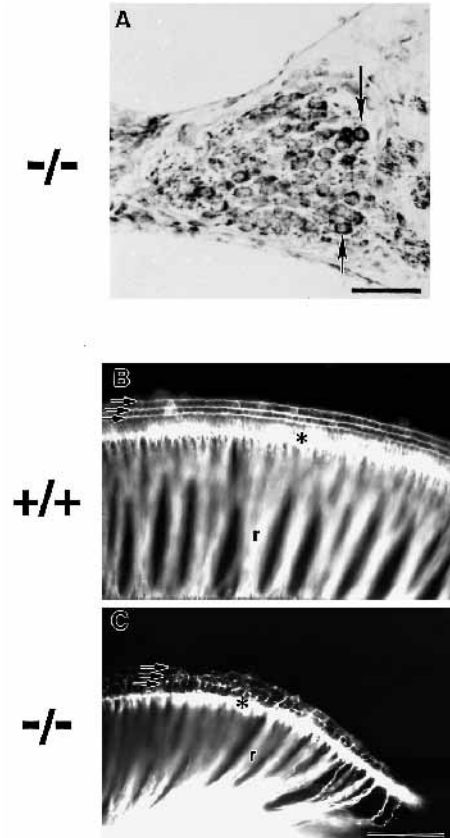
a greater reduction in vestibular neurons in *bdnf*$^{−/−}$: *nt-4/5*$^{−/−}$ double knockout mice. Therefore, we examined the vestibular ganglia in mice lacking expression of both *BDNF* and *NT-4/5* at P1, at a stage when vestibular neurons were reduced, but not completely lost in *bdnf*$^{−/−}$ mice.

Double mutant mice (P1) were generated from mice heterozygous for *BDNF* and *NT-4/5*. The mice resulting from these crosses carried various combinations of wild-type (+/+), heterozygote (+/−) and homozygote (−/−) genes for BDNF and NT-4/5 (Table 2). Mice lacking both BDNF and NT-4/5 did not show any greater reduction in vestibular ganglion size than mice lacking only BDNF (Table 2). Cochlear structures appeared normal in mice lacking both BDNF and NT-4/5. Thus, NT-4/5 alone, or in combination with BDNF, does not appear to play a role in development of vestibular or cochlear

Table 2. Vestibular ganglia in double neurotrophin mutant mice

Genotype		Volume (×10⁶ µm³)	% of Control (+/+)/(+/+)
(bdnf) / (nt-4/5)			
(+/+)/(+/+)	(n=3)	11.6±0.92	100%
(+/+)/(−/−)	(n=3)	12.8±1.63	110%
(+/−)/(−/−)	(n=2)	8.39±4.44	73%
(−/−)/(+/+)	(n=3)	1.28±0.01	11%
(−/−)/(−/−)	(n=4)	1.26±0.07	11%

Loss of both BDNF and NT-4/5 did not lead to a greater reduction in the volume of vestibular ganglia in P1 mice. Genotypes of the mice are indicated with the *bdnf* genotype first and the *nt-4/5* genotype second. The size of the vestibular ganglia is similar in mice with either a wild-type or disrupted copy of the NT-4/5 gene.

neurons and is unable to compensate for the lack of BDNF in the vestibular system.

DISCUSSION

The present study revealed both age-dependent and dose-dependent effects of BDNF on developing vestibular neurons. Our results indicate that neurons within the statoacoustic (vestibulocochlear) complex do not require BDNF during neurogenesis or in the initial stages of neurite outgrowth. However, neurons within the vestibular component appear to become dependent on BDNF by mid-stages of inner ear development. At early stages of development, the SAG complex consists of both vestibular and cochlear portions of the ganglia. By E12.5-13, both cochlear and vestibular regions of the SAG undergo proliferation and begin to extend processes to the undifferentiated sensory epithelium of the otocyst (see Fritzsch and Nichols, 1993; Fritzsch et al., 1995). In the present study, we found that the SAG of *bdnf*^(−/−) mice formed normally and at E12.5-13 was similar in size and appearance to that of *bdnf*^(+/+) mice. Although the mouse otocyst contains *BDNF* mRNA transcripts in presumptive vestibular regions at these early stages (Schecterson and Bothwell, 1994), the initial production of SAG neurons appears normal in the absence of BDNF. The present in vivo results are consistent with previous in vitro studies that demonstrated that BDNF did not influence the survival or outgrowth of early stage SAG neurons (Davies et al., 1986; Bianchi and Cohan, 1993) and that BDNF-like activity was not detected in media conditioned by otocyst tissue (Bianchi and Cohan, 1993).

