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Developing inner ear sensory neurons require TrkB and TrkC receptors for innervation of their peripheral targets

Thomas Schimmang[1], Liliana Minichiello[3], Esther Vazquez[1], Isabel San Jose[2], Fernando Giraldez[1], Rüdiger Klein[3] and Juan Represa[2]

[1]Departamento Bioquimica, Biologia Molecular y Fisiologia y [2]Departamento Anatomia, Facultad de Medicina, Universidad de Valladolid, CSIC, 47005 Valladolid, Spain
[3]Differentiation Programme, European Molecular Biology Laboratory, 69117 Heidelberg, Germany

SUMMARY

The *trkB* and *trkC* genes are expressed during the formation of the vestibular and auditory system. To elucidate the function of *trkB* and *trkC* during this process, we have analysed mice carrying a germline mutation in the tyrosine kinase catalytic domain of these genes. Neuroanatomical analysis of homozygous mutant mice revealed neuronal deficiencies in the vestibular and cochlear ganglia. In *trkB* (–/–) animals vestibular neurons and a subset of cochlear neurons responsible for the innervation of outer hair cells were drastically reduced. The peripheral targets of the respective neurons showed severe innervation defects. A comparative analysis of ganglia from *trkC* (–/–) mutants revealed a moderate reduction of vestibular neurons and a specific loss of cochlear neurons innervating inner hair cells. No nerve fibres were detected in the sensory epithelium containing inner hair cells.

A developmental study of *trkB* (–/–) and *trkC* (–/–) mice showed that some vestibular and cochlear fibres initially reached their peripheral targets but failed to maintain innervation and degenerated. TrkB and TrkC receptors are therefore required for the survival of specific neuronal populations and the maintenance of target innervation in the peripheral sensory system of the inner ear.

Key words: *trkB*, *trkC*, mouse mutant, inner ear, vestibular ganglion, cochlear ganglion, hair cell, target innervation

INTRODUCTION

The Trk family of protein tyrosine kinase receptors mediate the biological effects of the nerve growth factor (NGF) family of neurotrophins (Meakin and Shooter, 1992; Glass and Yancopoulos, 1993). The *trkA* and *trkC* genes encode high-affinity receptors for NGF (Kaplan et al., 1991; Klein et al., 1991a) and neurotrophin-3 (NT-3; Lamballe et al., 1991), respectively. The product of the *trkB* gene serves as the receptor for two related neurotrophins, brain derived neurotrophic factor (BDNF; Klein et al., 1991b; Soppet et al., 1991; Squinto et al., 1991) and neurotrophin 4 (NT-4; Berkemeier et al., 1991; Klein et al., 1992; Ip et al., 1992). TrkB also binds NT-3, although the extent to which this occurs in vivo is controversial (Ip et al., 1993). In addition to the Trks, all neurotrophins bind to another cell surface receptor, known as p75 or the low affinity NGF receptor (LNGFR) whose role may be to modulate Trk signalling (Meakin and Shooter, 1992; Glass and Yancopoulos, 1993; Davies et al., 1994; Hantzopoulos et al., 1994; Verdi et al., 1994).

Cranial sensory neurons have been especially useful for studying the specificity and time-course of the survival-promoting effects of neurotrophins (Davies, 1987; Vogel and Davies, 1991; Davies, 1994). The cochleovestibular nerve (cranial nerve VIII) contains afferent fibres whose targets are the sensory epithelia of the inner ear. Cochlear fibres commence in the organ of Corti, and vestibular fibres originate in the utricular and saccular maculae as well as the ampullary cristae of the semicircular canals. The somata of all of these afferent fibres are located in the cochlear and vestibular ganglia. Most of the cochlear and vestibular neurons are bipolar, with a peripheral process contacting the hair cells in their respective sensory epithelia, and a central process that projects in a specific topographical fashion to the cochlear and vestibular nuclei of the medulla (Spoendlin, 1988). TrkB, TrkC as well as LNGFR are expressed in the cochlear and vestibular ganglion before and during innervation of their target fields, whereas TrkA is expressed only transiently and at a low level (Von Bartheld, 1991; Represa et al., 1991; Ernfors et al., 1992; Tessarollo et al., 1993; Pirvola et al., 1994; Schecterson and Bothwell, 1994; Vazquez et al., 1994). BDNF and NT-3, but not NT-4 or NGF, are detected in the sensory epithelia (Pirvola et al., 1992, 1994; Schecterson and Bothwell, 1994). In explant cultures, exogenous BDNF and NT-4 promote neurite outgrowth from the cochlear and vestibular ganglion neurons, while NT-3 is mainly effective on cochlear neurons (Pirvola et al., 1994; Vazquez et al., 1994). In dissociated neuron cultures, BDNF and NT-3 promote survival of cochlear and vestibular neurons (Davies et al., 1986; Pirvola et al., 1994; Vazquez et al., 1994). Results of the effectiveness of NGF in bioassays are controversial (Lefebvre et al., 1990, 1991; Pirvola et al., 1992, 1994). Based on spatiotemporal expression patterns and bio-

Table 1. Volumes of vestibular and cochlear ganglia in *trkB* mutant mice

Age	Ganglion	wild-type	*trkB* (–/–)	Reduction (%)
14.5 p.c.	cochlear	17.6±0.7 (4)	17.3±0.1 (4)	not significant
	vestibular	13.2±0.4 (4)	12.9±0.2 (4)	not significant
18.5 p.c.	cochlear	28.9±0.8 (4)	27.3±0.5 (4)	6*
	vestibular	19.3±0.5 (4)	11.9±0.3 (4)	38**
P1	cochlear	26.8±0.3 (4)	23.9±0.4 (4)	11**
	vestibular	17.6±0.2 (4)	7.5±0.3 (4)	57**

The volume in 10^6 μm^3 (mean ± s.e.m.) of ganglia were calculated in the developmental stages indicated. The sampling number is shown in parentheses. Student's two-tailed *t*-test determined significance levels. **P*<0.005, ***P*<0.00005.

logical effects, BDNF and NT-3, rather than NGF and NT-4, are considered to be responsible for the survival and neurite outgrowth of cochlear and vestibular neurons, acting via their high-affinity receptors TrkB and TrkC, respectively.

Recently, the phenotypes of germline-targeted mutant mice lacking functional neurotrophins and their receptors have allowed the identification of the specific neuron populations that are dependent on neurotrophin action during development (Barbacid, 1994; Klein, 1994a; Snider, 1994). Consistent with the NT-3 expression pattern and in vitro experiments, NT-3 (–/–) mutant mice show reductions in the cochlear and vestibular ganglion (Fariñas et al., 1994). Mice carrying an inactive form of *trkC* are available, but have not been analysed for inner ear defects (Klein et al., 1994b). BDNF (–/–) mutant mice have severe deficiencies in coordination and balance, suggesting abnormalities in the functioning of the vestibular system (Ernfors et al., 1994a; Jones et al., 1994). Vestibular ganglia neurons are drastically depleted and the vestibular fibres fail to innervate the sensory epithelia in postnatal animals. Together with the known expression pattern of BDNF and its effects in in vitro systems, the results suggest that most of the vestibular ganglion cells require BDNF for survival. In contrast, the cochlear ganglion, the cochlea and the innervation of inner and outer hair cells appear to be unaffected in BDNF (–/–) mice (Ernfors et al., 1994a).

In the present study, we have analysed the vestibular and auditory system in mice mutant for the TrkB and TrkC receptor, respectively (Klein et al., 1993, 1994b). Our results reveal that TrkB and TrkC are required for the survival of specific neuronal populations and the innervation of their peripheral targets. A developmental study of *trkB* (–/–) and *trkC* (–/–) mice demonstrates that these neurons do not depend on functional Trk receptors for target encounter of peripheral nerve processes, but rather that they are required for neuronal survival and maintenance of target innervation.

