Auditory Neuroscience, 1995, Vol 1, pp. 401–417
Reprints available directly from the publisher
Photocopying permitted by license only

Reduction and Loss of Inner Ear Innervation in *trk*B and *trk*C Receptor Knockout Mice: A Whole Mount DiI and Scanning Electron Microscopic Analysis

B. FRITZSCH, I. SILOS-SANTIAGO,* R. SMEYNE,† A. M. FAGAN,* and M. BARBACID*

Creighton University, Omaha, Nebraska 68104; Bristol-Myers Squibb, Pharmaceutical Research Institute Princeton, New Jersey 08543; and Roche Institute for Molecular Biology, Nutley, New Jersey 07110

(Received April 24, 1995; accepted July 11, 1995)

The pattern of afferent and efferent innervation of the ear as well as the degree of differentiation was investigated in embryonic and neonatal mice homozygotic for the *trk*B or *trk*C gene deletions or various combinations of homozygosity and heterozygosity of both neurotrophin receptor knockouts (*trk*B$^{-/-}$/*trk*C$^{-/-}$, *trk*B$^{-/-}$/*trk*C$^{+/-}$, *trk*B$^{+/-}$/*trk*C$^{-/-}$). Mice were fixed by cardiac perfusion with 4% paraformaldehyde in phosphate-buffered saline, and DiI soaked filter strips were inserted at various positions in the ear or the brain to label afferent or efferent projections. *trk*B$^{-/-}$ mice developed innervation to all sensory epithelia by 12.5 days postcortum (dpc) but lost all innervation of sensory epithelia of the semicircular canals by 13.5 dpc. Furthermore, the utricle and the utricular branch to the saccule showed a pronounced reduction in both afferent and efferent innervation. The vestibular ganglion was reduced to 35% of its volume by 17.5 dpc. Efferent innervation to the vestibular region developed with a 1 day delay and was always confined to areas that had an afferent innervation. Neither cochlear afferent nor efferent innervation showed gross abnormalities. *trk*C$^{-/-}$ mice showed no alteration in the vestibular region and only minor effects in the cochlea: the basal turn showed a reduction of innervation density to both inner and outer hair cells. In addition, the efferent system showed many fewer intraganglionic and inner spiral fibers at birth. *trk*B$^{-/-}$/*trk*C$^{-/-}$ double knockout mice had no innervation to any sensory epithelium by postnatal day 0 (P0). Mice homozygotic for *trk*B$^{-/-}$ and heterozygotic for *trk*C$^{+/-}$ displayed the *trk*B$^{-/-}$ vestibular phenotype but showed only a patchy innervation of the cochlea by very few spiral ganglion cells. In contrast, mice heterozygotic for *trk*B$^{+/-}$ and homozygotic for *trk*C$^{-/-}$ showed only the phenotype of a simple *trk*C$^{-/-}$ mutant. Together, these data suggest that neither of these two neurotrophin receptors is important for initial fiber outgrowth to all sensory epithelia of the ear but that TrkB and TrkC are both necessary for later survival of ganglion cells. TrkB signaling plays a major role for the survival of sensory neurons in the vestibular ganglion and TrkC signaling for cochlear ganglia. The delayed outgrowth and reduction of efferent innervation may come through impaired pathfinding along the lethally damaged afferents.

Key words: Ear development, neurotrophin receptor knockouts, TrkB, TrkC, ganglion cell survival, efferent development

BASED PREDOMINANTLY ON HIS STUDIES of ear development, Ramon y Cajal (1960) suggested that neurotropic activity attracts wandering fibers to the sensory epithelia of the ear. This chemotropic hypothesis thus created has received much support for the ear in recent years based on data generated from co-cultivation of the ear and the otic ganglion (Hemond and Morest, 1992; van de Water *et al.*, 1992; Bianchi and Cohan, 1993; Staecker *et al.*, 1994). However, owing to the various neurotrophins expressed in the developing ear, the major factor for this neurotropic action has not yet been identified, nor is it known whether it is distinct from the neurotrophic action generally ascribed to a set of related molecules (Snider, 1994).

Among the numerous neurotrophic and putative neurotropic factors discovered in the developing ear, two, brain-derived neurotrophic factor (BDNF) and neurotrophin-3 (NT-3), show a partially overlapping distribution in the vestibular and cochlear sensory epithelia of the ear of rats (Pirvola *et al.*, 1992, 1994; Schecterson and Bothwell, 1992, 1994; Wheeler *et al.*, 1994). In addi-

*Corresponding author: B. Fritzsch, Ph.D., Creighton University, Department of Biomedical Sciences, Omaha, NE 68178.

tion, messenger RNA for the appropriate receptors (*trk*B for BDNF and *trk*C for NT-3) are expressed in the otic (statoacoustic) ganglion (Ernfors *et al.*, 1992; Ylikoski *et al.*, 1993; Pirvola *et al.*, 1994) (see Fig. 1b). Some *in vitro* studies have generated evidence suggesting that BDNF and NT-3 may be the likely neurotrophic and neurotropic factors produced by the developing ear (Pirvola *et al.*, 1992; van de Water *et al.*, 1992; Staecker *et al.*, 1994). Other *in vitro* data suggest that additional factor or factors may be necessary to attract the growing neurites of the otic ganglion (Bianchi and Cohan, 1993). At least the neurotrophic role for these factors was recently confirmed in mice lacking functional genes for the neurotrophins BDNF or NT-3. BDNF knockout mice were almost completely devoid of any innervation in all vestibular sensory epithelia and had a reduced number of vestibular ganglion cells (Ernfors *et al.*, 1994). In contrast, NT-3 knockouts showed a severe reduction in cochlear spiral ganglion cells (Farinas *et al.*, 1994). Unfortunately, these data do not fully agree with the known distribution of NT-3 and BDNF in the ear. This distribution would suggest that the semicircular canals, which express only BDNF, should be more affected than the other vestibular epithelia in BDNF knockouts, if BDNF absence is not compensated for by NT-3, as is suggested for the central nervous system (CNS) (Silos-Santiago *et al.*, 1995).

We have further tested this neurotrophic hypothesis of vestibular and cochlear ganglion cell survival by examining the innervation pattern of mice homozygous for a targeted gene disruption of the BDNF receptor, *trk*B (Klein *et al.*, 1993), the NT-3 receptor *trk*C (Klein *et al.*, 1994), and in *trk*B/C double knockout mice. Our data suggest that the two different receptors (Ylikoski *et al.*, 1993; Pirvola *et al.*, 1994) expressed by the afferents to the ears of mice are both necessary to mediate the trophic support of afferent and efferent fibers but play no role for the neurotropic effect exerted by the developing ear.

MATERIAL AND METHODS

Transgenic Mice

Mice heterozygous for *trk*B and *trk*C were generated from timed matings of heterozygous mice as described previously (Klein *et al.*, 1993, 1994) to obtain mice homozygous for *trk* gene deletion. The day of the vaginal plug was considered to be 0.5 days postcortum (dpc). Double homozygous mutants and animals with various combinations of homozygosity of one receptor combined with heterozygosity of the other receptor were obtained by crossing mice heterozygotic for both *trk*B and *trk*C.

trk$B^{-/-}$

A total of 21 *trk*$B^{-/-}$ embryos and newborns were analyzed using implantation of 1,1'-dioctadecyl-3,3,3',3'-teramethylindocarbocyanine perchlorate (DiI; Molecular Probes) into the ear or the brainstem of these aldehyde-fixed embryos and pups (four animals at 12.5, three animals each at 13.5 and 16.5, six animals at 17.5 dpc and five newborns [P0]).

trk$C^{-/-}$

We investigated 3 P0, two P9, and two 1 year old *trk*$C^{-/-}$ animals.

trk$B^{-/-}/C^{-/-}$

One neonate double knockout mice (*trk*$B^{-/-}$/*trk*$C^{-/-}$) was available.

trk$B^{-/-}/C^{-/+}$

Seven neonatal animals of this genotype were available.

trk$B^{-/+}/C^{-/-}$

Three neonatal mice of this genotype were available.

trk$B^{+/-}/C^{+/-}$

Five P0 mice were available.

Unless otherwise noted, wildtype or heterozygotic littermates served as controls.

Fixation and Tissue Processing

The litters of defined developmental stages younger than 16.5 dpc were fixed by immersion in 4% paraformaldehyde in phosphate-buffered saline, and all older animals were fixed by perfusion through the heart. The animals were then stored cold (4°C) in this fixative until further use. The care and the use of the animals in this study were in agreement with the guidelines of the Declaration of Helsinki and were approved by the Animal Care Committee of Bristol-Myers Squibb where all these manipulations were performed.

DiI implantation

An analysis of afferent and efferent connections of the ear was performed as previously described (Fritzsch and Nichols, 1993). Briefly, DiI was dissolved in methanol and filter strips were soaked in this solution. After the strips were dry they were applied to cuts in either the alar plate (for afferent labeling), the basal plate (for efferent labeling), the statoacoustic nerve (for afferent and efferent labeling), or into the cochlea (for retrograde filling of the efferents and intrinsic cochlear connections). After appropriate diffusion time (3 to 8 days at 36°C) the ears, with the otic ganglion and the facial nerve attached, were dissected free from adjacent periotic mesenchyme and viewed as whole mounts with an Olympus compound microscope. Some of the images were captured and processed to enhance the resolution

using the Waytek deconvolution algorithm. Volumetric measures of the otic ganglion were performed on these captured images using the ImagePro software.

After viewing for DiI fluorescence, some ears and ganglia were embedded in gelatin and sectioned frozen at 20 to 60 μm. Some sections were processed for silver staining of nerve fibers as previously described (Fritzsch and Zakon, 1988). Others were counterstained with cresyl violet for cellular cytoarchitecture. The sections and ears were then dehydrated, mounted in Permount and viewed using an Olympus compound microscope.

Other ears were processed for scanning electron microscopy (SEM). In brief, for SEM, the ears were critical point dried, coated with gold-paladium, and viewed and photographed with a Hitachi scanning microscope.

RESULTS

To help understand the organization of ear innervation that we show here as whole mounts, we will first describe the pattern of innervation of an ear as seen in wildtype 14 dpc mouse (Fig. 1a). We will subsequently describe all *trk*B knockouts in comparison with the wild types in chronological order, then the *trk*C knockouts followed by the *trk*B/C double knockouts and the homozygote/heterozygote combination animals. We will always describe the afferents first and then the efferents as revealed with DiI or silver stain followed by the SEM analysis.