By E13.5-14, afferent innervation to vestibular sensory epithelia was reduced in *bdnf*^(−/−) mice. Fibers to the posterior vertical canal and most afferent fibers to the anterior and horizontal canals were absent, while the utricle and saccule had only a decrease in innervation density. All innervation to the semicircular canals was lost by E14.5-15. These results are consistent with a recent report on the fiber innervation to vestibular epithelia in mice lacking the Trk B receptor (Fritzsch et al., 1995). In that report, afferent fibers were seen extending to vestibular regions at E12.5, but were first decreased at E13.5. As with *bdnf*^(−/−) mice, the innervation to the semicircular canals was more severely affected than innervation to the utricle and saccule. These findings are consistent with the previous findings that semicircular canals express only BDNF,

whereas all other regions of the inner ear express both BDNF and NT-3 (Hallböök et al., 1993; Schecterson and Bothwell, 1994; Pirvola et al., 1992, 1994).

Although BDNF does not appear to be required early in vestibular neurogenesis, our results indicate that BDNF functions as a survival factor for vestibular neurons by mid-stages of inner ear development. By E16.5-17, corresponding to the period of mid-synaptogenesis in the mouse vestibular system (Mbeine et al., 1988; Desmadryl et al., 1990), the number of vestibular neurons in *bdnf*^(−/−) mice was reduced. Vestibular ganglia of *bdnf*^(−/−) mice continued to decrease in size, as well as in the number of vestibular neurons, from E16.5-P15. Similarly, Fritzsch et al. (1995) reported pycnotic cells in the vestibular ganglia of both wild-type and Trk B^(−/−) mice at E17.5 and P0. These results suggest that programmed cell death of vestibular neurons continues through at least early neonatal stages. Schimmang et al. (1995) also reported a reduction in the number of vestibular neurons in Trk B^(−/−) from E18.5 to P1. In contrast to these results, Ernfors et al. (1995) reported no further decrease in vestibular neurons from E16 through P14 in *bdnf*^(−/−) mice, suggesting that all vestibular neuronal death occurs between E13 and E16. In the mouse, synaptogenesis of vestibular afferent fibers on peripheral target cells is largely completed between E18 and the first postnatal week (Desmadryl et al., 1992). The continued decrease in vestibular neurons observed in the present study of *bdnf*^(−/−) mice, as well as recent studies of Trk B^(−/−) mice (Fritzsch et al., 1995; Schimmang et al., 1995), would be consistent with this timecourse of innervation and suggests that vestibular neurons degenerate during the period that peripheral synaptic contacts are normally made.

Although the timing and expression of *BDNF* mRNA in peripheral vestibular structures is consistent with the idea that BDNF functions as a target-derived factor for vestibular neurons, additional sources of trophic support may also be available. Expression of *BDNF* has not been reported in central vestibular targets; however, expression of *BDNF*, *NGF* and *NT-3* mRNA has been detected in embryonic mouse and quail SAG (Schecterson and Bothwell, 1994; Bernd et al., 1994). These factors may act at later stages of development to support survival of vestibular or cochlear neurons in an autocrine or paracrine manner as recently described for other sensory neurons (Acheson et al., 1995).

