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BDNF gene replacement reveals multiple mechanisms for establishing neurotrophin specificity during sensory nervous system development

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SUMMARY

Neurotrophins have multiple functions during peripheral nervous system development such as controlling neuronal survival, target innervation and synaptogenesis. Neurotrophin specificity has been attributed to the selective expression of the Trk tyrosine kinase receptors in different neuronal subpopulations. However, despite overlapping expression of TrkB and TrkC in many sensory ganglia, brain-derived neurotrophic factor (BDNF) and neurotrophin 3 (NT3) null mutant mice display selective losses in neuronal subpopulations. In the present study we have replaced the coding part of the *BDNF* gene in mice with that of NT3 ($BDNF^{NT3/NT3}$) to analyse the specificity and selective roles of BDNF and NT3 during development. Analysis of $BDNF^{NT3/NT3}$ mice showed striking differences in the ability of NT3 to promote survival, short-range innervation and synaptogenesis in different sensory systems. In the cochlea, specificity is achieved by a tightly controlled spatial and temporal ligand expression. In the vestibular system TrkB or TrkC activation is sufficient to promote vestibular ganglion neuron survival, while TrkB activation is required to promote proper innervation and synaptogenesis. In the gustatory system, NT3 is unable to replace the actions of BDNF possibly because of a temporally selective expression of TrkB in taste neurons. We conclude that there is no general mechanism by which neurotrophin specificity is attained and that specificity is achieved by (i) a tightly controlled spatial and temporal expression of ligands, (ii) different Trk receptors playing distinct roles within the same neuronal subpopulation, or (iii) selective receptor expression in sensory neuron subpopulations.

Key words: BDNF, NT3, Sensory neurons, Ccochlea, Neurotrophin, Taste, Mouse

INTRODUCTION

Neurotrophins are required for the development and maintenance of the nervous system. The neurotrophin family consists of nerve growth factor (NGF), brain derived neurotrophic factor (BDNF), neurotrophin 3 (NT3), and neurotrophin 4 (NT4). The functions of the neurotrophins are mediated by their interactions with the Trk tyrosine kinase receptors. NGF binds and activates the TrkA receptor (also known as Ntrk1), BDNF and NT4 binds the TrkB receptor (Ntrk2), and NT3 (Ntf3) predominantly binds the TrkC receptor (Ntrk3), but can also interact with the TrkA and TrkB receptors (Barbacid, 1994; Davies et al., 1995; Farinas et al., 1998).

The analysis of knockout mice for the neurotrophins and their receptors conveyed important information on the requirement of neurotrophins for selective neuronal subpopulations in the peripheral nervous system (Bibel and Barde, 2000; Snider and Wright, 1996). These knockout mice show losses of different subpopulations of sensory neurons in the dorsal root ganglia. $NGF^{-/-}$ and $TrkA^{-/-}$ mice show a similar phenotype with a loss predominantly in the unmyelinated and the small myelinated dorsal root ganglia neurons conveying nociceptive information, while the NT3 and TrkC deficient mice lose the large myelinated neurons that convey limb proprioception. The selective neuronal loss of these specific dorsal root ganglia subpopulations is correlated with the expression of one particular Trk receptor in each of these subclasses of neurons. Furthermore, NT3 is expressed in the intrafusal muscle fibres and in golgi tendon organs, the target tissues of the large myelinated neurons that convey limb proprioception (Copray and Brouwer, 1997; Oakley et al., 1995; Oakley et al., 1997) and NGF in the skin, which is a major target of the nociceptive neurons (Davies et al., 1987; Schornig et al., 1993). Thus, specificity of these subclasses of dorsal root ganglia neurons is attained by a selective expression of Trk receptors and by a spatially regulated expression of their ligands in the respective target tissues.

Unexpectedly, the mechanism for achieving specificity of dorsal root ganglia neuron subpopulations to neurotrophins is not generic. In most cranial sensory ganglia, Trk receptors are more widely expressed than in the dorsal root ganglia. For instance, in both the vestibular and spiral ganglia, TrkB and TrkC are ubiquitously expressed and BDNF and NT3 are present in both vestibular and auditory sensory epithelia. Yet, ligand and receptor null mutant mice display distinct and specific deficits. BDNF is of major importance for the development of the vestibular system. In *BDNF*$^{-/-}$ mice there is an 80% loss of vestibular ganglion neurons and a complete loss of innervation of the target sensory epithelia at birth, while in *NT3*$^{-/-}$ mice only 20% of the vestibular neurons are lost (Bianchi et al., 1996; Ernfors et al., 1995a; Ernfors et al., 1994a; Ernfors et al., 1995b). In contrast to the vestibular neurons, the majority of spiral ganglion neurons depend on NT3 for survival (90% loss in *NT3*$^{-/-}$ mice) while BDNF supports only about 10% at birth (Ernfors et al., 1994a; Ernfors et al., 1995b; Farinas et al., 1994; Fritzsch et al., 1997a). Most spiral ganglion neurons lost in *NT3*$^{-/-}$ mice are type I neurons that constitute around 90% of the ganglion and that innervate inner hair cells (IHC) while nearly all type II neurons are lost in *BDNF*$^{-/-}$ mice. These amount to about 10% of the ganglion and innervate outer hair cells (OHC) (Ernfors et al., 1995b). In addition to this specific action of selective neurotrophins upon distinct spiral neuron subclasses, BDNF and NT3 might also act in a spatial gradient. Neuronal loss in *NT3*$^{-/-}$ mice is almost complete in the basal part of the cochlea with the remaining few percent are localised apically. In contrast, *BDNF*$^{-/-}$ mice an almost complete loss of type II neurons and OHC innervation. However, in these mice a minor residual OHC innervation is more pronounced in the base than in the apex (Farinas et al., 2001; Fritzsch et al., 1997a). A similar degree of specificity has been observed in *TrkB*$^{-/-}$ and *TrkC*$^{-/-}$ mice (Fritzsch et al., 1998). These results suggest that BDNF and NT3 are not functionally redundant in the inner ear even though TrkB and TrkC have overlapping expression in individual neurons of both the vestibular and spiral ganglia (Ernfors et al., 1992; Ylikoski et al., 1993).

TrkB and TrkC but not TrkA expression has been demonstrated in the geniculate ganglion, which largely provides innervation for the tongue (Ernfors et al., 1992). The expression of TrkB in the geniculate ganglion agrees with results showing expression of BDNF selectively in taste buds of gustatory papillae. Consistently, there is also a distinct difference in the lingual phenotypes in the *BDNF*$^{-/-}$ and *NT3*$^{-/-}$ mice. *BDNF*$^{-/-}$ mice display a specific set of deficits in their peripheral gustatory system, including general loss of taste buds, innervation deficits in the remaining taste buds, and fewer and malformed tongue papillae. Instead, *NT3*$^{-/-}$ animals lose their lingual somatosensory innervation (Nosrat et al., 1997; Zhang et al., 1997).

We have generated mice in which the coding exon of the *BDNF* gene has been replaced with *NT3*. Thus, the normal temporal and spatial expression of BDNF during development has been replaced by NT3. In the auditory system, we find that replacement with NT3 largely restores neuronal survival, target innervation, and hearing, indicating that the specificity of BDNF in the inner ear is attained by the temporal and spatial ligand availability. In the vestibular system we find a rescue from neuronal death but a loss of innervation and function (and as a consequence an additional delayed loss of neurons). Loss of geniculate taste neurons and inappropriate target innervation in the gustatory system of *BDNF*$^{NT3/NT3}$ mice were similar to those of *BDNF*$^{-/-}$ mice, indicating that a specific BDNF-TrkB activation is required for proper development of the peripheral gustatory system.

MATERIALS AND METHODS

Generation of *BDNF*$^{NT3/NT3}$ mice

BDNF$^{NT3/NT3}$ mice were generated using ES cell technology. A target replacement vector was generated in which exon V of the *BDNF* gene was opened and the protein coding part of the *NT3* gene was fused in-frame with *BDNF*. The vector included a *neomycin* expression cassette, with the phosphoglycerate kinase promoter, flanked with *loxP* sites. The targeting vector was electroporated into the ES cell line E14. DNA from G418-resistant clones were digested with *Bgl*II and analysed by Southern blot using an external probe. Two clones showing homologous recombination were injected into blastocysts to generate chimeric mice. Genotyping of mice was performed by Southern blot analyses and later also by PCR. Offsprings from mating of heterozygous mice showed a Mendelian distribution of the alleles at birth. *BDNF*$^{NT3/+}$ mice were mated with deleter-cre mice (Schwenk et al., 1995) to generate animals without the neomycin cassette.

PCR

DNA was extracted from tails and used for genotyping with the following primers:

BL1: 5′-ATGAAAGAAGTAAACGTCCAC-3′,
BL2: 5′-CCAGCAGAAAGAGTAGAGGAG-3′
PGK: 5′-GGGAACTTCCTGACTAGGGG-3′
NT3-04: 5′-TGGAGGATTATGTGGGCAAC-3′
Neo-3′: 5′-GATCCCCTCAGAAGAACTCGT-3′
Neo-5′: 5′-CTGTGCTCGACGTTGTCACTG-3′

A mixture of BL1, BL2 and PGK was used to determine the *BDNF*$^{NT3/NT3}$ and wild-type alleles. NT3-04 and BL2 as well as Neo-3′ and Neo-5′ were used to distinguish between loxed, *BDNF*$^{NT3lox/NT3lox}$, and not loxed, *BDNF*$^{NT3/NT3}$, animals.

In situ hybridisation

For in situ hybridisation fresh frozen brains from postnatal day 14 (P14) mice were used. ^{35}S-labeled riboprobe in situ hybridisation was performed as previously described (Trupp et al., 1997). Riboprobes complementary to rat *BDNF* and mouse *NT3* coding sequence were used. The probes were generated from linearised plasmids using T3 and T7 RNA polymerase and ^{35}S-labelled α-UTP. Slides were exposed to β-max X-ray film (Amersham) for 4 days.

