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REVIEW

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Making and breaking the innervation of the ear: neurotrophic support during ear development and its clinical implications

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Abstract Analyses of single and double mutants of members of the neurotrophin family and their receptors are reviewed. These data demonstrate that the two neurotrophins, brain-derived neurotrophic factor (BDNF) and neurotrophin 3 (NT-3), and their high-affinity receptors trkB and trkC, are the sole support for the developing afferent innervation of the ear. Neurotrophins are first expressed in the otocyst around the time afferent sensory neurons become postmitotic. They are crucial for the survival of certain topologically distinct populations of sensory neurons. BDNF supports all sensory neurons to the semicircular canals, most sensory neurons to the saccule and utricle, and many sensory neurons to the apex and middle turn of the cochlea. In contrast, NT-3 supports few sensory neurons to the utricle and saccule, all sensory neurons to the basal turn of the cochlea and most sensory neurons to the middle and apical turn. Some topologically restricted effects reflect the pattern of neurotrophin distribution as revealed by in situ hybridization (e.g., loss of all innervation to the semicircular canal sensory epithelia in BDNF or trkB mutants). However, other topologically restricted effects cannot be explained on the basis of current knowledge of neurotrophin or neurotrophin receptor distribution. Data on mutants also support the notion that BDNF may play a role in neonatal plastic reorganization of the pattern of innervation in the ear and possibly the brainstem. In contrast, data obtained thus far on the ability of neurotrophins to rescue adult sensory neuron after insults to cochlear hair cells are less compelling. The ear is a model system to test the interactions of the two neurotrophins, BDNF and NT-3, with their two high-affinity receptors, trkB and trkC.

Key words Neurotrophins · Ear development · trk receptors · Mutants

Introduction

In recent years the molecular characterization of multiple families of neurotrophic substances and receptors has partly resolved the long-standing debate of neurotrophic interactions between hair cells and their innervating afferent nerve fibers. The neurotrophins, best known through the nerve growth factor (NGF; Levi-Montalcini 1987; Reichardt and Fariñas 1998), have six family members among vertebrates (Lai et al. 1998) with a well-characterized molecular structure (Ibañez 1998) and an emerging evolutionary history (Hallböök et al. 1998). In situ hybridization has shown that two neurotrophins (brain-derived neurotrophic factor, BDNF; and neurotrophin 3, NT-3) and their specific high-affinity tyrosine kinase receptors (trkB and trkC) are expressed in the ear and its innervating sensory neurons (Ernfors et al. 1992; Pirvola et al. 1992, 1994; Ylikoski et al. 1993). Several studies on various neurotrophin and neurotrophin receptor mutants (Ernfors et al. 1995; Fritzsch et al. 1995; Liebl et al. 1997; Silos-Santiago et al. 1997) have established a crucial role for these two neurotrophins and their receptors for the embryonic survival of the afferent innervation of the ear. These studies on mutants, some of which have little or no afferent innerva-

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tion of the ear left, show that hair cells predominantly support sensory ganglion cells during embryonic development of the ear (see Fritzsch et al. 1997b, 1998a). Based on these data, the role of neurotrophic factors in the development and maintenance of the afferent ear innervation may be subdivided into three phases:

1. An early phase in which neurotrophins and their receptors are expressed in the otocyst but may not play a critical role in the survival of the newly formed spiral and vestibular neurons. These neurons can extend neurites to the sensory epithelium even in mutants genetically engineered to lack one neurotrophin or its receptor (Bianchi et al. 1996; Fritzsch et al. 1995).
2. A later phase in which spiral and vestibular neurons depend critically on the two neurotrophins and their cognate receptors (Pirvola et al. 1992) for their survival (Liebl et al. 1997; Silos-Santiago et al. 1997).
3. A neonatal phase, which possibly extends into adulthood, in which these neurotrophins may be less critical for survival of ganglion cells and more important for plasticity of afferent connections. This suggestion is based on the novel functions found recently for the neurotrophin BDNF in this context (Timmusk et al. 1993; Shieh et al. 1998).

The data derived from these developmental studies provided the stimulation for several investigations into the functional role of various neurotrophic substances in the adult cochleovestibular ganglia as well as their possible clinical application (see Miller et al. 1997 for review). The present review will critically assess issues that have been partially or completely resolved. We will also highlight open questions for further studies on early developmental aspects of neurotrophins. In addition, and based on the insights gained from these developmental studies, this review will propose new research directions for future clinical trials into the use of neurotrophically active substances for the rescue of inner ear sensory neurons.

Early development of the ear and its ganglia

The ear develops from a cake-like ectodermal thickening named the otic placode by von Kupffer (1895). Neither the induction nor the transformation of the otic placode into the otic vesicle is fully understood in its molecular governance. However, several molecules that play some role have been identified in recent years (Fritzsch et al. 1998a; Whitfield et al. 1997; Torres and Giraldez 1998). For example, several transcription factors such as Pax 2 and 3, GATA 3 and Dlx 3 have been shown to be expressed in the early-forming otocyst and otic placode (Fritzsch et al. 1998a; Torres and Giraldez 1998). Eventually, the invaginated otocyst becomes compartmentalized through differential gene expression, into neurogenic and non-neurogenic areas (Fekete 1996; Fritzsch et al. 1998a; Torres and Giraldez 1998). Genes that appear to characterize the early phases of sensory patch differentiation in mammals are *int-2* (Wilkinson et al. 1989), bone morphogenetic factor 4 (*BMP-4*) and lunatic fringe (*Fng*; Morsli et al. 1998). One of the earliest effects of this compartmentalization of the otocyst is the formation of the cochleovestibular (otic) ganglion cells by the otocyst. The cochlear and vestibular ganglia form in mice between E9.5 and E14.5 (Ruben 1967), in rats between E11.5 and E15.5 (Altman and Bayer 1982). This formation is apparently under the influence of the basic helix-loop-helix transcription factor neurogenin 1 (ngn-1; Ma et al. 1998). Mutants of ngn-1 never develop an afferent innervation of the ear (Ma et al. 1998; Fritzsch et al., unpublished data).

Tissue culture experiments suggest that ganglion cell precursors emerge from the anteroventral area of the otocyst (see Fritzsch et al. 1998a for review). Detailed histological analysis suggests that these cells migrate away from the otocyst and undergo further proliferation before they aggregate to form the postmitotic otic ganglion cells (Altman and Bayer 1982). Expression of NeuroD, a crucial transcription factor that acts downstream from ngn-1 (Ma et al. 1998), suggests that ganglion cells may in fact derive from the same neurogenic anlage as the future sensory hair cells of the utricle, saccule and cochlea. NeuroD expression has been shown in cells inside the otic vesicle and outside in what appears to be ganglion cells. Likewise, in situ hybridization studies of the expression of int-2 suggest that ganglion cells derive from the int-2-expressing ventroanterior patch of the otocyst and retain their int-2 expression while migrating away from the otocyst (McKay et al. 1996). In fact, sensory hair cells and sensory ganglion cells may be clonally related in mammals and in birds (Fekete et al. 1998). However, at least some ganglion cells appear to differentiate while within the sensory epithelium (Bruce et al. 1997) but will translocate their perikarya into the spiral ganglion later. Our data on proliferation and expression of neuronal markers such as neurofilaments (Pirvola et al. 1994) agree with this scenario of migration of precursor cells initially proposed by Altman and Bayer (1982; Carney and Silver 1983; McKay et al. 1995). Neurofilament-positive neurons are more distal whereas cells between them and the otocyst are negative for this early neuronal marker (Fig. 1). We suggest that cells between the otocyst and the more distal cells that do express neuronal markers are neuronal precursor cells that are migrating away from the otocyst (Fig. 1).