In the present study, analysis of *bdnf*^(+/−) mice revealed a loss of vestibular neurons that was remarkably intermediate to the neuronal loss observed in *bdnf*^(+/+) and *bdnf*^(−/−) mice. The intermediate decrease in vestibular ganglia and the time course of degeneration of vestibular neurons is consistent with classical ideas of the role of neurotrophic factors in neuronal development. According to this model, neurons become dependent upon neurotrophic factors when they reach their target cells and compete for limited amounts of these survival factors (Levi-Montalcini, 1987; Davies, 1988). Thus, the number of surviving vestibular neurons appears to be regulated in proportion to the amounts of available BDNF. ELISA measurements of BDNF protein content from DRG revealed a 'dose-response curve' for BDNF protein in *bdnf*^(+/+), *bdnf*^(+/−) and *bdnf*^(−/−) mice and indicated BDNF protein levels were reduced by approximately half in *bdnf*^(+/−) mice compared to *bdnf*^(+/+) mice. Due to the small size of vestibular sensory epithelia, it was not possible to measure BDNF protein levels from these

tissues. While we cannot be certain that the same dose-response would be found for vestibular tissues, the results from DRG strongly support the idea that BDNF protein is produced in more limited quantities in $bdnf^{+/-}$ mice compared to $bdnf^{+/+}$ mice.

Recent studies of mice lacking neurotrophins have provided indirect evidence for the target-derived neurotrophic factor hypothesis (Crowley et al., 1994; Ernfors et al., 1994b, 1995). Evidence for a 'gene dosage' effect of a neurotrophic factor was first obtained following evaluation of muscle spindle formation in mice homozygous or heterozygous for the *NT-3* null mutation (Ernfors et al., 1994b). Interestingly, muscle spindles, which rely on afferent innervation for formation, were absent in nt-$3^{-/-}$ mice and reduced by 50% in nt-$3^{+/-}$ mice. In contrast to muscle spindle formation, formation of hair cells does not appear to be dependent on afferent innervation (Corwin and Cotanche, 1989; Swanson et al., 1990). In the present study, vestibular hair cells were detected in all vestibular sensory epithelia in both $bdnf^{+/-}$ and $bdnf^{-/-}$ mice, consistent with earlier reports that innervation is not required for hair cell differentiation. A recent report (Fritzsch et al., 1995), utilizing light and scanning electron microscopy, examined hair cell formation in mice lacking the neurotrophin receptors Trk B and Trk C. These investigators found that hair cells formed normally throughout the vestibular and cochlear regions in mice lacking one or both of these receptors, at least until P0, the latest stage examined. Even in the Trk B/C double mutant mice, where all innervation to the inner ear was lost, hair cell formation proceeded normally. Another recent report (Ernfors et al., 1995) revealed that vestibular hair cells were present in normal numbers in mice lacking BDNF, but suggested that the vestibular hair cells had an 'immature' appearance at the ultrastructural level in these mice. Schimmang et al. (1995) also reported a reduction in the thickness of cochlear sensory epithelium in mice lacking Trk C. Thus, the possibility that subtle morphological changes occur in the sensory epithelium of mice lacking neurotrophins or their associated receptors cannot be ruled out.

Despite the presence of *BDNF* mRNA transcripts in inner and outer hair cells of the cochlea and the expression of *Trk B* mRNA in cochlear neurons (Pirvola et al., 1992, 1994; Schecterson and Bothwell, 1994), no severe morphological deficits were detected in the cochlear neurons of mice lacking either BDNF or NT-4/5. In the present study, the density of myelinated nerve fibers to the apical region of the organ of Corti appeared reduced in P15 $bdnf^{-/-}$ mice compared to $bdnf^{+/+}$ mice. Although nerve fiber density increased in this region from P11 to P15, it is unclear whether nerve fiber density ever achieves a normal pattern in this region due to the short lifespan of the $bdnf^{-/-}$ mice. Temporally, the apex is the last region to become fully differentiated. Thus, the decrease in innervation may represent a developmental delay, consistent with previous reports showing that $bdnf^{-/-}$ mice are retarded in growth compared to wild-type littermates (Ernfors et al., 1994a; Jones et al., 1994; Conover et al., 1995). The present study also revealed that the unmyelinated, type II cochlear afferent fibers to the outer hair cells of $bdnf^{-/-}$ mice were reduced, particularly in the apical and middle turns of the cochlea. Interestingly, in mice lacking either Trk B or Trk C, afferent nerve fiber densities also varied at specific cochlear regions (Fritzsch et al., 1995). Mice lacking Trk B had reduced innervation to the apical and middle turns of the cochlea, whereas mice lacking Trk C had reduced innervation to the basal turn of the cochlea. Together, these results suggest the possibility of gradients along the cochlea with Trk B/BDNF being more important at the apical-middle regions and Trk C/NT-3 more important for the basal regions. A previous report (Ernfors et al., 1995) that the type II afferents were completely absent in $bdnf^{-/-}$ mice may have been a result of examining only the apical region of the cochlea.