After implanting DiI into the efferent vestibulocochlear bundle near the internal facial genu, the whole mounted ear of a 14 dpc mouse shows three major fiber tracts of centrifugally labeled efferent fibers (Fig. 1a). Most dorsoanterior are the fibers passing through the vestibular foramen to the utricle, anterior vertical canal (AC), horizontal canal (HC), and the vestibular part of the saccule. The most posterior and ventral cochlear foramen leads efferent fibers to the posterior vertical canal (PC), the cochlea and the cochlear part of the saccule. The most anterior foramen leads the combined visceral and branchial motor fibers of the facial nerve to the external genu of the facial nerve.

trk$B^{-/-}$ *Effects*

12.5 dpc. At this stage, all future sensory epithelia receive afferent innervation. All four *trk*$B^{-/-}$ ears also had afferent innervation to all sensory epithelia of the ear except for one animal in which the afferent fibers did not reach the posterior vertical canal (Figs. 2,3). This latter animal was slightly advanced in its development, whereas the others were slightly delayed. According to a staging table, they would agree with 12 and 13 dpc animals (Theiler, 1989). Compared to control littermates the innervation to the PC was, if at all present, much reduced (Figs. 2,3). The innervation to the AC and HC showed abberations and an apparent reduction in particular in the most advanced specimen (Figs. 2,3).

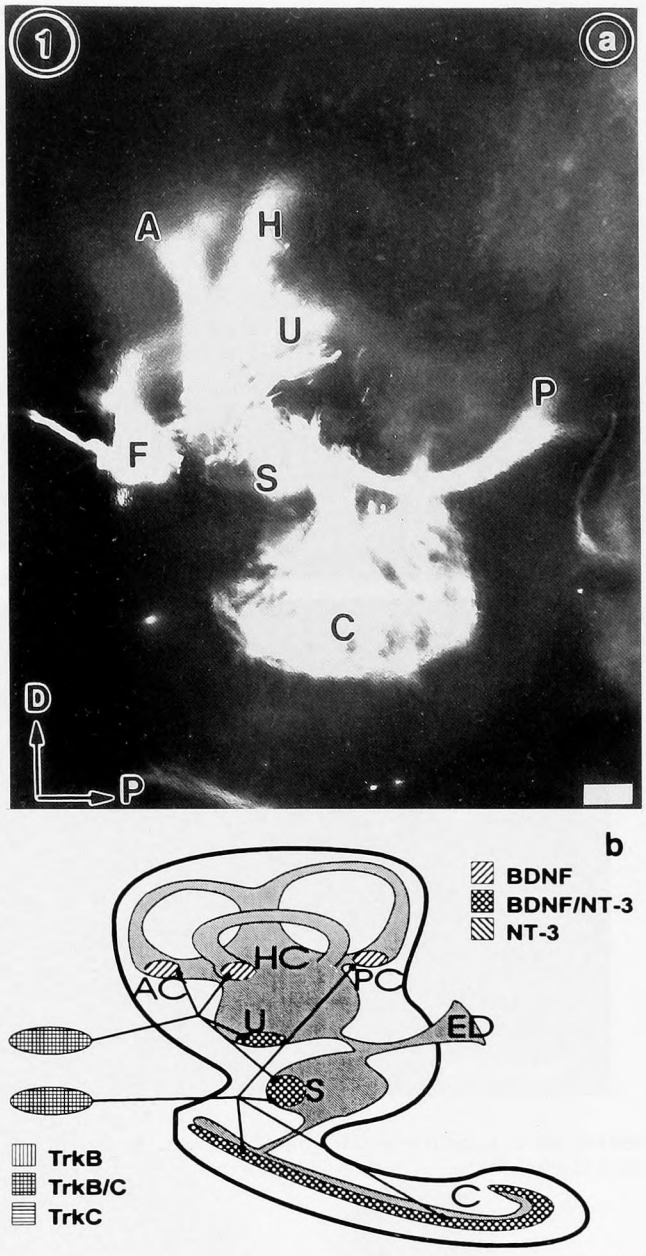

FIGURE 1 **The right ear of a 14 dpc control mouse is shown in this epifluorescence photograph with efferent fibers labeled with DiI from the basal plate (a) and the distribution of neurotrophins and their receptors (b).** The vestibular ganglion and the intermediate nerve have been removed for clarity. Three major fiber tracts are obvious. The most rostral one is the facial nerve (F), the most caudal and ventral one goes to the cochlea (C), the posterior vertical canal (PC) and the saccule (S). In between these two roots is the vestibular root to the utricle (U), the anterior vertical canal (AC) and the horizontal canal (HC). Arrows indicate posterior (P) and dorsal (D). Bar equals 100 μm. The scheme (b) is derived from the work of Pirvola *et al.* (1992, 1994) and shows the discrete distribution of BDNF in the semicircular sensory epithelia, the overlapping distribution of BDNF and NT-3 in all other sensory epithelia, and the likely overlapping distribution of trkB and trkC in the vestibular and cochlear ganglion cells. ED = endolymphatic duct.

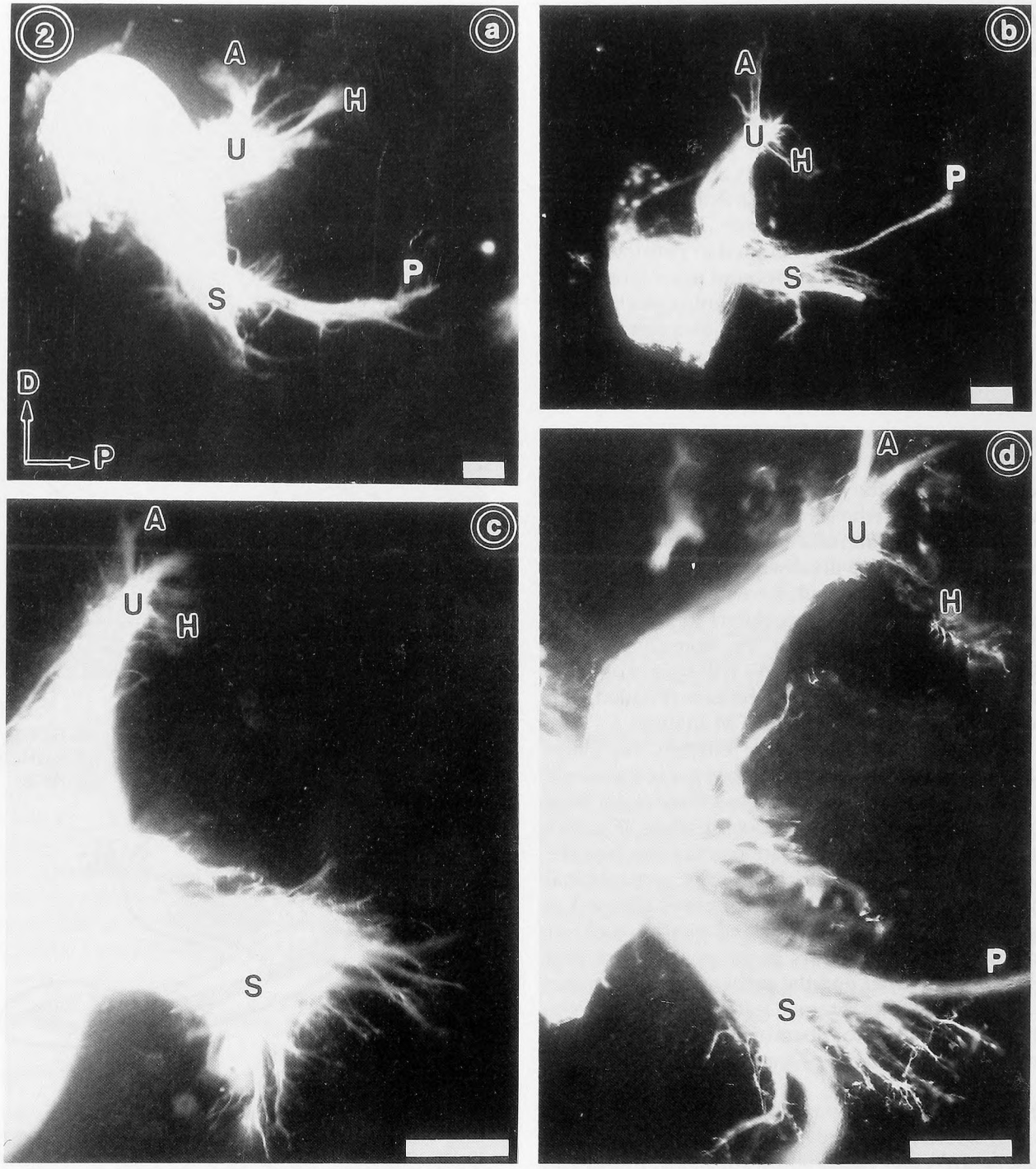

FIGURE 2 **The right ears of 12.5 dpc *trk*B$^{-/-}$ (b,c,d) and one control littermate (a) are shown.** Notice that in all mutants there are fewer fibers extending to the utricle (U) and fibers to semicircular canals (A,H,P) are either reduced (b,d) or absent (no P in c). In contrast fiber outgrowth to the saccule (S) and the cochlea shows much less effect. Arrows indicate posterior (P) and dorsal (D). Bar equals 100 μm.

Higher power analysis of the growth cones showed that the *trk*B knockouts had fewer and less sophisticated growth cones extending toward the semicircular canals (Fig. 3). In summary, there was a remarkable reduction in the innervation of all semicircular canals, whereas all other sensory epithelial showed much less pronounced effects.

By putting DiI into rhombomere 4 of the brainstem near the floor plate, we labeled exclusively the efferent innervation. In all control animals we observed massive fiber outgrowth throughout the vestibular ganglion toward the cochlea and the vestibular sensory epithelia. Growth cones of these efferent fibers were rather simple enlargements with few, if any, filopodia (Fig. 4a,b). In