In situ hybridization data (Pirvola et al. 1994) suggest that a ventral and anteriorly located neuroepithelial sensory patch of the otocyst expresses the neurotrophic factor NT-3 at a time when ganglion cells or their precursors emigrate from the ear in rats (Fig. 1). This same patch appears also to be positive for int-2 (FGF-3; McKay et al. 1996) and likely also for lunatic fringe (Morsli et al. 1998). BDNF is also expressed very early in the otocyst (E12), but its expression does not overlap with the NT-3 signal (Pirvola et al. 1994). Instead, it may overlap with BMP-4 expression (Morsli et al. 1998). Cells, which apparently migrate from the NT-3-expressing future sensory epithelia to the statoacoustic ganglion (Altman and Bayer 1982; Carney and Silver 1983; McKay et al. 1996), do not express detectable levels of the neurotrophins BDNF and NT-3 or of their high-affinity receptors (Fig. 1). In fact, these migratory cells appear to upregulate transiently the high-affinity NGF receptor, trkA (Fig. 1). Other cells, more distal to the otocyst, express neurotrophin receptors trkB and trkC (Fig. 1). All of these ganglion cells appear to be positive for int-2 (McKay et al.

1996), and int-2 mutation appears to downsize the number of ganglion cells, at least in the most severely defected cases (Mansour et al. 1993). These cells also express Islet-1 as soon as they leave the otocyst (Whitfield et al. 1997).

These expression data thus suggest a rapid downregulation of NT-3 mRNA expression shortly before or immediately after emigration of these cells from the future sensory epithelia (Fig. 1). This is followed by a transient upregulation of the neurotrophic receptor trkA while migrating, and a subsequent upregulation of the neutrophin receptors trkB and trkC as well as neurofilament protein after the postmitotic cells have migrated to, and start to differentiate at their future position. Rapid developmental changes in neurotrophin receptor expression are also known in other developing sensory systems (Reichardt and Fariñas 1998). The transient expression of trkA may in fact relate to withdrawal from the cell cycle and onset of neuronal differentiation (Reichardt and Fariñas 1998; for review). More tests involving early markers for proliferation and differentiation are needed to further resolve this process.

Based on published data, there could be a topological similarity of BMP-4 and lunatic fringe expression with BDNF and NT-3 expression, respectively. Overlapping expression with NT-3 may exist for int-2 (FGF-3; McKay et al. 1996; Fig. 1c,f). An analysis of lunatic fringe, int-2, and NT-3 expression in mutants that lack cochlear duct formation such as the Hox-a1 (Chisaka et al. 1992) or Pax-2 (Torres et al. 1996) can show whether these genes are all affected simultaneously by these mutations.

The functional significance of the restricted expression of neurotrophins in the otocyst prior to the arrival of afferent and efferent processes (E12; Pirvola et al. 1994) remains elusive at the moment. It is possible that NT-3 serves in this system as a regulator for proliferation of ganglion cell precursors, analogous to dorsal root ganglia (Reichardt and Fariñas 1998). Immunocytochemical data using well-characterized antibodies against trk receptors show that these antibodies stain growth cones (Tuttle and O'Leary 1998). A possible role in growth cone steering of dorsal root ganglia has been suggested for neurotrophins (Paves and Saarma 1997; Tuttle and O'Leary 1998). Unfortunately, this potential role of neurotrophins for neurite guidance has not received compelling support from data generated in mutant mice ears. In fact, at least some neurites can reach their target even in the absence of neurotrophins or their receptors (Fritzsch et al. 1995, 1997a). A similar conclusion was reached in tissue culture for otic ganglion cells (Bianchi and Cohan 1993) and seems to be the case for other developing sensory neurons as well (Reichardt and Fariñas 1998).

Neurotrophins and ganglion cell survival

The second phase of the neurotrophin role in ear development is characterized by specific survival of topographically distinct populations of vestibular and spiral neurons. The initial formation of ganglion cells is clearly under the control of transcription factors such as ngn-1 and neuroD (Ma et al. 1998). However, neurons may become dependent on neurotrophic support very soon after they become postmitotic, reach their final position, and express the high-affinity neurotrophin receptors (Pirvola et al. 1994; Fig. 1). For example, counting of ganglion cell numbers in neurotrophin and neurotrophin receptor mutants throughout embryonic development suggests a rapid disappearance of ganglion cells (Fariñas et al. 1994; Ernfors et al. 1995; Schimmang et al. 1995; Bianchi et al. 1996). This demise of ganglion cells in BDNF and trkB mutants appears to be accompanied by a loss of the early fibers extending initially to the sensory epithelia. Continuing cell death may leave, for example, the semicircular canals without and the utricle and saccule with a rudimentary number of fibers (Fritzsch et al. 1995; Bianchi et al. 1996).

Specifically, in BDNF mutants the majority of vestibular neurons degenerate rapidly leaving only about 20% of neurons in late embryos and neonates (Bianchi et al. 1996). A comparable neuronal loss was reported for mutants of the high-affinity BDNF receptor trkB (Schimmang et al. 1995). Conversely, the majority of ganglion neurons innervating the auditory organ appear to depend on the second neurotrophin expressed in the ear, NT-3 (Fariñas et al. 1994), and its receptor trkC (Schimmang et al. 1995; Silos-Santiago et al. 1997; Fritzsch et al. 1998b). In contrast, BDNF seems to play only a minor role in about 10% of the spiral ganglion cells (Jones et al. 1994; Ernfors et al. 1995). This effect is apparently mediated through the trkB receptor (Schimmang et al. 1995; Fritzsch et al. 1998b).

The cochlea is innervated by two types of sensory neurons: type I innervates inner hair cells and type II innervates outer hair cells. In fact, more than 90% of all sensory neurons to the cochlea are type I sensory neurons. Initial data suggested a differential effect of the two neurotrophins and their receptors on the two classes of auditory sensory neurons. It was suggested that NT-3/trkC supports all inner hair cell innervation whereas BDNF/trkB supports all outer hair cell innervation (Ernfors et al. 1995; Schimmang et al. 1995, 1997; Minichiello et al. 1995). These data were not fully confirmed by later, more detailed analyses (Fritzsch et al. 1997a, 1998b). In fact, recent data obtained in some ligand null mutations and in various combinations of trkB and trkC homo- and heterozygotic mutations stress a topologically restricted effect of these receptor mutations in the cochlea (Fritzsch et al. 1997a,b, 1998b):

1. BDNF$^{-/-}$ null mutation causes loss of outer hair cell afferent innervation in the apical turn of the cochlea, which is quantitatively more pronounced in trkB mutants (Bianchi et al. 1996; Fritzsch et al. 1997b).
2. trkB$^{-/-}$, if combined with trkC$^{+/-}$, always retains the basal turn spiral neurons but may lose most of the middle and apical turn spiral neurons and their innervation, in particular to outer hair cells (Fig. 2).
3. trkC$^{-/-}$ causes a complete absence of the most basal spiral neurons (Fritzsch et al. 1998b; Fig. 2).
4. trkC$^{-/-}$ if combined with trkB$^{+/-}$ extends spiral neuron loss further toward the middle turn (Fritzsch et al. 1998b; Fig. 2).
5. NT-3$^{-/-}$ null mutation causes the most severe loss of all single mutations (Fariñas et al. 1994). All spiral neurons in the basal turn are lost, comparable to the trkC$^{-/-}$/trkB$^{+/-}$ phenotype (Fritzsch et al. 1997c; Fig. 2).