BDNF and NT3 ELISA

Brains from P14 mice were removed and the hippocampus and part of the cortex were dissected out, frozen fresh and kept at –70°C until further processing. The tissue was homogenised using syringes with decreasing diameter down to 0.7 mm. BDNF and NT3 ELISA were then performed according to the manufacturer's protocol (Promega).

Auditory brainstem response (ABR)

ABR was recorded in anaesthetised mice (0.05 mg ketalar and 0.003 mg rompun per gram body weight i.p.) as previously described (Duan et al., 2000). The stimuli consisted of full cycle sine waves at 6.3, 8, 12.5 and 20 kHz synthesised digitally by SigGen software [Tucker Davis Technologies (TDT), Florida, USA]. The potentials were amplified by 100,000, averaged for 1000 sweeps, and then processed with BioSig software (TDT, Florida, USA). Threshold was defined as the lowest intensity at which a visible ABR wave was seen in two averaged runs.

DiI tracing

Wild type (n=14), $BDNF^{NT3/NT3}$ (n=11), and $BDNF^{-/-}$ (n=7), were sacrificed by cervical dislocation at P5. The heads were removed and the brain lifted out. The intact fifth and seventh nerve were identified and cut to reveal the eighth cranial nerve. The eighth nerve was cut and DiI crystals (1, 1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate; Molecular Probes) were applied directly to the end of the eighth nerve. The tissue was incubated in 4% PFA at 37°C for 2 days. The cochleae were dissected into 3 pieces each being approximately 2 mm long and analysed using fluorescence microscopy. Confocal images were acquired and the number of OHCs in row one was estimated in each dissected part. The number of nerve fibres projecting to the first, second and third row of OHCs was then determined and results presented as percentage of the number of OHCs per dissected part (apex, middle or base).

Semithin sections

Semithin 1 μm plastic sections were prepared as previously described (Ernfors et al., 1995b).

Tissue preparation

Embryos were obtained from overnight mating and the morning of the appearance of the vaginal plug was considered as embryonic day 0 (E0). Tissues were immersion fixed in 4% PFA overnight, equilibrated in 10% PFA, followed by 20% sucrose, frozen and sectioned in a cryostat at a thickness of 14 μm.

Immunohistochemistry

Immunohistochemistry was performed on 14 μm sections or on whole mounts of the inner ear and on primary cell cultures using primary antibodies against the p75 receptor (rabbit 9651; 1:200) or monoclonal acetylated tubulin 6-11B1 (1:500; Sigma) together with FITC-conjugated phalloidin (1:80; Molecular Probes) or calretinin (1:250; Swant Antibodies) and NF200 N52 (1:400; Sigma) as previously described (Ernfors et al., 1995b). Immunohistochemistry on tongue with antiserum against protein gene product 9.5 (PGP 9.5; Chemicon) was performed as previously described (Nosrat et al., 1997). Photomicrographs were obtained on a confocal microscope (Zeiss LSM510 or BioRad Radiance 2100). Selected levels or single projections of stacked images were used.

Quantification

14 μm cryostat serial sections were stained with Cresyl Violet. Neuronal numbers were established by counting neurons with a clear nucleus and nucleoli in every third section in spiral and vestibular ganglia (in the DRG and geniculate ganglia all sections were counted, in the trigeminal ganglion every 12th section and in the nodose ganglion every sixth). The number of neurons counted was multiplied by section separation to give a total estimated number of profiles (n). This number was multiplied by section thickness (T), divided by T plus the average diameter of the nuclei (D) to give the neuronal number (N); $N=nxT/(T+D)$ (Abercrombie, 1946; Coggeshall, 1992).

Cell culture

Isolated vestibular ganglion cells were obtained from 3-day old wild-type Balb/C mice via enzymatic (trypsin and collagenase, 1 mg/ml each, for 5 minutes at 37°C) and mechanical dissociation. The cells were plated at a density of 500 cells/mm^2 on laminin (10 μg/ml) coated coverslips in serum-free MEM Earle’s (GIBCO BRL) supplemented with defined additives N2 (2%), glucose (4.5 g/l), glutamine (0.3 g/l), pyruvate (0.1 g/l) and penicillin (100 U/ml). All additives were purchased from Life Technologies, penicillin and enzymes from Sigma. Vestibular sensory epithelial cells from wild type, $BDNF^{-/-}$, or from $BDNF^{NT3/NT3}$ mice were seeded on wild-type vestibular ganglion cell cultures after dissociation with DNAse (0.5 mg/ml; Roche Diagnostics), protease type X and collagenase (both at 1 mg/ml; Sigma) in MEM Earl’s (GIBCO). Co-cultures were fixed after 6 to 9 days of development in vitro and stained using immunohistochemistry for neurons and hair cells.

Scanning electron microscopy (SEM)

Tongues were fixed in 4% PFA, rinsed in PBS and kept at 4°C. Upon use, tongues were dehydrated in a graded serie of ethanol that was exchanged during three subsequent washes in hexamethyldisilazane (HMDS). The HMDS was allowed to evaporate in a fume hood overnight. The tongues were then mounted on stubs, lightly sputter-coated with gold/palladium, and studied in a scanning electron microscope.

RESULTS

Gene replacement of the *BDNF* coding sequence with *NT3*

A target replacement vector was generated in which the protein coding part of the *NT3* gene was fused in-frame with *BDNF* in exon V of the *BDNF* gene followed by a *neomycin* expression cassette flanked with *loxP* sites (Fig. 1A). Exon V of both the *BDNF* and *NT3* genes contains all the protein coding sequence. ES cells were electroporated, selected, and analysed with Southern blot for homologous recombination. ES clones carrying the $BDNF^{NT3/+}$ alleles were injected into blastocysts. Offspring were characterised by Southern blotting (Fig. 1B). Offspring from matings of heterozygous mice showed a Mendelian distribution of the alleles at birth. $BDNF^{NT3/+}$ mice were crossed with deleter-cre mice (Schwenk et al., 1995) to recombine out the neomycin cassette, $BDNF^{NT3lox/+}$ mice (Fig. 1C-E).

mRNA and protein expression in the $BDNF^{NT3/NT3}$ mice

We next investigated the expression pattern of the *NT3/NT3* alleles to confirm that it fully corresponded to the endogenous expression of the *BDNF* gene. BDNF and NT3 are expressed principally in a non-overlapping pattern in the brain. BDNF mRNA is strongly expressed in cerebral cortex, dentate gyrus and CA1-CA3 of the hippocampus while NT3 is expressed in dentate gyrus, medial CA1 and CA2, and with little expression in the cerebral cortex (Ernfors et al., 1990; Phillips et al., 1990). Both BDNF and NT3 expression in P14 wild-type mice was similar to that previously reported (Fig. 2A,B). NT3 mRNA expression was changed from the NT3 pattern to the BDNF expression pattern, although the overall level was reduced in $BDNF^{NT3/+}$ mice (Fig. 2C). The $BDNF^{NT3/NT3}$ mice displayed an expression of NT3 mRNA that completely corresponded with both the pattern and level of the normal expression of BDNF mRNA (Fig. 2A,D). We then examined whether the mRNA for NT3 in these mice was appropriately translated into the protein. The protein levels were examined using ELISA (Fig. 2E,F). Cerebral cortex (not shown) and hippocampus dissected from P14 mice were analysed. BDNF protein was completely absent in $BDNF^{NT3/NT3}$ mice and reduced to half in $BDNF^{NT3/+}$ mice, compared to wild-type mice (Fig. 2E). Consistent with a less abundant and more restricted pattern of NT3 mRNA expression in the hippocampus, NT3 protein levels were much lower than BDNF protein in wild-type mice. $BDNF^{NT3/+}$ mice showed an increased production of NT3, which was doubled in $BDNF^{NT3/NT3}$ mice, reaching a level remarkably similar to the level of BDNF in wild-type mice (Fig. 2F).