In contrast to BDNF, lack of NT-3 leads to a significant loss of cochlear neurons (Farinas et al., 1994; Ernfors et al., 1995). The developmental timecourse for NT-3 dependence, as well as the effect on type I and type II cochlear neuron populations, has not yet been examined. In mice lacking Trk C, the receptor for NT-3, only diminished innervation to inner and outer hair cells in the basal turn of the cochlea was detected by DiI labeling (Fritzsch et al., 1995). Although the survival of a portion of vestibular neurons may be mediated by NT-3 (Ernfors et al., 1995; Fritzsch et al., 1995, Schimmang et al., 1995), NT-3 and its associated receptor, Trk C, appear to play a greater role in cochlear development.

Although Trk B has been detected on both vestibular and cochlear neurons, mice lacking NT-4/5 did not show a reduction in either neuronal population. These results are consistent with the observation that *NT-4/5* mRNA is not detected in inner ear tissues (Ylikoski et al., 1993; Pirvola et al., 1994).

In conclusion, the present experiments reveal that vestibular neurons have an age-dependent requirement for BDNF. Vestibular neurons are produced normally in $bdnf^{-/-}$ mice and do not appear to require BDNF until mid-stages of inner ear development. Prior to the period of initial synaptogenesis, neurons within the SAG complex were not dependent upon BDNF for their survival. However, beginning at the period when vestibular fibers approach their target cells, vestibular neurons become dependent on BDNF for their survival. Furthermore, comparison of $bdnf^{+/-}$ and $bdnf^{-/-}$ mice provide in vivo evidence supporting the neurotrophic hypothesis and suggest that the final complement of mature vestibular neurons is the result of competition for limiting amounts of BDNF.

REFERENCES

Abercrombie, M. (1946). Estimation of nuclear population from microtome sections. *Anat. Rec.* **94**, 239-247.

Acheson, A., Conover, J. C., Fandl, J. P., DeChiara, T. M., Russell, M., Thadani, A., Squinto, S. P., Yancopoulos, G. D. and Lindsay, R. M. (1995). A BDNF autocrine loop in adult sensory neurons prevents cell death. *Nature* **374**, 450-453.

Alvarez, I. S. and Navascues, J. (1990). Shaping, invagination, and closure of the chick embryo otic vesicle: Scanning electron microscopic and quantitative study. *Anat. Rec.* **228**, 315-326.

Avila, M. A., Varela-Nieto, I., Romero, G., Mato, J. M., Giraldez, F., Van De Water, T. R. and Represa, J. (1993). Brain-derived neurotrophic factor and neurotrophin-3 support the survival and neuritogenesis response of developing cochleovestibular ganglion neurons. *Dev. Biol.* **159**, 266-275.

Berglund, A. M. and Ryugo, D. K. (1986). A monoclonal antibody labels type II neurons of the spiral ganglion. *Hear. Res.* **383**, 327-332.

Bernd, P., Zhang, D., Yao, L. and Rozenberg, I. (1994). The potential role of nerve growth factor, brain-derived neurotrophic factor and neurotrophin-3 in avian cochlear and vestibular ganglia development. *Int. J. Devl. Neuroscience* **12**, 709-723.