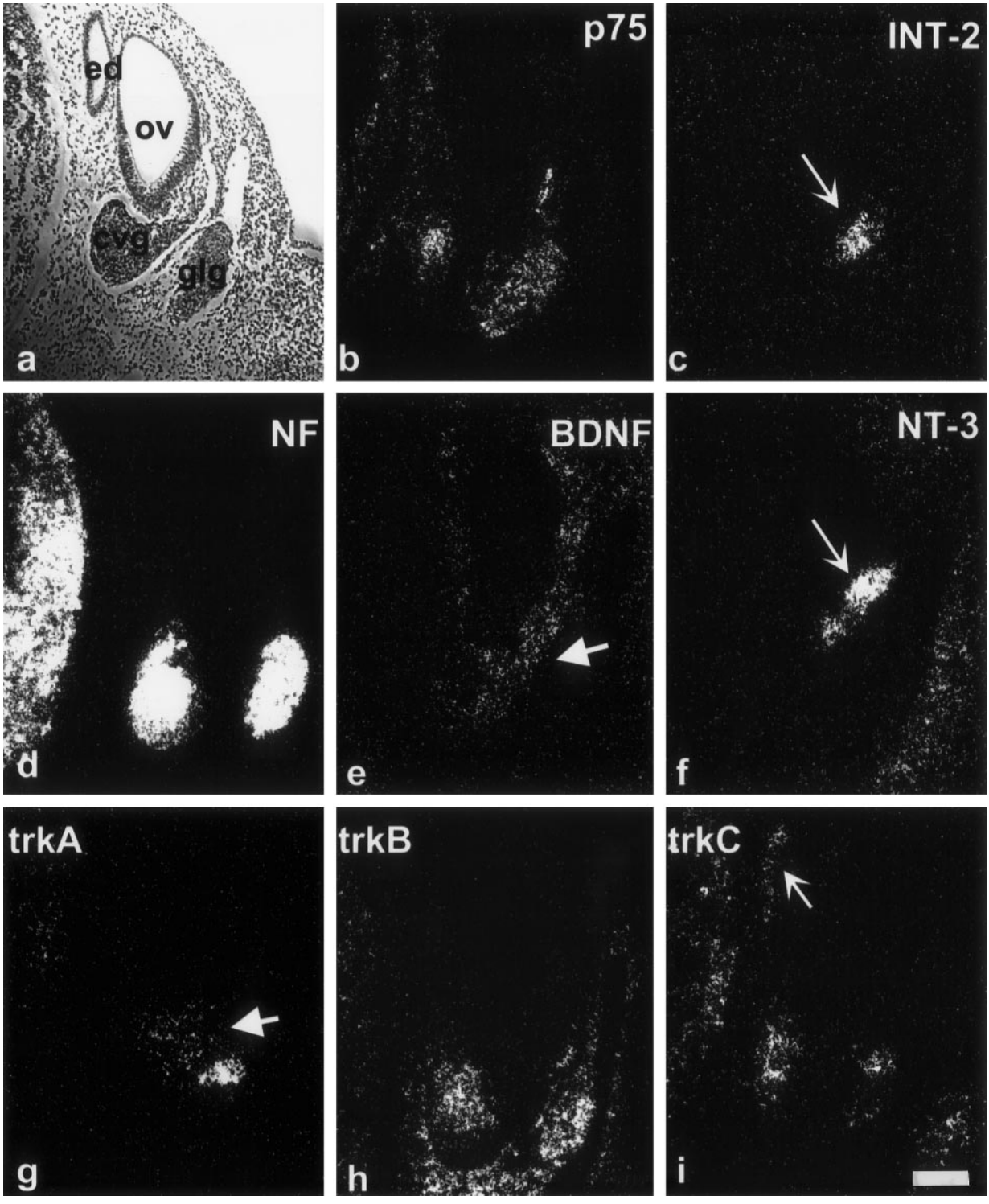


Fig. 1a–i The early phase is shown of NT-3, BDNF, trkC, trkB, trkA and neurofilament expression as revealed by in situ hybridization. Transverse sections of comparable levels through the otic region of 13.5-day-old rat embryos are shown. Notice that BDNF **(e)** and NT-3 **(f)** expression are both differently distributed in the otocyst wall. NT-3 expression **(f)** appears to overlap with int-2 **(c)** expression. trkB **(h)** and trkC **(i)** expression overlap in the distal part of the forming cochleovestibular ganglion and to a lesser extent in the geniculate ganglion. In contrast, trkA **(g)** is transiently expressed on a population of ganglion cells located between the otocyst and the mature cochleovestibular ganglion cells. These cells are here interpreted as migratory, undifferentiated ganglion cell precursors comparable to migrating neural crest cells. The small trkA-positive population near the geniculate ganglion may represent the neural-crest-derived proximal ganglion cells. Expression of neurofilament message **(d)** is restricted to the postmitotic otic and geniculate neurons that also express trkB **(h)** and trkC **(i)** and largely overlaps with the expression of the low-affinity receptor p75 **(b)** (*glg* geniculate ganglion, *ov* otic vesicle, *cvg* cochleovestibular ganglion, *ed* endolymphatic duct, *thin arrows* expression in sensory epithelium of the otic vesicle, *bold arrows* migrating cells). *Bar in* **i** 100 μm **(a–i)**

These data strongly suggest that the most prominent effects of either NT-3 or trkC occur in the basal turn of the cochlea. Here, spiral neurons will be maintained in the presence of only a single allele of trkC (even in the absence of trkB) but are invariably lost in all NT-3$^{-/-}$ or trkC$^{-/-}$ mutations (Fig. 2). Conversely, BDNF$^{-/-}$ exerts its most pronounced effect on the outer hair cell innervation in the apex (Ernfors et al. 1995; Bianchi et al. 1996). trkB$^{-/-}$ also acts predominantly on the apex and causes complete loss of outer hair cell innervation (Fritzsch et al. 1997c, 1998b).

It is unclear how this differential spatial loss of spiral and vestibular neurons relates to the spatiotemporal distribution of neurotrophins and neurotrophin receptors. Available data on neurotrophin and neurotrophin receptor expression are only compatible with the complete loss of afferent innervation of the semicircular canal epithelia. These epithelia express only BDNF (Pirvola et al. 1992, 1994) and lose all afferent innervation rapidly in BDNF$^{-/-}$ (Bianchi et al. 1996) and trkB$^{-/-}$ mutations (Fritzsch et al. 1995). In contrast, BDNF and NT-3 are overlappingly expressed in the utricle, saccule and cochlea (Pirvola et al. 1992). However, data on the expression dynamics suggest that BDNF is always limited to hair cells, both in the cochlea and in vestibular sensory epithelia (Pirvola et al. 1992, 1994). In contrast, NT-3 appears initially throughout the cochlea and becomes restricted to inner hair cells only as development progresses (Pirvola et al. 1994; Wheeler et al. 1994). The dynamics of NT-3 expression changes in the utricle and saccule have not been fully worked out. Whether this dynamic change in NT-3 expression pattern in the cochlea is causally related to the apparent specific spatial losses of spiral neurons observed in NT-3/trkC mutant mice is presently unknown.

In this context, it should be pointed out that the distribution of neurotrophins is not universally agreed upon. However, when comparing data generated using different techniques, problems unique to the neurotrophins should be kept in mind. Neurotrophins are actively transported (von Bartheld 1998). This may provide false-positive data in fibers or even cells when revealed with immunocytochemistry (Conner et al. 1997). Thus, additional independent controls are necessary when elucidating distribution of neurotrophins with immunocytochemistry. The rapid onset of expression and the comparatively low abundance of mRNA (Jones et al. 1994) may impose constraints on the sensitivity of the current in situ techniques. While the use of the Lac-Z reporter system may overcome the initial low abundance problem, the comparatively long lived β-galactosidase enzyme may provide a positive signal at least hours after the mRNAs for neurotrophins have been metabolized. This could provide a false-positive signal about the presence of a signal that is in fact already downregulated. Also, β-galactosidase, if released by hair cells, may be accumulated by nearby cells such as supporting cells and thus generate a false-positive signal. Clearly, combinations of all these approaches linked with a keen awareness of the limitations of each approach are necessary to resolve some of the discrepancies on expression of neurotrophins and their receptors that are still in the literature.

