Lack of Bdnf and TrkB signalling in the postnatal cochlea leads to a spatial reshaping of innervation along the tonotopic axis and hearing loss

Thomas Schimmang[2], Justin Tan[1], Marcus Müller[1], Ulrike Zimmermann[1], Karin Rohbock[1], Iris Köpschall[1], Annette Limberger[1], Liliana Minichiello[3] and Marlies Knipper[1,*]

[1]Tübingen Hearing Research Center (THRC), University of Tübingen, Department of Otolaryngology, Molecular Neurobiology, Elfriede-Aulhorn-Strasse 5, D-72076 Tübingen
[2]Center for Molecular Neurobiology Hamburg, University of Hamburg, Falkenried 94, D-20251 Hamburg, Germany
[3]European Molecular Biology Laboratory, via Ramarini 32, 00016 Monterotondo, Italy
*Author for correspondence (e-mail: marlies.knipper@uni-tuebingen.de)

Accepted 18 June 2003

Development 130, 4741-4750

doi:10.1242/dev.00676

Summary

Members of the neurotrophin gene family and their high-affinity Trk receptors control innervation of the cochlea during embryonic development. Lack of neurotrophin signalling in the cochlea has been well documented for early postnatal animals, resulting in a loss of cochlear sensory neurones and a region-specific reduction of target innervation along the tonotopic axis. However, how reduced neurotrophin signalling affects the innervation of the mature cochlea is currently unknown. Here, we have analysed the consequences of a lack of the TrkB receptor and its ligand, the neurotrophin brain-derived neurotrophic factor (Bdnf), in the late postnatal or adult cochlea using mouse mutants. During early postnatal development, mutant animals show a lack of afferent innervation of outer hair cells in the apical part of the cochlea, whereas nerve fibres in the basal part are maintained. Strikingly, this phenotype is reversed during subsequent maturation of the cochlea, which results in a normal pattern of outer hair cell innervation in the apex and loss of nerve fibres at the base in adult mutants. Measurements of auditory brain stem responses of these mice revealed a significant hearing loss. The observed innervation patterns correlate with opposing gradients of Bdnf and Nt3 expression in cochlear neurones along the tonotopic axis. Thus, the reshaping of innervation may be controlled by autocrine signalling between neurotrophins and their receptors in cochlear neurones. Our results indicate a substantial potential for re-innervation processes in the mature cochlea, which may also be of relevance for treatment of hearing loss in humans.

Key words: Bdnf, TrkB, Cochlea, Hearing loss, Mice

Introduction

Signalling of the neurotrophin gene family through their high-affinity receptors, termed Trks, is responsible for neuronal survival and/or target innervation in the peripheral nervous system (Bibel and Barde, 2000). In the inner ear, the neurotrophins brain-derived neurotrophic factor (Bdnf) and neurotrophin 3 (Nt3; now known as Ntf3), and their corresponding receptors TrkB (Ntrk2) and TrkC (Ntrk3), are responsible for the survival of cochlear and vestibular neurones (Rubel and Fritzsch, 2002). In mammals, the dependence of these neurones on Bdnf or Nt3 signalling through their receptors has been demonstrated by the study of mouse mutants in which the corresponding genes have been inactivated by homologous recombination (Ernfors et al., 1995; Schimmang et al., 1995; Bianchi et al., 1996; Fritzsch et al., 1997a). In these studies, it was shown that Bdnf and TrkB mutants lose the majority of vestibular neurones, whereas Nt3- and TrkC-deficient mice revealed a severe reduction of neurones in the cochlea.

Cochlea type I sensory neurones, comprising approximately 90% of the cochlear ganglion, provide afferent innervation to the inner hair cells (IHCs). The remaining part of the ganglion consists of small-sized type II sensory neurones, which innervate outer hair cells (OHCs). Initial studies of knockout animals for Bdnf or TrkB showed a severe reduction of type II sensory neurones, whereas Nt3 and TrkC mouse mutants lost the majority of type I neurones (Ernfors et al., 1995; Schimmang et al., 1995). From these results, and from the study of the afferent innervation patterns using neurofilament antibodies, it was suggested that survival of specific neurone types and their targets was under the control of particular neurotrophins and their receptors.

More detailed studies using DiI as a tracer revealed that innervation in the cochlear sensory epithelium shows a more complex pattern in neurotrophin and Trk receptor mutants (Fritzsch et al., 1997a). Bdnf and TrkB mutants showed the most pronounced defects in the apex, whereas the lack of innervation of TrkC and Nt3 mutants was most severe at the cochlear base (Bianchi et al., 1996; Fritzsch et al., 1997b; Fritzsch et al., 1998). Moreover, these studies demonstrated that both neurotrophins and their receptors are responsible for the innervation of OHCs (Rubel and Fritzsch, 2002).

Recent data suggest that the spatial gradients of neuronal loss observed in neurotrophin and Trk receptor mutants is attributable to the spatial-temporal gradients of neurotrophin expression during embryonic development (Fariñas et al., 2001). Based on this study, Bdnf is localised predominantly in the apex of the cochlea, whereas Nt3 is localised in the base. Additionally, Bdnf expression has been shown to correlate with the rearrangement of fibres during postnatal development of the cochlea, and this reorganisation is disturbed in Bdnf knockout mice (Wiechers et al., 1999). However, because Bdnf or TrkB receptor mouse mutants do not survive past the first postnatal weeks (Klein et al., 1993; Ernfors et al., 1994; Jones et al., 1994; Korte et al., 1995), the consequences of a severe loss of TrkB signalling in the mature cochlea could not be analysed.

To study the importance of particular signalling pathways used by Trk receptors for neuronal survival and target innervation, specific point mutations have recently been introduced in the docking site for the Shc adapter protein on the TrkB and TrkC receptor (Minichiello et al., 1998; Postigo et al., 2002). Although in both *TrkB*$^{shc/shc}$- and *TrkC*$^{shc/shc}$-mutant mice the RAS/MAPK and phosphoinositide-3-kinase pathways are similarly affected, neuronal survival in the peripheral nervous system, including the inner ear, is only modestly reduced. However, whereas target innervation in *TrkC*$^{shc/shc}$ mice is maintained, in *TrkB*$^{shc/shc}$ mice several cranial sensory populations, including vestibular neurones, lose innervation to their peripheral targets (Postigo et al., 2002).

In the present study, we used these animals to create adult mice with a severe lack of TrkB receptor signalling, by crossing them to *TrkB*$^{+/-}$ mutants with a targeted deletion within the tyrosine kinase domain of the receptor (Klein et al., 1993). Similar to *TrkB*$^{-/-}$ and *Bdnf*$^{-/-}$ mutants (Bianchi et al., 1996; Fritzsch et al., 1998), mice carrying the mutant shc allele and the mutated tyrosine kinase domain of the TrkB receptor (*TrkB*$^{shc/-}$) show a region-specific absence of outer hair cell innervation in the apical part of the cochlea during early postnatal development.

Further analysis of *Bdnf*$^{-/-}$ and *TrkB*$^{shc/-}$ animals revealed that the observed defect is reversed during maturation of the cochlea, thus leading to normal innervation of OHCs in the apex but absence of afferent and retardation of efferent nerve fibres in the basal turn. Although the cellular integrity of OHCs appeared unaffected in the mutant animals in all cochlear turns, hearing thresholds of *TrkB*$^{shc/-}$ mice were significantly reduced compared with control mice. The present data are consistent with recent results that suggest that neurotrophins in the mature hearing organ may have a distribution opposite to that found during development (Adamson et al., 2002).

Materials and methods

Animals

Mice heterozygous for a deletion of the *Bdnf* gene were kindly provided by Patrick Carroll and Hans Thoenen (MPI Martinsried, Munich, Germany). The offspring of these mice were genotyped by PCR according to Korte et al. (Korte et al., 1995). Heterozygous *TrkB*$^{+/-}$-mutant mice were obtained from Jackson Laboratory and their offspring were genotyped by PCR according to Schimmang et al. (Schimmang et al., 1995).

Mice carrying the mutant shc allele of the TrkB receptor (*TrkB*$^{shc/shc}$) have been described in Minichiello et al., 1998. Mutant *TrkB*$^{shc/-}$ mice were created by crossing *TrkB*$^{+/-}$ mice with *TrkB*$^{shc/shc}$ mutants.

Tissue preparation

Cochleae were isolated and dissected as previously described (Knipper et al., 2000). Briefly, cochleae were fixed by immersion in 2% paraformaldehyde, 125 mM sucrose in 100 mM phosphate buffered saline (PBS; pH 7.4), for 2 hours, followed by overnight incubation in 25% sucrose, 1 mM protease inhibitor (Pefabloc, Roche) in PBS (pH 7.4). Cochleae of animals older than postnatal day 10 (P10) were decalcified after fixation for 15 minutes to 2 hours in Rapid bone decalcifier (#904687, Eurobio, Fisher-Scientific, 61130 Nidderau, Germany). After overnight incubation, cochleae were embedded in O.C.T. compound (Miles Laboratories, Elkhart, Ind., USA). Tissues were then cryosectioned at 10 μm thickness for in situ hybridisation and immunohistochemistry, mounted on SuperFrost*/plus microscope slides, dried for 1 hour and stored at –20°C before use.

