Survival of inner ear sensory neurons depends on two neurotrophins, BDNF and NT-3, and their respective receptors, TrkB and TrkC. Because both receptors are present in the same neuron, it has been suggested that BDNF and NT-3 are functionally redundant in promoting neuronal survival. Knock-in of one ligand into the locus of the other one confirmed this hypothesis for the cochlea, leaving open the question of why two neurotrophins are required for proper innervation of the mammalian ear. Here, we show that the precise spatiotemporal pattern of expression of the two neurotrophins is essential for proper patterning of the inner ear innervation. Mice expressing BDNF under the control of the NT-3 promoter develop exuberant projections of vestibular sensory neurons to the basal turn of the cochlea. This projection can be enhanced by combining the transgene with a null mutation of BDNF. However, vestibular fibers rerouted into the cochlea do not reach hair cells and remain outside the organ of Corti, suggesting a chemotactic role for neurotrophins on these fibers. Our data provide genetic evidence that neurotrophins in the ear exert both survival and axon guidance roles.

Key words: cochlea; ear; guidance; neurotrophic; neurotropic; vestibular

Introduction

The inner ear of vertebrates is composed of three different sensory systems dedicated to the perception of sound, gravity, and various head movements (Lewis et al., 1985). Keeping the information gathered by these different systems distinct requires that connections of the ear with the brain develop such that each of the six end organs communicates its information selectively to specific areas of the brain for sensory information processing (Maklad and Fritzsch, 2003b; Newlands and Perachio, 2003; Ryugo and Parks, 2003). Therefore, connections of the cochlea have to develop in such a way that specific areas are mapped in a precise topological manner onto auditory nuclei and higher-auditory centers (Rubel and Fritzsch, 2002) to ensure appropriate development of the tonotopic neuronal network that decodes direction-specific information (Kapfer et al., 2002). Recent evidence on the distribution of sensory neurons connecting various end organs suggests that matching sensory neurons to specific epithelia and central targets may not rely on mechanisms known from other sensory systems (Maklad and Fritzsch, 2003a). At the moment, it is fair to say that the molecular basis for the development of specific inner ear connections is still unknown (Fritzsch, 2003).

One aspect unique to the development of the mammalian ear is a complicated spatiotemporal pattern of expression of two neurotrophins, BDNF and NT-3 (Ernfors et al., 1992; Pirvola et al., 1992; Farinas et al., 2001) and an overlapping distribution of their specific receptors, TrkB and TrkC (Ernfors et al., 1992; Ylikoski et al., 1993; Farinas et al., 2001). What exactly the function of two neurotrophins is in the mammalian ear remains somewhat unclear. Initial data suggested a hair cell-specific projection (Ernfors et al., 1995). Closer analyses of single null neurotrophin mutants showed that the developmental dynamics of neurotrophin expression leads to specific loss of sensory neurons to parts or entire sensory organs in which the other neurotrophin cannot compensate for its absence (Bianchi et al., 1996; Fritzsch et al., 1997). It has been suggested that the two neurotrophins of the ear may be functionally replaceable because their specific function appears to be related to their respective expression profile rather than any specific signaling capacity (Farinas et al., 2001). Indeed, recent studies using replacement of either neurotrophin by the other indicates functional equivalence of either neurotrophin for the cochlea (Coppola et al., 2001; Agerman et al., 2003). Interestingly, although replacement of NT-3 with BDNF rescues the survival of the cochlea basal turn sensory neurons, lost in NT-3 null mutant mice, it also generates an exuberant innervation of the basal turn (Coppola et al., 2001).

Using this mouse model (Coppola et al., 2001), we now provide evidence that the delayed expression of BDNF in the basal turn of the cochlea is crucial to avoid redirection of vestibular fibers into the cochlea to generate an exuberant innervation. We also provide evidence that in the ear neurotrophins are not necessary for initial pathfinding. Together, these findings suggest that in the ear there are two distinct molecular guidance phases that control the pattern of innervation.