In contrast to the rapid changes in neurotrophin expression, no major reorganization of trkB or trkC expression pattern has been reported for vestibular and spiral neurons (Pirvola et al. 1992, 1994). In addition, these receptors appear to be overlappingly expressed in individual ganglion cells (Pirvola et al. 1992, 1994; Ylikoski et al. 1993). Therefore, it appears at the moment unlikely that the differential spatial loss of, for example, spiral neurons in the basal turn of NT-3 mutants is mediated through selective expression of one or the other neurotrophin receptor. Since all but the semicircular canal epithelia express both neurotrophins (Pirvola et al. 1992, 1994), this overlapping expression could indicate that both receptors contribute to the survival of sensory neurons. Clearly, in situ hybridization for one neurotrophin receptor combined with immunocytochemistry for the second receptor needs to be performed to analyze in more detail the temporal and spatial distribution of trkB and trkC expression.

It remains unclear at the moment why there is an apparent overlapping expression of both high-affinity neurotrophin receptors in the semicircular canal sensory neurons (Pirvola et al. 1994; Ylikoski et al. 1993). This epithelium expresses only the neurotrophin BDNF (Pirvola et al. 1992). Moreover, BDNF and trkB mutations alike lose all innervation to the semicircular canals (Bianchi et al. 1996; Fritzsch et al. 1995). Thus expression of trkB in semicircular canal sensory neurons would suffice to explain the loss of these neurons in mutants. However, in chickens, which have a very limited expression of NT-3 in the ear, trkC is apparently expressed in all sensory neurons (Pirvola et al. 1997). Using a combination of tract tracing and in situ hybridization (Fritzsch and Hallböck 1996), one could elucidate whether all semicircular canal ganglion cells do in fact express trkC at the same level as utricular or saccular ganglion cells. Since nothing is known about the regulation of the expression of these receptors, it could be that their promoter similarity (Salin et al. 1997) simply causes a simultaneous upregulation of both in the developing ear.

The effects of NT-3/trkC and BDNF/trkB mutations, respectively, differ not only in their distribution (cochlea or vestibular system, respectively) but show opposite quantitative trends. BDNF mutation causes slightly less severe reduction of spiral neurons than trkB mutation (Ernfors et al. 1995; Schimmang et al. 1995; Bianchi et al. 1996; Silos-Santiago et al. 1997). In contrast, NT-3 mutations show a more pronounced reduction of the total number of spiral neurons than trkC mutations (Fariñas et al. 1994; Schimmang et al. 1995; Silos-Santiago et al. 1997). These differences may be brought about through the limited signaling of NT-3 through trkB (Barbacid 1994; Nakatani et al. 1998). This may cause the less severe phenotype in BDNF compared to trkB mutations, but a more severe phenotype in the NT-3 mutation compared to the trkC mutation. Generating BDNF/trkC double mutants, in which only NT-3 signaling through trkB would be possible, could test this hypothesis. The spatial and quantitative loss of spiral neurons would be expected to differ from NT-3/trkB double

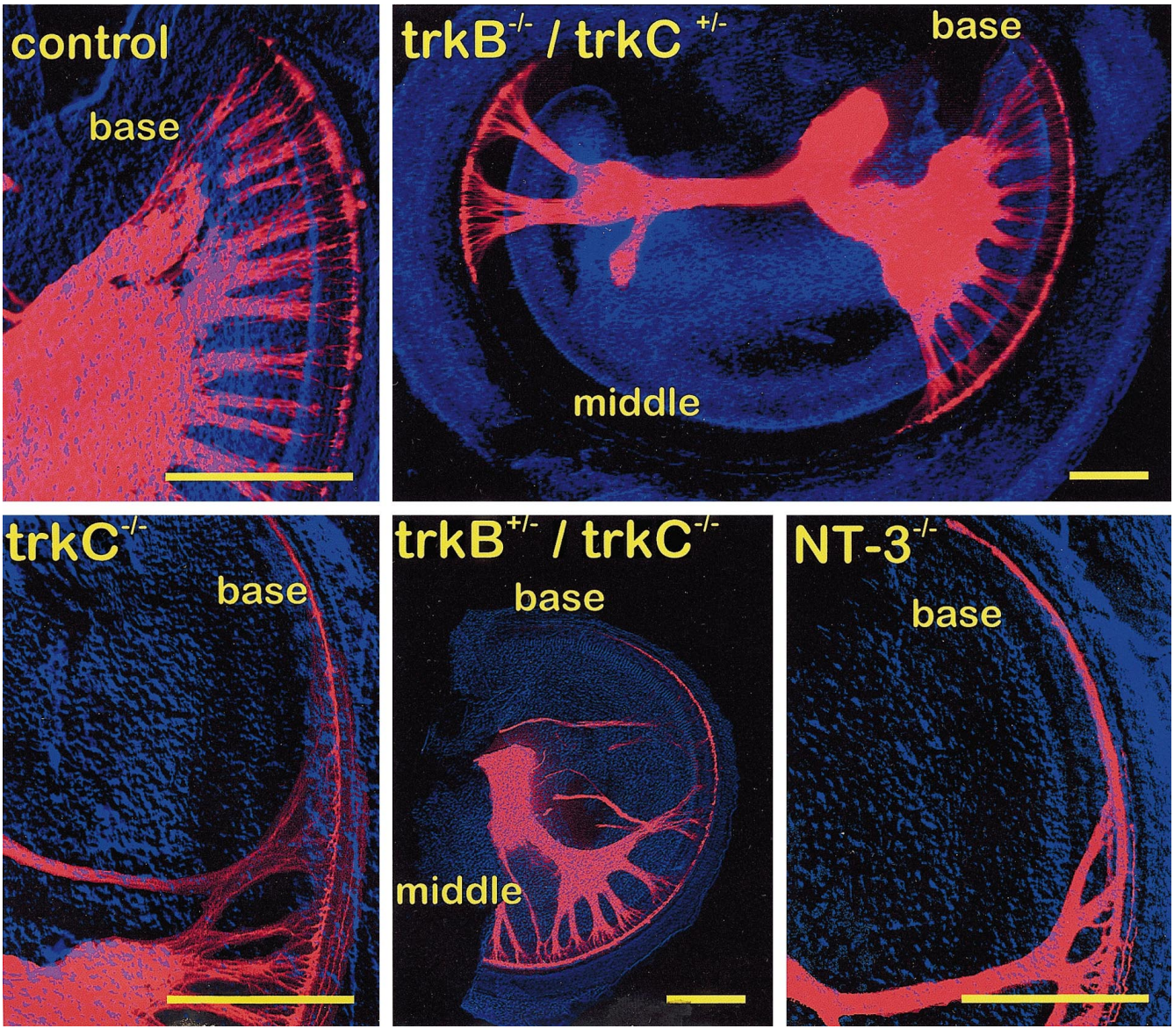


Fig. 2 Effects of various combinations of neurotrophin and neurotrophin receptor mutations on the pattern of afferent innervation of the cochlear base are shown. Note that a dense innervation of the basal turn of the organ of Corti by radial fibers exists in the control mice. In the trkB$^{-/-}$/trkC$^{+/-}$ double mutant the base may be the only part of the cochlea that is innervated in some mice whereas others of this genotype show some innervation of the apex. However, in contrast to the control and all other mutants, the trkB$^{-/-}$/trkC$^{+/-}$ mutant lacks both radial fibers and spiral neurons in the middle turn. The apparent retention of spiral neurons and radial fibers in trkB$^{-/-}$/trkC$^{+/-}$ mutants contrasts with any combination of trkC$^{-/-}$ mutation alone or combined with trkB $^{+/-}$ heterozygosity. All of the latter mutations result in a loss of spiral neurons and radial fibers near the base in a topologically comparable pattern to NT-3 mutation. These images of the cochlea were generated by combining the DiI epifluorescence signal (*red*) with the differential interference contrast image (*blue*). *Bar* 100 μm

mutants. In these mutants no signaling through the trkC receptor by BDNF is to be expected and they should lose all innervation, comparable to double receptor mutants (Fritzsch et al. 1995; Silos-Santiago et al. 1997) or double ligand mutants (Ernfors et al. 1995; Liebl et al. 1997).