Immunohistochemical staining for fluorescence microscopy

For immunohistochemistry, cochlear sections from different postnatal stages and adult mice or rats were thawed, permeabilised with 0.1% Triton X-100 for 3 minutes at room temperature, preblocked with 1% bovine serum albumin in PBS and incubated overnight at 4°C with primary antibodies. The following primary antibodies were used: anti-NF200 (Sigma N4142); anti-synaptophysin (The Binding Site #PH510) anti-peripherin (Chemicon #AB1530), anti-prestin (Weber et al., 2002) and anti-potassium channel SK2 (Sigma, #P0483). Expression of full-length TrkB in cochlear neurones was confirmed by antibodies directed against aminoacids 794-808 of the TrkB receptor (#794, Santa Cruz Biotechnology). Primary antibodies were visualised with either Cy3-conjugated goat anti-rabbit Ig (0.35 μg/ml; Jackson Immuno Research Laboratories, PA, USA) or with Alexa-Green conjugated goat-anti-mouse antibodies (1:1500, Molecular Probes, Leiden, The Netherlands). Sections were rinsed, mounted with Vectashield (Vector Laboratories, Burlingame, CA, USA) and viewed using an Olympus AX70 microscope equipped with epifluorescence illumination.

Bdnf probe isolation and riboprobe synthesis

Genomic DNA from rat liver was isolated by Easy-DNA kit from Invitrogen, following the protocol provided by the manufacturer. Polymerase chain reaction was used to amplify exon 4 of the *Bdnf* gene (Timmusk et al., 1993). For the exon 4-specific probe, a sense primer (5′-cca atc gaa gct caa ccg aa-3′) and an antisense primer (5′-tca ggg tcc aca caa agc tc-3′) corresponding respectively to nucleotide position 1732-1751 and 2059-2078 from genomic fragment B were used. During the PCR reaction, genomic DNA was first denatured for 4 minutes at 94°C, followed by 30 cycles consisting of 1 minute at 94°C, 1 minute at 55°C and 1 minute at 72°C. The extension reaction was carried out at 72°C for 10 minutes. The amplified fragment corresponding to the expected length of 347 nucleotides was extracted and sequenced. Clones of full-length rat *Nt3* and full-length *TrkB* were supplied by Regeneron Pharmaceuticals. In situ hybridisation was performed as described (Wiechers et al., 1999). Sections were mounted with Moviol (Sigma) and viewed using an Olympus AX70 microscope.

Auditory brainstem response measurements

Auditory evoked brainstem responses (ABR) were recorded in anaesthetised mice. Anaesthesia was achieved by the intraperitoneal injection of 65 mg/kg ketamin hydrochloride (Ketamin 50 Curamed, CuraMED Pharma, Germany), 10.5 mg/kg xylazin hydrochloride (Rompun 290, Bayer Leverkusen, Germany) and 0.33 mg/kg atropine sulphate (Atropinsulfat, Braun, Germany). Recordings were carried

out in a sound-proof chamber (IAC, Type 400-A). A Multi IO Card (National Instruments MIO 16 E1) was used for the generation of stimuli and recording of evoked potentials. Tone pips of 3 ms duration (1 ms rise and fall time, cosine-shaped) were presented at a rate of 60/second with alternating phase. Clicks with duration of 100 μs of alternating phase were also used. Sound pressure level was adjusted with a custom-made attenuator and tone pips were amplified with a custom-made amplifier. Stimuli were delivered to the ear in a calibrated (Bruel&Kjaer 2610, 4191) closed system by a Beyer DT911 loudspeaker. In the case of tone pips, the sound pressure was calibrated in situ at all frequencies recorded prior to each measurement taken. As this is not possible for clicks, sound pressure was calibrated off-line once prior to the experiments. In this case, 0 dB attenuation corresponds to 112 dB SPL root means square (rms). To record bio-electrical potentials, subdermal silver wire electrodes were inserted at the vertex (reference), ventro-lateral to the measured ear (active) and at the back of the animal (ground). After amplification (100 dB) and bandpass filtering (0.3-5.0 kHz), electrical signals were averaged (128 repetitions). ABR responses were recorded for frequencies between 2.8 kHz and 45.2 kHz at a resolution of 2 steps per octave. At each frequency ABRs were collected from 20 dB to 100 dB SPL in 5 dB steps. The software averager included an artefact rejection code (all waveforms with a peak to peak voltage exceeding a defined voltage were rejected) to eliminate the ECG and muscle activity. Thresholds were defined as the sound pressure level where a stimulus correlated response was clearly identified in the recorded signal. If no response was obtained we used 100 dB SPL as the threshold value volume for further calculations.

Measurements of distortion product otoacoustic emissions (DPOAE)

Anaesthesia was performed as described for the ABR measurements. For DPOAE measurements acoustic stimulations were performed by a PC-based system with an A/D converter board using LabWindows software, which also performed fast Fourier transformation (FFT) for data analysis. Pure tone sound stimuli were generated and delivered on two separate channels using dynamic speakers (Beyer DT48). Sound levels were measured with a probe microphone (Bruel&Kjaer 4135) and a measuring amplifier (Bruel&Kjaer 2610). The speakers and microphone were coupled together, with the closed field into the ear canal of the animal, and the sound system calibrated with white noise (50 Hz-20 kHz). DPOAEs were measured as cubic distortion products at the frequency 2f1-f2. For each frequency f1 (7, 12 or 18 kHz), the individual best ratio (BR) f2/f1 was determined using an intermediate sound level (60 dB SPL or higher, if necessary), varying the ratio from 1.06 to 1.44 in steps of 0.02. This ratio was used for the following input/output measurement. The sound level of f1 was kept at a constant 10 dB louder than the f2 level. Sound was initially presented at the lowest level and increased in steps of 5 dB. Recording windows were averaged in order to decrease the background noise to at least 10 dB below the level of emission, but not over more than 1000 repetitions.

Results

Innervation defects in mice lacking TrkB signalling

As previously reported, we observed defects of the afferent and efferent innervation in all vestibular sensory epithelia in adult *TrkB*$^{shc/shc}$ mice (Fig. 1A,B) (Postigo et al., 2002). By contrast, innervation of the cochlear sensory epithelium was found to be normal in these mutants (data not shown). As *TrkB*$^{-/-}$ mouse mutants, which carry a defective tyrosine kinase, die shortly after the first or second postnatal week (Klein et al., 1993), an analysis of the lack of TrkB function during further maturation of the inner ear in vivo has not been possible. To monitor the consequences of a severe reduction of TrkB signalling during later development, we crossed *TrkB*$^{shc/shc}$ mice with viable heterozygous *TrkB*$^{+/-}$ mice, to produce *TrkB*$^{shc/-}$ mice. *TrkB*$^{shc/-}$ mice were found to have a normal lifespan, thus permitting a further analysis of the inner ear during later postnatal development and adulthood.

Examination of the vestibular system of adult *TrkB*$^{shc/-}$ mice revealed a similar reduction of afferent innervation of target sensory epithelia to that observed in *TrkB*$^{shc/shc}$ animals. In *TrkB*$^{shc/+}$ control animals bundles of neurofilament-positive afferent fibres project to the vestibular hair cells as shown for the sensory cells of the sacculus, reaching the target hair cells