Table 2. Neuron cell numbers in the vestibular and cochlear ganglia of *trkB* mutant mice

Age	Ganglion	wild-type	*trkB* (–/–)	Reduction (%)
14.5 p.c.	cochlear	4570±260 (4)	4477±165 (4)	not significant
	vestibular	3411±170 (4)	3366±184 (4)	not significant
18.5 p.c.	cochlear	8001±259 (4)	7652±133 (4)	not significant
	vestibular	5330±189 (4)	3343±101 (4)	37**
P1	cochlear	7950±236 (8)	6765±119 (4)	15*
	vestibular	4819±115 (8)	2128±113 (4)	56**

Ganglia were dissected and sectioned at 8 μm thickness. Neurons were counted in every sixth section. Values were not corrected for split nucleoli. Mean number of neurons (± s.e.m.) are listed and the sampling number is shown in parentheses. Differences were tested using a two-tailed Student's *t*-test. **P*<0.01, ***P*<0.00005.

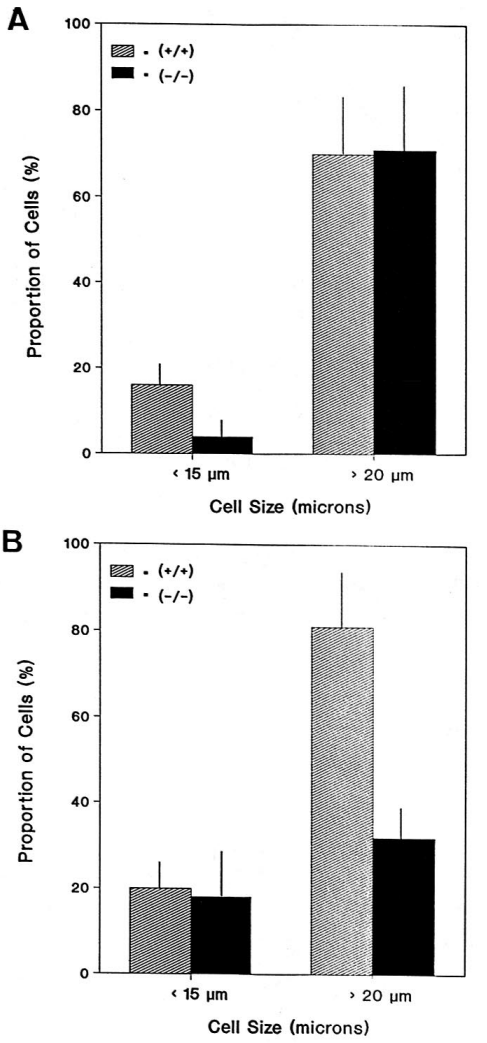

Fig. 1. Cell size histograms of cochlear ganglion neurons of wild-type (+/+), *trkB* (–/–) (A) and *trkC* (–/–) (B) mice. The mean number of cells falling into each size category is expressed as a percentage of total cells in wild-type ganglia. (A) Only small sized (<15 μm) neurons are reduced in *trkB* (–/–) mutant mice. (B) *TrkC* (–/–) animals specifically lack large sized (>20 μm) neurons compared to wild-type (+/+) mice.

MATERIALS AND METHODS

Genotyping of animals

TrkB and TrkC mutant mice were generated by breeding heterozygous mutant mice kept on a mixed 129/sv × C57Bl/6 background. Genomic DNA was extracted from tail biopsies of mouse embryos using standard procedures (Laird et al., 1991). The *trkB* genotype was determined by PCR amplification (94°C, 1 minute; 65°C, 2 minutes; 72°C, 3 minutes for 40 cycles) using a common 5′ primer (5′-TCGCGTAAAGACGGAACATGATCC-3′) and either a 3′ primer

for the wild-type allele (5′-AGACCATGATGAGTGGGTCGCC-3′) or a 3′ primer from the pgk-1 promoter of the neo cassette (5′-GATGTGGAATGTGTGCGAGGCC-3′). The *trkC* genotype was determined using a common 5′ primer (5′-CTGAAGTCACTGGCTAGAGTCTGGG-3′) and either a 3′ primer for the wild-type allele (5′-GTCCCATCTTGCTTACCCTGAGG-3′) or a 3′ primer from the pgk-1 promoter of the neo cassette (5′-CCAGCCTCTGAGCCCAGAAAGC-3′). PCR amplified DNA was analyzed on a 1.5% agarose gel.

Histological analysis

Entire temporal bone primordia were dissected and fixed in 4% paraformaldehyde in 0.1 M sodium phosphate (pH.7.2) for between 6-12 hours, dehydrated in ethanol, embedded in paraffin, serially sectioned at 8 μm and stained with 0.1% cresyl violet. The volume of each ganglion was measured using a morphometric analysis program (VIDS Image Analyzer, Synoptics Ltd, Cambridge). For counts of cochlear and vestibular neuron nuclei were counted from cresyl violet and hematoxilyn and eosin-stained sections. Neurons were identified by their size and distinct nucleus. No correction was made for split nucleoli. Nuclei were counted at 200× magnification in 4 randomly chosen fields in every section of 8 μm thickness. Up to about 80 fields per ganglion in sections that were 40 μm apart were analysed. No corrections were used because neuronal size (max. 25 μm) was always smaller than the intersection interval, making it impossible to count the same neuron twice. The cell size histograms were generated from computer aided drawings of neuronal profiles from cochlear ganglia of P1 mice. At least 500 profiles were drawn and the profiles were corrected as described by Bolender (1983). Immunohistochemistry was carried out using the ABC method (Vectastain Kit, Vector Laboratories) on sections cut at 25 μm on a cryostat or on paraffin sections (8 μm). Sections were incubated in TBS solution (50 mM Tris-HCl buffer (pH 7.5) containing 0.1% sheep serum, 0.1% bovine serum albumin, 0.1% Triton X-100), quenched in 3% H_2O_2, blocked with serum and left overnight at 4°C in TBS solution containing 2-4 μg/ml of a mouse anti-200K neurofilament monoclonal antibody (Boehringer, Mannheim). After incubation with an biotinylated antibody and the ABC reagent, peroxidase was reacted with 0.05% diaminobenzidine tetrahydrochloride and 0.003% hydrogen peroxide.

Transmission electron microscopy

Membranous labyrinths were isolated and fixed for 2 hours at 4°C in 2% glutaraldehyde, rinsed twice for 15 minutes in 0.1 M cacodylate buffer, postfixed in 2% osmium tetroxide for 30 minutes, dehydrated in a graded series of acetone and embedded in plastic. Transverse thin sections were taken at various levels, stained with uranyl acetate and lead citrate and examined under a JEOL 1200EX electron microscope at 80 kV.

RESULTS

Homozygous *trk*B (–/–) mutant mice exhibit cell loss in vestibular and cochlear ganglia

To elucidate the role of TrkB in the auditory and vestibular system, we studied mice carrying a *trkB* locus specifically targeted within its protein tyrosine kinase sequences (Klein et