Neurotrophins (Bianchi et al. 1996; Liebl et al. 1997) and trkB receptors in the CNS (Minichiello and Klein 1996) seem to generate a threshold effect. Thus, heterozygotic animals, despite their apparently normal pattern of innervation, nevertheless have a reduced number of vestibular and spiral neurons. It is likely that the differential enhancements of spiral neuron loss in various combinations of homo- and heterozygotic mutations of neurotrophin receptors may relate to this threshold effect (Fritzsch et al. 1998b). A critical test would be the finding of a comparable spatial loss of spiral neurons in various NT-3/BDNF homo- and heterozygotic combinations.

The bewildering variations in the patterns of spiral neuron loss in various combinations of neurotrophin receptor mutations (Fig. 2) stress the importance of a test system to establish the multiple levels of interactions between the four alleles of the two neurotrophin receptors as well as the four alleles of neurotrophins. The developing ear appears to be the perfect in vivo model system to further test the effects of the two relevant neurotrophins and neurotrophin receptors in various combinations of homo- and heterozygosity. Such tests are more complicated in most other cranial ganglia or the CNS, which express all three neurotrophin receptors to some extent overlappingly (Silos-Santiago et al. 1997). However, the epibranchial-placode-derived ganglion cells innervating the taste buds appear to be even simpler in that they require almost exclusively trkB (Fritzsch et al. 1997e; Silos-Santiago et al. 1997).

Beyond simple survival: a possible role of neurotrophins in plasticity during neonatal phases?

The data on neurotrophins and neurotrophin receptor distribution and the effects of various null mutants establish beyond reasonable doubt the crucial role of the two neurotrophins and their receptors in early embryonic support of the afferent innervation. In contrast, the late embryonic and neonatal role of neurotrophins is less well understood. For one thing, largely depending on the technique employed, various neonatal and adult patterns of neurotrophins and their receptors have been described. We will here rely predominantly on in situ hybridization data, which most likely reflect the physiologically relevant signaling.

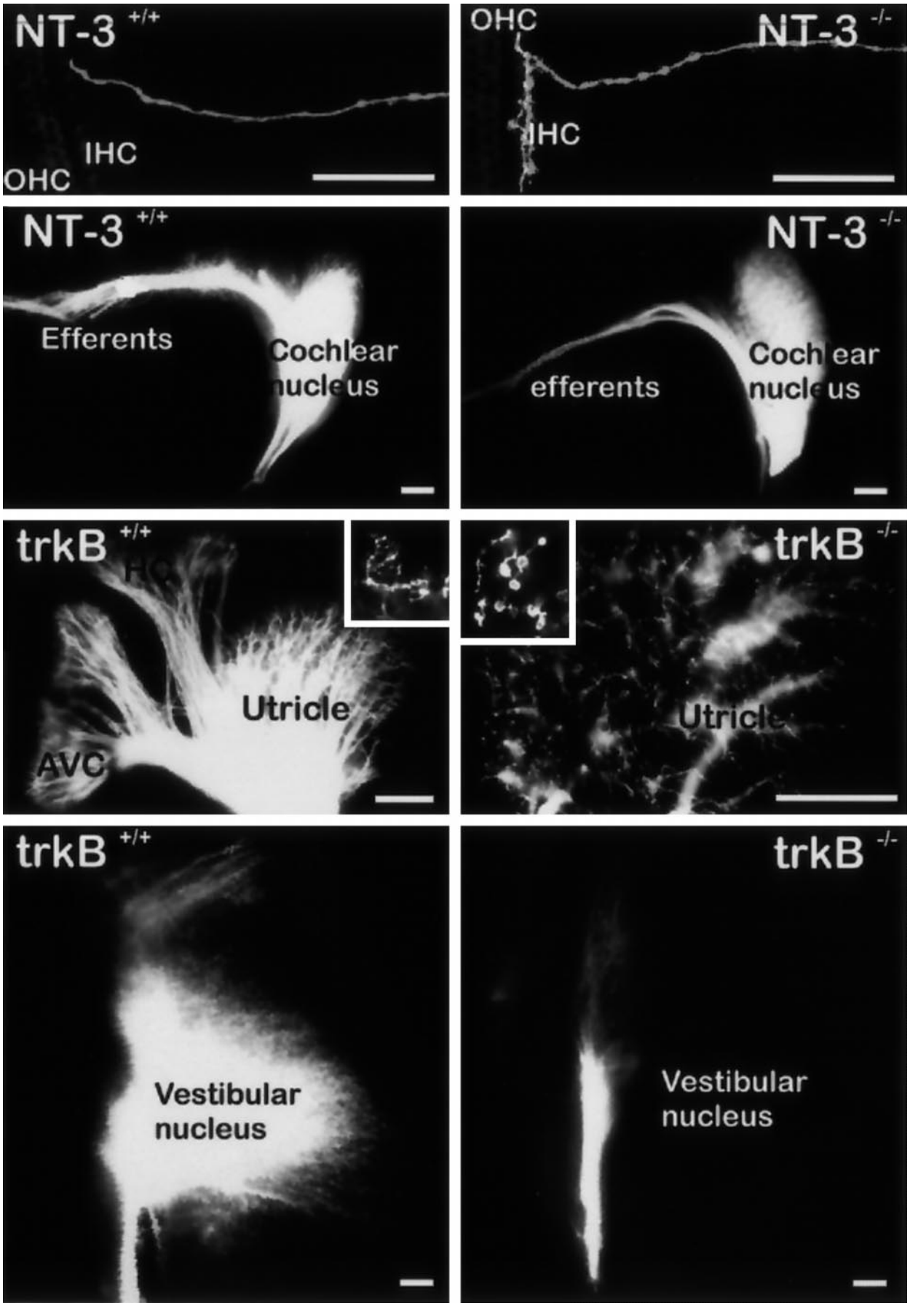
Based on in situ hybridization, neurotrophins appear to be downregulated in late neonatal cochleae in all sensory epithelia. However, while BDNF seems to disappear from outer and subsequently from inner hair cells of the cochlea (Ylikoski et al. 1993; Pirvola et al. 1994), NT-3 seems to shift its expression from outer hair cells (OHCs) to inner hair cells (IHCs) and reduces its non-sensory cell expression (Pirvola et al. 1994). Data on vestibular sensory epithelia suggest an expression of BDNF exclusively in sensory cells (Pirvola et al. 1994). Recent immunocytochemical data suggest an early expression of BDNF in both sensory and supporting cells followed later by an expression only in supporting cells (Montcouquiol et al. 1998). Data obtained with the lac-Z reporter for NT-3 suggest a differential expression of NT-3 in supporting cells only near the striola as well as around the sensory patches of the utricle and saccule but not in hair cells (Fritzsch et al. 1997d; Fariñas et al., in preparation). The NT-3 lac-Z reaction also shows a weak signal for NT-3 in the semicircular canals near the dark cells (Fariñas et al., in preparation). In the cochlea, the NT-3 lac-Z data suggest a much longer persistence of expression in the inner sulcus cells adjacent to the inner hair cells, as well as in inner hair cells (Fariñas et al., in preparation). How much of this NT-3 lac-Z expression is due to transcellular transfer of the β-galactosidase molecule remains unclear.