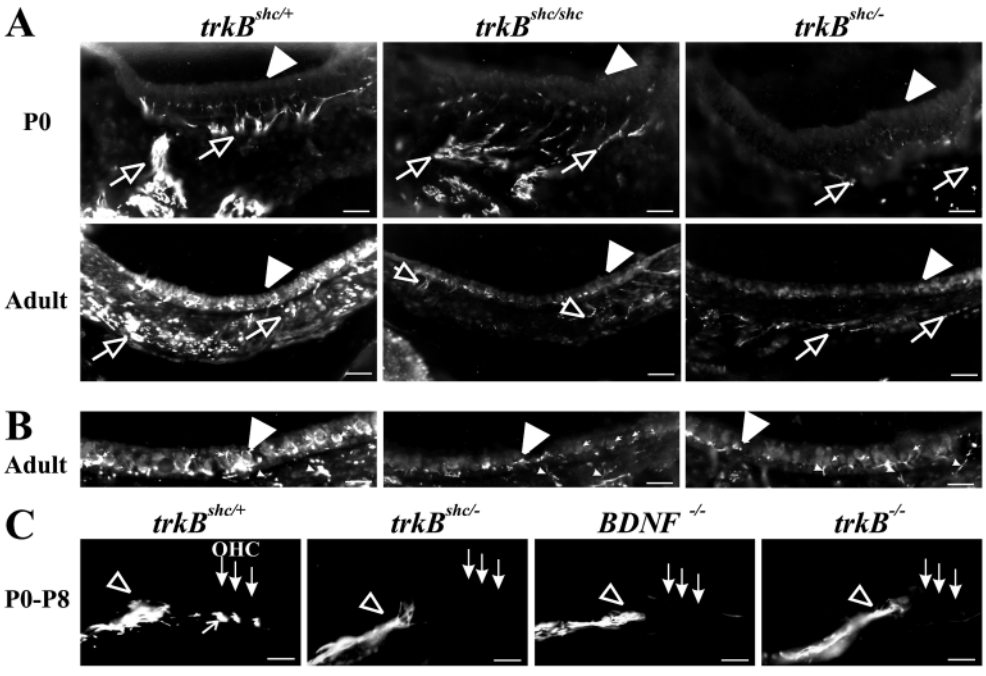

Fig. 1. Innervation of vestibular (A,B) and cochlear (C) sensory epithelia in TrkB- and Bdnf-mutant mice. Cochlear sections through the labyrinth of newborn (P0) and adult animals were labelled with anti-NF200 antibody to visualise afferent innervations. Arrows point to fibres; filled arrowheads mark hair cells (A,B). (A) *TrkB*$^{shc/shc}$ mice maintain innervation of the sacculus at birth but show a severe reduction of NF200-positive fibres opposite the sensory epithelia in adults compared with *TrkB*$^{shc/+}$ controls. In *TrkB*$^{shc-/-}$ mutants innervation is already lost at birth. (B) In the utriculus, both *TrkB*$^{shc/shc}$ and *TrkB*$^{shc/-}$ mutants lack afferent innervation at adulthood. (C) In the first postnatal week (P0-P8) neurofilament-positive projections are detected in the cochlea opposite all three rows of outer hair cells (OHC) in *TrkB*$^{shc/+}$ controls, but not in *TrkB*$^{shc/-}$, *Bdnf*$^{-/-}$ or *TrkB*$^{-/-}$ mutants. Scale bars: 20 μm.

at P0 and forming a calyx-like structure around type I hair cells in adult specimens (Fig. 1A). In both P0 and adult *TrkB*$^{shc/shc}$ and *TrkB*$^{shc/–}$ mice, the number of neurofilament-positive fibres contacting the target hair cells in the sacculus and utriculus is significantly reduced, and calyx-like structures are not formed in adults (Fig. 1A,B). Similar to *TrkB*$^{shc/shc}$ mice, the ampullary cristae show the most severe innervation defects in the *TrkB*$^{shc/–}$ mutants (data not shown) (Postigo et al., 2002). Moreover, we observed a lack of nerve fibres in the cochlear sensory epithelium at the level of the OHCs in *TrkB*$^{shc/–}$ mice: the medial turns of early postnatal cochleae in *TrkB*$^{shc/–}$ mice are shown for comparison with *TrkB*$^{shc/+}$ controls (Fig. 1C). In agreement with earlier findings, a similar phenotype could be observed in *Bdnf*$^{–/–}$ and *TrkB*$^{–/–}$ mouse mutants (Fig. 1C) (Bianchi et al., 1996; Fritzsch et al., 1998; Wiechers et al., 1999). Thus, the *TrkB*$^{shc/–}$ mutant offers a useful model to study the consequences of defective TrkB signalling in the cochlea during further maturation and in adulthood. We therefore decided to perform a detailed analysis of the innervation patterns and the cochlear morphology *TrkB*$^{shc/–}$ mice during these timepoints and to compare them with the defects of *TrkB*$^{–/–}$ and *Bdnf*$^{–/–}$ mutants.

Neuronal loss and hair cell maintenance in adult TrkB mutants

Microscopical examination on serial sections revealed no apparent morphological changes in the organ of Corti of *TrkB*$^{shc/–}$ mice compared with control littermates (Fig. 2A). To further confirm the intactness of OHCs, we used antibodies directed against the outer hair cell motor protein prestin (Weber et al., 2002). Similar to *TrkB*$^{shc/+}$ control animals, the characteristic staining pattern of prestin localised in the lateral membrane of the outer hair cell was observed in all cochlear turns of adult *TrkB*$^{shc/–}$ mutants (Fig. 2B). Previous studies had reported a specific loss of type II sensory neurones, innervating OHCs in TrkB and Bdnf mutants (Ernfors et al., 1995; Schimmang et al., 1995). To monitor the presence of type II sensory neurones in *TrkB*$^{shc/–}$ mice we used antibodies directed against peripherin, a 57 kDa type III intermediate filament that has been defined as a specific marker for these neurones (Hafidi, 1998). On serial sections of P19 and adult control mice distinct small-sized peripherin-positive neurones were detected (Fig. 3A,C). No peripherin-positive neurones could be detected in the cochlear ganglion along the entire length of the hearing organ in adult *TrkB*$^{shc/–}$ mutants (Fig. 3B). This type of neurone was also not detected in *Bdnf*$^{–/–}$ mutants at P19 (Fig. 3D). Therefore, the absence of type II sensory neurones in *TrkB*$^{shc/–}$ mutants confirms the dependence of these neurones on TrkB signalling and underlines the validity of *TrkB*$^{shc/–}$ mice as a model for a severe loss of TrkB function in the cochlea.

Spatial reversal of innervation patterns during postnatal development in TrkB mutants

TrkB$^{–/–}$ and *Bdnf*$^{–/–}$ mutants showed a lack of afferent type II fibres to OHCs at birth and after the first postnatal week (Fig. 1) (Bianchi et al., 1996; Fritzsch et al., 1998; Wiechers et al., 1999). Furthermore, a region-specific absence of nerve fibres in the apex could be observed. In agreement with these data, we were unable to detect neurofilament type II fibres in the medial and apical turn of cochleae of P0-P8 of *TrkB*$^{–/–}$ and *Bdnf*$^{–/–}$ mice (Fig. 4). In comparison, a dense pattern of fibres was observed in these cochlear turns at the base of OHCs in *TrkB*$^{shc/+}$ (Fig. 4A) and *TrkB*$^{+/–}$ mice (data not shown). Similar to *TrkB*$^{–/–}$ and *Bdnf*$^{–/–}$ mice, neurofilament-positive projections towards OHCs were not detected in the medial and apical turn in *TrkB*$^{shc/–}$ mutants, whereas fibres of this phenotype were found opposite of OHCs in the basal cochlear turn (Fig. 4A). No innervation defects were observed at the level of IHCs in *TrkB*$^{shc/–}$ mutants.

To analyse the further development of the innervation

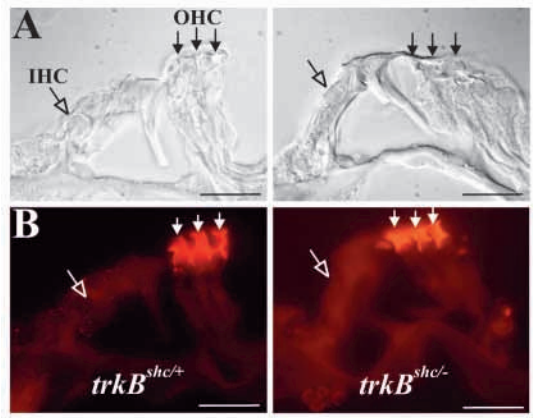

Fig. 2. Normal morphology and prestin expression in hair cells of TrkB-mutant mice. (A) Representative micrographs (DIC) showing the organ of Corti of adult control *TrkB*$^{shc/+}$ and *TrkB*$^{shc/–}$ mutant mice. The inner (IHC) and outer hair cells (OHC) are indicated by arrows. (B) Sections were stained with anti-prestin antibodies. No significant differences were noted between either the intensity or expression pattern of prestin in OHCs of *TrkB*$^{shc/+}$ and *TrkB*$^{shc/–}$ mice. Scale bars: 20 μm.

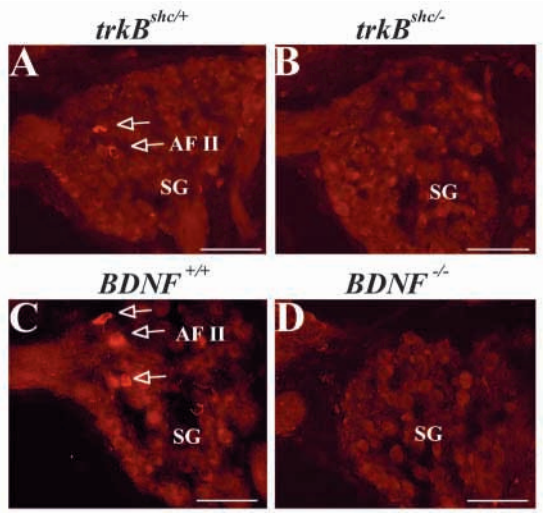

Fig. 3. Loss of peripherin-positive type II afferent neurones in the cochleae of TrkB and Bdnf mutants. Cochleae were isolated, sectioned and stained with antibodies against peripherin as described under Materials and methods. Representative micrographs illustrating the presence of type II spiral ganglion neurones (SG) in the midbasal cochlear turn of the organ of Corti in adult *TrkB*$^{shc/+}$ (A) and P19 *Bdnf*$^{+/+}$ (C) control mice; no peripherin-positive neurones were detected in *TrkB*$^{shc/–}$ (B) and *Bdnf*$^{–/–}$ mutants (D). Scale bars: 50 μm.