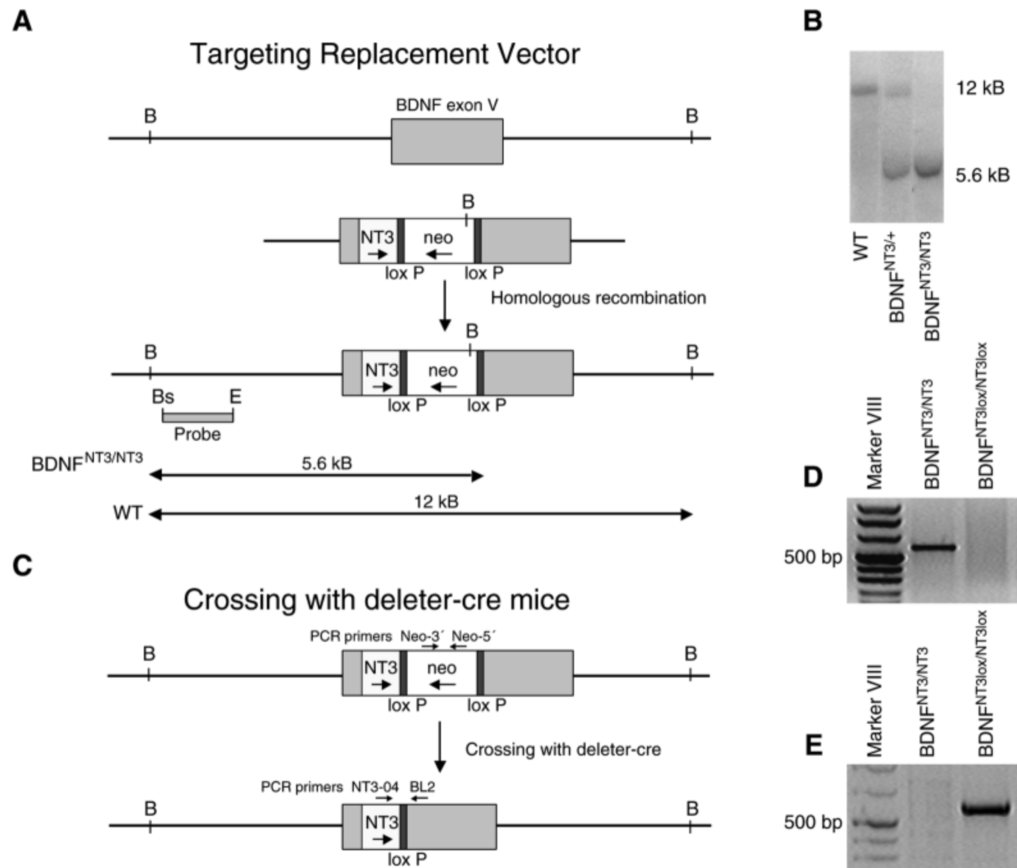

Fig. 1. Gene replacement of the *BDNF* coding sequence with *NT3*. (A) Schematic diagram of the approach to change the *BDNF* loci to *NT3*. The *BDNF* exon V (shaded box) was opened with *Bgl*II (B) and the *NT3* coding region was fused in-frame followed by a *neomycin* cassette. (B) ES cells were analysed for homologous recombination using an external probe for Southern blot analysis (Bs; *Bst*XI and E; *Eco*RV). The wild-type (WT) allele revealed a 12 kb band while the mutant band was 5.6 kb. (C) Animals were mated with deleter-cre mice to lox out the *neomycin* cassette. (D-E) Two PCRs were performed to distinguish between animals containing or not containing the *neomycin* cassette. The PCR primers used for this analysis are depicted in (C). (D) The first PCR primers (Neo-3′, Neo-5′) are designed to amplify a fragment within the *neomycin* cassette of about 600 bp. The neomycin fragment is amplified in $BDNF^{NT3/NT3}$ mice while no fragment is amplified in mice crossed with deleter-cre mice ($BDNF^{NT3lox/NT3lox}$ mice). (E) In the $BDNF^{NT3lox/NT3lox}$ mice a fragment of about 600 bp is instead amplified using the NT3-04 and BL2 primers, this band is not amplified in animals still carrying the *neomycin* cassette.

The $BDNF^{NT3/NT3}$ mice showed similar phenotypes to the $BDNF^{-/-}$ mice, with a few exceptions. It was common for the $BDNF^{NT3/NT3}$ mice to live for 3-4 weeks, compared to the $BDNF^{-/-}$ mice that almost invariably died during the third postnatal week. $BDNF^{NT3/NT3}$ mice also appeared to have increased body weight compared to $BDNF^{-/-}$ mice. The $BDNF^{NT3/NT3}$ mice, as the $BDNF^{-/-}$ mice (Ernfors et al., 1994a; Jones et al., 1994), suffered from defective coordination of balance and movement. Measurements of neuronal numbers in the trigeminal, nodose, dorsal root, spiral, vestibular and geniculate ganglia of $BDNF^{-/-}$, $BDNF^{NT3/NT3}$ and wild-type mice revealed differences in neuronal survival (Table 1, counts of neuronal numbers in trigeminal, nodose, and dorsal root ganglia at P0; data on spiral, vestibular and geniculate ganglia are described below). A partial rescue of neuronal survival is seen in nodose and dorsal root ganglia in the $BDNF^{NT3/NT3}$ mice compared to $BDNF^{-/-}$ mice.

Rescue of neuronal numbers, target innervation and function in the cochlea

BDNF is expressed in the hair cells, while NT3 is expressed in supporting cells and hair cells during embryogenesis (Fig. 3A) (Ernfors et al., 1992; Farinas et al., 2001; Pirvola et al., 1992; Ylikoski et al., 1993). TrkB and TrkC are expressed during embryogenesis and this continues into adulthood in the spiral ganglia where it is evenly distributed over the entire ganglia (Ernfors et al., 1992; Ylikoski et al., 1993). $BDNF^{-/-}$ mice showed almost complete loss of innervation of the three rows of OHCs (Ernfors et al., 1995b) and a reduction in neuronal numbers (Fig. 3E) similar to previous results (Ernfors et al., 1995b; Farinas et al., 2001), as well as a subsequent loss

Table 1. Neuronal cell counts in sensory ganglia in wild-type, $BDNF^{-/-}$ and $BDNF^{NT3/NT3}$ mice at P0

Ganglion	Mean number of neurons ± s.e.m.	Percentage of control ± s.e.m.
Trigeminal		
Wild type	30 183±1524 (*n*=6)	100±5
$BDNF^{-/-}$	27 610±985 (*n*=5)	91±3
$BDNF^{NT3/NT3}$	33 013±1097 (*n*=5)	109±4†
Nodose		
Wild type	11 604±439 (*n*=7)	100±4
$BDNF^{-/-}$	7 076±252 (*n*=4)	61±2***
$BDNF^{NT3/NT3}$	9 315±548 (*n*=4)	80±5†, **
Dorsal root (L4)		
Wild type	9 045±592 (*n*=4)	100±7
$BDNF^{-/-}$	5 984±542 (*n*=4)	66±6*
$BDNF^{NT3/NT3}$	7 835±585 (*n*=5)	87±6†

Cryostat sections from newborn mice of the indicated genotypes were prepared and stained with Cresyl Violet. Trigeminal ganglion neurons with a clear nucleus and nucleoli were counted in every 12th section, nodose every sixth and DRG every second section. ANOVA Fischer's $^{\dagger}P<0.05$ between $BDNF^{NT3/NT3}$ and $BDNF^{-/-}$ mice; ANOVA Fischer's $*P<0.05$, $**P<0.005$ and $***P<0.001$ between mutant mice and wild-type mice.

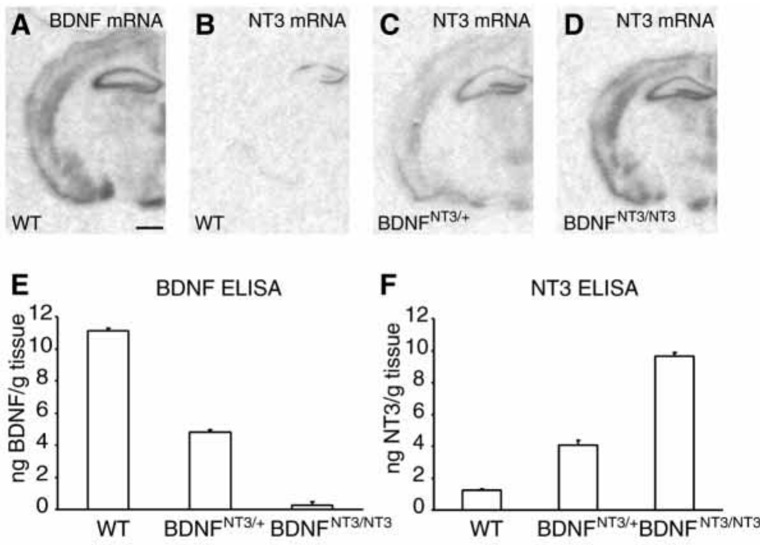

Fig. 2. mRNA and protein expression in the *BDNF*$^{NT3/NT3}$ mice at P14. (A-D) BDNF and NT3 in situ hybridisation of brain sections. Scale bars:100 μm in A-D. Note that NT3 mRNA expression changes in *BDNF*$^{NT3/+}$ (C) and *BDNF*$^{NT3/NT3}$ (D) mice towards the same expression pattern as BDNF in wild-type animals (A). (E-F) Analyses of protein levels in hippocampus using BDNF and NT3 ELISA. (E) BDNF protein levels decrease in *BDNF*$^{NT3/+}$ mice and are completely absent in *BDNF*$^{NT3/NT3}$ animals. (F) NT3 protein levels increase in *BDNF*$^{NT3/+}$ and *BDNF*$^{NT3/NT3}$ mice to reach a similar level as BDNF protein in wild-type animals.