In addition, numerous data on expression of trk receptors in the sensory epithelium of the cochlea have been published using commercially available antibodies against trk receptors (Knipper et al. 1996). Unfortunately, others using in situ hybridization in neonates (Ylikoski et al. 1993) have not yet confirmed the expression of full-length trk receptors in any sensory epithelium. However, in situ data show expression of truncated trkB receptors in sensory epithelia (Pirvola et al. 1994). Clearly, the data on trk receptor expression require a more detailed analysis using the recently available highly specific antibodies (Reichardt et al., in preparation) in combination with in situ hybridization.

Immunocytochemical data on expression of trk receptors (Knipper et al. 1996) and of BDNF (Montcouquiol et al. 1998) in the sensory epithelia have been used to speculate about a possible involvement of these neurotrophins in neonatal plasticity in the vestibular and cochlear sensory epithelia (Pujol 1986). While the suggestions are important, current data on neurotrophin and neurotrophin receptor mutants either do not provide any support or they fully refute these ideas. However, the mutant data suggest that at least hair cells and their surrounding supporting cells develop fairly normally even in the absence of any innervation (Fritzsch et al. 1997b,c). Also, even in BDNF or NT-3 mutants with partial loss of innervation, the remaining fibers appear to be able to establish normal synapses (Fritzsch et al. 1997b). More recent data on ngn-1 mutants suggest that they never develop any innervation. These data also suggest no dramatic effect of afferent innervation on hair cell maturation in neonates (Fritzsch et al., in preparation). Together, the ngn-1 as well as double neurotrophin and neurotrophin receptor mutant data strongly support a notion of trophic support in one direction only (Fritzsch et al. 1997b): from the sensory epithelia to the afferents. Whether any reciprocity exists requires further investigations.

Nevertheless, there is growing evidence in other systems that BDNF may be involved in activity-mediated neuronal plasticity in postnatal animals (Cabelli et al. 1997; Shieh et al. 1998). Hair cells do express the relevant L-type voltage-gated calcium channel (Boyer et al. 1998) that could help transform enhanced electrical activity into an upregulation of BDNF production and release (Shieh et al. 1998). This could, in turn, provide more neurotrophin to more active afferents and stimulate their sprouting. In addition, the expression of truncated trkB and trkC receptors in the sensory epithelium (Pirvola et al. 1994) could act to minimize access of BDNF to axons, which are not in immediate contact with hair cells producing BDNF (Freyer et al. 1997). Moreover, the unique presence of four exons each with its own set of promoters makes BDNF different from other neurotrophins (Timmusk et al. 1993; Salin et al. 1997).

The finding that BDNF expression becomes downregulated in the inner ear of late neonates (Pirvola et al. 1994; Xing-Qun et al. 1998) would be consistent with the idea that this molecule may play a role in activity-mediated neonatal neuronal plasticity. In the visual system, BDNF has been related to a critical phase in early contact forma-


NT-3 +/+
IHC
OHC
OHC
NT-3 -/-
IHC
NT-3 +/+
Efferents
Cochlear nucleus
NT-3 -/-
efferents
Cochlear nucleus
trkB +/+
Utricle
AVC
trkB -/-
Utricle
trkB +/+
Vestibular nucleus
trkB -/-
Vestibular nucleus

tion (Cabelli et al. 1997). Specific tests showing activity-dependent upregulation of BDNF, such as those described by Conner et al. (1997) in hair cells and perhaps vestibular nuclei in neonatal animals, are needed to make these suggestions more substantial. Nevertheless, some data on neurotrophin and neurotrophin receptor mutants are compatible with such a scenario. For example, the few spiral neurons that survive in NT-3 and trkC mutants (Fritzsch et al. 1997c, 1998b) clearly branch more extensively to supply innervation to about ten inner hair cells each (Fig. 3). In contrast, about ten sensory neurons converge onto a single hair cell in control animals (Fig. 3). We would like to suggest that this excessive divergence of a single afferent fiber in the cochlea may be due to the BDNF produced by the denervated hair cells in the basal turn regardless of the absence of innervation, thus promoting sprouting of surviving fibers in NT-3 mutants.

In contrast, the few vestibular ganglion cells that survive in BDNF and trkB mutations do not increase their area of innervation in the utricular and saccular sensory epithelia, equally depleted of afferent innervation (Fig. 3). We suggest that the NT-3 expressed in these sensory epithelia may only be able to sustain those few sensory neurons but is apparently unable to cause their sprouting. Instead, the surviving vestibular sensory neurons appear to even reduce both the numbers of surviving sensory cells (Bianchi et al. 1996) and the territory innervated by them over postnatal time (Fig. 3). The implications of these data are that, for example, the afferent branching pattern in the vestibular sensory epithelia could be reduced in BDNF and trkB doubly heterozygotic mutants, a prediction that should be tested in the appropriate mutant.

In situ hybridization data show expression of BDNF and NT-3 in the cochlear and vestibular nuclei of neonates (Rocamora et al. 1993; Pirvola et al., unpublished). The expression of these neurotrophins is at the same level as the surrounding tissue. In adults, BDNF appears to be present only in a limited amount in some cells of adult vestibular nuclei (Conner et al. 1997). It would be important to show that BDNF expressed in neonatal vestibular nuclei (Rocamora et al. 1993) is regulated by the activity-driven exon III (Shieh et al. 1998). Nevertheless, it is clear that the 15% remaining cochlear afferents in NT-3 mutants sprout to supply the entire cochlear nuclei (Fritzsch et al. 1997c). Comparable to the peripheral sprouting of individual afferents in the NT-3 mutant, we would like to suggest that the central sprouting is also mediated by the activity-driven BDNF expression.

The reduction of vestibular afferents in BDNF and trkB mutants is comparable to the cochlear afferent reduction in NT-3 mutants (Bianchi et al. 1996; Schimmang et al. 1995; Fariñas et al. 1994). However, there is no sprouting of vestibular afferents to expand into denervated areas of the vestibular nuclei (Fig. 3). This absence of sprouting of the remaining central afferents in BDNF or trkB mutants supports the notion that BDNF regulates neonatal plasticity, probably through an activity-driven BDNF upregulation, as in the visual system (Shieh et al. 1998).

An area largely unexplored concerns the effects of various neurotrophin mutations on vestibular and cochlear nucleus neuron survival and maturation. Data obtained in chicken after otocyst ablation were the first to demonstrate a trophic dependency of these neurons on their afferent supply (Levi-Montalcini 1949). Similar data were later reported for frogs (Fritzsch 1990) and mammals (Moore 1992). Conceivably, the effects on cochlear nuclei described after neonatal ablation of the cochlea should be more extensive in trkB/trkC or BDNF/NT-3 double mutants, in which no spiral neurons survive past embryonic day 18 (Ernfors et al. 1995; Silos-Santiago et al. 1997; Liebl et al. 1997). However, thus far only a reduction of the cerebellum, which receives direct vestibular afferents, has been noted (Silos-Santiago et al. 1997). Clearly, morphometric analysis of vestibular and cochlear nuclei in double neurotrophin mutants is needed to put mammals onto an equal footing with the pioneering work of Levi-Montalcini (1949) in the chicken. We are currently conducting this analysis in double trkB/trkC mutants (Fritzsch et al. 1995; Silos-Santiago et al. 1997), comparing the data with ngn-1 mutants (Ma et al. 1998) in which no vestibular or spiral neurons ever form.