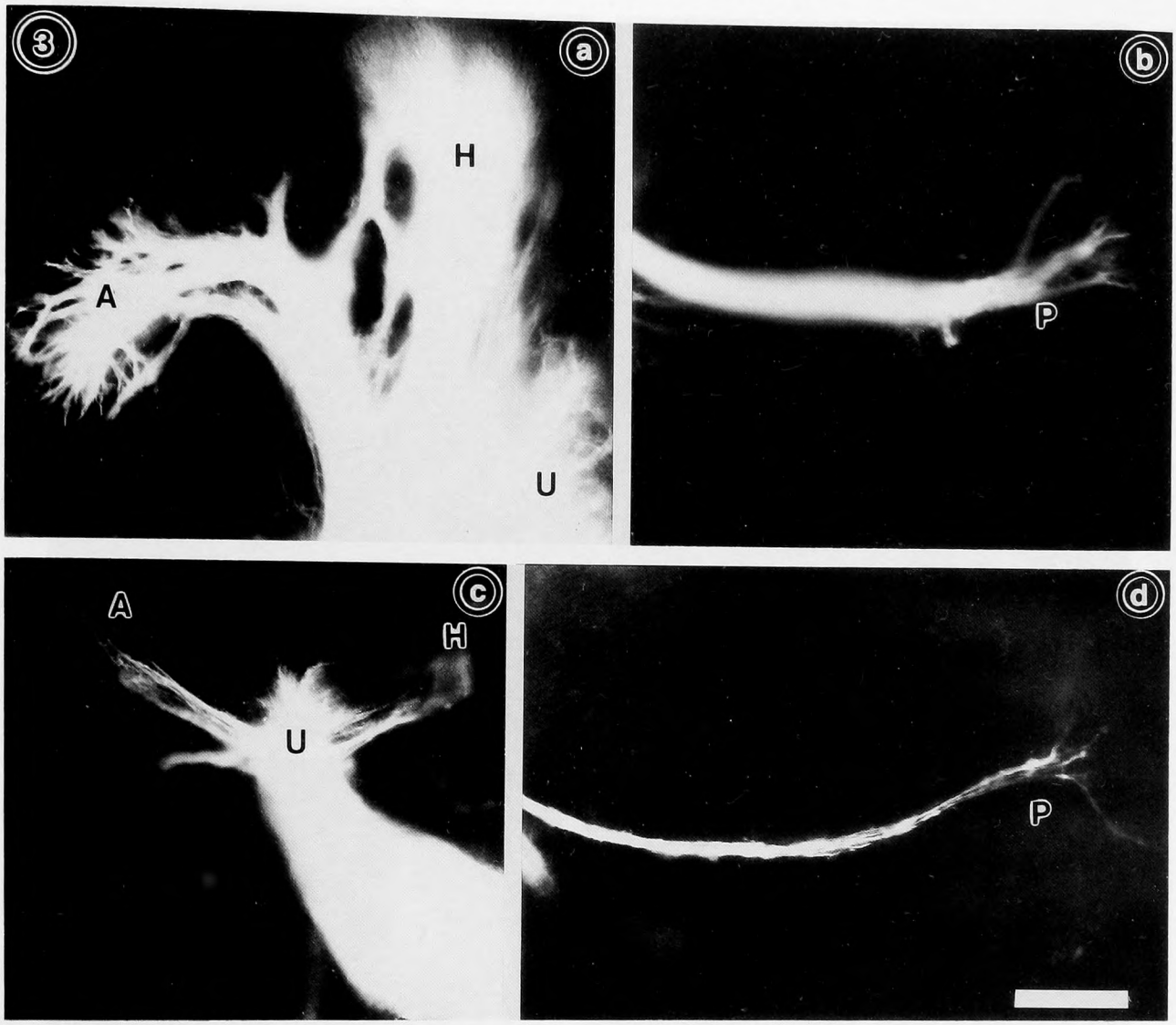

FIGURE 3 **Higher power epifluorescence micrographs that show the enormous difference in density of innervation of vestibular sensory epithelia at 12.5 dpc control littermates (a,b) and *trk*B$^{-/-}$ knockouts (c,d).** Nevertheless, some fibers clearly reach the area of semicircular sensory epithelia (A,H,P). Bar in d equals 100 μm for all micrographs.

contrast, three of four *trk*B$^{-/-}$ cases showed not a single fiber growing toward the vestibular sensory epithelia but a robust outgrowth of facial motoneurons and of efferent fibers to the cochlea and fewer toward the saccule (Fig. 4c,d). Growth cones of these fibers were much more complex, with numerous filopodia extending from the enlarged growth cone (Fig. 4d). Only one case showed a few fibers growing toward the utricle. Although no formal counting was performed, the efferent outgrowth to the cochlea was in most cases as far and almost as robust in the *trk*B$^{-/-}$ as in the control littermates (Fig. 4a,c).

13.5 dpc. When afferents in wild type animals were labeled with DiI through application to the ascending tract in the brain, it revealed innervation to all sensory epithelia with fibers approaching hair cells. The AC and HC innervation was clearly distinct from the utricle (Fig. 5b). In contrast, two of three 13.5 dpc *trk*B$^{-/-}$ had no innervation to all three semicircular canals (Fig. 5a). However, they had strong innervation of all other sensory epithelia of the ear. In the third animal, which appeared to be slightly less developed than its littermates based on the development of the auricle and the limbs (Theiler, 1989), there were a few fibers extending in the direction of the HC and AC from the utricle, but they did not reach them (Fig. 5a). The pattern of innervation of the utricle in this and all older animals showed a peculiar paucity compared with the utricle of control littermates (Fig. 5a,b). The saccule showed only a rare fiber entering the epithelium from the vestibular branch. In contrast, fibers running with the cochlear branch to the saccule showed little if any reduction compared to control animals (data now shown).

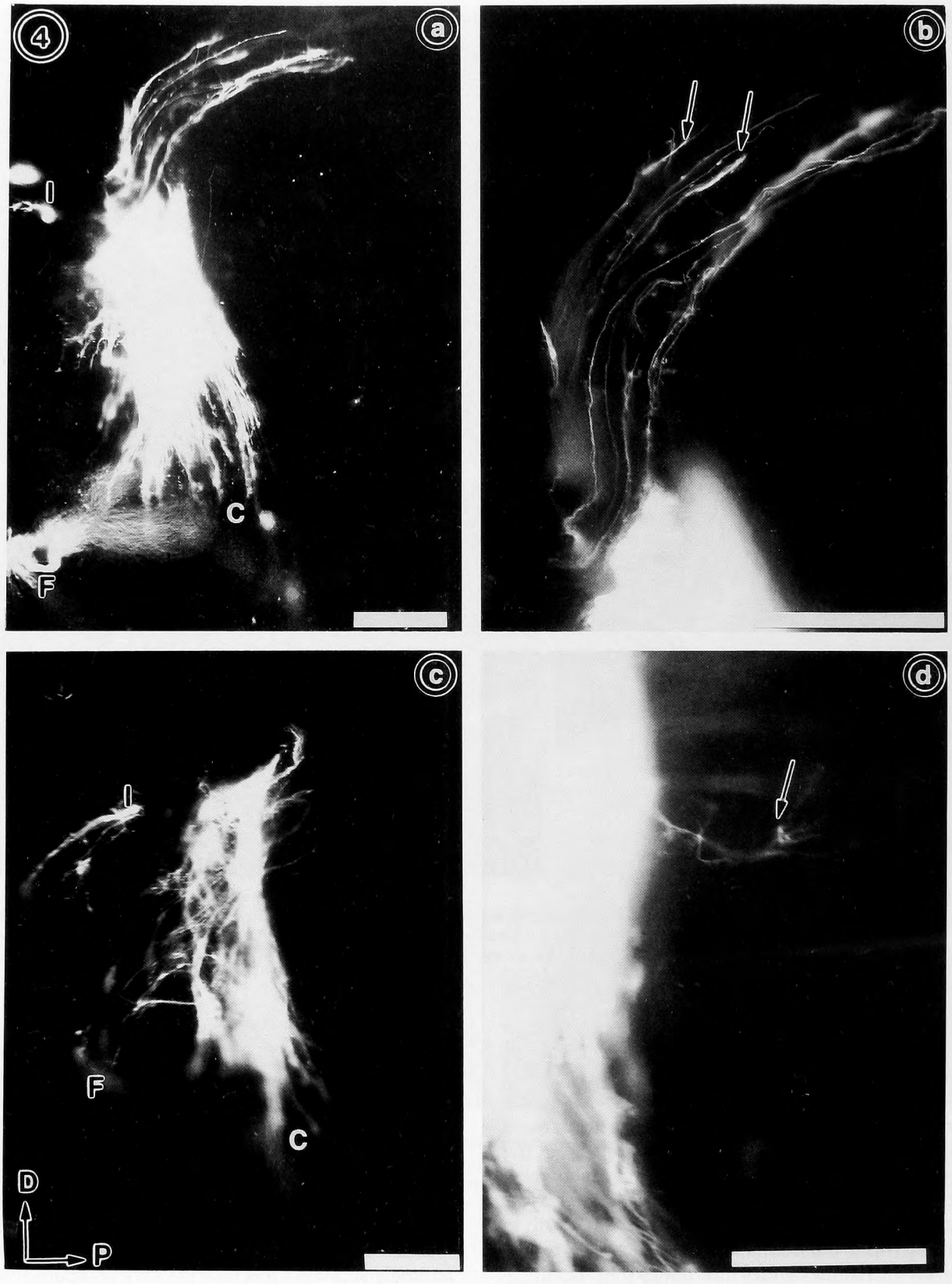

FIGURE 4 **The outgrowth of efferent fibers in the vestibular ganglion and toward the sensory epithelia as revealed with DiI is shown in 12.5 dpc and control littermates (a,b) and *trk*B$^{-/-}$ (c,d).** Although there is a massive fiber outgrowth from the brain to the vestibular ganglion toward the developing cochlea (C) in both mutant (c) and littermates (a), fibers reach only in control littermates into the vestibular root (a,c). Growth cones of efferents in the littermates are rather simple (b, arrows) whereas they are more complex in the mutants (d, arrow). I indicates intermediate nerve, F the branchiomotor part of the facial nerve. Bar equals 100 μm.