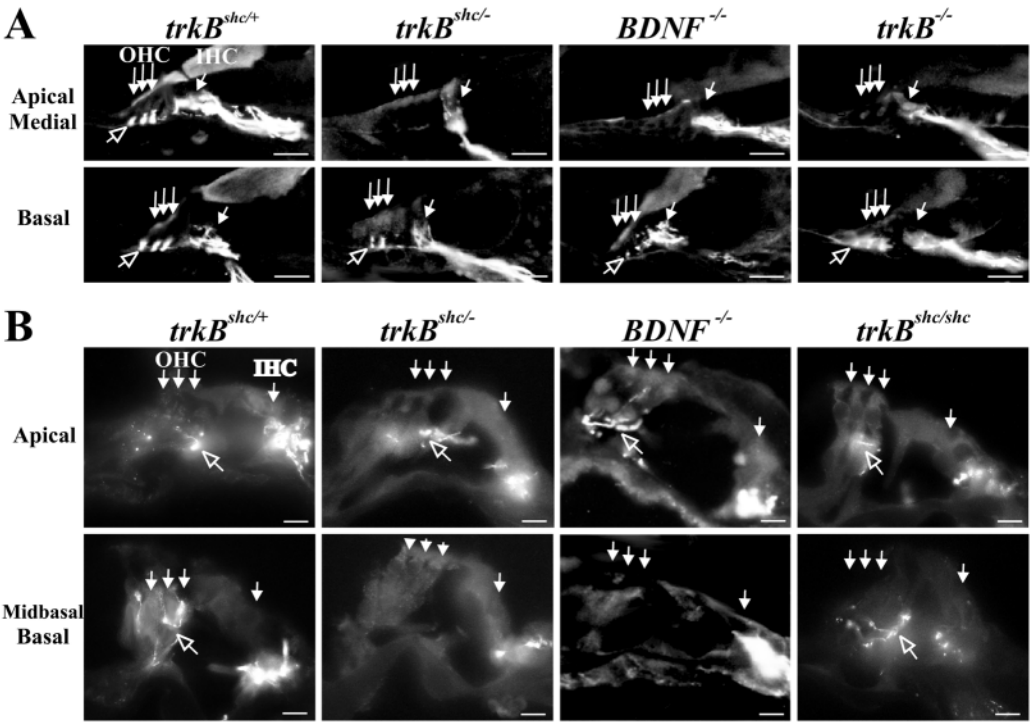

Fig. 4. Spatial reversal of innervation patterns during postnatal development in TrkB and Bdnf mutants. Representative images of sections through the cochlear turns at the indicated levels (apical/medial/midbasal/basal) of animals with the indicated genotypes, labelled with anti-NF200 antibodies as described under Materials and methods. Arrows point to the level of the three rows of outer hair cells (OHC) and the single row of inner hair cells (IHC). The open arrows mark the position of afferent fibres innervating OHCs. Note the absence of NF200-positive fibres below OHCs in the apical and medial cochlear turns of *TrkB*$^{shc/-}$, *Bdnf*$^{-/-}$ or *TrkB*$^{-/-}$ mutants compared with *TrkB*$^{shc/+}$ control animals during the first postnatal week (A). Sections through basal and midbasal cochlear turns of adult *TrkB*$^{shc/-}$ and P19 *Bdnf*$^{-/-}$ mice lack afferent innervation to OHCs, compared with their respective age-matched controls, shown for *TrkB*$^{shc/+}$ (B). Scale bars: 20 μm.

pattern in the hearing organ with a severe lack of TrkB signalling, we examined the cochlear sensory epithelium of P19 *Bdnf*$^{-/-}$ mutants and adult *TrkB*$^{shc/-}$ mice. Surprisingly, at these timepoints, *Bdnf*$^{-/-}$ and *TrkB*$^{shc/-}$ mice revealed the opposite innervation pattern to the one observed during early postnatal development (Fig. 4B). Apical turns of P19 *Bdnf*$^{-/-}$ and adult *TrkB*$^{shc/-}$ mice showed a normal pattern of afferent fibres below OHCs, similar to *TrkB*$^{shc/+}$, *TrkB*$^{shc/shc}$ and *TrkB*$^{+/-}$ mice (Fig. 4B and data not shown). On the contrary, in basal turns, no contacts of fibres with OHCs could be detected in *TrkB*$^{shc/-}$ and *Bdnf*$^{-/-}$ animals, whereas *TrkB*$^{shc/+}$, *TrkB*$^{shc/shc}$ and *TrkB*$^{+/-}$ mice showed a normal innervation pattern (Fig. 4B and data not shown). These results demonstrate that the initial innervation pattern observed during early postnatal development is reversed during maturation of the cochlea in mutants deficient for Bdnf or TrkB.

Loss of efferent innervation in Bdnf and TrkB mutants

Upon analysis of the efferent innervation pattern using synaptophysin as a marker protein (Knipper et al., 2000), we observed that the loss of afferent type II fibres projecting to OHCs in basal cochlear turns of *TrkB*$^{shc/-}$ mice was associated with a severe reduction in the number and size of synaptophysin-positive boutons below these cells (Fig. 5). To further define this defect on the postsynaptic site of the cell membrane, we studied the localisation of the apamin-sensitive, small-conductance Ca^{2+}-activated potassium channel SK2, which has been localised in OHCs, in a localisation opposite to the efferents (Fig. 5) (Oliver et al., 2000). In the basal turn of adult *TrkB*$^{shc/+}$ animals, synaptophysin-positive boutons were detected below each hair cell, opposite SK2 channels and restricted to the area of efferent contact. In adult *TrkB*$^{shc/-}$ mice, we observed only a few small synaptophysin-positive boutons, with either a very weak or no SK2-positive staining at the outer hair cell contact zone. These findings were also confirmed in the basal turn of control *Bdnf*$^{+/+}$ and *Bdnf*$^{-/-}$ mice at P19. These results indicate that the loss of afferents below OHCs in the basal cochlear turns of Bdnf- and TrkB-deficient mice may have consequences on the size of the efferent contact zone on the hair cell, and that the reduced size of the presynaptic efferent contact zone may subsequently result in a reduction or absence of the postsynaptic field, characterised by the expression of SK2 ion channels.

Neurotrophin and *TrkB* mRNA along the tonotopic axis

The switch of the dependence on Bdnf observed in afferent fibres along the tonotopic axis during postnatal cochlear development, and the resulting reversal of the innervation pattern, may be explained by the differential expression of Bdnf or its receptor, TrkB, along this axis. Additionally, the expression of Nt3, which is also able to signal through TrkB (Davies et al., 1995; Kaplan and Miller, 1997), may influence the reorganisation of afferent fibres. Although it has been shown that Nt3 is expressed by adult inner hair cells (see also Fig. 6A), neither Bdnf nor TrkB are expressed in the adult sensory epithelium (Ylikoski et al., 1993; Wheeler et al., 1994; Fritzsch et al., 1999). However, mammalian spiral ganglion neurones have been shown to express *Nt3*, *Bdnf* and *TrkB* either postnatally or in adulthood (Mou et al., 1997; Wiechers et al., 1999; Oestreicher et al., 2000; Zha et al., 2001). Transcripts of *Nt3*, *Bdnf* and *TrkB* were also found to be expressed in adult cochlear neurones of rats (Fig. 6A). We next studied the