◀ **Fig. 3** This plate shows the sprouting of remaining fibers in the cochlea and cochlear nuclei in NT-3 mutants (*top four images*) and the lack of such sprouting in the utricle and vestibular nuclei of trkB mutants (*bottom four images*). Note that despite a comparable reduction of 85% of ganglion cells in each of the two mutations (Fariñas et al. 1994; Silos-Santiago et al. 1997), the behavior of the remaining fibers is different. We suggest that the potential for sprouting in the NT-3 mutant both in the ear and in the brain may relate to the normal BDNF expression in this mutant. In contrast, trkB mutants show no sprouting despite a comparable opportunity to expand onto denervated vestibular hair cells or vestibular neurons (*inserts*) probably because a trkB mutation precludes any reaction to BDNF to increase sprouting (*IHC* inner hair cells, *OHC* outer hair cells, *HC* horizontal canal, *AVC* anterior vertical canal). *Bars* 100 μm

The role of neurotrophins and GDNF in adult sensory neuron and hair cell protection

Significant hearing loss occurs in more than 10% of the adult human population and more than one-third will have substantial hearing impairment at 65 years of age. In most cases, auditory impairment results from the death of sensory hair cells in the organ of Corti. The auditory nerve at first remains intact after its peripheral targets, the inner hair cells, are lost. However, the auditory sensory neurons will undergo a slow, nearly complete degeneration in the absence of hair cells, which will accelerate when the entire organ of Corti degenerates (Ylikoski 1974; Miller et al. 1997). Communication disabilities of these profoundly deaf individuals can be alleviated by cochlear implants, electromagnetic devices that directly stimulate the sensory neurons. Cochlear implants work only if suitable numbers of sensory neurons are preserved. Prevention of death of auditory sensory neurons is thus of great therapeutic significance, even if hair cells cannot be rescued or regenerated.

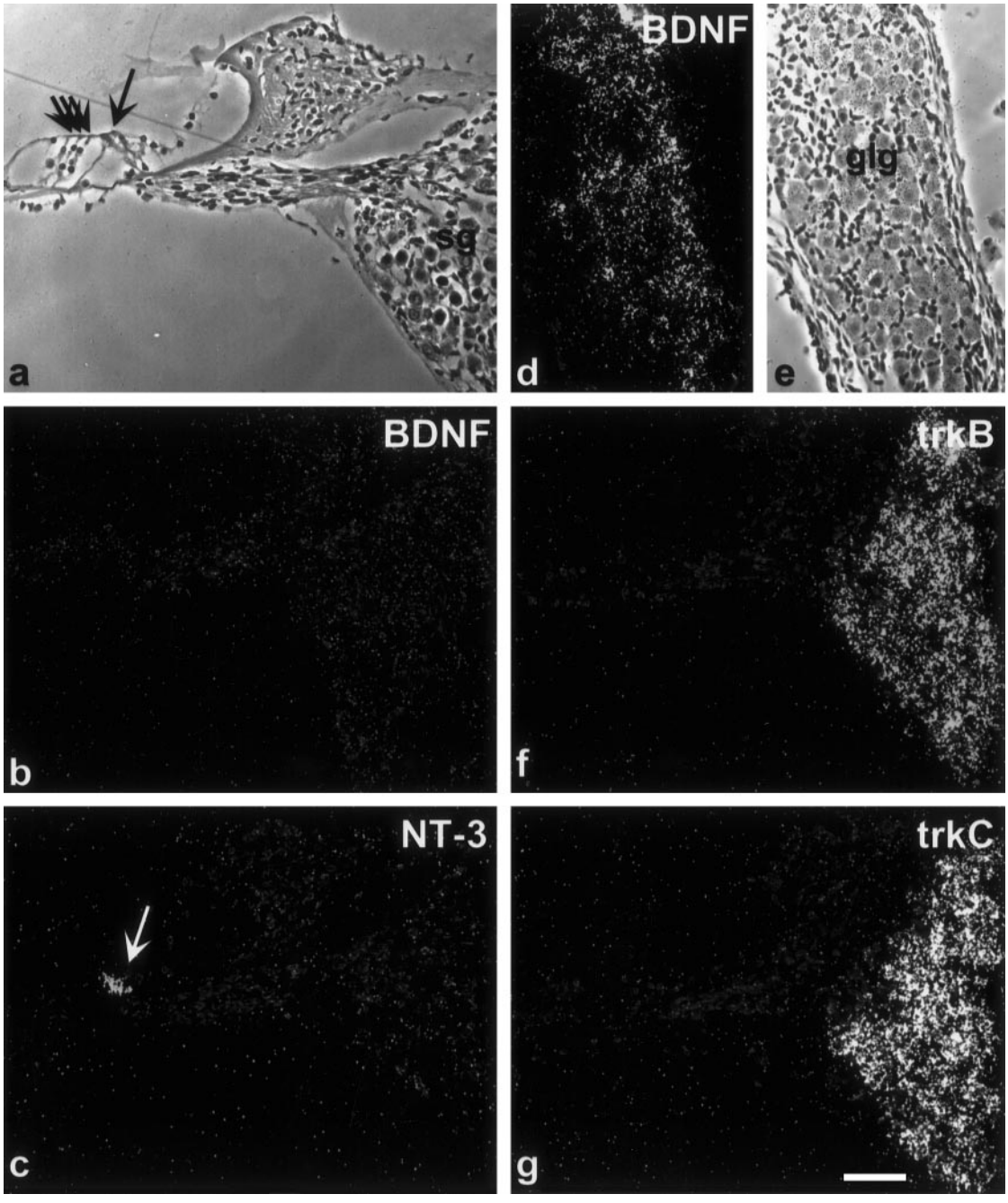


Fig. 4 The expressions of neurotrophin and neurotrophin receptor mRNAs in adult sensory epithelia and neurons of the cochlea **(a–c,f,g)** and the geniculate ganglion **(d,e)** are shown. Note that BDNF is not expressed in sensory epithelia or neurons **(b)** but is present in the nearby geniculate ganglion **(d,e)**, where it may form an autocrine loop, as trkB is also present in these sensory neurons (Pirvola et al. 1994). While the kinase domain of the high-affinity neurotrophin receptors trkB and trkC is expressed in the sensory neurons **(f,g)**, only truncated forms of trkB appear to be present in the sensory epithelia (data not shown). Note that the expression of NT-3 in the adult cochlea is restricted to the inner hair cells **(c)** if studied with in situ hybridization (*sg* spiral ganglion cells, *glg* geniculate ganglion cells, *short arrows* outer hair cells, *large arrow* inner hair cells). *Bar* in **(g)** 100 μm

Expression studies using in situ hybridization show that NT-3 mRNA is strongly expressed in IHCs of the adult rat. mRNA of the high-affinity receptors trkC and trkB is expressed in the sensory neurons (Fig. 4). The mature cochlear hair cells do not express mRNAs of BDNF, trkB or trkC (Fig. 4) and neither BDNF nor NT-3 mRNA is expressed in sensory neurons (Fig. 4). Thus, an autocrine loop, similar to that reported for dorsal root ganglia (Acheson et al. 1995), may not exist in inner ear sensory neurons. Therefore, cochlear sensory neurons are critically dependent on trophic support from their peripheral field (the inner and outer hair cells). Consequently, damage to the IHCs stops the supply

of NT-3 (and BDNF) and causes a nearly complete retrograde degeneration of cochlear neurons (Ylikoski et al. 1998). The crucial role of NT-3 and BDNF in the development of auditory neurons has been discussed in the previous paragraphs. Together, this suggests that NT-3 and BDNF could be used as therapeutic agents to protect cochlear neurons from degeneration. Thus, it comes as no surprise that several studies have attempted to show a protective effect of neurotrophins after various toxic actions of different classes of ototoxic substances and noise.