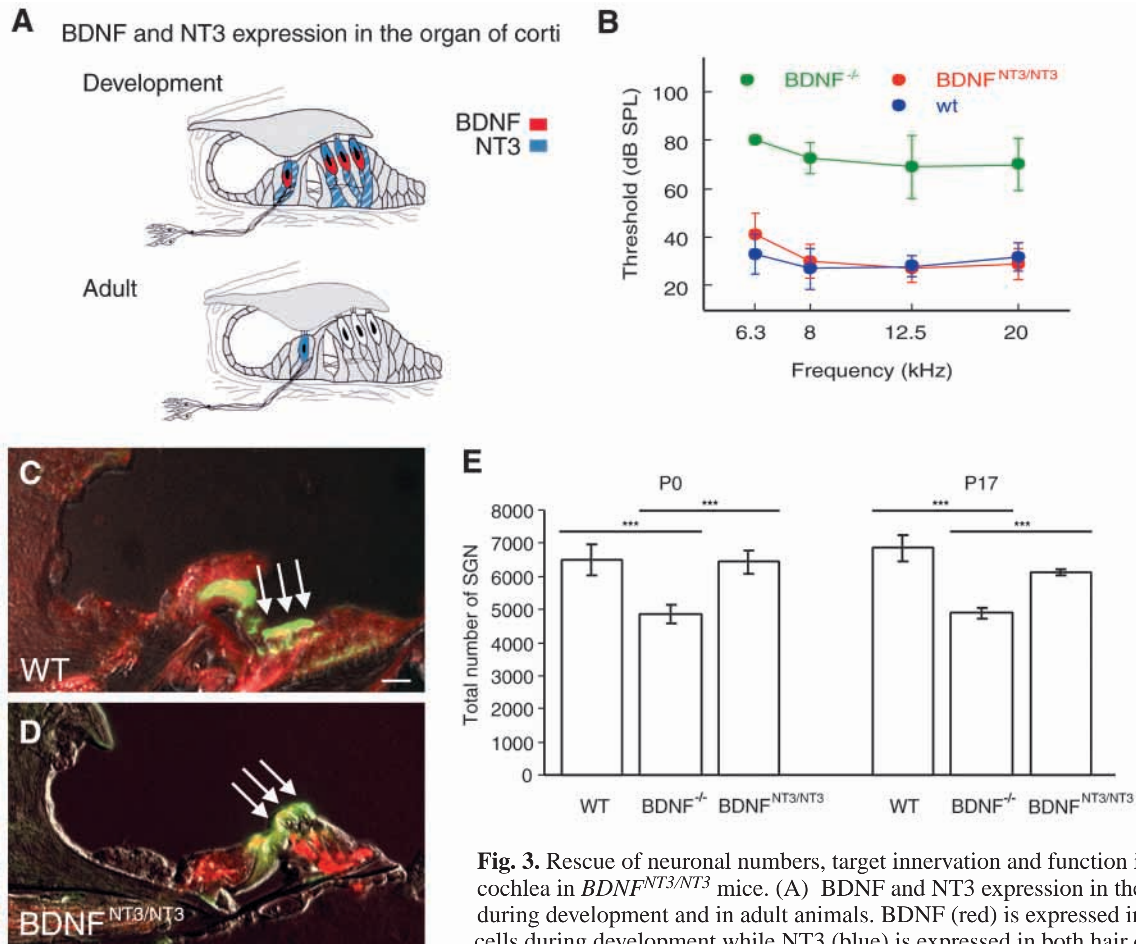

Fig. 3. Rescue of neuronal numbers, target innervation and function in the cochlea in *BDNF*$^{NT3/NT3}$ mice. (A) BDNF and NT3 expression in the cochlea during development and in adult animals. BDNF (red) is expressed in the hair cells during development while NT3 (blue) is expressed in both hair cells and supporting cells. In adult animals NT3 is only expressed in the IHC. (B) Auditory brainstem response in *BDNF*$^{-/-}$ (green), *BDNF*$^{NT3/NT3}$ (red) and wild-type (blue) mice. Note that the *BDNF*$^{-/-}$ mice suffer from a hearing loss that is completely rescued in the *BDNF*$^{NT3/NT3}$ mice. (C,D) Afferent innervation in sections from the apical to middle part of the cochlea stained with an antibody directed against p75^{NTR} (red) counterstained with phalloidin (green) to visualise the hair cells. Scale bars: 20 μm. The arrows indicate the OHC. Note innervation of the three rows of OHCs in the *BDNF*$^{NT3/NT3}$ mice. (E) Spiral ganglion neurons (SGN) were counted at P0 and P17. At P0 25% (n=3) and at P17 29% (n=4) of the neurons were lost in *BDNF*$^{-/-}$ mice compared to wild-type mice. This loss was rescued at P0 in *BDNF*$^{NT3/NT3}$ mice (n=3) and at P17 there was still no significant difference between the wild-type (n=4) and *BDNF*$^{NT3/NT3}$ mice (n=3). ANOVA Fischer's ***P<0.001; ±s.e.m.

of hearing (Fig. 3B, green line). In contrast to *BDNF*$^{–/–}$ mice, the *BDNF*$^{NT3/NT3}$ mice responded to snapping of the fingers by showing a Preyer's reflex. These findings directed us to further examine the auditory function of *BDNF*$^{NT3/NT3}$ mice using ABR. The study was conducted on 17-days old wild-type (n=5), *BDNF*$^{NT3/NT3}$ (n=5) and *BDNF*$^{–/–}$ mice (n=5 except at 8 kHz n=4). The response of the *BDNF*$^{NT3/NT3}$ mice was equal to that of the wild-type mice throughout all measured frequencies (Fig. 3B). The innervation of the OHCs (arrows) was rescued at P17 in *BDNF*$^{NT3/NT3}$ mice as seen by immunohistochemistry with an antibody directed against the p75 receptor specifically visualising the afferent nerve fibres (Fig. 3C,D). Thus, these results show that NT3 can support innervation of OHCs and normal auditory function in the absence of BDNF.

Previous results from another group (Bianchi et al., 1996; Farinas et al., 2001) suggested a general loss of both OHC and IHC innervation in *BDNF*$^{–/–}$ mice as seen by a pronounced reduction in radial fibres specifically in the apical part of the cochlea in *BDNF*$^{–/–}$ mice, while we have reported that there is a specific loss of type II neurons and OHC innervation in these mice (Ernfors et al., 1995a; Ernfors et al., 1995b). We therefore conducted a DiI tracing study of both *BDNF*$^{–/–}$ and *BDNF*$^{NT3/NT3}$ mice, as well as performing immunohistochemical analyses of radial fibres using an antibody against acetylated tubulin throughout the entire longitudinal axis of the cochlea. A large number of animals were traced with DiI to discount technical and biological variation. Radial fibres (rf) were not diminished in either *BDNF*$^{–/–}$ or *BDNF*$^{NT3/NT3}$ mice compared with wild-type mice as detected with DiI tracing (Fig. 4A-C) and with immunohistochemistry for acetylated tubulin (Fig. 4M,N,O). We next analysed OHC innervation in *BDNF*$^{–/–}$ and *BDNF*$^{NT3/NT3}$ mice. In wild-type mice, DiI labelled nerve fibres projecting through the spiral lamina, branched and reached all three rows of OHCs. The projection was robust and we were

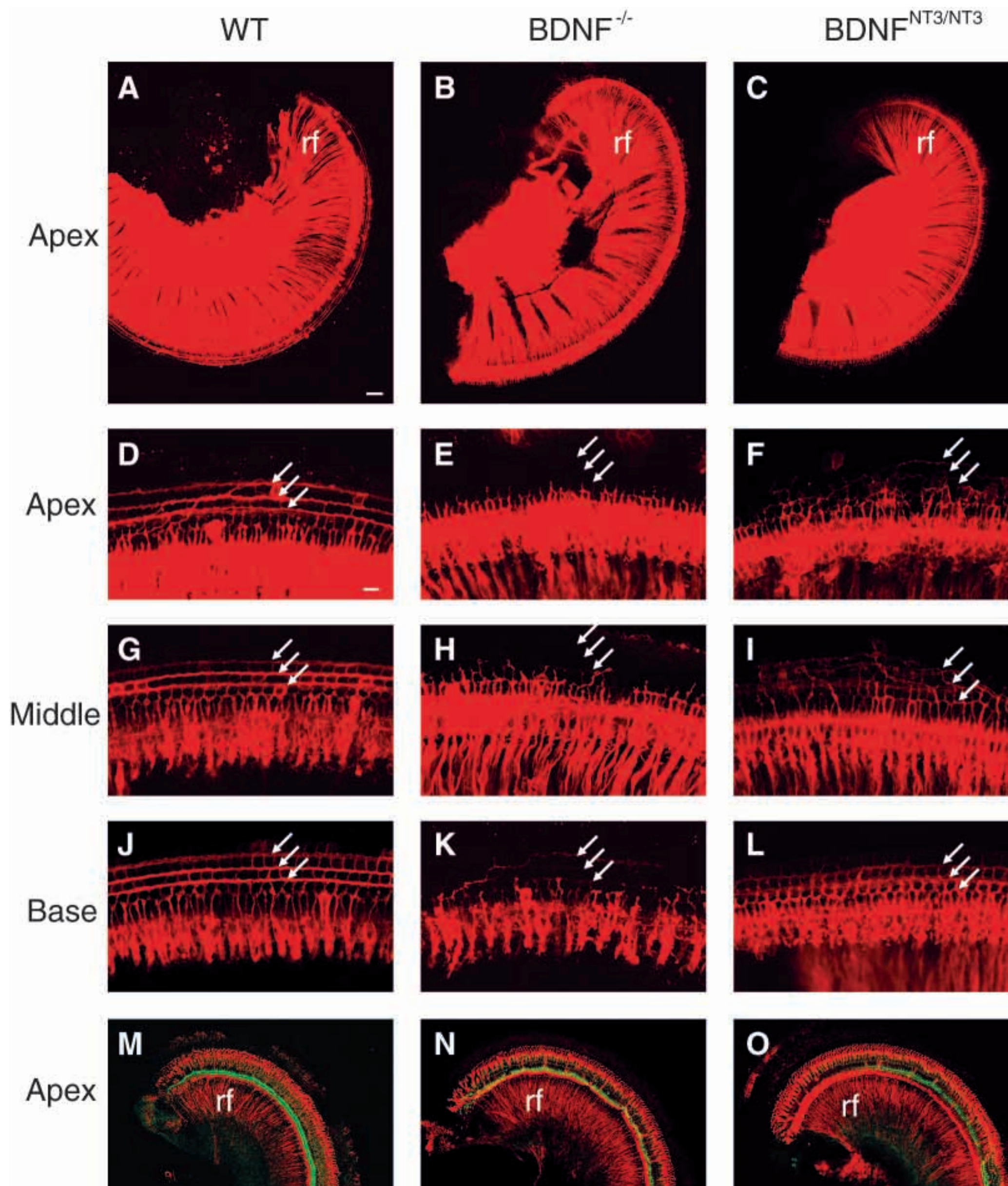

Fig. 4. DiI tracing of afferent innervation in wild type (A,D,G,J) *BDNF*$^{–/–}$ (B,E,H,K) and *BDNF*$^{NT3/NT3}$ (C,F,I,L), and whole mount immunohistochemistry to detect radial fibres in wild type (M) *BDNF*$^{–/–}$ (N) and *BDNF*$^{NT3/NT3}$ (O). Micrographs illustrate the apical (A-F,M-O), middle (G-I), and base (J-L) of the cochlea. Scale bars: 40 μm in A-C and M-O and 10 μm in D-L. There is no reduction in the density of radial fibres (rf) in either *BDNF*$^{–/–}$ (B) or *BDNF*$^{NT3/NT3}$ (C) compared to wild-type (A) mice as seen by DiI tracing. This is also shown by the distribution of nerve fibres stained for acetylated tubulin (red) and counterstained with FITC-conjugated phalloidin (green) to visualise the hair cells (M-O). Note the innervation of the three rows of OHCs in wild-type mice (D,G,J), loss of innervation of all three rows of OHCs in *BDNF*$^{–/–}$ mice (E,H,K) and rescue of innervation of the OHCs in the *BDNF*$^{NT3/NT3}$ mice (F,I,L). Arrows indicate the three rows of OHC.