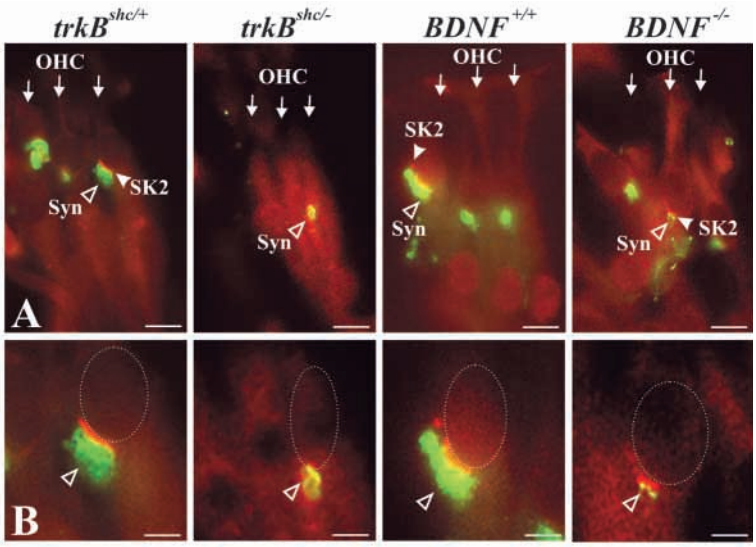

Fig. 5. Reduced size of efferent synapses in the absence of Bdnf and TrkB signalling. Cochleae of adult *TrkB*$^{shc/+}$, *TrkB*$^{shc/–}$, *Bdnf*$^{+/+}$ and *Bdnf*$^{–/–}$ mice at P19 were isolated, sectioned, and double-immunostained with anti-synaptophysin and anti-SK2 antibodies as described under Materials and methods. (A) Representative micrographs illustrating synaptophysin (Syn; green) and SK2 (red) staining at the outer hair cell (OHC) level of the basal cochlear turn in mice with the indicated genotypes. Note the severe reduction of synaptophysin-positive boutons and of SK2 staining opposite the synaptic bouton in *TrkB*$^{shc/–}$ and *Bdnf*$^{–/–}$ mutant mice compared with their respective controls. (B) Higher magnification view of a representative OHC from A. Area outlined by dotted line represents the position of the OHC body. Scale bars: 10 μm in A; 5 μm in B.

distribution of these transcripts along the tonotopic axis. For *Bdnf* and *TrkB*, we observed a gradient of expression increasing from the apical and medial turns towards the midbasal and basal parts of the cochlea (Fig. 6B). By contrast, *Nt3* mRNA showed an opposite gradient of expression, with more transcripts present in spiral ganglia neurones of the apical and medial turns compared with the midbasal and basal parts (Fig. 6B). This distribution was first observed around the end of the second postnatal week, and the described expression patterns were also confirmed in mice and gerbils (data not shown). Consequently, the lack of innervation in the more basal part of TrkB and Bdnf mutants during maturation of the cochlea most likely reflects the dependence of afferent fibres on increased levels of Bdnf and TrkB expression.

Hearing loss in TrkB-mutant mice

To monitor the importance of the observed mutant phenotype in *TrkB*$^{shc/–}$ mice for function of the cochlea, we performed hearing tests. Frequency-dependent auditory brain stem responses were determined in adult *TrkB*$^{shc/–}$ mutant and *TrkB*$^{shc/+}$ control animals. Mutant mice showed significantly elevated thresholds at all frequencies tested (Fig. 7A). Moreover, distortion product otoacoustic emissions above the background level were not detectable in *TrkB*$^{shc/–}$ animals (Fig. 7B). These results thus suggest that the observed lack of outer hair cell innervation in *TrkB*$^{shc/–}$ mice leads to a qualitative loss of hearing performance.

Discussion

Consequences of the lack of TrkB signalling for the adult inner ear

Until recently, the consequences of a lack of Bdnf or TrkB signalling for the mature inner ear could not be analysed because mouse mutants for these genes die within the first postnatal weeks (Klein et al., 1993; Ernfors et al., 1994; Jones et al., 1994; Korte et al., 1995). The creation of mouse mutants that lack specific parts of the signalling cascade controlled by the TrkB and TrkC receptor results in mice with a normal lifespan, and thus has allowed us to study the phenotypes of these mice (Minichiello et al., 1998; Postigo et al., 2002). Specifically *TrkB*$^{shc/shc}$ mice, which lack the binding site of the Shc adapter protein of TrkB, appear to offer a useful model to investigate a reduction of TrkB signalling during further maturation of the inner ear.

In the present study, and a previous study (Postigo et al., 2002), we observed severe afferent and efferent innervation defects in the vestibular sensory epithelia of *TrkB*$^{shc/shc}$ mice, which are similar to the ones found in *TrkB*$^{–/–}$ mice that lack the entire tyrosine kinase domain (Schimmang et al., 1995; Fritzsch et al., 1998). By contrast, no defects could be observed in cochlear neurones of *TrkB*$^{shc/shc}$ mutants. Cochlear neurones may therefore depend on other signalling domains of the TrkB receptor (Minichiello et al., 2002), or on additional growth factors (Fritzsch et al., 1999). By crossing *TrkB*$^{shc/shc}$ with *TrkB*$^{+/–}$ mice we have created *TrkB*$^{shc/–}$ mutants with a further reduction in TrkB signalling but a normal life span. In the vestibular system of *TrkB*$^{shc/–}$ mice, we observed similar innervation defects to the ones observed in *TrkB*$^{shc/shc}$ mutants (Postigo et al., 2002). Interestingly however, in contrast to *TrkB*$^{shc/shc}$ mutants, *TrkB*$^{shc/–}$ mice also show a loss of afferent type II fibres to the OHCs of the adult cochlea, which had so far only been reported in *TrkB*$^{–/–}$ mutants during early postnatal development (Schimmang et al., 1995; Fritzsch et al., 1998). *TrkB*$^{shc/–}$ mice thus offered an interesting model to study the consequences of severely reduced TrkB signalling in the adult cochlea. The validity of this model was established by the parallel analysis of postnatal Bdnf-mutant mice, which show an expanded postnatal lifespan compared with *TrkB*$^{–/–}$ mutants (Klein et al., 1993; Ernfors et al., 1994; Jones et al., 1994; Korte et al., 1995).

Similar to results obtained by neuronal cell counts in TrkB and Bdnf mutants (Schimmang et al., 1995; Ernfors et al., 1995), we observed a loss of type II cochlear sensory neurones using a cell-type specific marker for these neurones (Hafidi, 1998) in *TrkB*$^{shc/–}$ mice. This result thus further confirms that the primary defect in Bdnf and TrkB mutants is a loss of type II sensory neurones, and a defect in the innervation of their targets, the OHCs (see below). However, the morphology and

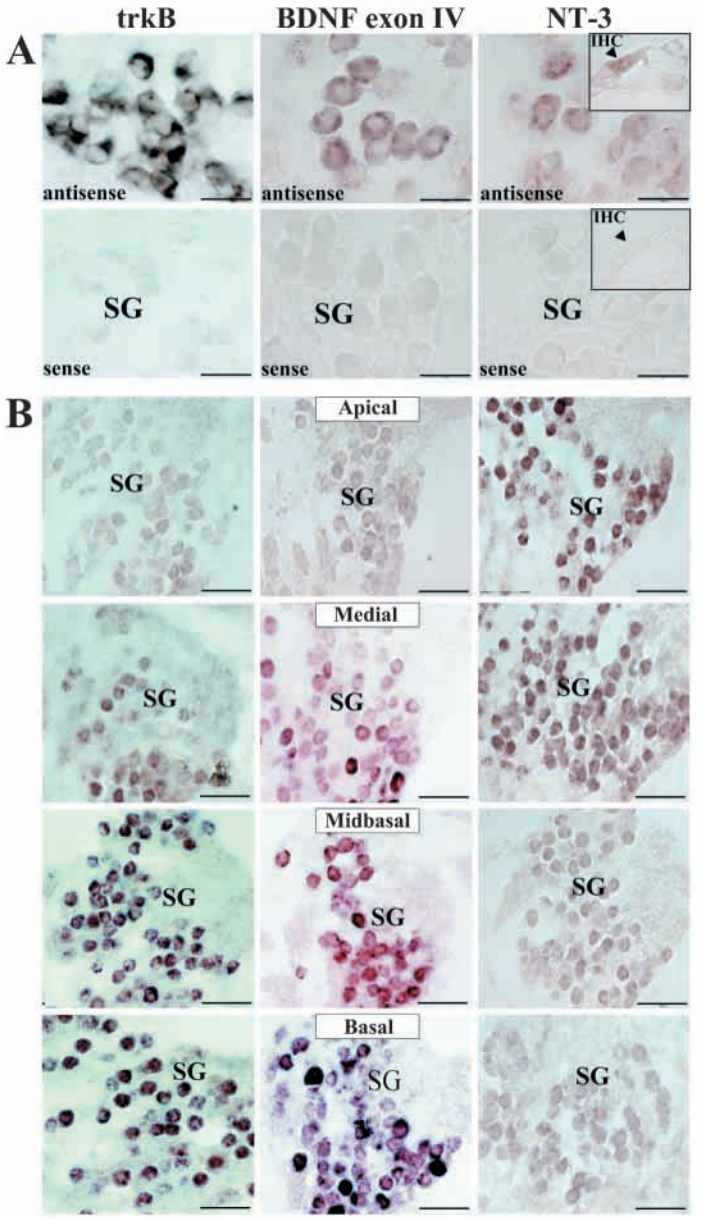

Fig. 6. Expression of *Bdnf*, *Nt3* and *TrkB* mRNA in the spiral ganglion of adult rats. (A) *TrkB*, *Bdnf* and *Nt3* riboprobes specifically hybridise with spiral ganglia of the midbasal cochlea turn of P35 rat cochlea. Note the expression of Nt3 in the IHCs (inset). (B) The expression of *Bdnf*, *Nt3* and *TrkB* was analysed in cochlear neurones along the tonotopic axis at the indicated levels (apical/medial/midbasal/basal) in rats. *Bdnf* and *TrkB* are expressed with increasing levels towards the basal part of the cochlea, whereas *Nt3* mRNA shows an opposite gradient of expression, with the highest amounts of mRNA detected in the apex. SG, spiral ganglion. Scale bars: 10 μm in inset in A; 20 μm in B.