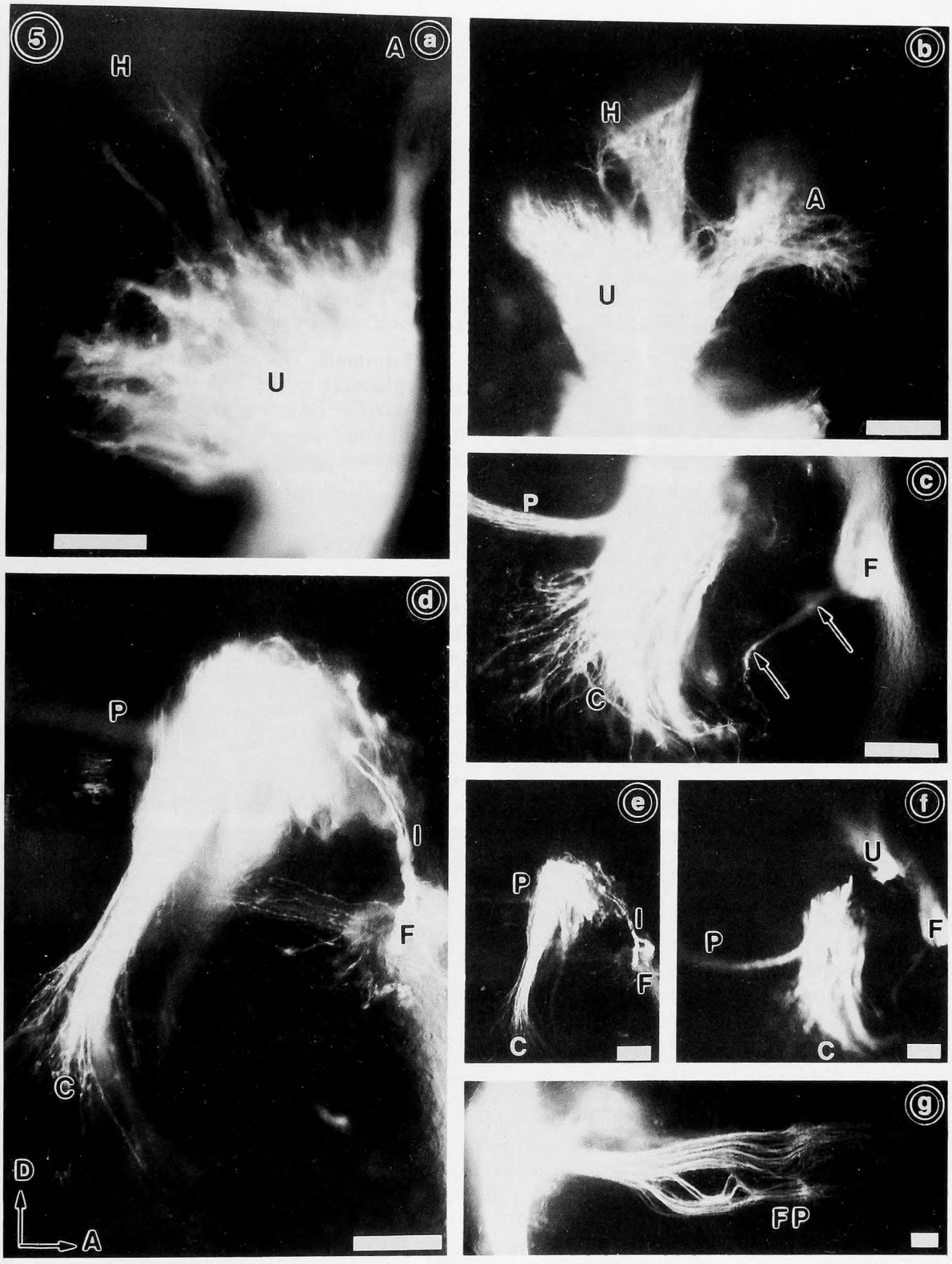

FIGURE 5 **The outgrowth of afferents (a,b) and efferents (c,d,e,f) and retrogradely filed efferent axons in the brainstem (g) are shown for 13.5 dpc *trk*B$^{-/-}$ (a,d,e,g) and control littermates (b,c,f).** Whereas afferents to the utricle (U) and the horizontal (H) and anterior vertical canal (A) have further matured in control littermates (b), there are virtually no fibers to the semicircular canals left in the mutant, and the utricle receives a less dense innervation (a). Likewise, although efferent fibers have reached all epithelia in littermates (c,f), there are no efferent fibers to the semicircular canals or the utricle in the mutant (d,e). The development of facial branchial (F) and visceral motoneurons (I) seems to be little effected at this developmental stage. Retrograde filling shows that the initial bilateral distribution of efferent axons with a crossing in the floor plate (FP) of rhombomere 4 (g) shows no deviation from published data. Arrows indicate anterior (A) and dorsal (D). Bar equals 100 μm.

In control animals, efferents extended to the cochlea, all semicircular canals, utricle, and saccule (Fig. 5c,f). In the *trk*B knockout mice the efferents to the cochlea were about as advanced as in control littermates, but appeared less numerous (Fig. 5d,e). The efferent innervation also showed no fibers reaching the semicircular canals and very few fibers entering the vestibular root (Fig. 5d,e). Efferents to the facial nerve, both the branchial and the visceral fibers in the intermediate nerve, were as well developed in the mutants as in the control animals (Fig. 5c–f). Retrograde filling of the efferents in the brainstem from the vestibulocochlear nerve displayed a pattern of fibers crossing the floor plate as previously described (Fritzsch and Nichols, 1993) in same aged control animals (Fig. 5g). Owing to the incomplete segregation of vestibular and cochlear efferents, it remains unclear whether vestibular efferents show any numerical reduction at this stage.

16.5 dpc. In control animals all sensory epithelia receive innervation and fibers extend to hair cells. In contrast, two of three 16.5 dpc *trk*B$^{-/-}$ mice had no innervation to the semicircular canals, and one had only a very few fibers directed toward the horizontal canal (data not shown). In addition, there was diminished innervation of the utricle and the fibers were less directed in their course. The saccule also showed reduced innervation from the vestibular branch, whereas the cochlear branch to the saccule, as well as the cochlea itself, showed no apparent deficits in the innervation. No separate analysis of the efferent innervation was performed at this stage. The ears were sectioned and processed for silver stain, which confirmed the data obtained with DiI, that is, no fibers to the semicircular canals, some fibers to the utricle and saccule, and substantial fibers to the cochlea (data not shown).

17.5 dpc. Compared with wild type mice (Fig. 6a), all six 17.5 dpc *trk*B$^{-/-}$ had no afferent or efferent innervation of any semicircular canal (Fig. 6b,e). The innervation to the utricle showed the same differences noted previously: many fewer fibers were present and they had a different course underneath the epithelium (Fig. 6b,f). A detailed analysis using a computerized reconstruction showed a large reduction in fibers that fanned out from the thin layer of fibers. However, the remaining fibers branched profusely and may reach all hair cells (Fig. 6f). As in the younger stages, there was a reduction of the vestibular innervation to the saccule, but the fibers to the cochlear side of the saccule appeared normal (data not shown, but see Fig. 11a). The innervation to the cochlea appeared superficially normal with only some minute differences in the spacing of radial fibers (Fig. 7a,b,d). However, fibers to the outer hair cells appeared less well developed in the *trk*B$^{-/-}$ mice compared with littermates.

The efferent innervation to the utricle consisted of very few fibers that radiated in a pattern closely comparable to the afferent innervation (Fig. 6b,e). In contrast, there was no apparent difference in the efferent innervation to the cochlea, which formed a well-organized intraganglionic fiber bundle with radial fibers extending to the inner and occasionally the outer hair cells (Fig. 7c). Analysis of three control and three *trk*B$^{-/-}$ mice showed that the area of the vestibular ganglion was much reduced (35%, SD = 4, n = 3; Fig. 6c,d). Individual ganglion cells were also much smaller. In addition, we found many more cells in the smaller vestibular ganglion of the mutants than in the control that showed disintegrated nuclear chromatin (217 *trk*B$^{-/-}$ and 7 in control mice; n = 3). Thus, at 17.5 dpc the ongoing natural and *trk*B$^{-/-}$-induced cell death was not yet completed.

PO Animals. The pattern of innervation was essentially identical to the 17.5 dpc animals and the PO *trk*B$^{-/-}$/*trk*C$^{+/-}$ described later. In brief, both efferent and afferent innervation were absent from all semicircular canals, whereas the utricle received reduced innervation, as did the saccule.

trkC$^{-/-}$ *Effects*

PO mice. In all three mutants and the control littermate DiI was inserted into the cochlear and vestibular nerve, so both afferents and efferents were labeled simultaneously. In all three *trk*C knockout neonates there were no large-scale defects apparent in the innervation to the ear. In particular, the vestibular sensory epithelia showed no apparent deviation from the normal pattern of innervation. However, there was an apparent reduction of the density of innervation to the hair cells of the cochlea. Instead of radial bundles being extremely dense and straight to the inner hair cells (Fig. 8e), we found anastomosis between radial fibers that, in addition, extended to hair cells considerable distances away from the original course of the radial fibers. This phenomenon was particularly obvious in the basal turn of the cochlea, where we observed many fewer radial bundles that branched and supplied a much larger area of the cochlea than normal. There was also an apparent reduction in spiral ganglia cells (Fig. 8a,b). In addition to a reduction of the density of inner hair cell innervation, there was also many fewer type II ganglion cell fibers projecting to the three rows of outer hair cells in the mutant (Fig. 8a,b).

P9 mice. Two *trk*C$^{-/-}$ and two control littermates were analyzed. As in the younger stages, there was no deficit in the innervation to the vestibular sensory epithelia, and the cochlea showed only a reduction in the basal turn, with fewer radial bundles supplying most of the basal turn rather than many parallel bundles (Fig. 8c,e). Closer examination of the innervation of the outer and inner hair cells showed that the innervation to both was somewhat reduced (Fig. 8d,f). We therefore inserted DiI into one turn of the cochlea to examine the pattern of efferent innervation in the spiral bundles. Surprisingly, we could not detect intraganglionic spiral bundles and very few inner