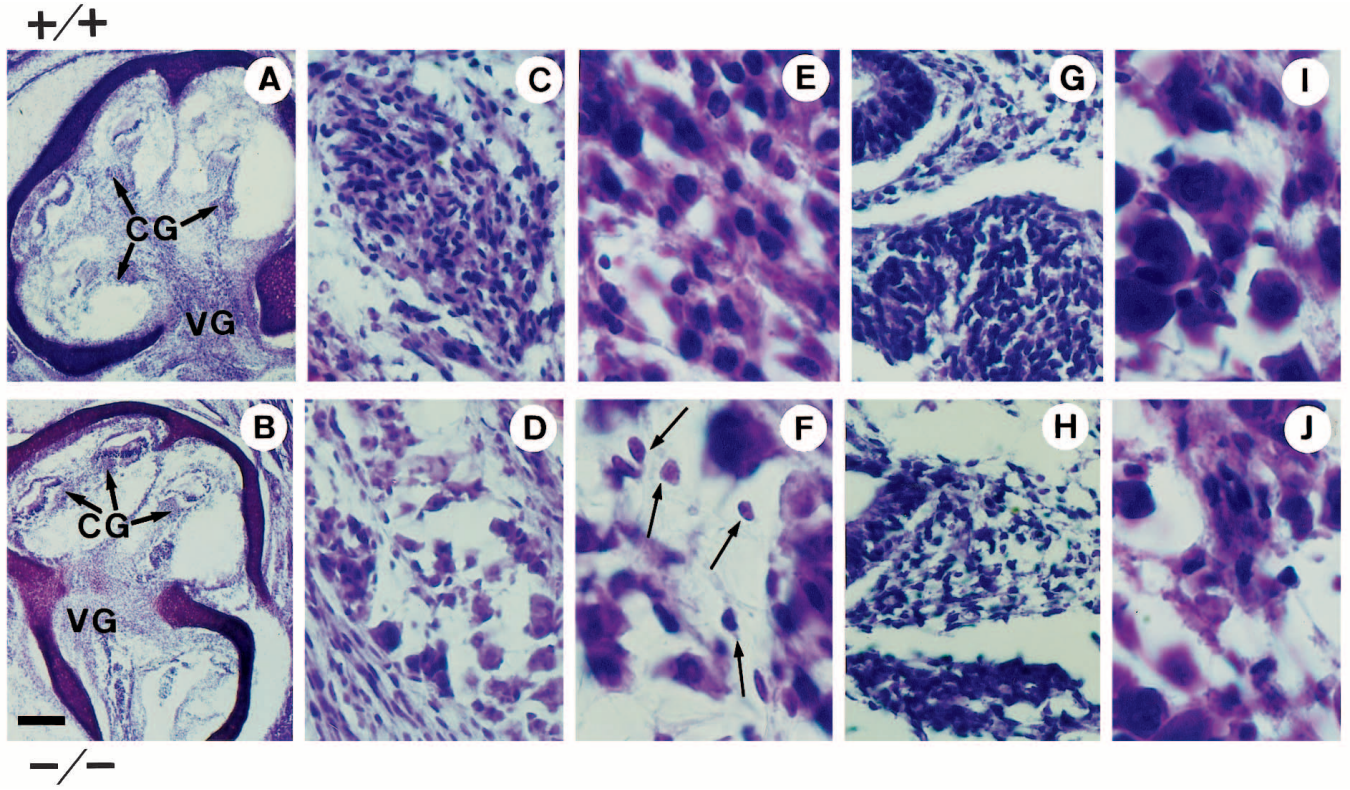

Fig. 2. Abnormalities in the vestibular and cochlear ganglia of *trkB* (–/–) mice. Sections through the inner ear of wild-type (+/+) (A,C,E,G,I) and *trkB* (–/–) mice (B,D,F,H,J). The vestibular (VG) and cochlear ganglion (CG, arrows) are indicated in A and B and are shown in detail (C,D and G,H, respectively). Note the reduced cell density and enlarged extracellular spaces in *trkB* (–/–) mutant animals. (E,F,I,J) High power magnification of C,D,G and H, respectively. Arrows in F point to cells with nuclei surrounded by a very small amount of cytoplasm. Scale bar in B corresponds to 450 μm (A,B); 100 μm (C,D,G,H); 20 μm (E,F,I,J).

al., 1993). To examine defects in vestibular and cochlear ganglia, their volumes were calculated and neurons were counted on serial sections. At day 14.5 post coitum (p.c.), most cochlear and vestibular neurons have become postmitotic (Ruben, 1967). No differences in sizes and neuronal profiles of the ganglia were detected in wild-type or homozygous *trkB* (–/–) mutant mice (Tables 1 and 2). However, at day 18.5 p.c. vestibular ganglia of *trkB* (–/–) mice were smaller than those of wild-type animals and the numbers of neurons were reduced. Also, the volume of the cochlear ganglion of *trkB* (–/–) animals was found to be reduced to a small but significant extent. At postnatal day 1 (P1) neuronal defects had become more dramatic in the vestibular ganglion. The ganglion had shrunk to 43% of its volume and had undergone a neuronal cell loss of 56% when compared to wild-type animals. Neuronal cell counts and volume calculations of the cochlear ganglion revealed a 15% and 11% reduction, respectively. Cell size histograms of the cochlear ganglion showed that the modest reduction of cells in *trkB* (–/–) mutant mice was due to the loss (77% reduction) of small sized (<15 μm) type II neurons, whereas large sized (>20 μm) type I neurons were unaffected (Fig. 1). Type I and type II neuronal cells are responsible for the innervation of inner and outer hair cells in the sensory epithelium of the cochlea, respectively (Spoendlin, 1969; Kiang et al., 1982; Sobkowicz, 1992).

Cresyl violet staining of sections from *trkB* (–/–) mice at P1 revealed areas of reduced cell density and large extracellular spaces in the vestibular ganglion (Fig. 2D) in addition to its smaller size compared to wild-type mice (Fig. 2C). At the cellular level, nuclei surrounded by a very small amount of cytoplasm were more abundant in the ganglia of *trkB* (–/–) mutants (Fig. 2F) than in wild-type animals (Fig. 2E). A similar situation, although to a lesser extent, was also observed in the cochlear ganglion (Fig. 2G-J). These results indicate that the TrkB signalling pathway is not required for the initial phase of cell proliferation and ganglion formation. The TrkB receptor is, however, critically important for survival of the majority of vestibular and a small subset of cochlear ganglion neurons during development.

Neonatal *trkB* mutant mice lack vestibular fibres and exhibit selective loss of outer hair cell innervation in the cochlea

To study target innervation of the vestibular and cochlear neurons, an antibody (RT97) directed against phosphorylated epitopes on neurofilaments was used (Wood and Anderton, 1981). In the inner ear of P1 wild-type mice, RT97 immunoreactive fibres contacted the utricular macula and the ampullary crista of the semicircular canals (Fig. 3A,E). Fibre bundles reached and entered the sensory epithelium, where they innervated hair cells. At the cellular level, innervation of type II hair cells and nerve chalicles characteristic of type I hair cells were found (Fig. 3C,G). *TrkB* (–/–) mutant mice revealed less immuno-