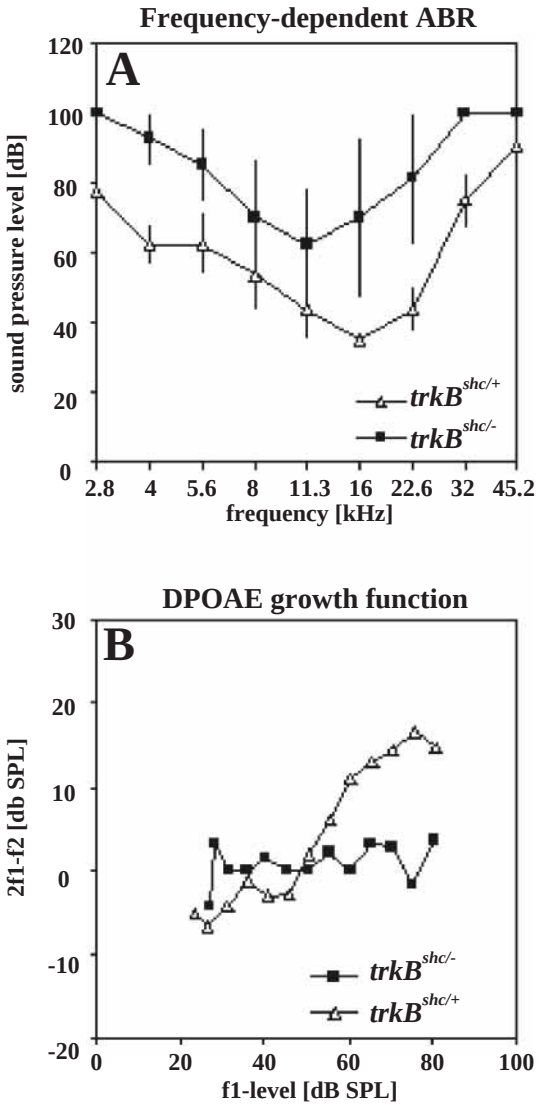

Fig. 7. Auditory brainstem response thresholds (A) and distortion product otoacoustic emissions (B) in adult *TrkB*$^{shc/-}$ mutant and *TrkB*$^{shc/+}$ control mice. ABR thresholds were elevated at all frequencies tested, and DPOAEs were absent in *TrkB*$^{shc/-}$ mutants compared with controls. Amplitude of DPOAE determined at 2×f2-f1 (f1=8.86 kHz; f2=10.99 kHz; delta I1-I2=10 dB). DPOAE, distortion product otoacoustic emission; SPL, sound pressure level; ABR, auditory brainstem response.

structural integrity of OHCs, as demonstrated by staining with antibodies against the OHC motor protein prestin, appears unaffected in *TrkB*$^{shc/-}$ mutants. Likewise, only minor or no structural changes of cochlear morphology have been reported in previous analyses of neurotrophin or Trk receptor mutants (Schimmang et al., 1995; Fritzsch et al., 1997b).

Dynamics of neurotrophin signalling and innervation in the prenatal and postnatal cochlea

As the major defect in TrkB and Bdnf mutants appears restricted to the innervation of OHCs, we have analysed in detail the spatiotemporal pattern of innervation to these cells during postnatal development along the tonotopic axis of the cochlea. As previously reported, we observed a lack of afferent fibres to OHCs in the medial and apical part of the cochlea in Bdnf and TrkB mutants during the first postnatal week (Bianchi et al., 1996; Fritzsch et al., 1998; Wiechers et al., 1999). A similar defect was also observed in *TrkB*$^{shc/-}$ mice, once more confirming the validity of these mice as a

model for a severe reduction of TrkB signalling in the postnatal cochlea.

The early postnatal phenotype of TrkB and Bdnf mutants is most likely explained by an apical-to-basal gradient of Bdnf expression in the cochlear sensory epithelia during embryonic development (Fariñas et al., 2001). Strikingly, we observed a mirror-image of this phenotype during subsequent postnatal maturation in the adult cochlea of *Bdnf*$^{-/-}$ and *TrkB*$^{shc/-}$ mutants, respectively. Towards the end of the third postnatal week, Bdnf mutants showed a complete absence of basal afferent innervation, whereas the apical part of the cochlea showed a normal pattern of afferent fibres on OHCs. Likewise, an identical pattern of innervation was observed in *TrkB*$^{shc/-}$ adult mutants. How can the reversal of the phenotype observed in Bdnf and TrkB mutants during postnatal development be explained?

At birth, a longitudinal expression of Bdnf with highest expression in the apex (Wheeler et al., 1994; Wiechers et al., 1999) or a homogenous pattern of expression (Fariñas et al., 2001) has been described in the cochlear sensory epithelium. Subsequently, Bdnf has been detected in OHCs of the apical to midbasal turn, but not of the basal turn during the first postnatal week (Wiechers et al., 1999). After this timepoint, and in adulthood, no Bdnf expression has been observed in OHCs (Wheeler et al., 1994; Fritzsch et al., 1999; Wiechers et al., 1999). In summary, these data provide no explanation for the basal loss of afferent innervation of OHCs observed towards the end of the third postnatal week and in adulthood.

However, the basal-to-apical gradient of Bdnf and TrkB expression detected in cochlear neurones in the present report offers an attractive explanation for the dependence of afferent innervation on Bdnf and TrkB signalling in the more basal cochlear turns. Specifically, the co-expression of TrkB and Bdnf suggests an autocrine mechanism that maintains the innervation of the basal turn during further maturation of the cochlea. This process may be initiated by the expression of Bdnf in cochlear ganglion neurones during the first postnatal week (Wiechers et al., 1999). The gradient of Bdnf expression is first observed at the end of the second postnatal week (M.K., unpublished observation). Therefore, the spatial and temporal expression of Bdnf in cochlear neurones during postnatal development and in adulthood fits perfectly with the gradual reversal of innervation of OHCs observed in Bdnf and TrkB mutants during the second and third postnatal weeks. In this context, it is worthwhile mentioning that Bdnf is not expressed in differentiated neurones that express TrkB during embryonic development (Fariñas et al., 2001). Therefore, the postulated autocrine mechanism for the maintenance of basal outer hair cell innervation is restricted to the postnatal and adult phases of life. How can the re-innervation of the medial and apical turns of the cochlea in Bdnf and TrkB mutants be explained?

The most attractive model may be an apical-to-basal gradient of expression of Nt3 in the postnatal inner ear. Indeed, such a gradient with the highest expression in the apical part has been described for the cochlear sensory epithelium at birth (Pirvola et al., 1992; Wheeler et al., 1994; Fritzsch et al., 1997b). However, recently this gradient could not be reconfirmed (Fariñas et al., 2001). In the present analysis we have detected an apical-to-basal gradient of Nt3 expression in cochlear neurones, which develops postnatally in the opposite direction to the Bdnf gradient described above. The increased expression of Nt3 in cochlear neurones in the apical turn thus offers an attractive explanation for the re-innervation of OHCs in this part of the cochlea in Bdnf and TrkB mutants. At present we are analysing whether this expression is accompanied by the expression of its high-affinity receptor TrkC. Similar to the situation in the base, co-expression of the neurotrophin and its high-affinity receptor may initiate an autocrine mechanism. Thus, in the apical turns of the cochleae of TrkB and Bdnf mutants, a presumptive Bdnf-independent, Nt3-dependent process of re-innervation of OHCs may be maintained.

At present, it is unclear which neurones are involved in the process of reinnervation. In theory, one would postulate the involvement of two classes of type II sensory neurones, which naturally may provide embryonal and adult innervation to OHCs. In support of this, ‘embryonal’ type II neurones have been shown to be severely reduced in the absence of Bdnf, as assessed using cell counts in early postnatal Bdnf and TrkB mutants (Ernfors et al., 1995; Schimmang et al., 1995). In the present study, using peripherin as a marker, we also found no evidence for the presence of type II sensory neurones in adult *TrkB*$^{shc/-}$ mutants. However, we cannot rule out the existence of type II neurones that have failed to express peripherin in the Bdnf and TrkB mutants analysed in the present study. Moreover, the presence of type II sensory neurones in *Bdnf*$^{-/-}$ mutants has been postulated based on the expression of the neurofilament marker RT97 (Bianchi et al., 1996). Therefore, two populations of these neurones may exist, which may be involved in initiating the process of embryonal innervation and postnatal re-innervation of OHCs.