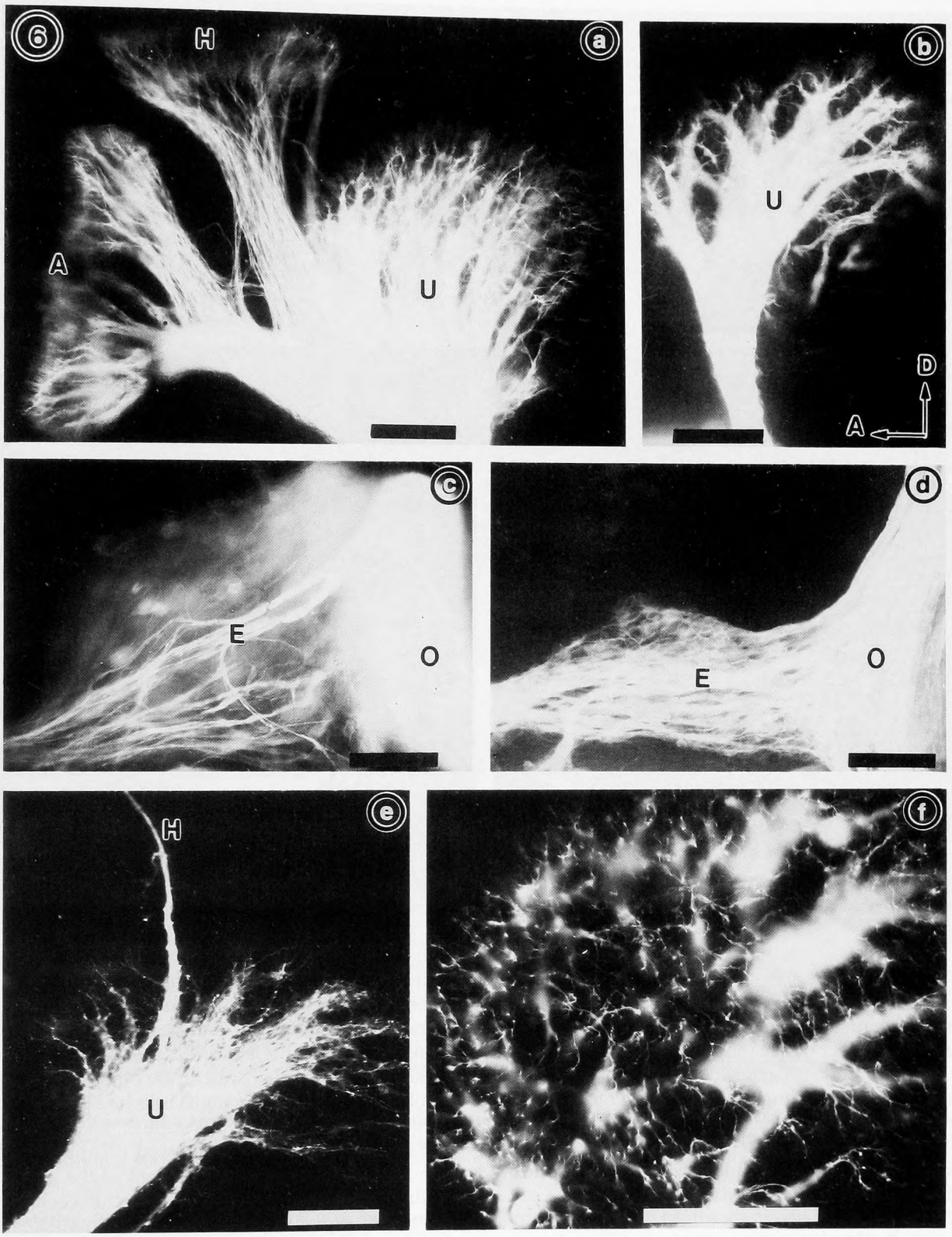

FIGURE 6 **The pattern of afferent (a,b,e,f) and efferent (e) innervation and of the vestibular ganglion (c,d) is shown for 17.5 dpc *trk*B$^{-/-}$ (b,d,e,f) and control littermates (a,c).** Note the reduced innervation of both afferents (b,f) and efferents (e) exclusively to the utricle in mutant. In contrast, control littermates have a more prominent innervation also of the semicircular canal cristae (A,H;a). In many mutants we found a few efferent fibers (e) extending toward the horizontal canal (H) but they did not reach the hair cells. The whole-mounted vestibular ganglia (c,d) show the course of efferent fibers (E) through it and the commissure of Oort (O) leading to the cochlea. Note the reduction of size of the mutant ganglion (d). Bar equals 100 μm.

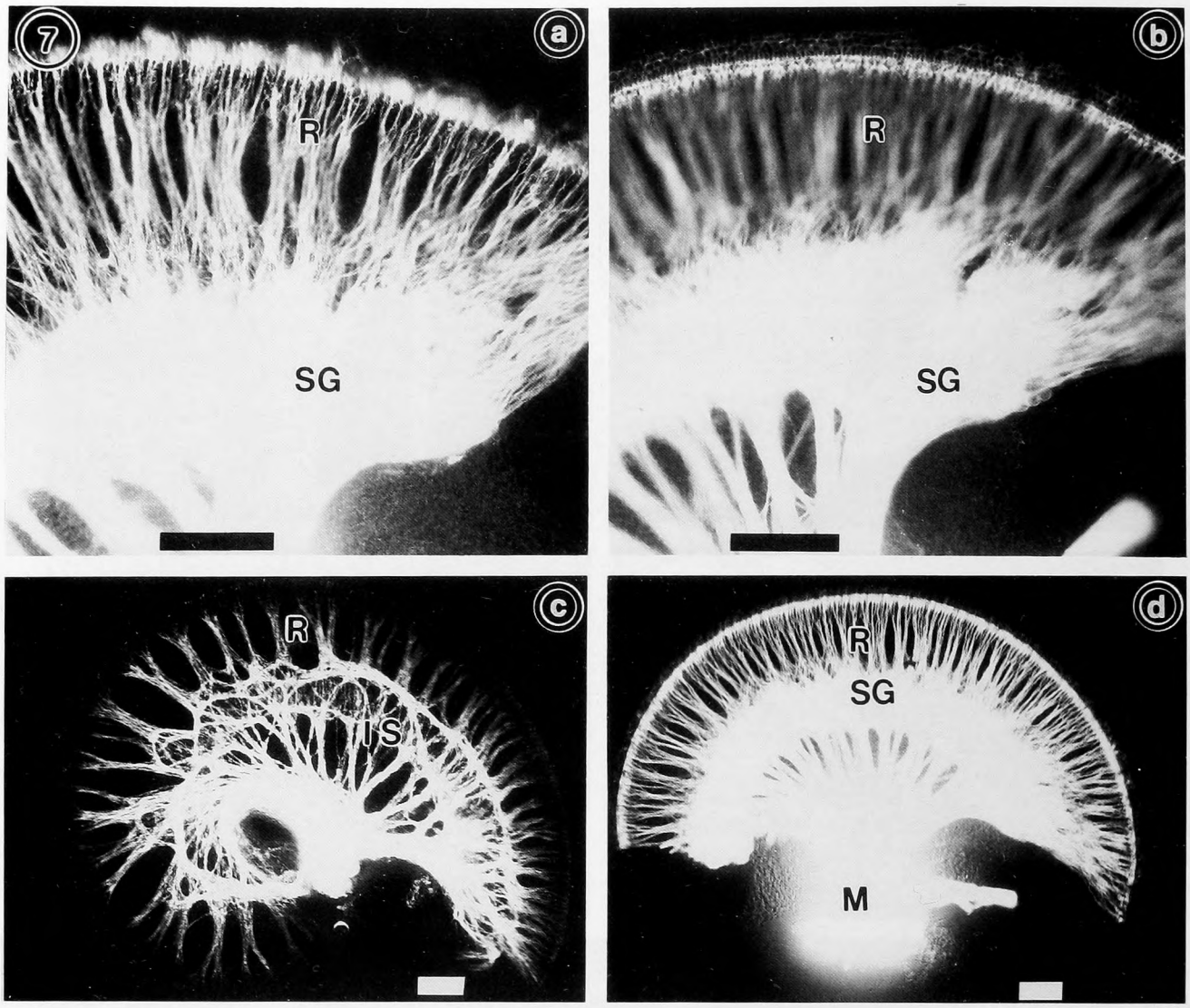

FIGURE 7 **These micrographs show the development of afferent (a,b,d) and efferent innervation (c) to the cochlea in 17.5 dpc *trk*B$^{-/-}$ (a,c) and control littermates (b,d).** Note the well-developed spiral ganglia (SG) and the radial fibers (R) extending to the hair cells. The efferents (c) form the complicated intraganglionic spiral (IS) fibers and extend as radial fibers to the inner hair cells. M = madiolus. Bar equals 100 μm.

spiral bundles (Fig. 9a,c), both of which could be labeled easily in control animals (Fig. 9b,d). This suggests that the efferent innervation to the cochlea is reduced and remains so even up to 1 year of age (Fig. 9e). In addition, we labeled spiral ganglion cells outside the area of DiI application, confirming the impression that the radial fibers were not as topologically restricted as in control animals (Fig. 9a,b). Younger stages of *trk*C knockouts, (such as 12.5 dpc) should be examined to identify the time at which deficits of the cochlear efferents appear.

trk*B and* trkC *Double Mutants*

PO **trk*B*$^{-/-}$/trk*C*$^{-/-}$.** This double mutant completely lacked innervation to the ear. A facial nerve was present, as was an indentation in the wall of the otocyst where the vestibular ganglion should be (data not shown). Close examination of the otic capsule showed only the openings of the perilymphatic and endolymphatic system (oval and round window, cochlear and vestibular aqueduct) and small openings for the labyrinthine vessels. The empty depression in the medial wall of the otocyst suggests that vestibular and cochlear ganglia cells had probably developed and had all died by this stage of development.

Examination with SEM showed no apparent deviation from the normal development of the sensory epithelia (Fig. 10b). All aspects of the cochlea were developed equally to the control littermate, including the delayed maturation of hair cells in the apex (Fig. 10c,d). In contrast to control animals, there were no spiral ganglion fibers or cells in the modiolus (Fig. 10a). Thus, development of the ear proceeded normally in pace and structural maturation even in the absence of ganglion cells in these animals at PO. We are currently

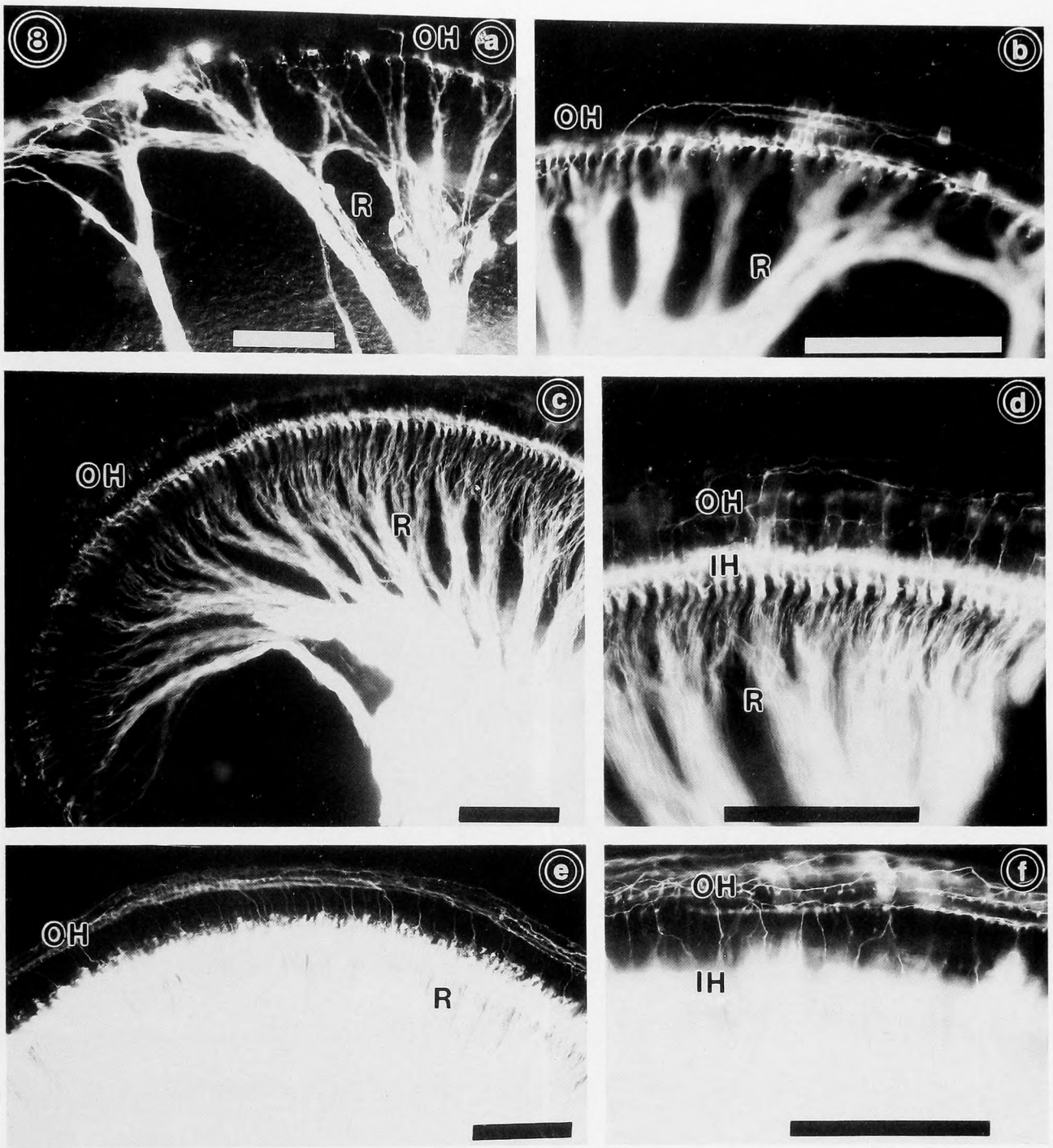

FIGURE 8 **The effect of *trk*C knockout is shown on the afferent innervation of P0 (a,b) and P9 (c,d) and P9 control littermates (e,f).** Note the much denser innervation of inner (IH) and outer hair cells (OH) and the close spacing of the radial fibers (R) in the control animal. Radial fibers near the base form anastomoses with each other in the mutant and appear to extend radially for a longer distance then in controls. Bar equals 100 μm.

examining the effects on the efferent system in early outgrowth and the time course of ganglion cell degeneration in these double knockouts.