Table 2. Semi-quantitative analysis of percentage DiI-labelled nerve fibres reaching hair cells in each of the three rows of OHCs

	Apex			Middle			Base		
Row of OHC	1	2	3	1	2	3	1	2	3
BDNF$^{-/-}$	0.1	0	0	2.9	1.2	0	18	10	0.1
BDNF$^{NT3/NT3}$	2.6	0.2	0	46	35	32	88	84	56

The 8th cranial nerve was traced using DiI in the indicated genotypes and the cochleae was dissected into three portions, apex, middle and base. Confocal images were acquired and the OHCs were counted in each dissected part the cochlea. The number of nerve fibres projecting to the 1st, 2nd and 3rd row of OHCs were then determined and are presented as percentage of the number of OHCs in row one (wild type n=14; *BDNF*$^{NT3/NT3}$ n=11; *BDNF*$^{-/-}$ n=7). In wild-type mice, traced fibres reaching the three rows of OHCs was seen throughout the longitudinal axis (i.e. 100%).

unable to identify single nerve fibres, this innervation was considered to be 100%. *BDNF*$^{-/-}$ mice showed loss of OHC innervation in the apex, middle, and basal turns of the cochlea (Fig. 4E,H,K arrows). Semi-quantitative measurements of the number of nerve fibres projecting to the three rows of OHCs confirmed this result (Table 2). *BDNF*$^{NT3/NT3}$ mice showed a rescue of innervation throughout all turns of the cochlea and most OHCs received innervation, although the fibres appeared not to branch and project spirally to the same degree as in wild-type mice (Fig. 4F,I,L arrows). The OHC innervation of both *BDNF*$^{NT3/NT3}$ and *BDNF*$^{-/-}$ mice (the few remaining fibres), showed a gradient in the loss. Fewer nerve fibres reached the third row of OHCs (virtually no fibres in *BDNF*$^{-/-}$ mice) than the second, and fewer reached the second than the first row. Furthermore, fewer nerve fibres were seen in the apex than in the base (Table 2).

Further investigation of the cochlea revealed a loss of spiral ganglion neurons by 25% (n=3) at P0 and 29% (n=4) at P17 in *BDNF*$^{-/-}$ mice (Fig. 3E). This loss was rescued at P0 in *BDNF*$^{NT3/NT3}$ mice (100%; n=3) and at P17 there was still no significant difference between wild-type (n=4) and *BDNF*$^{NT3/NT3}$ mice (n=3) (Fig. 3E). Similar results were seen at P0 in *BDNF*$^{NT3lox/NT3lox}$ mice (data not shown). Analyses of semi-thin sections (1 μm sections) revealed type II neurons in *BDNF*$^{NT3/NT3}$ and wild-type mice (data not shown). Thus, these results show that NT3, if expressed in the temporal and spatial pattern of BDNF in the cochlea, preserves neuronal numbers and target innervation with subsequent normal development of function.

Neuronal survival but not innervation and function is rescued in the vestibular system

Both NT3 and BDNF mRNA are strongly expressed in the sensory epithelia of the saccular and utricular maculae during development (Ernfors et al., 1992; Pirvola et al., 1992). The levels of NT3 decrease with maturation and at P9 the expression is almost gone (Fig. 5A). BDNF mRNA, but not NT3 mRNA is also seen in the ampullar cristae (Pirvola et al., 1992). As in the spiral ganglion, both TrkB and TrkC are expressed in the embryonic, postnatal as well as adult vestibular ganglion with an evenly distributed pattern throughout most or all neurons (Ylikoski et al., 1993). If the mechanisms establishing specificity of the neurotrophins and their receptors were the same in the vestibular system as in the

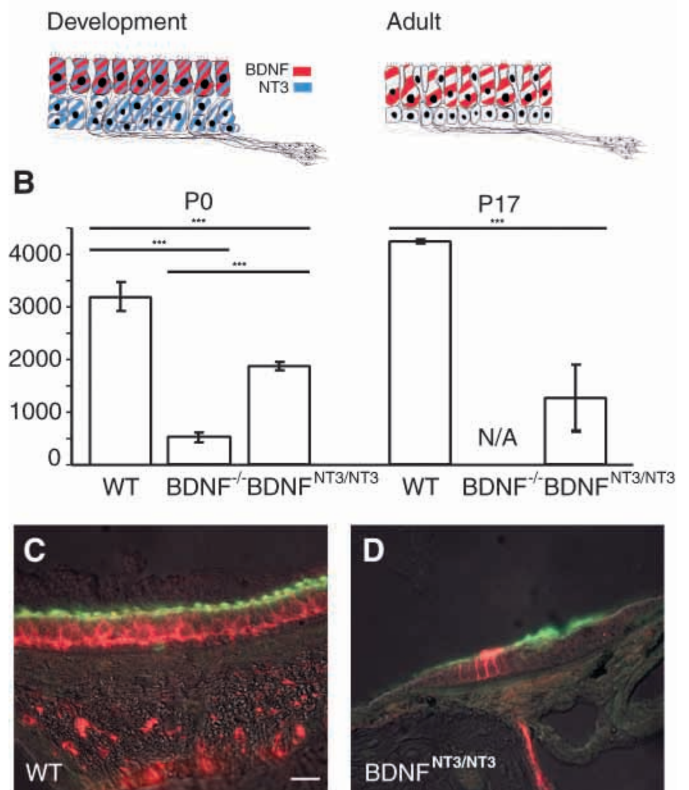

Fig. 5. Neuronal survival but not innervation is rescued in the vestibular system. (A) BDNF and NT3 expression in the vestibule during development and in adult animals. Both BDNF (red) and NT3 (blue) are expressed in the sensory epithelia during development, while only BDNF is expressed in the adult. (B) In P0 *BDNF*$^{NT3/NT3}$ mice, 60% of the neurons survived compared to only 16% survival in *BDNF*$^{-/-}$ mice. At P17 half of the neurons seen at P0 were lost in *BDNF*$^{NT3/NT3}$ mice. (C-D) Sections of utricular maculae immunohistochemically stained with an antibody directed against p75^{NTR} (red) that specifically stains the afferents and with phalloidin (green) to visualise the hair cells. Scale bars: 25 μm in C and D. Only a sparse innervation that does not form calyces is seen in the *BDNF*$^{NT3/NT3}$ mice (D) compared to wild-type animals (C). ANOVA Fischer's ***P<0.001; ±s.e.m.

cochlea, the balance defects of *BDNF*$^{-/-}$ mice should be rescued in *BDNF*$^{NT3/NT3}$ mice. However, *BDNF*$^{NT3/NT3}$ mice displayed a similar defect in balance as *BDNF*$^{-/-}$ mice.

BDNF$^{-/-}$ mice are severely affected in the vestibular system with up to 80% loss of the vestibular ganglion neurons, and a subsequent loss of innervation of both the utricular and saccular maculae, occurring largely between E13 and E16 (Bianchi et al., 1996; Ernfors et al., 1995b). *BDNF*$^{NT3/NT3}$ mice show about 60% neuronal rescue at P0 compared to wild-type mice (Fig. 5B, also confirmed in *BDNF*$^{NT3lox/NT3lox}$ mice). There was a slow gradual loss of the vestibular ganglion neurons in the *BDNF*$^{NT3/NT3}$ mice, since at P17 nearly half of the neurons seen at P0 were lost (Fig. 5B). We next examined the innervation of the vestibular sensory epithelia. Unexpectedly, in *BDNF*$^{NT3/NT3}$ mice innervation at P17 was strongly reduced (Fig. 5C,D). Only occasional sections were

found that retained a sparse innervation (Fig. 5D). The afferent fibres that reached the sensory epithelia of the utricular and saccular maculae showed no indication of differentiating into calyces (nerve endings forming a cup-shaped structure around the base of the hair cell) around the type I hair cells at P17 (Fig. 5D), but appeared to project through the epithelia.

BDNF/TrkB but not NT3/TrkC elicit terminal innervation and maturation of functional sensory nerve endings

The failure to innervate the saccular and utricular maculae despite the survival of many vestibular ganglion neurons suggested that BDNF and NT3 play different roles in the vestibular ganglion neurons. We therefore first determined whether the reduced innervation is caused by a failure to maintain the nerves or whether the sensory nerve fibres never innervate the saccular and utricular maculae. Already at E16, at which time the fibres should have reached the epithelium but not yet formed calyces, $BDNF^{NT3/NT3}$ mice showed less innervation (Fig. 6C) compared with wild-type mice (Fig. 6A). Similar results were obtained in neonatal mice (Fig. 6D-F). At all these stages, nerve fibres were seen in the subepithelial layer with sparse innervation of the sensory epithelia, suggesting that BDNF is only required for terminal innervation and formation of functional nerve endings, and not for nerve fibres to project to the utricular and saccular compartments. The ampullar cristae completely lacked innervation in both $BDNF^{-/-}$ and $BDNF^{NT3/NT3}$ mice as shown by p75 immunohistochemistry (Fig. 6G-I).