Bianchi, L. M. and Cohan, C. S. (1993). The effects of neurotrophins and CNTF on developing statoacoustic neurons: Comparison with an otocyst-derived factor. *Dev. Biol.* **159**, 353-365.

Conover, J. C., Erickson, J. T., Katz, D. M., Bianchi, L. M., Poueymirou, W. T., McClain, J., Pan, L., Helgren, M., Ip, N. Y., Boland, P., Freidman, B., Weigand, S., Vejsada, R., Kato, A., DeChiara, T. M. and Yancopoulos, G. D. (1995). Neuronal deficits, not involving motor neurons, in mice lacking BDNF and/or NT-4. *Nature* **375**, 235-238.

Corwin, J. T. and Cotanche, D. A. (1989). Development of location-specific hair cell stereocilia in denervated embryonic ears. *J. Comp. Neurol.* **288**, 529-537.

Crowley, C., Spencer, S. D., Nishimura, M. C., Chen, K. S., Pitts-Meek, S., Armani, M. P., Ling, L. H., McMahon, S. B., Shelton, D. L., Levinson, A. D. and Phillips, H. S. (1994). Mice lacking nerve growth factor display perinatal loss of sensory and sympathetic neurons yet develop basal forebrain cholinergic neurons. *Cell* **76**, 1001-1011.

Davies, A., Thoenen, H. and Barde, Y.-A. (1986). The response of chick sensory neurons to brain-derived neurotrophic factor. *J. Neurosci.* **6**, 1897-1904.

Davies A. (1988). Role of neurotrophic factors in development. *Trends in Genet.* **4**, 139-143.

Dechant, G., Biffo, S., Okazawa, H., Kolbeck, R., Pottgiesser, J. and Barde, Y.-A. (1993). Expression and binding characteristics of the BDNF receptor chick TrkB. *Development* **119**, 545-558.

Desmadryl, G. and Sans, A. (1990). Afferent innervation in crista ampullaris of the mouse during ontogenesis. *Dev. Brain Res.* **52**, 183-189.

Desmadryl, G., Dechesne, C. J., Raymond, J. (1992) Recent aspects of development of the vestibular sense organs and their innervation. In *Development of Auditory and Vestibular Systems* **2** (ed. R. Romand). pp 461-487. Amsterdam: Elsevier.

Engstrom, H., Ades, H. W. and Andersson, A. (1968). *Structural Pattern of the Organ of Corti.* Baltimore: Williams and Wilkins Co.

Ernfors, P., Lee, K-F and Jaenisch, R. (1994a). Mice lacking brain-derived neurotrophic factor develop with sensory deficits. *Nature* **368**, 147-150.

Ernfors, P., Lee, K-F., Kucera, J. and Jaenisch, R. (1994b). Lack of neurotrophin-3 leads to deficiencies in the peripheral nervous system and loss of limb proprioceptive afferents. *Cell* **77**, 503-512.

Ernfors, P., Van De Water, T., Loring, J. and Jaenisch, R. (1995). Complementary roles of BDNF and NT3 in vestibular and auditory development. *Neuron* **14**, 1153-1164.

Farinas, I., Jones, K. R., Backus, C., Wanf, X-Y and Reichardt, L. F. (1994). Severe sensory and sympathetic deficits in mice lacking neurotrophin-3. *Nature* **369**, 658-661.

Fritzsch, B. and Nichols, D. H. (1993). DiI reveals a prenatal arrival of efferents at the differentiating otocyst of mice. *Hear. Res.* **65**, 51-60.

Fritzsch, B., Silos-Santiago, I., Smeyne, R., Fagan, A. M. and Barbacid, M. (1995). Reduction and loss of inner ear innervation in trkB and trkC receptor knockout mice: A whole-mount DiI and scanning electron microscopic analysis. *Aud. Neurosci.* **1**, 401-417.

Hallböök, F., Ibáñez, C. F., Ebendal, T. and Persson, H. (1993). Cellular localization of brain-derived neurotrophic factor and neurotrophin-3 mRNA expression in the early chicken embryo. *Eur. J. Neurosci.* **5**, 1-14.