***PO* trk*B*$^{-/-}$trk*C*$^{+/-}$.** *trk*B knockout animals heterozygous for the *trk*C deletion were analyzed to investigate any putative *trk*C gene dosage effect. Compared to wild type animals (Fig. 6a) these animals had the vestibular innervation defects of the *trk*B knockouts, that is, they lacked innervation to the semicircular canals, had a reduced innervation to the vestibular side of the saccule (Fig. 11a), and had reduced innervation to the utricul (Fig. 11b). As in many *trk*B$^{-/-}$ mutants, there was frequently an extension of a few fibers toward the HC that did not pen-

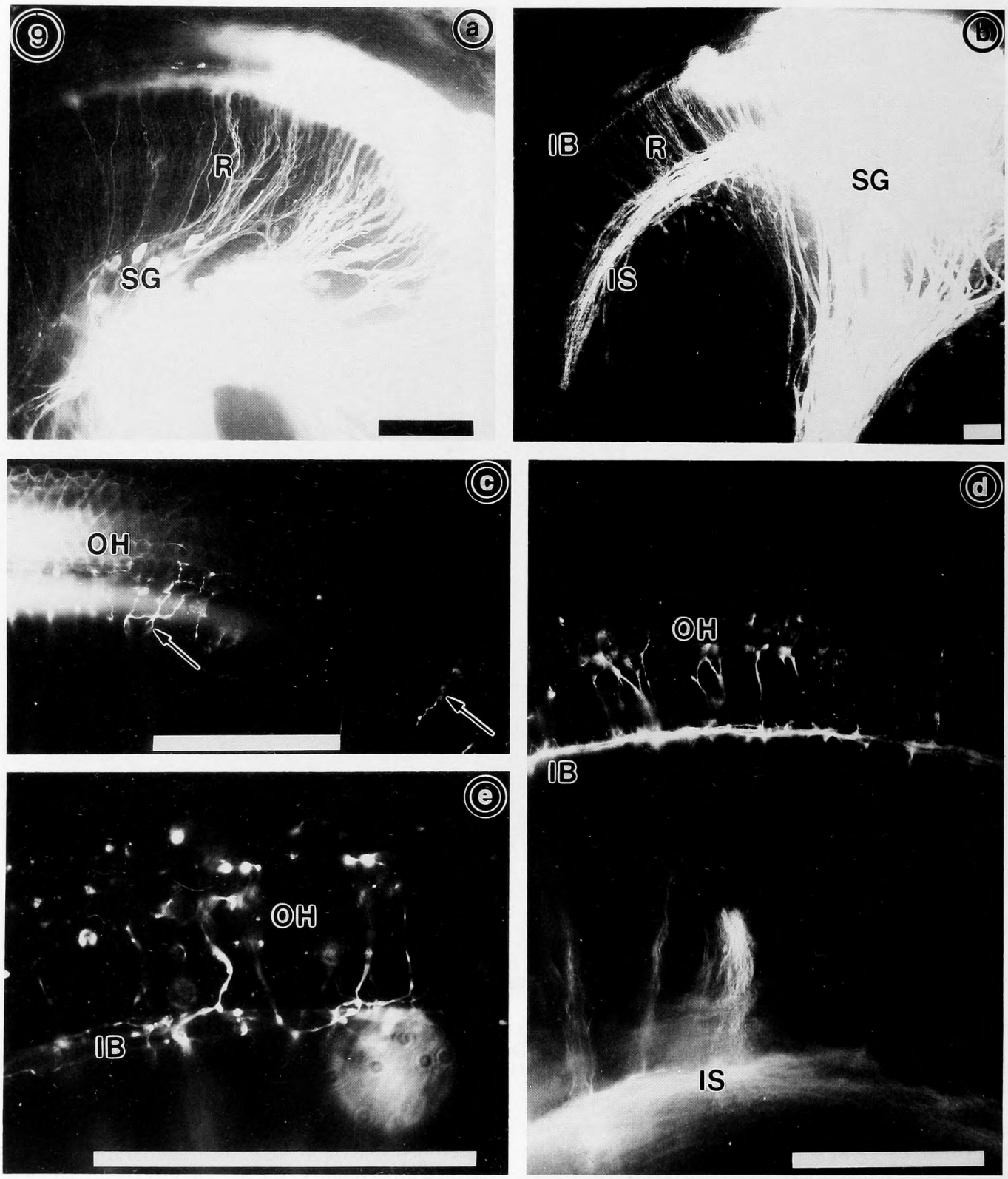

FIGURE 9 **The organization of the efferent fibers in 9-days-old (a–d) and 1-year-old (e) *trk*C knockout (a,c,e) and control littermate (b,d).** Note the prominent intraganglionic spiral bundle (IS) and inner spiral bundle (IB) in the control animals (b,d) as compared to same aged mutant littermates (a,c) or a 1-year-old mutant (e). Only in the mutant are spiral ganglion cells labeled (SG) which form radial fibers (R) outside the DiI application side (a,b). Bar equals 100 μm.

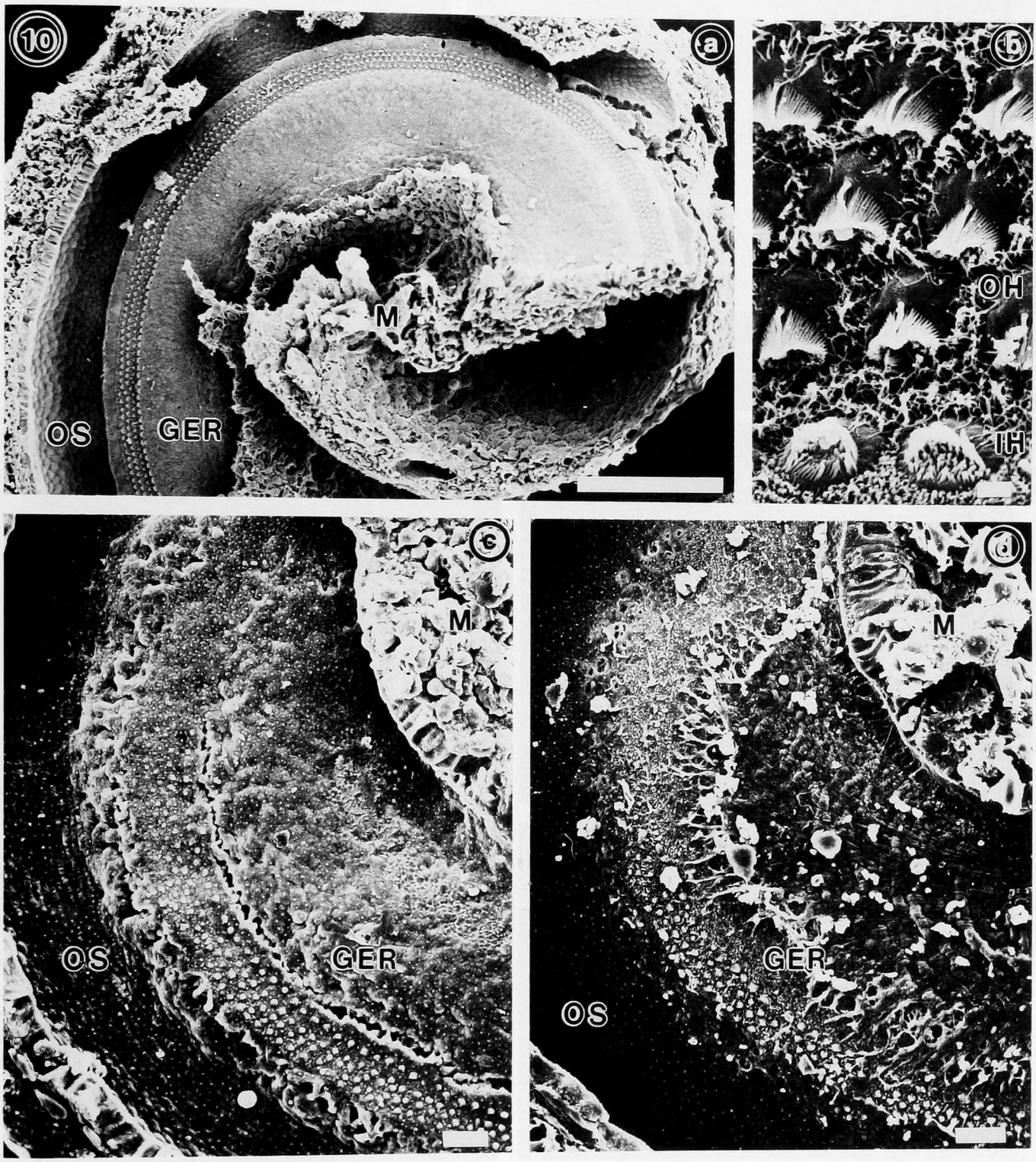

FIGURE 10 **The differentiation of cochlea hair cells of a *trk*B$^{-/-}$/*trk*C$^{-/-}$ double knockout mutant (a,b,d) and a P0 control littermate (c) is shown in these SEM micrographs.** The double knockout has no innervation of the cochlea at P0 and consequently shows no spiral ganglia in the modiolus (M), whereas these cells can be seen in the modiolus of the control animal (c). Nevertheless the hair cell maturation of outer (OH) and inner hair cells (IH) shows no difference between the mutant and control littermate even at the growing apical tip (c,d). GER indicates greater epithelial ridge, OS indicates outer spiral sulcus. Bars equal 100 μm (a), 1 μm (b), and 10 μm (c,d).