Analysis of whole-mount utricular maculae using confocal microscopy confirmed a sparse innervation of the sensory epithelia in the $BDNF^{NT3/NT3}$ mice, a failure of these fibres to form calyces and it also revealed that the nerve fibres in the subepithelial layer were highly disorganised (Fig. 7B). In order to confirm a direct role for BDNF in sensory hair cell innervation and nerve ending formation we established an in vitro system. In this assay, hair cells dissected from wild-type mice were seeded over laminin-coated slides containing vestibular ganglion neurons. This assay allows a qualitative measurement of hair cell innervation and calyx formation. In all co-cultures (*n*=50) of wild-type vestibular neurons and wild-type hair cells, the vestibular ganglion neurons successfully innervated the hair cells. The nerve endings branched extensively and wrapped around the hair cells forming digitations reminiscent of the process of calyx formation in vivo (Fig. 7C,D). Conversely only two out of 200 cases showed neuronal contacts between wild-type neurons co-cultured with $BDNF^{-/-}$ hair cells. The diameter of the nerve fibre in these cases is thinner and there is little branching on the surface of the hair cell (Fig. 7E,F). In contrast to the $BDNF^{-/-}$ hair cells, vestibular neurons reached nearly all $BDNF^{NT3/NT3}$ hair cells (11 out of 12). However, similar to $BDNF^{-/-}$ hair cells, the nerve fibres were thin, showed little branching, and did not wrap around the hair cells (Fig. 7G,H).

Combined, the in vivo and in vitro results provide evidence that both BDNF/TrkB and NT3/TrkC can sustain vestibular neuronal survival but BDNF/TrkB are required for proper terminal innervation and synaptogenesis in the vestibular system. We conclude that the differences in the vestibular compartment between BDNF/TrkB and NT3/TrkC knockout mice is caused by different intracellular activities of TrkB and TrkC, and TrkB activation is exclusively necessary for short range (terminal) innervation and formation of synaptic contacts.

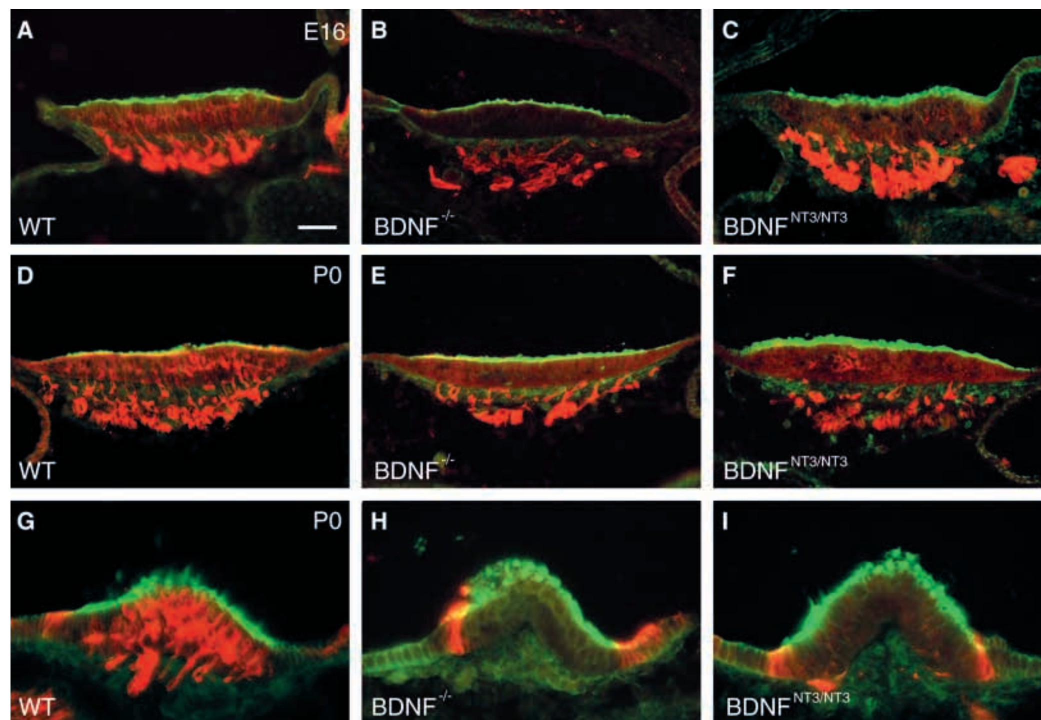

Fig. 6. Failure of terminal nerve in-growth in $BDNF^{-/-}$ and $BDNF^{NT3/NT3}$ mice. (A-I) Immunohistochemistry for p75 to detect afferent innervation (red) and FITC-conjugated phalloidin (green), which visualises the hair cells. (A-C) Already at E16 when the fibres have reached the epithelium of the utricular and saccular maculae in wild-type mice (A), $BDNF^{NT3/NT3}$ mice showed a comparable failure of innervation (C) to $BDNF^{-/-}$ mice (B). (D-F) Similar results were obtained at P0. Note that only a few fibres have reached the hair cells in the $BDNF^{NT3/NT3}$ mice (F) and none has reached the hair cells in $BDNF^{-/-}$ mice (E). Nerve fibres are seen at all stages in the subepithelial layer, suggesting that BDNF is only required for terminal innervation and formation of functional nerve endings but that it is not required for nerve fibres to project to the utricular and saccular compartments. (G-I) The ampullar cristae completely lack innervation in both $BDNF^{-/-}$ (H) and $BDNF^{NT3/NT3}$ mice (I). Scale bars: 50 μm in A-I.

The *NT3/NT3* alleles supports neither survival of taste neurons, nor proper innervation of the peripheral gustatory system

In the rodent tongue, BDNF and NT3 show distinct non-overlapping expression patterns. BDNF mRNA is expressed in the epithelium of the developing gustatory papillae prior to the formation of taste buds and continues to be expressed throughout adulthood (Nosrat et al., 1996; Nosrat and Olson, 1995). NT3 mRNA is located in those areas were BDNF is not expressed, such as the superior surface and the lateral epithelium of the fungiform papillae (Nosrat et al., 1996) (Fig. 8A).

$BDNF^{-/-}$ mice exhibit a loss of gustatory innervation and a subsequent loss of taste buds and gustatory papillae (Nosrat et al., 1997; Zhang et al., 1997). Interestingly, similar deficits were seen in $BDNF^{NT3/NT3}$ mice with a distinct disruption of gustatory innervation while lingual somatosensory innervation appeared intact (Fig. 8C,D). Intriguingly, in $BDNF^{NT3/NT3}$ mice the NT3-dependent somatosensory innervation did not invade the taste buds, which in these mice express NT3. We examined the morphology of the tongue surface using SEM. A marked loss of fungiform papillae was seen in 2-weeks old $BDNF^{NT3/NT3}$ mice with only about a third of the papillae remaining (Fig. 8F), similar to the $BDNF^{-/-}$ mice (Fig. 8G). Furthermore, the remaining fungiform papillae were smaller in $BDNF^{NT3/NT3}$ mice compared to wild-type mice, again like that seen in $BDNF^{-/-}$ mice (8E-G). The number of taste buds in the circumvallate papillae were similar in $BDNF^{-/-}$ and $BDNF^{NT3/NT3}$ mice (data not shown).

In order to assess whether these $BDNF^{-/-}$-like deficits in $BDNF^{NT3/NT3}$ mice were caused by a loss of gustatory neurons, neurons in the geniculate ganglion were counted, which in part consists of neurons projecting to fungiform taste buds. The geniculate ganglion in $BDNF^{NT3/NT3}$ mice showed the same extent of neuronal loss as in $BDNF^{-/-}$ mice, compared to wild-type mice (Fig. 8H). We conclude that NT3 can neither preserve the BDNF-dependent gustatory innervation of the taste buds, nor rescue the neurons from death in the peripheral gustatory system, indicating that NT3 does not play a physiological role as a ligand for TrkB in vivo in this system.

DISCUSSION

Previous results showed that BDNF/TrkB and NT3/TrkC null mutant mice display different phenotypes in the cochlea, vestibular and lingual systems despite a ubiquitous expression of Trk receptors in spiral, vestibular, and at some stages in the geniculate ganglia. We have taken a genetic strategy by generating a mouse in which the coding sequence of the *BDNF* gene has been replaced by *NT3* to determine the functions of BDNF and NT3, and the specificity of BDNF in the peripheral nervous system. We obtained different results in the three peripheral cranial sensory systems. In the cochlea, NT3 can largely replace the actions of BDNF while in the vestibular system NT3 can promote neuronal survival with only sparse innervation rescued. In the tongue, NT3 is not able to replace the role of BDNF. We conclude that there is no generic mechanisms that cause specificity of neurotrophins, but rather that the specificity arise from either (i) spatial and temporal expression of the ligands, (ii) distinct intracellular pathways activated by TrkB and TrkC, or (iii) selective expression of the receptors in distinct subpopulations of neurons.