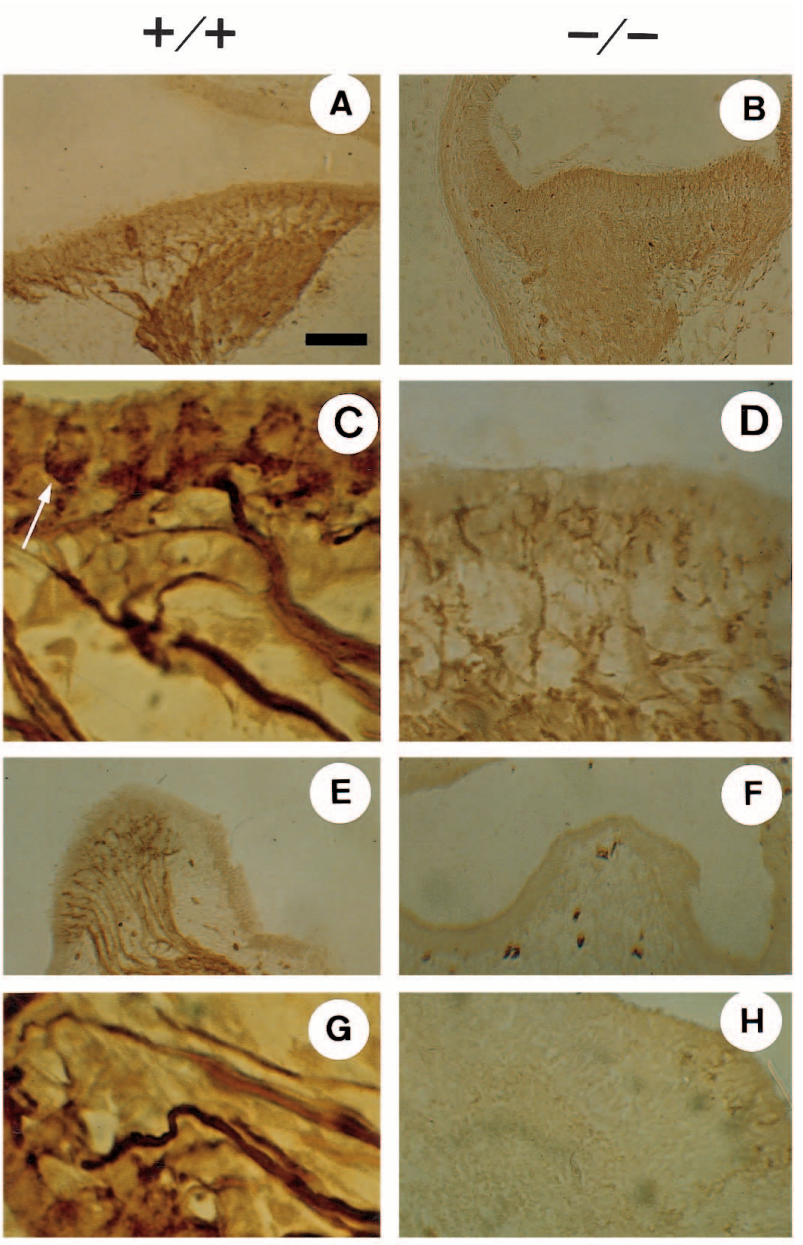

Fig. 3. Lack of *trkB* leads to deficiencies of innervation in the vestibular sensory epithelia. Sections of P1 wild-type (+/+) (A,C,E,G) and *trkB* (–/–) (B,D,F,H) mice were stained with a monoclonal antibody directed against phosphorylated epitopes on neurofilaments. The utricular macula (A-D) and the ampullary crista of the lateral semicircular canal (E-H) are shown. (A,C,E,G) Wild-type animals revealed a normal innervation pattern. (C,G) High power magnifications of the sensory epithelium show fibres entering the epithelium and innervation of hair cells. A nerve chalicle typical of the innervation of type I hair cells is indicated by an arrow. (B,D,F,H) Note the overall low immunoreactivity in mutant *trkB* (–/–) animals compared with wild-type mice. (D) In the utricular macula nerve fibres have reached the base of the hair cells, but are degenerating and retracting from their targets. (H) No innervation is observed in the sensory epithelia of the ampullary crista. Scale bar in A corresponds to 250 μm (A,B,E,F); 50 μm (G,H); 30 μm (C,D).

reactivity of neurofilaments. In the utricular macula, vestibular fibres were poorly stained and appeared fractionated and degenerate (Fig. 3B). The vestibular nerve terminals had reached the sensory epithelium, but appeared in the process of retraction from their targets (Fig. 3D). In the ampullary crista of *trkB* (–/–) mice, no innervation was visible, as revealed by the absence of immunoreactivity for neurofilaments (Fig. 3F,H).

We had observed a selective loss of small type II neurons in the cochlear ganglion of *trkB* (–/–) mutant mice. Lack of these neurons is expected to cause innervation defects of their corresponding peripheral sense organs, the outer hair cells. In wild-type mice, sections at the level of the outer hair cells revealed nerve fibres, underneath the sensory epithelium, which entered the epithelium and innervated hair cells (Fig. 4C). In *trkB* (–/–) mice the sensory epithelium was generally devoid of innervation (Fig. 4D). At a few sites, retracting neurites with residual synaptic contacts were detected by the neurofilament antibody. We also examined the innervation of inner hair cells. In both, wild-type and *trkB* (–/–) mutant animals thick fibres were entering the sensory epithelium and innervated hair cells (Fig. 4A,B).

To characterize further the innervation defects of neonatal *trkB* (–/–) animals at the ultrastructure level we analysed ultrathin sections using electron microscopy. Wild-type mice showed the normal pattern of synaptogenesis and myelination of the inner ear of newborns (Pujol and Sans, 1986; Dechesne et al., 1987). At the junction between nerve endings and hair cells high-density synaptic plates and membrane vesicles were detected, while in *trkB* (–/–) mutant mice these were absent or dramatically reduced (Fig. 5A,B). In wild-type specimen neurites were myelinated and associated with Schwann cells, whereas sections of *trkB* (–/–) animals revealed degenerating fibres, empty perineural sheets and no evidence for myelination (Fig. 5C,D).

TrkC mutant mice exhibit specific defects in the cochlear ganglion and innervation of inner hair cells

In addition to TrkB, the TrkC receptor is expressed during ganglion formation and target innervation of the inner ear (Ernfors et al., 1992; Tessarollo et al., 1993). To analyse its functional role during this process we examined mice in which sequences encoding the TrkC protein kinase had been inactivated by gene targeting (Klein et al., 1994b). Neuronal counts of cochlear ganglia showed that compared to wild-type animals the number of neurons was reduced by 51% in P1 *trkC* (–/–) mice (Table 3). Cell size histograms of the cochlear ganglion showed that the reduction of cells in *trkC* (–/–) mutant mice was due to the loss (62% reduction) of large sized (>20 μm) type I neurons (Fig. 1B). Cresyl violet stained sections from *trkC* (–/–) mutants also revealed that the cochlear ganglion specifically lacks large type I neurons, which are responsible for the innervation of inner hair cells in the sensory epithelium (Fig. 6A,B,E,F). Upon further histological examination, the formation and differentiation of the cochlear sensory epi-