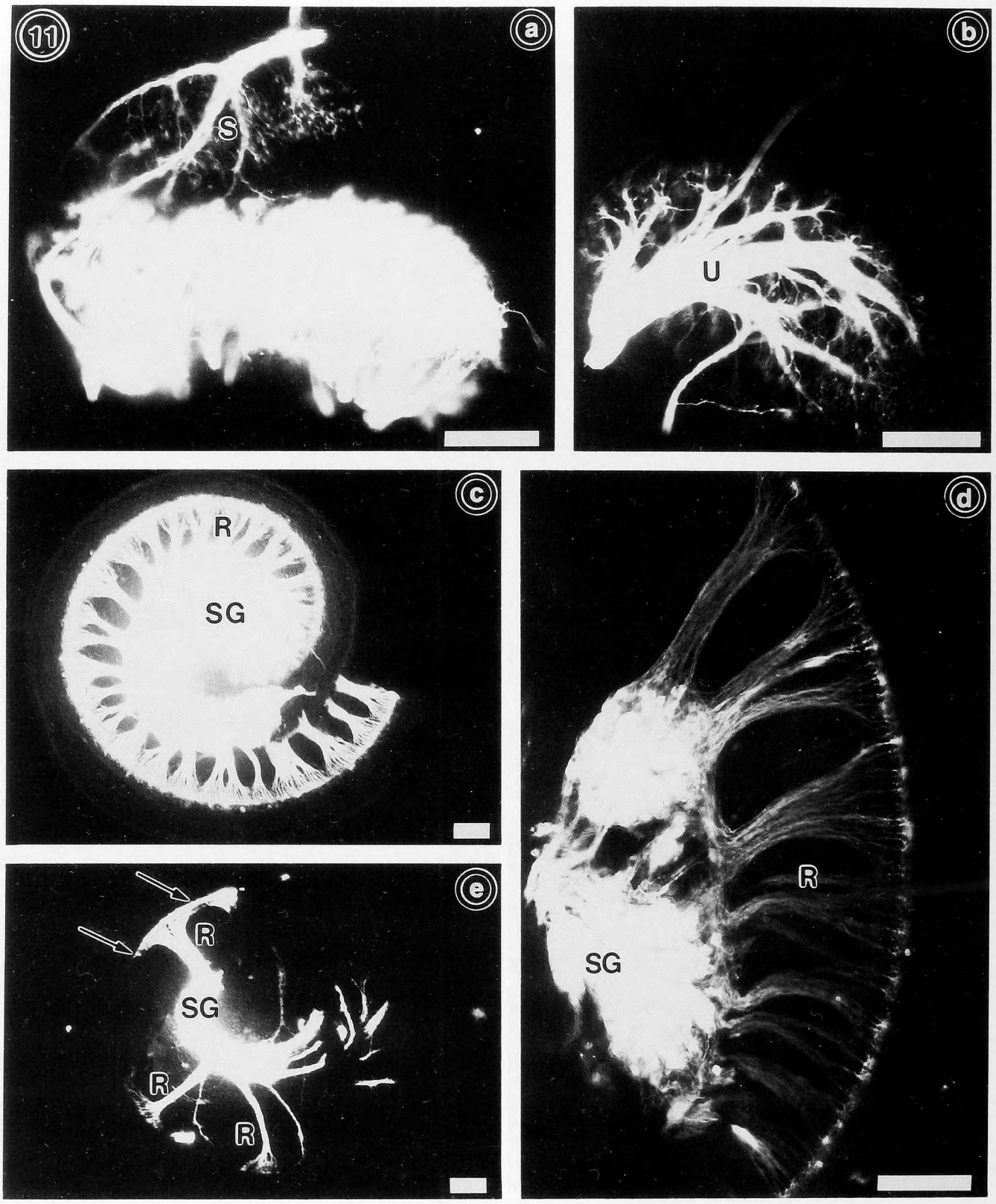

FIGURE 11 **The innervation of the saccule (a), utricle (b) and cochlea (d,e) of a P0*trk*B$^{-/-}$/*trk*C$^{+/-}$ mutant and a 17.5 dpc control animal (c).** The phenotype of vestibular epithelia innervation resembles closely the simple *trk*B$^{-/-}$ knockout with loss of semicircular canal innervation and a reduced innervation of the utricle (U) and the vestibular part of the saccule (S). In contrast, the phenotype of cochlear innervation shows that even loss of a single *trk*C allele causes major loss of spiral ganglia (SG) in a patchy distribution if combined with *trk*B$^{-/-}$ knockout. Radial fibers (R) tend to expand on reaching the inner hair cells (arrows, e). Bar equals 100 μm.

etrate this epithelium (Fig. 11b). There was no apparent additional effect of *trk*C heterozygosity on the vestibular innervation. In contrast, there was an interesting additional effect of *trk*C heterozygosity in the cochlea. This sensory epithelium is normally densely innervated, even in younger stages (Fig. 11c), but these mutants showed a patchy innervation, with areas being supplied by small groups of spiral ganglion cells (Fig. 11d,e). On reaching the inner hair cells, the fibers tended to spread both toward the base and the apex, a feature not found in control animals (arrows, Fig. 11e). Moreover, the innervation was most pronounced at the base and more patchy toward the apex. At the apex, fibers bypassed the immature hair cells and extended toward the outer spiral sulcus. Efferent fibers were detected only in patches that still had spiral ganglion cells and radial fiber bundles (data not shown). An SEM analysis of the cochlea showed no difference in hair cell development with or without innervation, thus confirming the data demonstrating hair cell maturation independent of innervation in the *trk*B/*trk*C double knockout mutant. We are currently investigating the earlier innervation of these *trk*B$^{-/-}$/*trk*C$^{+/-}$ animals to determine the time course of the presumed selective degeneration of some afferents, as well as the early pattern of efferent innervation.

***PO* trkB$^{+/-}$/trkC$^{-/-}$.** Three animals of this genotype were analyzed. They showed no apparent deficit in the innervation of the vestibular sensory epithelia and only a reduction of spiral ganglion cells in the base, comparable to the simple *trk*C$^{-/-}$ knockout mice (data not shown). Thus, the phenotype of this mutant indicates that a single functional allele of *trk*B is sufficient to maintain almost the entire innervation of the ear. More refined analyses in older animals are necessary to confirm this tentative conclusion.

***PO* trkB$^{+/-}$/trkC$^{+/-}$.** All five animals showed no apparent phenotype changes compared with controls (data not shown).

DISCUSSION

We report here that the effects of null mutations for the neurotrophin receptors *trk*B and *trk*C on ear innervation agree in detail with the known distribution of BDNF and NT-3, respectively (Fig. 1b): In *trk*B knockouts, sensory epithelia that are known to express only BDNF mRNA (Pirvola *et al.*, 1992; 1994) lose their innervation, whereas all epithelia that express both BDNF and NT-3 mRNA show at most a reduced innervation (utricle, saccule) or no apparent change at all (cochlea). The absence of large-scale effects on the innervation in the *trk*C mutants suggests that most afferent development can be compensated for by the presence of the TrkB receptor, which may bind both BDNF and NT-3 (Barbacid, 1993). Absence of any innervation in the *trk*B/C double knockout emphasizes the crucial need for both receptors to maintain the normal innervation of the ear. A comparable finding was recently obtained in BNDF/NT-3 double knockout mutants (Ernfors *et al.*, 1995).

Although the neurotrophins and their receptors are necessary for maintenance of ganglia and their fibers, they are not needed for initial fiber outgrowth: Afferent fibers extend initially to all sensory epithelia (Figs. 2,3), a finding also suggested for BDNF knockout mice (Ernfors *et al.*, 1994) and supported by some *in vitro* data (Bianchi and Cohan, 1993). The loss of all afferent innervation to the semicircular canals and the reduction of innervation of the utricle and the vestibular part of the saccule in all older animals appears to come about by excessive death of the projecting neurons themselves: the vestibular ganglion of 17.5 dpc *trk*B$^{-/-}$ is only 35% of the size of control animals (Fig. 6c,d). Thus, many vestibular ganglion cells apparently die in the absence of the *trk*B receptor more then 5 days after they become postmitotic (Ruben, 1967). The parallelism of *trk*B-induced and normal ontogenetic cell death is in agreement with the suggestion that neurotrophins, through their receptor signaling, may be involved in the onset of natural cell death (Silos-Santiago *et al.*, 1995). Owing to this ongoing degeneration, we refrained from a cell count of vestibular ganglion cells at 17.5 dpc, since we do not know how many of the ganglion cells in the mutant will ultimately survive. It is likely that these degenerating cells are utricular/saccular ganglion cells that may have been supported for some time through the *trk*C receptor and the neurotrophin NT-3 present in these epithelia. Developmental analysis of *trk*C expression in the *trk*B mutant will help resolve this issue.

Given that afferent fibers initially reach all vestibular sensory epithelia in both *trk*B and *trk*C knockouts, these data imply that factors that do not mediate their effect through the *trk*B/*trk*C receptors must play a role as neurotropic substances for afferents. Although other candidate neurotropic substances have been suggested (Bianchi and Cohan, 1993), it is possible that BDNF or NT-3 may play this role but mediate their effect through a different receptor. A possible candidate is the p75 receptor, which appears to support axonal outgrowth in some sympathetic neurons (Lee *et al.*, 1994), binds all neurotrophins thus far recognized (Hantzopoulos *et al.*, 1994) and is expressed in the otic ganglia (Hallböök *et al.*, 1993; Hallböök and Fritzsch, 1995).

The efferent fibers apparently show a different effect and are unable to innervate the semicircular canals at all (Figs. 4c, 5d,e). They reach the utricle only with a delay of at least 2 days (at least 14.5 dpc compared with 12.5 dpc in control animals) (Fritzsch and Nichols, 1993) in the *trk*B knockout mice. We cannot rule out that the TrkB receptor interacts with an as yet unidentified neurotropic factor to lure efferent fibers toward the semicircular canals. However, the neurotrophin NT-5 may not play a rule in this context (Conover *et al.*, 1995). Clearly, the presence of efferent fiber outgrowth exclusively toward epithelia where not all ganglion cells degenerate in the *trk*B

knockout mice suggests another scenario. It is possible that the intact afferents to the semicircular canals provide a pathway for ingrowing efferent fibers, thus causing their delayed and sparse arrival. We are currently comparing the number of retrogradely filled vestibular efferents to estimate when the *trk*B deficient mice show loss in these facial motoneuron-derived cells (Fritzsch and Nichols, 1993). Irrespective of these data, it is clear that the TrkB receptor plays a major role in the development of vestibular efferents, as it does for the survival of facial motoneurons (Oppenheim *et al.*, 1992; Snider, 1994; Silos-Santiago *et al.*, 1995), the ontogenetic source of all inner ear efferents (Fritzsch and Nichols, 1993; Fritzsch *et al.*, 1993; Fritzsch, 1995). Likewise, the apparent reduction of efferents to the cochlea in the *trk*C$^{-/-}$ animals suggests that *trk*C may be as essential for the *fi*ber outgrowth of the cochlear efferents as apparently *trk*B is for the vestibular efferents, or that efferents need a substrate provided only by nonlethally affected spiral ganglion cells. The presence of efferents exclusively on existing afferent bundles in the patchy innervated cochlea supports the latter hypothesis.