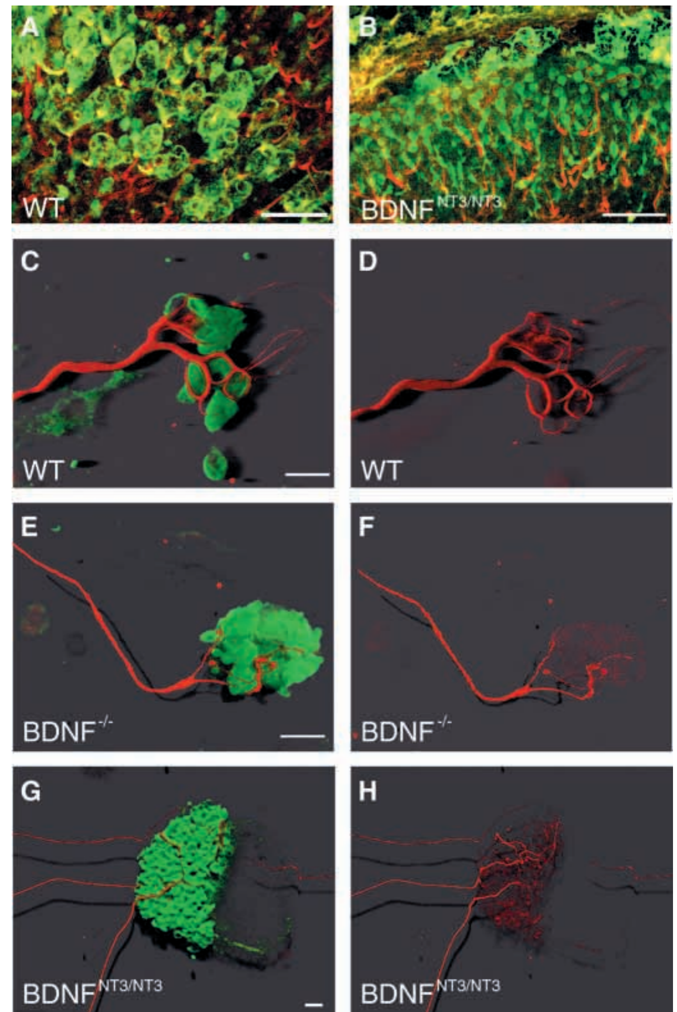

Fig. 7. BDNF/TrkB signalling is required for terminal innervation and formation of functional sensory nerve endings.
(A-H) Immunohistochemical double staining against NF200 to detect nerve fibres (red) and calretinin to detect calyces (green).
(A,B) Whole-mount preparations from the utricular maculae in wild-type (A) and $BDNF^{NT3/NT3}$ mice (B). Scale bars: 30 µm in A and B. The analyses using confocal microscopy confirmed a sparse innervation of the epithelia and also revealed that the nerve fibres in the subepithelial layer were highly disorganised in $BDNF^{NT3/NT3}$ mice (B). (C-H) Co-cultures of wild-type vestibular ganglion neurons with wild-type hair cells (C-D) $BDNF^{-/-}$ hair cells (E-F), or $BDNF^{NT3/NT3}$ hair cells (G-H). Scale bars: 20 µm in C-H. With wild-type hair cells the nerve endings branch extensively and wrap around the hair cells forming digitations similar of the process of calyx formation in vivo (C, nerve including calretinin-stained hair cells and D, nerve only). In only two out of 200 cases did wild-type neurons co-cultured with $BDNF^{-/-}$ hair cells contact a hair cell (E-F). Note that the diameter of the nerve fibre is much thinner and it shows less branching on the surface of the $BDNF^{-/-}$ hair cell. (G,H) In contrast to $BDNF^{-/-}$ hair cells, nearly all $BDNF^{NT3/NT3}$ hair cell fragments were contacted by vestibular neurons (11 out of 12). However the contacting neurites remained undifferentiated on the surface of the epithelia and did not develop digitations.

A selective expression of receptors in separate

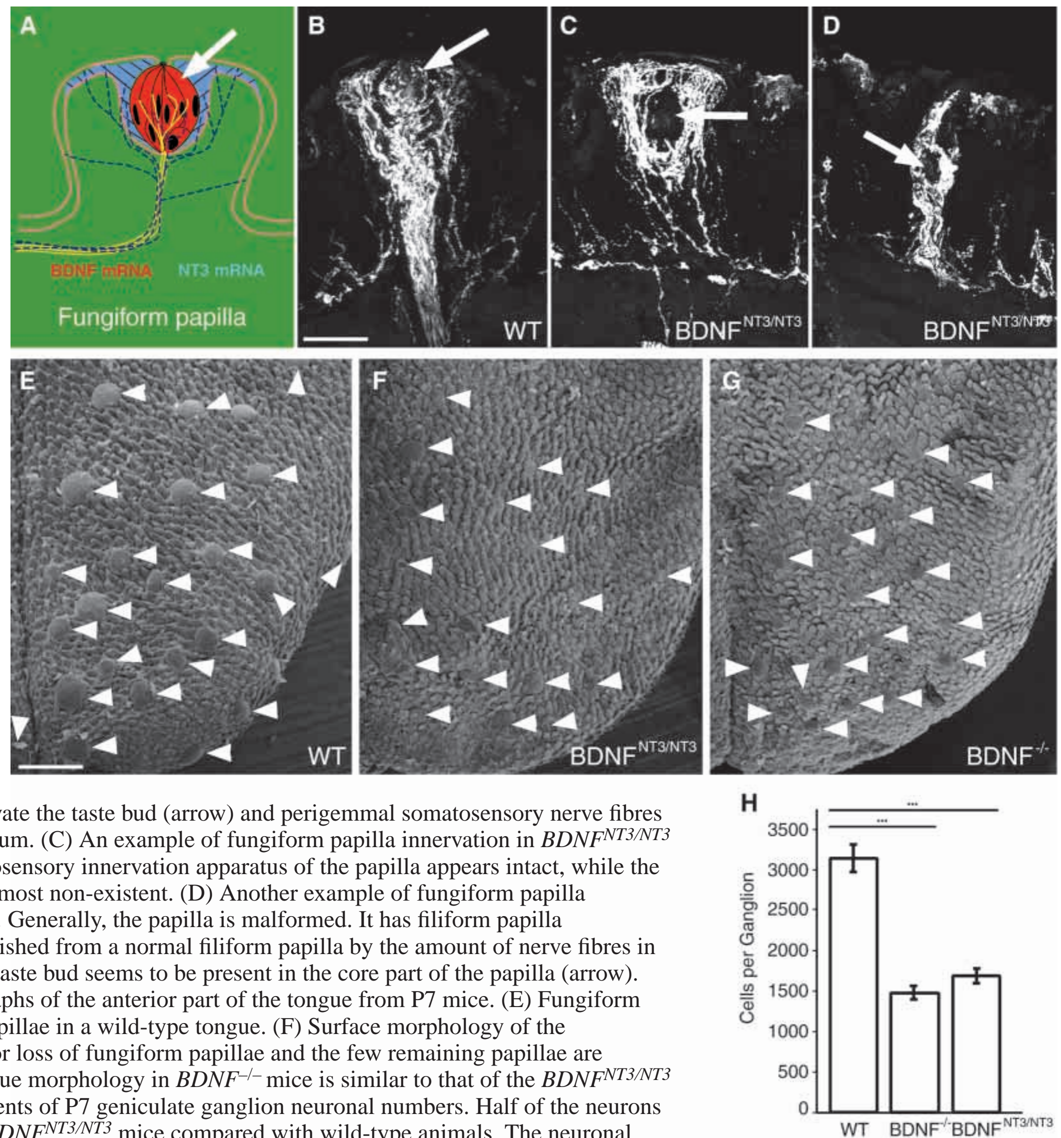

Fig. 8. NT3 cannot support neuronal survival or innervation in the tongue. (A) Schematic drawing of BDNF (red) and NT3 (light blue) mRNA expression in fungiform papilla in rodents. The arrow indicates the taste bud with innervation. The yellow lines represent the BDNF-dependent intragemmal fibres (gustatory fibres) and the dark blue dashed lines represent perigemmal fibres (somatosensory innervation). Gustatory nerve fibres innervate only the taste buds where BDNF is present while somatosensory nerve fibres predominantly innervate the surrounding epithelium where NT3 is present. (B) Typical innervation of a fungiform papilla and its taste bud (arrow) in a wild-type P7 mouse tongue, visualised by immunohistochemistry against protein gene product 9.5 (PGP). The papilla is well-innervated, gustatory fibres innervate the taste bud (arrow) and perigemmal somatosensory nerve fibres innervate the surrounding epithelium. (C) An example of fungiform papilla innervation in $BDNF^{NT3/NT3}$ mice. The NT3-dependent somatosensory innervation apparatus of the papilla appears intact, while the taste bud innervation (arrow) is almost non-existent. (D) Another example of fungiform papilla innervation in $BDNF^{NT3/NT3}$ mice. Generally, the papilla is malformed. It has filiform papilla morphology but could be distinguished from a normal filiform papilla by the amount of nerve fibres in this papilla. An under-developed taste bud seems to be present in the core part of the papilla (arrow). (E-G) Scanning electron micrographs of the anterior part of the tongue from P7 mice. (E) Fungiform papillae rise above the filiform papillae in a wild-type tongue. (F) Surface morphology of the $BDNF^{NT3/NT3}$ tongue reveals major loss of fungiform papillae and the few remaining papillae are reduced in size. (G) Anterior tongue morphology in $BDNF^{-/-}$ mice is similar to that of the $BDNF^{NT3/NT3}$ mice. (H) Quantitative measurements of P7 geniculate ganglion neuronal numbers. Half of the neurons are missing in the $BDNF^{-/-}$ and $BDNF^{NT3/NT3}$ mice compared with wild-type animals. The neuronal numbers show no significant difference between $BDNF^{-/-}$ and $BDNF^{NT3/NT3}$ mice. ANOVA Fischer's ***P<0.001; ±s.e.m. Scale bars: 100 μm in B-D and 200 μm in E-G.