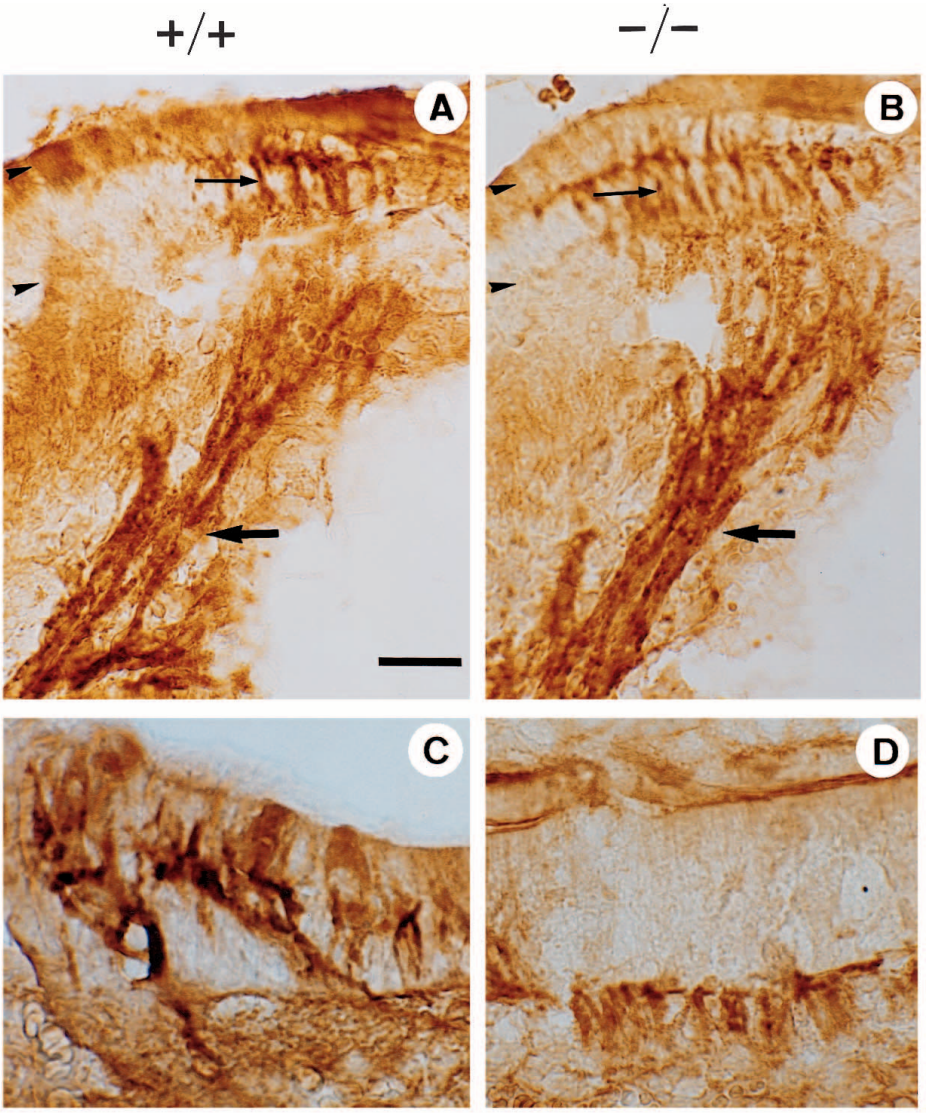

Fig. 4. *TrkB* (–/–) mutants fail to innervate the outer hair cells of the cochlea. Longitudinal sections through the medial part of the cochlea of P1 wild-type (+/+) (A,C) and *trkB* (–/–) (B,D) animals, which have been stained with an antibody raised against neurofilaments. (A,C) In wild-type mice, nerve fibres enter the sensory epithelium and innervate inner (A) and outer hair cells (C). Nerve fibres (arrows) and the sensory epithelium (arrowheads) are indicated in A and B. (B) The innervation pattern of inner hair cells of *trkB* (–/–) mutant animals appears normal. (D) The sensory epithelium containing outer hair cells is generally devoid of nerve fibres. Scale bar in A corresponds to 75 μm in A-D.

thelium of *trkC* (–/–) mice was found to be incomplete (Fig. 6B,D). The epithelium was thinner and less stratified compared to wild-type mice (Fig. 6A,C). Innervation of the sensory epithelium was again examined by using a neurofilament antibody, as described above. In wild-type animals nerve fibres reached and entered the sensory epithelium containing inner and outer hair cells (Fig. 7A,C). Inner hair cells of *trkC* (–/–) mice were devoid of innervation, whereas outer hair cells were properly innervated (Fig. 7B,D).

The vestibular ganglion of *trkC* (–/–) mice had undergone a moderate but significant reduction in neuronal cell number (16% reduction), when compared to wild-type animals (Table 3). Histological examination of the vestibular ganglia, the vestibular sensory epithelia and their innervation patterns revealed no apparent abnormalities in *trkC* (–/–) mutant mice (data not shown).

Affected nerve fibres in mice carrying a disrupted *trkB* or *trkC* allele are capable of target innervation

We were interested to know whether the severe reduction or absence of innervation in the vestibular and/or cochlear system of *trkB* (–/–) and *trkC* (–/–) mutant mice was caused by a failure of nerve fibres to reach their targets or by excessive cell death after they have contacted the sensory epithelia. Ultrastructural studies of the mouse during development of vestibular and cochlear hair cells and their afferent innervation have shown that most of the afferent contacts and early synapses are set up from day 14 p.c to day 18 p.c. (Anniko, 1983). Wild-type mice developed thin fibres, characteristic of this developmental stage, which innervated sensory cells of the utricular macula, the ampullary crista and at the level of the outer and inner hair cells of the cochlea (Figs 8A,C,E,G,I and 7E), respectively. Similarly, examination of *trkB* (–/–) and *trkC* (–/–) animals revealed the presence of vestibular fibres and cochlear nerve fibers contacting the base of the sensory epithelia, although they showed less immunoreactivity and some regressing nerve terminals (Figs 8B,D,F,H,J and 7F). However, compared with the mutant phenotypes observed in the utricular macula (Fig. 3D) and in the outer hair cells of the cochlea (Fig. 4D) of P1 *trkB* (–/–) mice, more and better developed nerve fibres were detected. In the sensory epithelium of the ampullary crista of P1 *trkB* (–/–) animals and the inner hair cells of P1 *trkC* (–/–) mutants no innervation was observed (Figs 3H and 7B). These results show that some inner ear sensory neurons of *trkB* (–/–) and *trkC* (–/–) mutant mice are initially capable of contacting their peripheral targets. Lacking neurotrophic signals, nerve fibres fail to maintain their innervation with the sensory epithelium and are retracted, paralleling an abnormal pattern of cell death in the vestibular and cochlear ganglion.

DISCUSSION

Survival of vestibular neurons is mainly dependent on functional TrkB receptors

The present study shows that, at P1, *trkB* (–/–) mutant vestibular ganglia reveal a dramatic reduction in neuron numbers

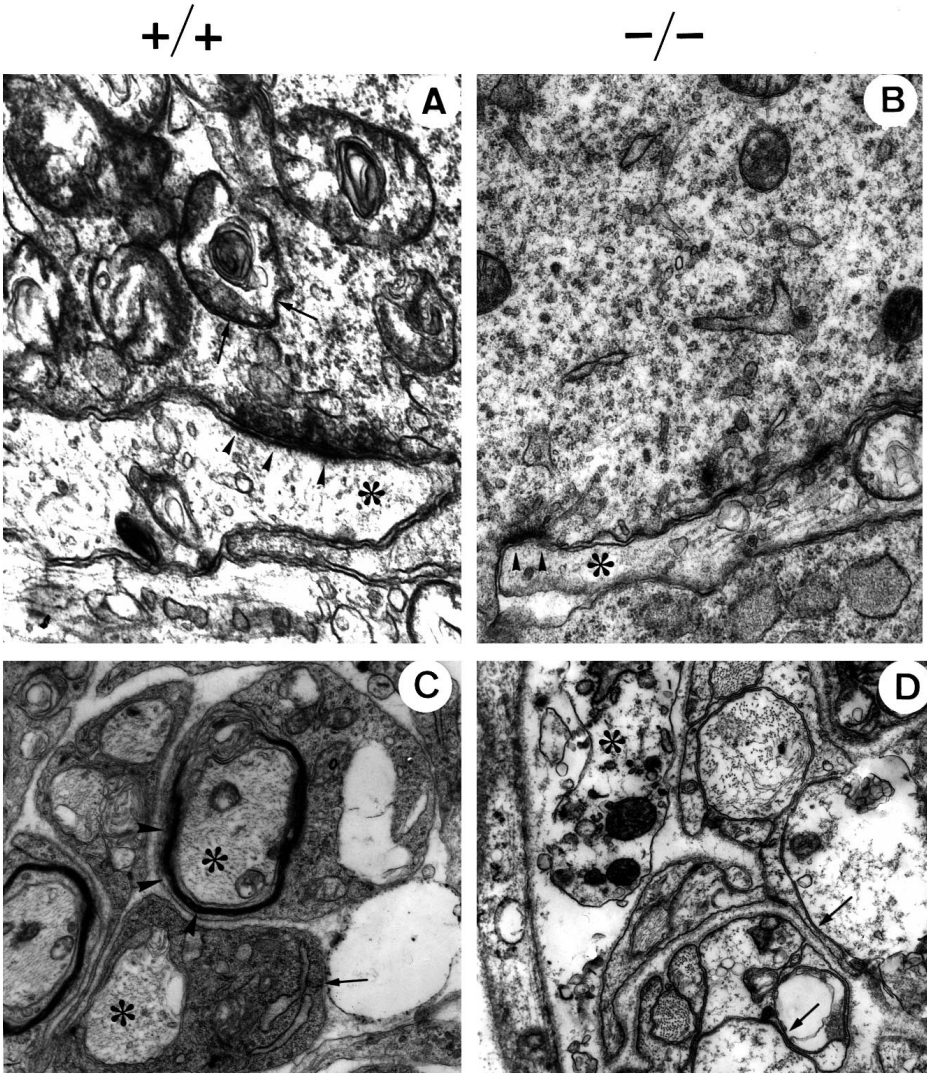

Fig. 5. Innervation defects of *trkB* (–/–) mice at the ultrastructural level. Transverse sections through the utricular macula (A,B) and the vestibular nerve (C,D) of a P1 wild-type (+/+) (A,C) and a *trkB* (–/–) mutant (B,D). (A) In wild-type mice nerve endings (asterisk) contact the base of the hair cells in the sensory epithelium and form synaptic plates (arrowheads). A synaptic vesicle is indicated by arrows. (B) *trkB* (–/–) animals lack membrane vesicles and contain residual synaptic plates at their nerve endings (asterisk). (C) Neurites (asterisks) of wild-type animals are associated with Schwann cells (arrow) and myelin (arrowheads). (D) *trkB* (–/–) mice showed degenerating nerve fibres (asterisk), empty perineural sheets (arrows) and no myelination. Magnification in A,B (12000×), in C,D (7500×).