Taken together, these data imply that the TrkB receptor is crucial for the survival of vestibular afferents. In contrast, the absence of the TrkC receptor alone has only minor effects on cochlear afferents and efferents. Clearly, lack of both receptors in double knockouts is incompatible with the maintenance of any innervation to the ear, thus showing that these two receptors are both necessary to maintain the innervation. Although the data on the single *trk*B$^{-/-}$/*trk*C$^{+/-}$ mouse is not conclusive, it suggests that in fact both receptors can mutually substitute for each other with respect to certain afferent ganglion cells, as already suggested for dorsal root ganglia and cells in the CNS (Silos-Santiago *et al.*, 1995). Thus, the otocyst-derived ganglia may fall into both discrete and overlapping subpopulations with respect to some neurotrophin receptor, as do the neural crest-derived dorsal root ganglia (Wright and Snider, 1995). A more detailed double-labeling study of neurotrophin receptors in the ear is necessary to prove this suggestion.

Effects comparable to the receptor knockouts, but with different magnitudes, have been reported for the ligand knockouts. Absence of BDNF (the ligand for TrkB) affects exclusively the vestibular innervation (a reduction in cells of 80%, Ernfors *et al.*, 1994; 95% reduction of cells by P15, Bianchi *et al.*, 1995). Although data on later stages are lacking, the reduction in size (65%) at 17.5 dpc is compatible with the noted reduction in volume in BDNF knockout mice around this stage (Bianchi *et al.*, 1995). However, although Ernfors *et al.* (1994) could not detect any innervation of hair cells in the vestibular epithelia in BDNF knockouts, our data show a sparing innervation of all sensory epithelia that co-express NT-3 (utricle, saccule) (Pirvola *et al.*, 1992, 1994) and loss of innervation in all semicircular canal cristae. Given that NT-3 is also a ligand for TrkB, whereas BDNF does not interact with TrkC (Barbacid, 1993; Snider, 1994), it is not apparent why the BDNF knockout should have a stronger effect than the knockout of its receptor that can bind both neurotrophins. We are currently investigating the pattern of innervation in BDNF null mutant mice (Fritzsch, B., Bianchi, L.M., and Conover, J.C., in preparation).

Our data on *trk*C knockouts agree qualitatively with those obtained in NT-3 knockouts (Farinas *et al.*, 1994) in that the effect, if at all apparent, is concentrated within the cochlea. This is particularly noticeable in the *trk*B$^{-/-}$/*trk*C$^{+/-}$ mutants, which apparently add the patchy innervation of the cochlea to the *trk*B knockout vestibular phenotype. An explanation for this patchiness could be a random assignment of the functional and nonfunctional *trk*C receptor allele, comparable to the inactivation of one female X chromosome, to descendants of specific clones of spiral ganglia. Alternatively, there may be a threshold effect of the TrkC receptor, as was recently found for BDNF heterozygotes (Bianchi *et al.*, 1995). A detailed mapping for the mutant mRNA in earlier stages should resolve that issue. The much more prominent quantitative effect of the NT-3 knockout mice on the cochlea (about 85% loss in spiral ganglia) (Farinas *et al.*, 1994) may come about through the interaction of NT-3 with other neurotrophin receptors like *trk*B (Barbacid, 1993). Thus, a *trk*C knockout will show apparent defects only if the *trk*B receptor is absent. Moreover, the single functional *trk*B allele present in the *trk*B$^{+/-}$/*trk*C$^{-/-}$ can maintain most of the innervation except for the small reduction in the basal turn of the cochlea. The absence of innervation in the double knockouts and the patchy innervation of the cochlea in the *trk*B$^{-/-}$/*trk*C$^{+/-}$ animal is in agreement with this suggestion of functional redundancy of the neurotrophin receptors in many but not all ganglion cells. Similar discrepancies in magnitude of receptor and ligand knockout were found in dorsal root ganglion cells (Farinas *et al.*, 1994; Silos-Santiago *et al.*, 1995).

Acknowledgement This work was in part supported by an National Institutes of Health grant P50 DC00215-09 (B.F.). We wish to express our gratitude to M. Christensen for excellent dark room work and C. Miller for her help with SEM, J.D. Wallace, A. Lewin, and M. Garber for technical assistance.

REFERENCES

Barbacid, M. (1993). The *trk* family of neurotrophin receptors: molecular characterization and ontogenetic activation in human tumors. In: *Molecular Genetics of Nervous System Tumors*, Levin, A. G., and Schmidek, H. H., eds. John Wiley & Sons, New York, pp. 123–136.

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

Bianchi, L. M., Conover, J. C., Lindsay, R. M., and Yancopoulos, G. D. (1995). Degeneration of vestibular neurons in late em-

bryogenesis of both heterozygous and homozygous BDNF null mutatn mice. (Submitted.)

Conover, J. C., Erickson, J. T., Katz, E. M., Bianchi, L. M., Poueymirou, W. T., McClain, J., Pan, L., Helgren, M., Ip, N. Y., Boland, P., Friedman, B., Wiegand, S., Vejsada, R., Kato, A. C., DeChiara, T. M., and Yancopoulos, G. D. (1995). Neuronal deficits, not involving motor neurons, in mice lacking BDNF and/or NT4. *Nature 375*, 235–238.

Ernfors, P., Merlio, J.-P., and Persson, H. (1992). Cells expressing mRNA for neurotrophins and their receptors during embryonic rat development. *Eur. J. Neurosci. 4*, 1140–1158.

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

Ernfors, P., Loring, J., Jaenisch, R., and van de Water, T. R. (1995). Function of the neurotrophins in the auditory and vestibular systems: Analysis of BDNF and NT-3 gene knockout mice. ARO abstracts, p. 190.

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

Fritzsch, B. (1995). Development of the labyrinthine efferent system. *NY Acad. Sci.* (in press)

Fritzsch, B., Christensen, M. A., and Nichols, D. H. (1993). Fiber pathways and positional changes in efferent perikarya of 2.5 to 7 day chick embryos as revealed with DiI and dextran amines. *J. Neurobiol. 24*, 1481–1499.

Fritzsch, B., and Nichols, D. H. (1993). DiI reveals a prenatal arrival of efferents at developing ears of mice. *Hear. Res. 65*, 51–60.

Fritzsch, B., and Zakon, H. H. (1988). A simple, reliable and inexpensive silver stain for nerve fibers in bleached skin. *Z. Naturforsch. 43*, 606–608.

Hallböök, F., and Fritzsch, B. (1995). The distribution of BDNF and *trk*B mRNA in the otic region of 4.5 day chicken as revealed with a combination of in situ hybridization and tract tracing. Submitted.

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

Hantzopoulos, P. A., Suri, C., Glass, D. J., Goldfarb, M. P., and Ynacopoulos, G. D. (1994). The low affinity NGF receptor, $p75^{LGNFR}$, can collaborate with each of the *trk*s to potentiate functional responses to the neurotrophins. *Neuron 13*, 187–210.

Hemond, S. G., and Morest, D. K. (1992). Trophic effects of otic epithelium on cochleovestibular ganglion fiber growth in vitro. *Anat. Rec. 232*, 273–284.

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

Klein, R., Silos-Santiago, I., Smeyne, R. J., Lira, S. A., Brambilla, R., Bryant, S., Zhang, L., Snider, W. D., and Barbacid, M. (1994). Disruption of the neurotrophin-3 receptor gene *trk*C eliminates Ia muscle afferents and results in abnormal movements. *Nature 368*, 249–251.

Lee, K.-F., Bachman, K., Landis, S., and Jaenisch, R. (1994). Dependence on p75 for innervation of some sympathetic targets. *Science 263*, 1447–1449.

Oppenheim, R. W., Qin-Wei, Y., Prevette, D., and Yan, Q. (1992). Brain-derived neurotrophic factor rescues developing avian motoneurons from cell death. *Nature 360*, 755–757.

Pirvola, U., Ylikoski, J., Palgi, J., Lehtonen, E., Arumae, U., and Saarma, M. (1992). Brain-derived neurotrophic factor and neurotrophin 3 mRNAs in the peripheral target fields of developing inner ear ganglia. *Proc. Natl. Acad. Sci. USA 89*, 9915–9919.

Pirvola, U., Arumae, U., Moshnyakov, M., Palgi, J., Saarma, M., and Ylikoski, J. (1994). Coordinated expression and function of neurotrophins and their receptors in the rat inner ear during target innervation. *Hear. Res. 75*, 131–144.

Ramón y Cajal, S. (1960). *Studies of Vertebrate Neurogenesis*. Translated by L. Guth. Charles C., Thomas, Springfield, II, pp 172–184.

Ruben, R. J. (1967). Development of the inner ear of the mouse: a radioautographic study of terminal mitosis. *Acta Otolaryngol. 220*, 1–44.

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

Schecterson, L. C., and Bothwell, M. (1994). Neurotrophin and neurotrophin mRNA expression in developing inner ear. *Hear. Res. 73*, 92–100.

Silos-Santiago, I., Greenlund, L. J. S., Johnson, E. M., and Snider, W. D. (1995). Molecular genetics of neuronal survival. *Curr. Opin. Neurobiol. 5*, 42–49.

Snider, W. D. (1994). Functions of neurotrophins during nervous system development: what the knockouts are teaching us. *Cell 77*, 627–638.

Staecker, H., Galinovic-Schwartz, V., Liu, W., Lefebvre, P. P., Malgrange, B., Moonen, G., and van de Water, T. R. (1994). Postnatal auditory neurons depend upon brain derived neurotrophic factor for survival and both nerve growth factor and neurotrophin-3 for the maintenance of peripheral neurites in vitro. *Soc. Neurosci. Abstr. 20*, 1094.

Theiler, K. (1989). *The House Mouse: Atlas of Embryonic Development*. Springer, New York.

van de Water, T. R., Frenz, D. A., Giraldez, F., Represa, J., Lefebvre, P. P., Rogister, B., and Moonen, G. (1992). Growth factors and development of the stato-acoustic system. In: Development of Auditory and Vestibular Systems 2, Romand R, ed. Elsevier, Amsterdam, pp. 1–32.

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

Wright, D. E., and Snider, W. D. (1995). Neurotrophin receptors mRNA expression defines distinct populations of neurons in adult rat dorsal root ganglia, largely comparable *J. Comp. Neurol. 351*, 329–338.

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