subpopulations of dorsal root ganglia has been shown to occur as a mechanism of creating specificity of neurotrophins. $BDNF^{-/-}$, $NT3^{-/-}$ and $TrkB^{-/-}$ mice (Fritzsch et al., 1997b; Nosrat, 1998; Nosrat et al., 1997), as well as transgenic mice overexpressing truncated TrkC receptors (Palko et al., 1999) show distinct differences in the role of BDNF and NT3 in the complex innervation of the tongue. In mice in which NT3 or its receptor has been manipulated, gustatory neurons and innervation are not affected, while manipulating the function of BDNF or TrkB leads to defects in gustatory innervation and loss of geniculate ganglion neurons. By placing *NT3* in the *BDNF* locus we have challenged this sensory system by expressing NT3 at the level, time and location where BDNF is normally present. Expression of NT3 in the place of BDNF did not rescue any of the observed phenotypes of $BDNF^{-/-}$ mice in the gustatory system. Thus, specificity of BDNF is not alone elicited by the distinct expression patterns of the ligands. In situ hybridisation studies have shown that TrkB mRNA is expressed in most cells in the geniculate ganglion at E13 to E18 in the rat while TrkC is expressed only at E13, with very few cells showing expression at later stages during embryonic development (Ernfors et al., 1992). NT3 mRNA is also expressed in the embryonic geniculate ganglion (Ernfors et al., 1992). The early expression of TrkC could therefore correlate with a local role for NT3 during neurogenesis, as previously shown for NT3 in the early forming dorsal root ganglia (ElShamy and Ernfors, 1996; Farinas et al., 1996). In agreement with this, the nestin-driven NT3 overexpressing transgenic mice, in which NT3 is expressed in neuronal precursor cells, appear to have a normal tongue surface morphology and innervation, while other sensory systems where NT3 is physiologically provided from the target show severe malformations (Ringstedt et al., 1997). Combined with the in situ hybridisation study for TrkB, our results suggest that the failure of rescue of the gustatory system in $BDNF^{NT3/NT3}$ mice is, just as in the dorsal root ganglia during target

innervation, caused by a temporally selective expression of TrkB in the gustatory neurons that prevent these neurons from responding to NT3 and as a consequence, the neurons fail to survive and innervate the target tissue. The failure of NT3-dependent perigemmal somatosensory nerve fibres to invade the intragemmal territory, which in these mice express NT3, indicates that in addition to neurotrophins stimulating innervation of these territories, other mechanisms participate in preventing innervation of a tissue by a particular class of sensory neurons (Rochlin and Farbman, 1998; Rochlin et al., 2000).

In *BDNF*$^{-/-}$ and *NT3*$^{-/-}$ mice, the loss of spiral ganglion neurons at birth coincide with the number of type II (8-10%) and type I neurons (around 90%) in the spiral ganglion, respectively. In both *BDNF*$^{-/-}$ and *TrkB*$^{-/-}$ mice there is a highly selective loss of OHC innervation and death of type II spiral ganglion neurons (Ernfors et al., 1995b; Minichiello et al., 1995; Schimmang et al., 1995). Based on the preferential loss of type II neurons in the BDNF mutant mice, the loss of 90% of spiral ganglion neurons in *NT3*$^{-/-}$ mice and the complete loss of all neurons in double mutant mice (Ernfors et al., 1995b), we hypothesised that NT3 must be acting primarily on type I neurons innervating IHCs (Ernfors et al., 1995b). Recent expression analysis using *lacZ* knock-in mice in the *NT3* and *BDNF* loci, suggest an apical-basal difference in the levels of BDNF and NT3 expression, with NT3 being preferentially expressed in the base and BDNF in the apex (Farinas et al., 2001). In contrast to the above studies, the most pronounced deficit in the *BDNF*$^{-/-}$ mice reported by Farinas et al. is that of a marked selective loss of radial fibres in the apex, indicating a general loss of both IHC and OHC innervation and type I and type II neurons in the apex, but not in the middle and basal turns of the cochlea (Bianchi et al., 1996; Farinas et al., 2001). Our results from a large number of DiI tracings (n=32) reveal that there is no loss of radial fibres or IHC innervation in either *BDNF*$^{-/-}$ or *BDNF*$^{NT3/NT3}$ mice. This finding is supported by an independent analysis of the presence of radial fibres using immunohistochemical staining. It has been shown that the number of radial fibres per IHC in the apex is only one third of the number in the middle and base of the cochlea (Liberman et al., 1990). It is also well known that the apex is not as well organised as the middle and basal regions of the cochlea; stereocilia can often be immature and the hair cell rows in the apex are sometimes disorganised with extra outer or inner hair cells added (Borg and Viberg, 1995; Bredberg, 1968; Roth and Bruns, 1992). It is possible that the variation of radial fibres in the apex reported in some previous studies (Bianchi et al., 1996; Farinas et al., 2001) is due to biological variation rather than a direct consequence of the BDNF null allele.

Another apical-basal difference reported in *BDNF*$^{-/-}$ and *TrkB*$^{-/-}$ mice is that some OHC innervation is retained in the basal portion of the cochlea (Bianchi et al., 1996; Fritzsch et al., 1997c). However, none of these studies have been quantitative and the extent of the retained innervation in *BDNF*$^{-/-}$ mice has thus been unknown. Our semi-quantitative measurements showed a very limited residual OHC innervation present at all turns of the cochlea, but those that were present showed an apical-basal difference in the amount of fibres. In addition, we found a gradient along the three rows of OHCs where the third row of OHCs did not receive innervation at any turn of the cochlea in the *BDNF*$^{-/-}$ mice. Taken together, the results reported here are consistent with our earlier results (Ernfors, 1995b), and demonstrate unequivocally that the main function of BDNF is to specifically support spiral type II neurons and OHC innervation.

Neuronal survival, innervation of the cochlea and hearing was rescued in *BDNF*$^{NT3/NT3}$ mice. These results imply that NT3/TrkC promotes spiral ganglion type II neuron survival and innervation similarly (but not equally) to BDNF/TrkB, provided that the ligand is expressed in the right place and at the right time. Thus, we conclude that the specificity of BDNF in the cochlea is dependent on a controlled spatial and temporal regulation of ligand expression. This finding raises another issue. Why does NT3 expressed by the IHC not attract innervation of the type II spiral ganglion neurons, which we show in this study responds to NT3 similarly to BDNF? The parsimonious explanation might be that type I and type II spiral ganglion neuron phenotypes are induced by the target they innervate, as are certain properties of both motor neurons and sensory dorsal root ganglia neurons determined by their targets (Arber et al., 2000). One major difference between the OHC and IHC innervation is that while many type I neurons converge onto each IHC, each type II neuron innervates at least 10 OHC through spirally running fibres. Consistent with results in the *BDNF*$^{NT3/NT3}$ mice, showing reduced branching and a reduction of spiral nerve fibres, mice in which NT3 has been replaced by BDNF display a massive hypertrophy of nerve fibres with spirally running fascicles at the level of the OHCs. These fibres appear to fail to innervate the hair cells. There also seems to be a recruitment of neurons projecting to the OHC as seen by thick fascicles of radial fibres projecting to the OHCs (Coppola et al., 2001).

Because there are both TrkB and TrkC mRNAs in the vestibular ganglion neurons (Pirvola et al., 1994), the simplest explanation for any specificity in the vestibular system would be, just as in the cochlea, a temporal and spatially restricted expression of the different ligands. In *BDNF*$^{-/-}$ mice 85% of the neurons are lost at birth and the remaining are atrophic, and at 2 weeks after birth all neurons are gone (Bianchi et al., 1996; Ernfors et al., 1994a; Ernfors et al., 1995b) while only 20-30% die in the *NT3*$^{-/-}$ mice (Ernfors et al., 1994b; Ernfors et al., 1995b; Farinas et al., 1996). We found that the *NT3/NT3* alleles rescued many of the vestibular ganglion neurons from death and some innervation of the utricular and saccular maculae but not the crista. We have however, with NF staining, observed one calyx formation in the crista of one *BDNF*$^{NT3/NT3}$ mouse (data not shown). It is conceivable that the progressive postnatal loss of neurons in the *BDNF*$^{NT3/NT3}$ mice is caused by reduced levels of NT3 reaching the vestibular axons as a consequence of the failure of the neurons to innervate the sensory epithelium.

It is intriguing that most or all vestibular afferents reach the subepithelial layer of the sensory organs but do not properly invade the epithelia in the *BDNF*$^{NT3/NT3}$ mice. We found occasional nerve fibres that did invade the epithelia, but these fibres shot through the hair cell layer and ended up on the surface of the epithelium and failed to form calyces. These results suggest that NT3 activation of TrkC is not equivalent to BDNF activation of TrkB and indicates that TrkB is required for two developmental processes that cannot be replaced by TrkC; (i) invasion of vestibular afferents into the sensory epithelium and (ii) inducing hair cell contact and formation of

the highly specialised sensory nerve endings in the vestibular system (calyces). Since these functions of BDNF have not previously been addressed we set up an in vitro model that would allow us to directly dissect the physiological role of BDNF produced in the target cells. These experiments confirmed a role for BDNF in terminal arborisation and maturation of calyces. There are several previous lines of evidence that has indicated the importance of BDNF activation of TrkB in target innervation. TrkB and TrkC have been proposed to elicit survival in vivo through similar intracellular signalling pathways and this pathway does not involve the Shc adaptor binding sites of the receptors (Postigo et al., 2002). In contrast, mice carrying a signalling mutation of the Shc adaptor-binding site showed a loss of target innervation in the case of the TrkB receptor whereas in the TrkC, the surviving neurons maintained target innervation. Consistently, although both BDNF and NT3 have been shown to cause growth cone turning in an in vitro assay, they do it by different intracellular signalling pathways (Song et al., 1997). Thus, activation of TrkB and TrkC is not equivalent and we show in this study that BDNF activation of TrkB, but not NT3 activation of TrkC, is required for target innervation and synaptic formation in vivo.

We show that specificity of neurotrophins is achieved by multiple mechanisms during development of the nervous system. These include selective receptor expression in neuronal populations, selective ligand expression in different targets and different signalling between Trk receptors. Conceivably, many other mechanisms can lead to distinct read-outs between different neurotrophins in selective neuronal populations, such as differences in levels of expression of both full-length receptors as well as differences in the proportion of endogenous truncated dominant negative and scavenging TrkB and TrkC receptors to full-length receptors (Biffo et al., 1995; Eide et al., 1996; Ninkina et al., 1996; Palko et al., 1999).

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