Table 3. Neuron cell numbers of vestibular and cochlear ganglia of *trkC* mutant mice

	Ganglion	wild-type	*trkC* (–/–)	Reduction (%)
P1	cochlear	7950±236 (8)	3901±157 (6)	51
	vestibular	4819±115 (8)	4059±76 (6)	16

Ganglia from P1 mice were dissected and sectioned at 8 μm thickness. Neurons were counted in every sixth section. Values were not corrected for split nucleoli. Mean number of neurons (± s.e.m.) are listed and the sampling number is shown in parentheses. Differences were tested using a two-tailed Student's *t*-test. $P<0.0002$.

(56%) and ganglion volume (57%). In comparison, *trkC* (–/–) mutant ganglia show a much less severe, yet significant reduction in vestibular neurons (16%). The more severe deficiency observed in *trkB* (–/–) mutant mice correlates well with the expression pattern of the TrkB ligand, BDNF, in the vestibular sensory epithelia of wild-type animals. BDNF is detected throughout embryonic development of the vestibular system (day 11.5 p.c. to P1). In contrast, the TrkC ligand, NT-3, has a more restricted temporal expression pattern (day 13.5 p.c. to 16.5 p.c.; Schecterson and Bothwell, 1994). Moreover, the ampullary crista which expresses BDNF, but not NT-3, is the main structure of the vestibular organ to be affected in *trkB* (–/–) mice (Pirvola et al., 1992; Schecterson and Bothwell, 1994).

Comparison of the phenotypes of *trkB* (–/–) and *trkC* (–/–) mutant mice with those of BDNF (–/–) and NT-3 (–/–) mutant mice, revealed interesting similarities, but also differences. Vestibular neurons of NT-3 (–/–) animals were shown to be

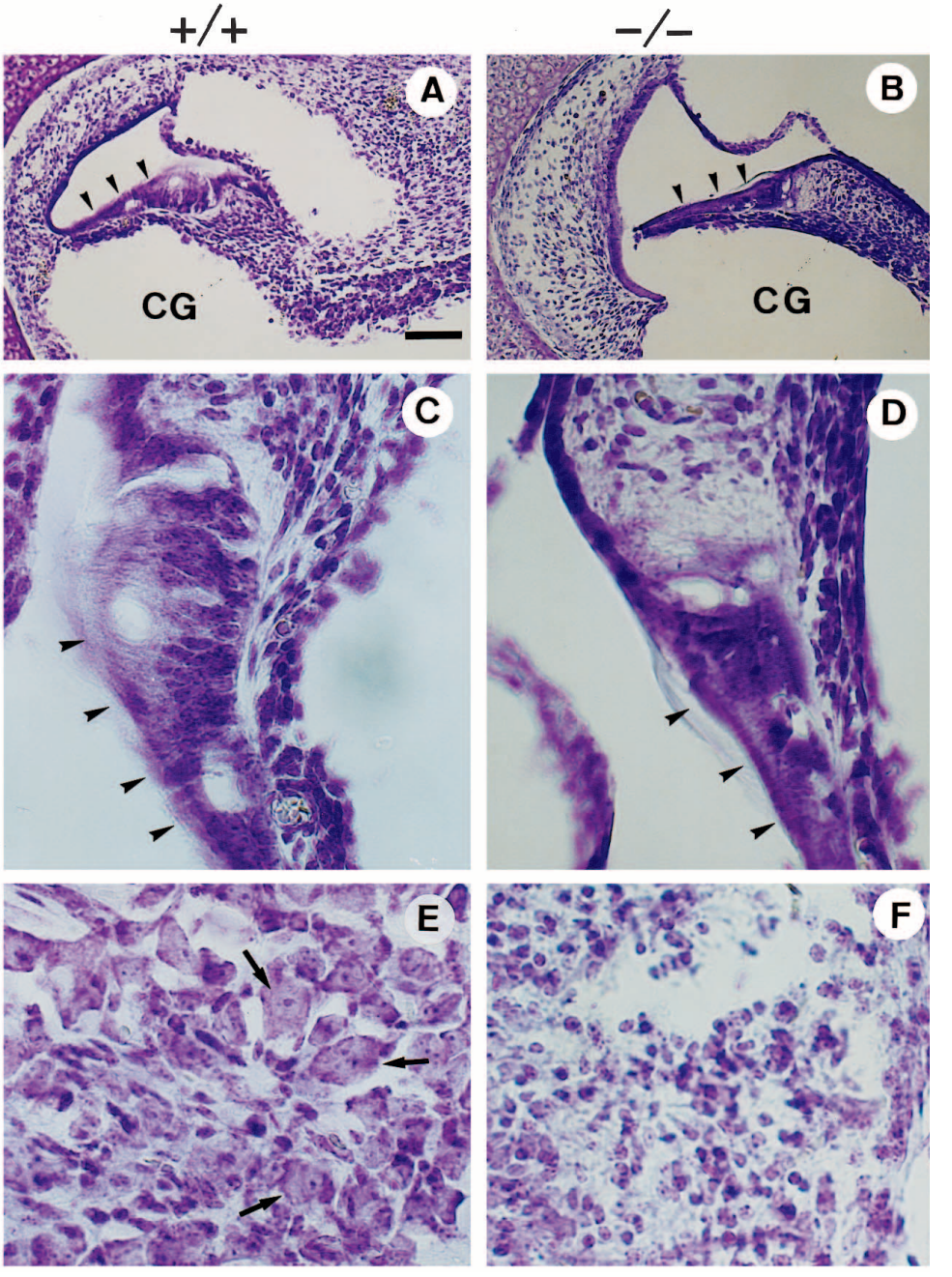

Fig. 6. Abnormalities in the cochlear sensory epithelium and ganglion of *trkC* (–/–) mice. Transverse sections through the cochlea of a P1 wild-type (+/+) (A,C,E) and a *trkC* (–/–) (B,D,F) mouse. The sensory epithelium (arrowheads) and ganglion (CG) are indicated in A and B. The sensory epithelium is shown in detail and indicated by arrowheads in C and D. Note the reduced thickness and stratification of the epithelium in *trkC* (–/–) mice compared to wild-type mice. (E,F) High power magnification of the cochlear ganglion. Note the absence of large type I cells (indicated in E by arrows) in *trkC* (–/–) mice. Scale bar in A corresponds to 200 μm (A,B); 100 μm (C,D); 25 μm (E,F).

reduced by 23% in newborns (Fariñas et al., 1994) This correlates very well with the observed 16% reduction of vestibular neurons in *trkC* (–/–) mice, indicating that the main NT-3 receptor in this sensory organ is indeed gp145TrkC. NT-3 (–/–) and *trkC* (–/–) mutant mice are characterized by abnormal movements and postures which has been explained by the deficiency of proprioceptive neurons (Ernfors et al., 1994b; Fariñas et al., 1994; Klein et al., 1994b). The observed loss of vestibular neurons possibly contributes to the abnormal behavioural phenotype of the mutant animals.