Hollyday, M. and Hamburger, V. (1976). Reduction of the naturally occurring motor neuron loss by enlargement of the periphery. *J. Comp. Neurol.* **170**, 311-320.

Jones, K. R., Farinas, I., Backus, C. and Reichardt, L. F. (1994). Targeted disruption of the *BDNF* gene perturbs brain and sensory neuron development but not motor neuron development. *Cell* **76**, 989-999.

Knowlton, V. Y. (1967). Correlation of the development of membranous and bony labyrinths, acoustic ganglia, nerves and brain centers of the chick embryo. *J. Morph.* **121**, 179-208.

Lefebvre, P. P., Van De Water, T. R., Weber, T., Rogister, B. and Moonen, G. (1991). Growth factor interactions in cultures of dissociated adult acoustic ganglia: neuronotrophic effects. *Brain Res.* **567**, 306-312.

Levi-Montalcini, R. and Booker, B. (1960). Destruction of the sympathetic ganglia in mammals by antiserum to a nerve growth factor protein. *Proc. Natl. Acad. Sci. USA* **46**, 384-391.

Levi-Montalcini, R. and Angeletti, P. U. (1966). Immunosympathectomy. *Pharmacol. Rev.* **10**, 619-628.

Levi-Montalcini, R. (1987). The nerve growth factor 35 years later. *Science* **237**, 1154-1162.

Li, C. W., Van De Water, T. R. and Ruben, R. J. (1978). The fate mapping of the eleventh and twelfth day mouse otocyst: An in vitro study of the sites of orgin of the embryonic inner ear sensory structures. *J. Morph.* **157**, 249-268.

Lindsay, R. M., Wiegand, S. J., Altar, C. A. and DiStefano, P. S. (1994). Neurotrophic factors: from molecule to man. *Trends Neurosci.* **17**, 182-190.

Liu, X., Ernfors, P., Wu, H. and Jaenisch, R. (1995). Sensory but not motor neuron deficits in mice lacking NT4 and *BDNF*. *Nature* **375**, 238-240.

Mbiene, J. P., Favre, D. and Sans, A. (1988). Early innervation and differentiation of hair cells in the vestibular epithelia of mouse embryos: SEM and TEM study. *Anat. Embryol.* **177**, 331-340.

Pirvola, U., Ylikoski, J., Palgi, J., Lehtonen, E., Arumae, U. and 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. and 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.

Radke, S., Holst, P., Fritsche, M. and Altar, C. A. (1996). Presence of BDNF protein in human and rat but not mouse plasma and in brain detected by a sensitive and specific enzyme-linked immunoassay. *Brain Res.* (In Press).

Represa, J. and Bernd, P. (1989). Nerve growth factor and serum differentially regulate development of embryonic otic vesicle and cochleovestibular ganglion in vitro. *Dev. Biol.* **134**, 21-29.

Schecterson, L. and Bothwell, M. (1992). Novel roles for neurotrophins are suggested by BDNF and NT-3 mRNA expression in developing neurons. *Neuron* **9**, 449-463.

Schimmang, T., Minichiello, L., Vazquez, E., San Jose, I., Giraldez, F., Klein, R. and Represa, J. (1995) Developing inner ear sensory neurons require Trk B and Trk C receptors for innervation of their peripheral targets. *Development* **121**, 3381-3391.

Swanson, G. J., Howard, M. and Lewis, J. (1990). Epithelial autonomy in the development of the inner ear of a bird embryo. *Dev. Biol.* **137**, 243-257.

Vogel, K. S. and Davies, A. M. (1991). The duration of neurotrophic factor independence in early sensory neurons is matched to the time course of target field innervation. *Neuron* **7**, 819-830.

Wheeler, E. F., Bothwell, M., Schecterson, L. C. and von Bartheld, C. S. (1994). Expression of BDNF and NT3 mRNA in hair cells of the organ of Corti: Quantitative analysis in developing rats. *Hear. Res.* **73**, 46-56.

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