Some authors, using physiological concentrations of BDNF and NT-3, report only a neuroprotective effect of BDNF on spiral neurons (Miller et al. 1997). In contrast, others using much higher concentrations reported a substantial neuroprotective effect subsequent to aminoglycoside treatment of NT-3, rescuing approximately 90% of the adult spiral ganglion cells (Ernfors et al. 1996; Staecker et al. 1996). BDNF was found to be less protective for spiral neurons. Clearly, specificity of neurotrophins to known receptors may become an issue if the concentration exceeds substantially the range normally found in the cochlea. It is therefore difficult to evaluate how the neuroprotective effect is achieved with these high concentrations of neurotrophins. In addition, administration of such high concentrations for therapeutic purposes may prove infeasible. Other, smaller and less expensive neuroprotective substances interfering with hair cell degeneration after ototoxic treatment may be more useful therapeutic means than neurotrophins.

Other factors such as activity (Hegarty et al. 1997) or other neurotrophic factors such as GDNF (Ylikoski et al. 1998) are effective in maintaining spiral neurons after hair cell destruction, or hair cells, respectively. However, there is a time delay in the onset of spiral neuron loss after hair cell destruction. Only when the entire organ of Corti degenerates is there a rapid decline in the numbers of spiral neurons (Miller et al. 1997). This delayed effect on spiral neurons suggests that factors supporting spiral neurons may be released not only from hair cells, but also from other cells of the organ of Corti. In particular NT-3 could be upregulated and released by supporting cells as its gene is known to be expressed in these cells during development. Ototoxic lesions of hair cells should be combined with NT-3 lac-Z expression to test for upregulated expression of NT-3 in these circumstances. Likewise, other factors such as FGFs could be released from non-sensory cells in the organ of Corti and form the molecular basis for the delay in spiral neuron demise after hair cell destruction (Miller et al. 1997). Clearly, further tests are needed to help understand the molecular nature of this delayed response of spiral neurons after hair cell destruction. Understanding this issue could be important for the long-term viability of cochlear implants.

Several authors have recently shown in animal model systems (using hair cell counts, cytocochleograms and hearing threshold measurements, e.g., by auditory brainstem responses) that the cochlear hair cells can be protected from both ototoxic and noise damage using various compounds. The most commonly used therapeutic compounds have been antioxidants or free radical scavengers. They have been tested because both ototoxic drug and noise damage have been postulated to produce an excess of reactive oxygen radicals (ROS) in the inner ear (Schacht 1998). Overproduction of ROS is thought to cause sensory hair cell damage by overwhelming the cochlea's antioxidant defense system (Ravi et al. 1995). Neurotrophins (e.g., NT-3 and BDNF) and other neurotrophic factors (GDNF), known to be important for protection of neurons within the ear, are now shown also to protect sensory hair cells from damage (Ernfors et al. 1996; Staecker et al. 1996; Gabaizadeh et al. 1997; Ernfors et al. 1998; Keithley et al. 1998; Shoji et al. 1998; Tay et al. 1998).

Unfortunately, it is still unclear how neurotrophins exert their neuroprotective effect either during development or in the adult ear after neurotoxic drug administration. It seems clear that the neurotrophin effect is not mediated through catalytic trk receptors, which are not present in the organ of Corti of the mammalian cochlea (Pirvola et al. 1994; Xing-Qun et al. 1998; Fig. 4). There may be specific pathways using non-trk receptors, e.g., through c-*jun* phosphorylation (Courtney et al. 1997), or the effect may be even more unspecific. Neurotrophins have been shown to act as free radical scavengers (Dugan et al. 1997), possibly through inhibiting cytotoxic nitric oxide (NO) synthesis (Klockner et al. 1997) or preventing neurotoxicity induced by NO donors (Yu and Chuang 1997). They may also modulate the NMDA receptors and prevent their activation by serving as glycine-like ligands for the NMDA receptors (Jarvis et al. 1997). Excitotoxic mechanisms mediated by activated glutamate receptors and subsequent release of cytotoxic NO have been suggested to be a pathogenic mechanism damaging both neurons and hair cells in the cochlea (Ernfors and Canlon 1996). Finally, one of the actions of the neurotrophins is to modulate and maintain intracellular calcium at appropriate levels in a number of in vivo and in vitro systems (Jiang and Guroff 1997; Holm et al. 1997). In the cochlea, neurotrophins may directly prevent an increase in intracellular Ca^{2+} and thus provide protection from cellular damage. The proposed unspecific protection of hair cells and auditory sensory neurons is also supported by observed protective effects by exogeneous applied NGF. Neither NGF nor its receptor trkA is present in the cochlea (Ylikoski et al. 1993), but it appears to be able to rescue auditory neurons from aminoglycoside-induced degeneration (Schindler et al. 1995).

Likewise, it is unknown how and where the intracellular cascade from the high-affinity receptor interferes with the intracellular cell death signaling. Release of cytochrome C from mitochondria has been frequently suggested as a cofactor for caspase-mediated apoptosis (Hengartner 1998). However, even cytochrome C in the cell can be blocked by Bcl-2, the most important inhibitor of cell death (Rosse et al. 1998) and a possible candidate to be activated in neurotrophin-mediated cell death inhibition. A more detailed understanding of the intracellular pathways involved in cell death is needed to elucidate the role of neurotrophins in developmental and induced cell death.

An interesting possibility is offered by the nearby geniculate ganglion sensory neurons. These cells express both

the neurotrophin BDNF (Fig. 4) and the high-affinity receptor trkB (Pirvola et al. 1994). Geniculate sensory neurons innervate taste buds on the fungiform papillae and can regenerate after transection of their fibers to reinnervate the taste buds (Oakley 1993; Fritzsch et al. 1997d). Much like some dorsal root ganglia (Acheson et al. 1995), they form an autocrine loop that allows them to sustain their viability after being disconnected from the periphery. Understanding the regulation of BDNF expression in these sensory neurons could enable us to induce a similar expression of BDNF in auditory sensory neurons. This would likely provide a similar resistance to loss of the peripheral target as is displayed by geniculate sensory neurons. In this context it should be pointed out that amphibians do have a substantial capacity to restore lost connections (Zakon 1988) and may be able to do so because they express both trkB and BDNF in their sensory neurons (Don et al. 1997). In fact, introduction of BDNF genes into spiral neurons using herpes vectors prevents spiral neuron degeneration after hair cell loss (Staecker et al. 1998). All the above data indicate that neurotrophins can protect auditory neurons from degeneration. It remains to be demonstrated whether these molecules are also capable of repairing these neurons.

Conclusion

A more detailed molecular analysis is needed to understand how the neurotrophins BDNF and NT-3 achieve their action in development and maintenance of inner ear afferents through their high-affinity receptors trkB and trkC. Such an understanding will be important to evaluate the interactions of these molecules in the model system inner ear, where these two neurotrophins and their high-affinity receptors are the most crucial molecules, at least during development. A more complete understanding, in particular of BDNF, is needed to elucidate the possible role of this neurotrophic factor in postnatal, activity-mediated plasticity and in therapeutic use for spiral and vestibular neuron protection. However, for the latter use, other molecules that interact directly with the cell death cascade (Hengartner 1998) may prove more useful in clinical trials.

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