There is a clear difference between the reduction in the volume of the vestibular ganglion and the number of vestibular neurons surviving in *trkB* (–/–) mice (57% and 44%, respectively; this study) and BDNF (–/–) mice (87% and 18%, respectively; Jones et al., 1994; Ernfors et al., 1994a). One possible explanation could be that at least in one study (Ernfors et al., 1994a) vestibular neuron numbers were determined in juvenile animals (postnatal days P14 to P16) rather than in neonates. More vestibular neurons may undergo cell death in *trkB* (–/–) mice during subsequent postnatal days. A further, although less likely explanation may be that BDNF would be able to exert its function in the vestibular system via an as yet unidentified receptor, distinct from TrkB. This would result in a higher percentage of neuronal cell death in BDNF (–/–) mutants compared with *trkB* (–/–) mice. Alternatively, the lack of functional TrkB receptors may cause a compensatory up-regulation of TrkC receptor expression in vestibular neurons which then could be rescued by the presence of NT-3 in their target fields. Once quantitative PCR-based expression assays for the TrkC receptor have been developed, it will be interesting to analyze the surviving vestibular neurons in *trkB* (–/–) mice for the expression of TrkC receptors. It is however, conceivable that vestibular neurons in *trkB* (–/–) mice up-regulate components of other, as yet undefined, signalling pathways than the NGF-like neurotrophins. It will therefore be crucial to generate double *trkB*/*trkC* mutant mice to analyze the interaction between TrkB and TrkC signalling pathways and to identify possible neurotrophin-independent survival signals for sensory neurons.

Inner and outer hair cells of the cochlea depend on the function of TrkC and TrkB, respectively

Recent experiments suggest that BDNF and NT-3 acting via their high-affinity receptors TrkB and TrkC are the dominant biologically active neurotrophins in the cochlea (Ernfors et al., 1992; Pirvola et al., 1992; Avila et al., 1993; Tessarollo, 1993; Pirvola et al.,

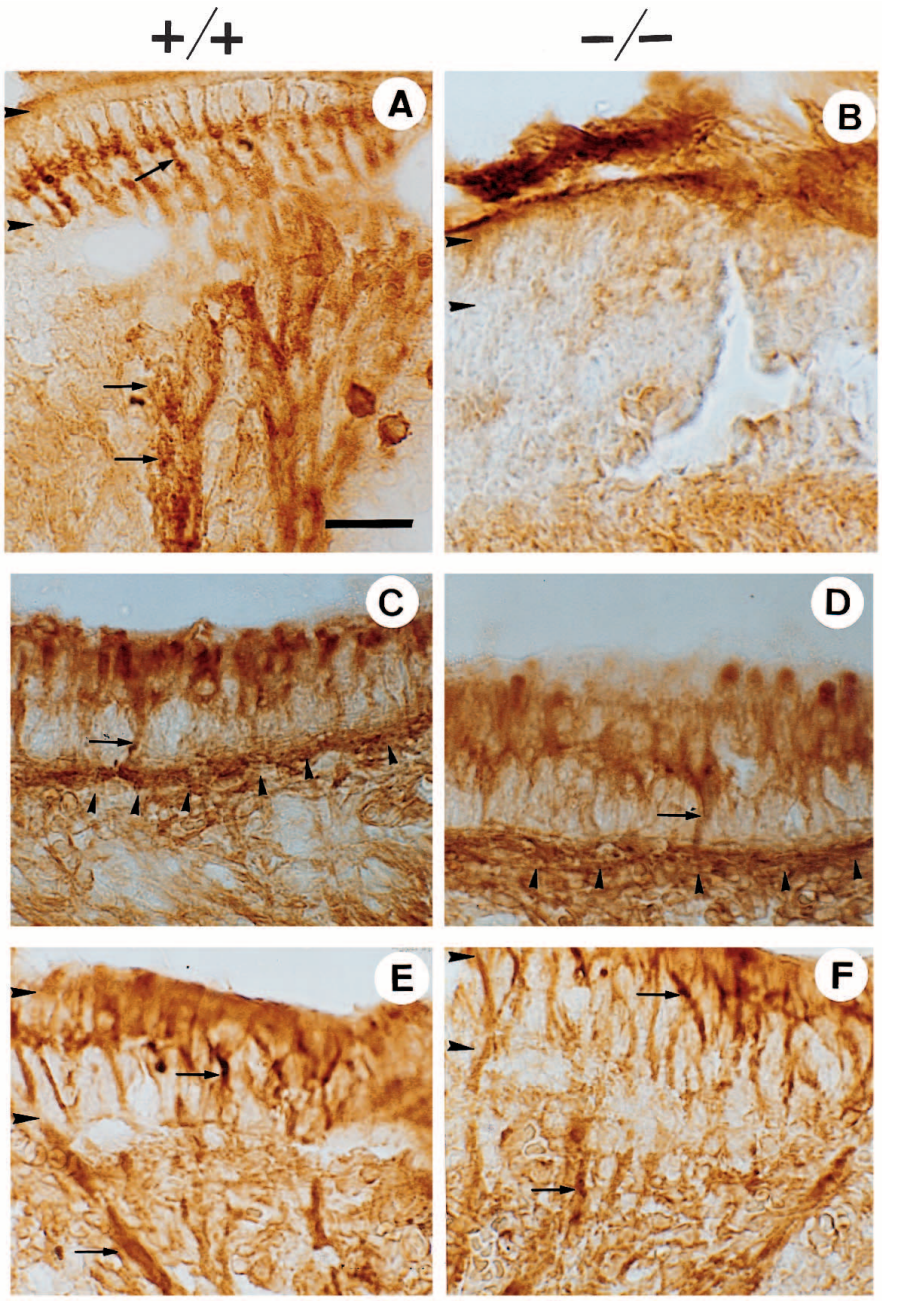

Fig. 7. Target innervation of *trkC* (–/–) mutants. Longitudinal sections at the level of inner (A,B,E,F) and outer hair cells (C,D) of wild-type (+/+) (A,C,E) and *trkC* (–/–) mice (B,D,F). Innervation of the sensory epithelium has been examined using a neurofilament antibody. (A,C) In P1 wild-type animals, nerve fibres reach and enter the sensory epithelium forming the typical radial (A) and spiral (C) nerve fiber systems. (B) The inner hair cells of P1 *trkC* (–/–) mice are devoid of innervation. Nerve fibres in A (arrows) and the sensory epithelium in A and B (arrowheads) are indicated. (D) The innervation pattern of the outer hair cells of P1 *trkC* (–/–) mice appears normal. A nerve fibre entering the sensory epithelium (arrow) and the underlying basal membrane (arrowheads) are indicated in C and D. In both, wild-type (E) and mutant *trkC* (–/–) (F) mice of day 16.5 p.c. nerve fibres (arrows) are detected underneath and within the sensory epithelium (arrowheads) at the level of the inner hair cells. Scale bar in A corresponds to 75 μm (A,B,E,F); 50 μm (C,D).

1994; Schecterson and Bothwell, 1994; Vazquez et al., 1994). Our analysis of the cochlear system of *trkC* (–/–) mutant mice shows that the number of neurons in the cochlear ganglion is reduced by 51%, due to the loss of large size type I neurons, which innervate inner hair cells with afferent fibres. *TrkC* (–/–) mice also revealed developmental defects in the cochlear sensory epithelium. The incomplete formation and differentiation of the epithelium may be a consequence of the innervation deficiences (Speidel, 1947, 1948; Guth, 1969).

TrkB (–/–) mutants specifically lack small size type II neurons which are responsible for the afferent innervation of the outer hair cells of the cochlea and therefore give a mirror image of the phenotype of *trkC* (–/–) mutants. In this context it will be important to confirm *trkB* and *trkC* expression in type II and type I neurons, respectively. Since no innervation of outer hair cells was detected in *trkB* (–/–) mice at P1, efferent fibres, which originate in the superior olivary complex and have reached the cochlea by P1 must have also been affected (Sobkowicz, 1992). Therefore, the modulation and feedback system of hearing, which controls the quality of hearing is most likely lost in *trkB* (–/–) mice.