It may be considered that neurones innervating OHCs in the apical region, which provide two times more synaptic contacts to OHCs than basally localised neurones of this type (Rubel and Fritzsch, 2002), are possibly Nt3-dependent, type I sensory neurones (Ernfors et al., 1995) sending out collaterals to the OHCs. As such, it has been suggested that initially individual ganglion cells innervate both IHCs and OHCs during development (Echteler, 1992; Pujol et al., 1998). In the mammalian cochlea, an extensive process of afferent and efferent fibre reorganisation takes place during the first and second postnatal week (Wiechers et al., 1999). Therefore, the possibility of an early innervation of OHCs by type I neurones, and a Bdnf-dependent phenotypic switch of type I to type II neurones in more basal and less apical turns during postnatal development, should be further investigated. Finally, as shown previously in Nt3 mutants (Fritzsch et al., 1997b; Coppola et al., 2001), tangential extensions of fibres running underneath the cochlea may also contribute to the innervation patterns observed.

The relevance of the neuronal expression of Bdnf described in the present article is also highlighted by recent results on the electrophysiological behaviour of postnatal cochlear neurones (Adamson et al., 2002). In these experiments it was shown that exposure to Bdnf caused all neurones, regardless of their original cochlear position, to display characteristics of the basal neurones. Conversely, Nt3 caused all neurones to show the properties of apical-cochlear neurones. These data fit perfectly with the opposing basal-to-apical gradients of Bdnf and Nt3 expression observed in the postnatal cochlea in the present study.

Defects in efferent innervation and hearing loss

Hair cells are the targets of olivocochlear fibres that control

efferent inhibitory feedback from the brain. Efferent feedback to OHCs is predominantly provided by the release of acetylcholine (ACh) from olivocochlear nerve fibres, which activates the characteristic high Ca^{2+} conductance hetero-oligomeric complex of ACh receptors (AChRs) (Elgoyhen et al., 1994; Elgoyhen et al., 2001). The Ca^{2+} flux through AChRs in turn, activates the small-conductance Ca^{2+}-activated potassium channel SK2. The fast coupling of both is reflected by their co-localisation (Oliver et al., 2000; Knipper and Zenner, 2003). Although current concepts indicate an autonomous morphological differentiation of hair cells independent from ganglion neurones (Sobkowicz, 1992; van de Water et al., 1992; Silos-Santiago et al., 1997; Ma et al., 2000), which acquire their basic physiological properties in the absence of innervation (He and Dallos, 1997; Rüsch et al., 1998; Rubel and Fritzsch, 2002), data in the present study indicate that distinct steps of the final functional phenotype of OHCs is influenced by the innervating fibres.

The loss of afferent type II fibres below OHCs in Bdnf and $TrkB^{shc/-}$ mutants is accompanied by a significant reduction in size of efferent synaptic endings and postsynaptic fields on OHCs, in which SK2 channels are inserted. As we observed no defects in the innervation of IHCs, the alteration of the OHC phenotype may directly be related with the observed hearing loss of 10-30 dB in $TrkB^{shc/-}$ mice across all frequencies tested. This hearing loss and the lack of DPOAEs suggest a common mechanism that is impaired in the mutant animals. As no DPOAEs were detectable, the defect may reside at the level of the OHC and may be caused by the reduction of efferent input. However, at present it remains unclear whether this defect is caused by structural or physiological changes of the OHC.

Previous analysis has shown that the neurotrophin receptor mutants lacking TrkB and TrkC develop central cochlear nuclei of a reduced size (Schimmang et al., 1997). The reduction of these nuclei may be explained by a direct trophic role of neurotrophins within these nuclei. Alternatively, a lack of trophic support of central auditory neurones by the peripheral auditory neurones may be responsible for the observed phenotype. In this context, it is also tempting to speculate that Bdnf expression, which is involved in several plasticity-driven processes (Berardi et al., 2000; Schinder and Poo, 2000), may also influence the tonotopic map present in the central part of the auditory system. Expression of Trk receptors and neurotrophins has been described in central auditory neurones (Hafidi et al., 1996; Hafidi, 1998), and their involvement in activity-dependent trophic interactions with the peripheral neurones has been suggested (Rubel and Fritzsch, 2002). The postnatal gradient of Bdnf expression in the cochlear ganglion may thus set up, and later reinforce or maintain, the tonotopic map in the central cochlear nuclei. Therefore, a central component may also be involved in the hearing loss of $TrkB^{shc/-}$ mice.

Finally, the observation that Bdnf and TrkB are co-expressed in cochlear neurones reinforces the idea of an autocrine mode to maintain functional activity of these neurones (Hansen et al., 2001). Treatment of cochlear neurones with neurotrophins has been shown to stimulate their survival and alter their functional activity (Hegarty et al., 1997; Adamson et al., 2002). Moreover, depolarisation of spiral ganglion neurones together with neurotrophins promotes survival in an additive manner in vitro (Hegarty et al., 1997). Our present results suggest that the interplay of an autocrine neurotrophic mechanism and neuronal activity may also maintain the survival and correct functioning of auditory neurones in vivo, thus ensuring the intactness of the sensory organ. In this context, it is worthwhile mentioning that the expression of Bdnf described in the present article may also give a functional explanation for the positive activity of neurotrophins in animal models of hearing loss. Application of Bdnf and Nt3 to the inner ear has been shown to ameliorate hearing loss in several of these models (Ernfors et al., 1996; Duan et al., 2002; Shinohara et al., 2002). Our results suggest that the underlying mechanism is the stimulation of cochlear neurones by neurotrophins, which may lead to a functional re-stimulation of neuronal activity. Further experiments will show if re-innervation processes, like the ones observed in the present study, can also be re-initiated in postnatal animals and adults. In summary, our results may provide an important step for understanding the basis of treatment of hearing loss by neurotrophins, and may eventually allow us to improve therapies by targeting specific neurotrophins to subsets of cochlear neurones.

We thank Rüdiger Klein for providing TrkB mutants and for critical reading of the manuscript, Bernd Fritzsch for help in the interpretation of the $TrkB^{shc/shc}$ mutant, and Michael Sendtner and Bettina Holtmann for providing the Bdnf mutants. This work was supported by the DFG-SFB444, DFG KN 316/3-2, fortüne Kni-F1251236, and fortüne Kni-F1251279.

References

Adamson, C. L., Reid, M. A. and Robin, L. D. (2002). Opposite actions of brain-derived neurotrophic factor and neurotrophin-3 firing features and ion channel composition of murine spiral ganglion neurons. *J. Neurosci.* **22**, 1385-1396.

Berardi, N. Pizzorusso, T. and Maffei, L. (2000). Critical periods during sensory development. *Curr. Opin. Neurobiol.* **10**, 138-145.

Bianchi, L. M., Conover, J. C., Fritzsch, B., DeChiara, T., Lindsay, R. M. and Yancopoulos, G. D. (1996). Degeneration of vestibular neurons in late embryogenesis of both heterozygous and homozygous BDNF null mutant mice. *Development* **122**, 1965-1973.

Bibel, M. and Barde, Y. A. (2000). Neurotrophins: key regulators of cell fate and cell shape in the vertebrate nervous system. *Genes Dev.* **14**, 2919-2937.

Coppola, V., Kucera, J., Palko, M. E., Martinez-De Velasco, J., Lyons, W. E., Fritzsch, B. and Tessarollo, L. (2001). Dissection of NT3 functions in vivo by gene replacement strategy. *Development* **128**, 4315-4327.

Davies, A. M., Minichiello, L. and Klein, R. (1995). Developmental changes in NT3 signalling via TrkA and TrkB in embryonic neurons. *EMBO J.* **14**, 4482-4489.

Duan, M. L., Ulfendahl, M., Laurell, G., Counter, A. S., Pyykko, I., Borg, E. and Rosenhall, U. (2002). Protection and treatment of sensorineural hearing disorders caused by exogenous factors: experimental findings and potential clinical application. *Hear. Res.* **169**, 169-178.

Echteler, S. M. (1992). Developmental segregation in the afferent projections to mammalian auditory hair cells. *Proc. Natl. Acad. Sci. USA* **89**, 6324-6327.

Elgoyhen, A. B., Johnson, D. S., Boulter, J., Vetter, D. E. and Heinemann, S. (1994). Alpha-9: an acetylcholine receptor with novel pharmacological properties expressed in rat cochlear hair cells. *Cell* **79**, 705-715.

Elgoyhen, A. B., Vetter, D. E., Katz, E., Rothlin, C. V., Heinemann, S. F. and Boulter, J. (2001). alpha 10: a determinant of nicotinic cholinergic receptor function in mammalian vestibular and cochlear mechanosensory hair cells. *Proc. Natl. Acad. Sci. USA* **98**, 3501-3506.

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

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

Ernfors, P., Duan, M. L., ElShamy, W. M. and Canlon, B. (1996). Protection of auditory neurons from aminoglycoside toxicity by neurotrophin-3. *Nat. Med.* **2**, 463-467.

Fariñas, I., Jones, K. R., Tessarollo, L., Vigers, A. J., Huang, E., Kirstein, M., de Caprona, D. C., Coppola, V., Backus, C., Reichardt, L. F. and Fritzsch, B. (2001). Spatial shaping of cochlear innervation by temporally regulated neurotrophin expression. *J. Neurosci.* **21**, 6170-6180.

Fritzsch, B., Silos-Santiago, I., Bianchi, L. M. and Fariñas, I. (1997a). The role of neurotrophic factors in regulating the development of inner ear innervation. *Trends Neurosci.* **20**, 159-164.

Fritzsch, B., Fariñas, I. and Reichardt, L. F. (1997b). Lack of neurotrophin 3 causes loss of both classes of spiral ganglion neurons in the cochlea in a region-specific fashion. *J. Neurosci*. **17**, 6213-6225.

Fritzsch, B., Barbacid, M. and Silos-Santiago, I. (1998). The combined effects of *trk*B and *trk*C mutations on the innervation of the inner ear. *Int. J. Dev. Neurosci*. **16**, 493-505.

Fritzsch, B., Pirvola, U. and Ylikoski, J. (1999). Making and breaking the innervation of the ear: neurotrophic support during ear development and its clinical implications. *Cell Tissue Res.* **295**, 369-382.

Hafidi, A., Moore, T. and Sanes, D. H. (1996). Regional distribution of neurotrophin receptors in the developing auditory brainstem. *J. Comp. Neurol.* **367**, 454-464.

Hafidi, A. (1998). Peripherin-like immunoreactivity in type II spiral ganglion cell body and projections. *Brain Res*. **14**, 181-190.

Hansen, M. R., Zha, X. M., Bok, J. and Green, S. H. (2001). Multiple distinct signal pathways, including an autocrine neurotrophic mechanism, contribute to the survival-promoting effect of depolarization on spiral ganglion neurons in vitro. *J. Neurosci.* **21**, 2256-2267.

He, D. Z. and Dallos, P. (1997). Expression of potassium channels in gerbil outer hair cells during development does not require neural induction. *Brain Res. Dev. Brain Res.* **103**, 95-97.

Hegarty, J. L., Kay, A. R. and Green, S. H. (1997). Trophic support of cultured spiral ganglion neurons by depolarisation exceeds and is additive with that by neurotrophins or cAMP and requires elevation of $[Ca^{2+}]_i$ within a set range. *J. Neurosci.* **17**, 1959-1970.

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

Kaplan, D. R. and Miller, F. D. (1997). Signal transduction by the neurotrophin receptors. *Curr. Opin. Cell. Biol.* **9**, 213-221.

Klein, R., Smeyne, R. J., Wurst, W., Long, L. K., Auerbach, B. A., Joyner, A. L. and Barbacid, M. (1993). Targeted disruption of the trkB neurotrophin receptor gene results in nervous system lesions and neonatal death. *Cell* **75**, 113-122.

Knipper, M., Zinn, C., Maier, H., Praetorius, M., Rohbock, K., Köpschall, I. and Zimmermann, U. (2000). Thyroid hormone deficiency before the onset of hearing causes irreversible damage to peripheral and central auditory systems. *J. Neurophysiol*. **83**, 3101-3112.

Knipper, M. and Zenner, H. P. (2003). Hearing and deafness: molecular elements operating in the organ of Corti. *Ann. Otol. Rhinol. Laryngol*. (in press).

Korte, M., Carroll, P., Wolf, E., Brem, G., Thoenen, H. and Bonhoeffer, T. (1995). Hippocampal long-term potentiation is impaired in mice lacking brain-derived neurotrophic factor. *Proc. Natl. Acad. Sci. USA* **92**, 8856-8860.

Ma, Q., Anderson, D. J. and Fritzsch, B. (2000). Neurogenin 1 null mutant ears develop fewer, morphologically normal hair cells in smaller sensory epithelia devoid of innervation. *J. Assoc. Res. Otolaryngol.* **1**, 129-143.

Minichiello, L., Casagrande, F., Soler-Tatche, R., Stucky, C. L., Postigo, A., Lewin, G. R., Davies, A. M. and Klein, R. (1998). Point mutation in trkB causes loss of NT4-dependent neurons without major defects on diverse BDNF responses. *Neuron* **21**, 335-345.

Minichiello, L., Calella, A. M., Medina, D. L., Bonhoeffer, T., Klein, R. and Korte, M. (2002). Mechanism of TrkB-mediated hippocampal long-term potentiation. *Neuron* **36**, 121-137.

Mou, K., Hunsberger, C. L., Cleary, J. M. and Davis, R. L. (1997). Synergistic effects of BDNF and NT-3 on postnatal spiral ganglion neurons. *J. Comp. Neurol.* **386**, 529-539.

Oestreicher, E., Knipper, M., Arnold, A., Zenner, H. P. and Felix, D. (2000). Neurotrophin 3 potentiates glutamatergic responses of IHC afferents in the cochlea in vivo. *Eur. J. Neurosci.* **12**, 1584-1590.

Oliver, D., Klöcker, N., Schuck, J., Baukrowitz, T., Ruppersberg, J. P. and Fakler, B. (2000). Gating of Ca^{2+}-activated K^+ channels controls fast inhibitory synaptic transmission at auditory outer hair cells. *Neuron* **26**, 595-601.

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.

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

Pujol, R., Lavigne-Rebillard, M. and Lenoir, M. (1998). Development of sensory and neural structures in the mammalian cochlea. In *Development of the Auditory System* (ed. E. W. Rubel, A. N. Popper and R. R. Fay), pp. 146-192. New York: Springer.

Rubel, E. W. and Fritzsch, B. (2002). Auditory system development: primary auditory neurons and their targets. *Annu. Rev. Neurosci*. **25**, 51-101.

Rüsch, A., Erway, L. C., Oliver, D., Vennström, B. and Forrest, D. (1998). Thyroid hormone receptor beta-dependent expression of a potassium conductance in inner hair cells at the onset of hearing. *Proc. Natl. Acad. Sci. USA* **95**, 15758-15762.

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

Schimmang, T., Alvarez-Bolado, G., Minichiello, L., Vazquez, E., Giraldez, F., Klein, R. and Represa, J. (1997). Survival of inner ear sensory neurons in trk mutant mice. *Mech. Dev.* **64**, 77-85.

Schinder, A. F. and Poo, M. (2000). The neurotrophin hypothesis for synaptic plasticity. *Trends Neurosci.* **23**, 639-645.

Shinohara, T., Bredberg, G., Ulfendahl, M., Pyykko, I., Olivius, N. P., Kaksonen, R., Lindström, B., Altschuler, R. and Miller, J. M. (2002). Neurotrophic factor intervention restores auditory function in deafened animals. *Proc. Natl. Acad. Sci. USA* **99**, 1657-1660.

Silos-Santiago, I., Fagan, A. M., Garber, M., Fritzsch, B. and Barbacid, M. (1997). Severe sensory deficits but normal CNS development in newborn mice lacking TrkB and TrkC tyrosine protein kinase receptors. *Eur. J. Neurosci.* **9**, 2045-2056.

Sobkowics, H. M. (1992). The development of innervation in the organ of Corti. In *Development of Auditory and Vestibular Systems*, Vol. 2 (ed. R. Romand), pp. 59-100. Amsterdam: Elsevier.

Timmusk, T., Palm, K., Metsis, M., Reintam, T., Paalme, V., Saarma, M. and Persson, H. (1993). Multiple promoters direct tissue-specific expression of the rat BDNF gene. *Neuron* **10**, 475-489.

van de Water, T. R., Frenz, D. A., Giraldez, F., Represa, J., Lefebre, P. P. et al. (1992). Growth factors and development of the stato-acoustic system. In *Development of Auditory and Vestibular Systems II* (ed. R. Romand), pp. 1-32. Amsterdam: Elsevier.

Weber, T., Zimmermann, U., Winter, H., Mack, A., Köpschall, I., Rohbock, K., Zenner, H. P. and Knipper, M. (2002). Thyroid hormone is a critical determinant for the regulation of the cochlear motor protein prestin. *Proc. Natl. Acad. Sci. USA* **99**, 2901-2906.

Wheeler, E. F., Bothwell, M., Schecterson, L. C. and von Bartheld, C. S. (1994). Expression of BDNF and NT-3 mRNA in hair cells of the organ of Corti: quantitative analysis in developing rats. *Hear. Res.* **73**, 46-56.

Wiechers, B., Gestwa, G., Mack, A., Carroll, P., Zenner, H. P. and Knipper, M. (1999). A changing pattern of brain-derived neurotrophic factor expression correlates with the rearrangement of fibres during cochlear development of rats and mice. *J. Neurosci*. **19**, 3033-3042.

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.

Zha, X. M., Bishop, J. F., Hansen, M. R., Victoria, L., Abbas, P. J., Mouradian, M. and Green, S. H. (2001). BDNF synthesis in spiral ganglion neurons is constitutive and CREB-dependent. *Hear. Res.* **156**, 53-68.