While in the cochlear ganglion TrkB and TrkC receptors have been detected (Ernfors et al., 1992; Tessarollo et al., 1993; Pirvola et al., 1994; Schecterson and Bothwell, 1994; Vazquez et al., 1994) inner and outer hair cells express both BDNF and NT-3 during auditory system development (Pirvola et al., 1992; Wheeler et al., 1994). NT-3 (–/–) mutant mice lack 85% of their cochlear neurons (Fariñas et al., 1994). The neuronal population affected has not been characterized. The fact that NT-3 (–/–) mutant mice loose more neurons than *trkC* (–/–) mice may be explained by the ability of NT-3 to exert its function via the TrkB receptor (Ip et al., 1993). The extra population of neurons surviving in *trkC* (–/–) animals may be rescued by this mechanism. Interestingly, the entire cochlear system, including cochlear ganglion neurons and the innervation pattern of outer hair cells are phenotypically normal in BDNF (–/–) mutants (Ernfors et al., 1994a). Considering the lack of the second TrkB ligand, NT-4 (Pirvola et al., 1992, 1994; Schecterson and Bothwell, 1994), these data suggest that NT-3, which has been shown to bind the TrkB receptor (Ip et al., 1993), may compensate for the loss of BDNF. Finally, phenotypic differences found between ligand and receptor knockouts may also be explained by the presence of non-catalytic isoforms of *trkB* and *trkC* in *trkB* (–/–) and *trkC* (–/–) mice, whose functions remain unclear (Klein et al., 1990; Middlemas et al., 1991; Tsoulfas et al., 1993; Valenzuela et al., 1993).

TrkB and TrkC are dispensable for target encounter but essential for maintenance of target innervation

The time course of TrkB, TrkC, BDNF and NT3 expression in vivo and BDNF and NT3 response in vitro is tightly correlated with the development of

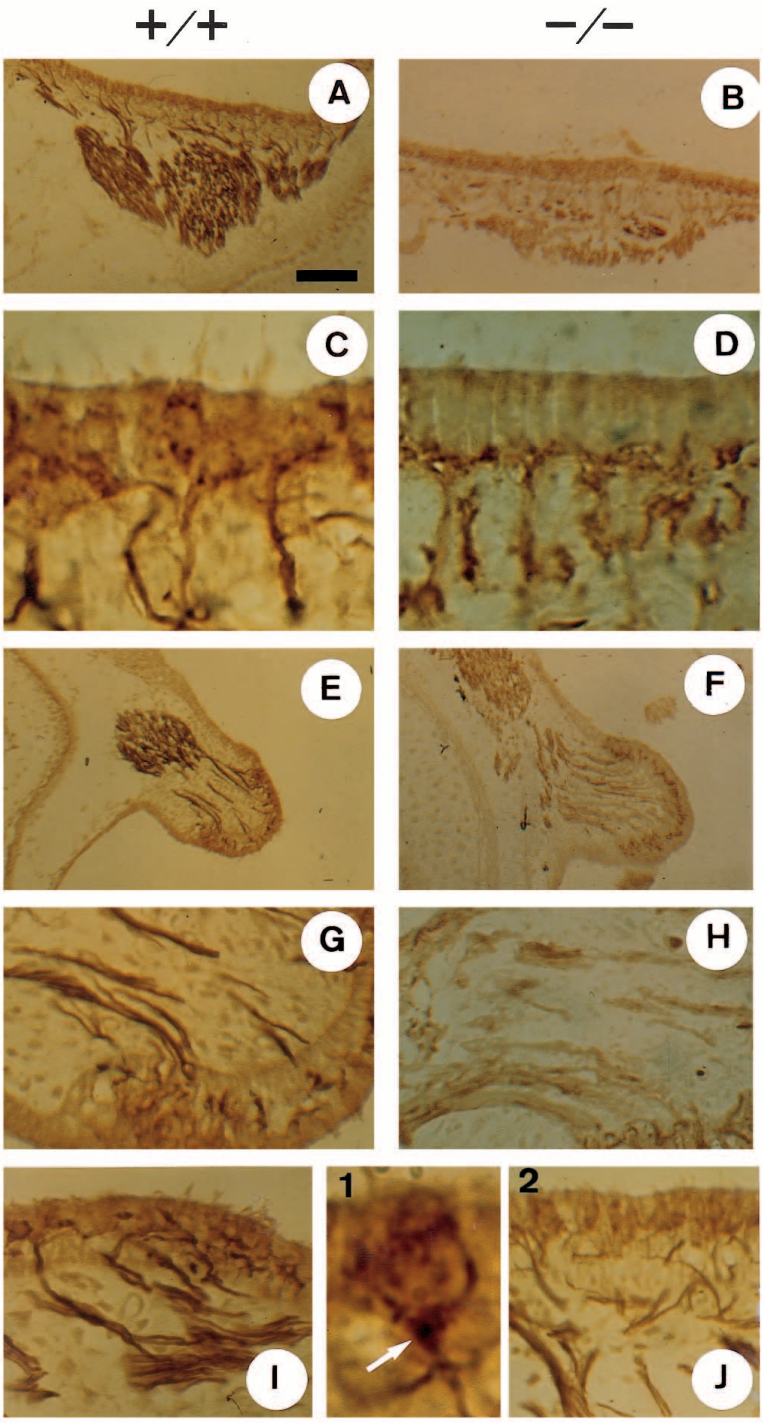

Fig. 8. Target innervation of *trkB* (–/–) mutants at day 18.5 p.c. Sections of the utricular macula (A-D), the ampullary crista (E-H) and the sensory epithelium of the cochlea containing outer hair cells (I,J) of wild-type (+/+) (A,C,E,G,I) and *trkB* (–/–) (B,D,F,H,J) mice of day 18.5 p.c. have been stained with a neurofilament antibody. In both, wild-type and *trkB* (–/–) animals nerve fibres are detected in the vestibular and cochlear system. High power magnifications of the sensory epithelia reveal that as in wild-type mice (C,G,I) nerve fibres reach and enter the epithelia in *trkB* (–/–) mutants (D,H,J). (J1) Typical image of an outer hair cell being innervated by a nerve fibre. The synaptic contact is indicated by an arrow. Scale bar in A corresponds to 250 μm (A,B,E,F); 50 μm (G,H,I,J2); 30 μm (C,D); 10 μm (J1).

inner ear afferent innervation, including the appearance and stabilization of presynaptic equipment (Pirvola et al., 1992, 1994; Tessarollo et al., 1993; Schecterson and Bothwell, 1994; Vazquez et al., 1994). This suggests a functionally relevant neurotrophin action during all these processes. Our results show that in the absence of a functional TrkB or TrkC receptor, nerve fibres can contact their peripheral targets. During later development neurite lysis and atrophy as well as synaptic disassembling is observed, leading to the observed neuronal cell death in vestibular and cochlear ganglia. Alternatively, loss of Trk signaling may trigger apoptosis which then leads to neurodegeneration. These data are consistent with current views about the relationship of target encounter to cell death saying that cranial sensory neurons survive independently of neurotrophins when their nerve fibres are growing and acquire neurotrophin dependence close to the time their neurites reach their targets (Davies and Lumsden, 1984; Vogel and Davies, 1991). Consequently, ganglion formation and afferent innervation may depend on the action of other yet unidentified growth factors in vivo.

In the inner ear ganglia of wild-type mice, *trkB* and *trkC* expression are maintained during postnatal development and adulthood (Ylikoski et al., 1993), possibly indicating a permanent role for innervation maintenance and/or neuronal survival. Consistent with this hypothesis, BDNF and NT-3 are expressed in the target fields of the ganglion cells during postnatal life (Ylikoski et al., 1993). Finally, neurotrophins have recently been found to regulate, among other properties, the synthesis of neuropeptides and the activity of voltage gated channels, the latter being crucial for neuronal survival (Ghosh et al., 1994; Snider et al., 1994). It will therefore be important to investigate, if the absence of functional TrkB receptors can cause additional phenotypic abnormalities in neurons.

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