Materials and Methods

Mice. NT-3<sup>−/−</sup> is a homozygous null mutation for the NT-3 gene that expresses BDNF under control of the NT-3 promoter. The
Results

We previously showed that loss of cochlear innervation caused by NT-3 deficiency can be rescued by BDNF expression under the NT-3 promoter (Coppola et al., 2001; Fritzsch et al., 2003). Paradoxically, we have also observed an extensive outgrowth of fibers selectively to the basal turn of the cochlea, despite some minor spiral ganglion neuron loss. Therefore, we have investigated whether these additional fibers came from sources other than the cochlea-specific spiral ganglion neurons. We focused on the vestibular afferents because only these ear afferents can reach the cerebellum and, thus, can readily be distinguished in retrograde labeling experiments (Dino et al., 2001; Barmack, 2003; Maklad and Fritzsch, 2003a; Newlands and Perachio, 2003). Because central expression of neurotrophins is upregulated only after initial fiber projections are established (Fritzsch, 2003), we reasoned that only peripheral projection of sensory neurons might be profoundly affected by the misexpression of BDNF under the control of the NT-3 promoter (NT-3/BDNF) (Fig. 1).

Table 1. Number of ears per genotype analyzed at various stages

<table>
<thead>
<tr>
<th>Genotype</th>
<th>E11.5</th>
<th>E12.5</th>
<th>E13.5</th>
<th>P0</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>8</td>
<td>20</td>
</tr>
<tr>
<td>NT-3tgBDNF+/+</td>
<td>2</td>
<td>2</td>
<td>6</td>
<td>12</td>
<td>22</td>
</tr>
<tr>
<td>NT-3tgBDNF+/-</td>
<td>2</td>
<td>4</td>
<td>8</td>
<td>18</td>
<td></td>
</tr>
<tr>
<td>NT-3tgBDNF-/-</td>
<td>6</td>
<td>10</td>
<td>10</td>
<td>12</td>
<td>38</td>
</tr>
<tr>
<td>NT-3tgBDNF+/BDNF+/+</td>
<td>0</td>
<td>2</td>
<td>6</td>
<td>14</td>
<td>22</td>
</tr>
<tr>
<td>NT-3tgBDNF+/BDNF-/-</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>8</td>
<td>14</td>
</tr>
<tr>
<td>NT-3tgBDNF-/-/BDNF-/+</td>
<td>2</td>
<td>4</td>
<td>6</td>
<td>16</td>
<td>28</td>
</tr>
<tr>
<td>BDNF+/+</td>
<td>4</td>
<td>4</td>
<td>2</td>
<td>8</td>
<td>18</td>
</tr>
<tr>
<td>BDNF-/-</td>
<td>4</td>
<td>4</td>
<td>8</td>
<td>6</td>
<td>24</td>
</tr>
<tr>
<td>Total</td>
<td>26</td>
<td>40</td>
<td>46</td>
<td>92</td>
<td>204</td>
</tr>
</tbody>
</table>

WT, Wild type.
Various combinations of NT-3 \textsuperscript{tgBDNF} and BDNF null mutations have limited effects on the pattern of inner ear innervation in early development compared with wild-type littermates (A, E, I). At E11.5, the overall pattern of innervation of mutant mice with different combinations of NT-3 \textsuperscript{tgBDNF} and BDNF null mutations (B–D) closely resembles that of wild type (A). Only a reduction of fiber growth to the horizontal (HC), anterior (AC), and posterior (PC) cristae is observed when an endogenous BDNF level is eliminated (B), even in the presence of NT-3 \textsuperscript{tgBDNF} expression (compare A and C and D). At E12.5, transgenic expression of BDNF starts to cause qualitative differences on inner ear innervation of mutant animals relative to that of controls (E–L). BDNF null mutants show no fibers extending to the posterior crista (F, J) even if combined with NT-3 \textsuperscript{tgBDNF} (G, H, K, L). Instead, these fibers are randomly oriented (K) or directed toward the cochlea (K, L, arrow). There is an obvious growth of fibers to the cochlea (G, H) in NT-3 \textsuperscript{tgBDNF} combined with BDNF null mutation. No such fibers are present in wild-type animals at this stage (E). There is an apparent dosage effect on the degree of branch formation of the posterior crista fibers to the basal turn of the cochlea (l, I, arrow), with the furthest extension of fibers in the simple BDNF null mutants. Scale bar, 100 \textmu m.

**Figure 2.** Various combinations of NT-3 \textsuperscript{tgBDNF} and BDNF null mutations have limited effects on the pattern of inner ear innervation in early development compared with wild-type littermates (A, E, I). At E11.5, the overall pattern of innervation of mutant mice with different combinations of NT-3 \textsuperscript{tgBDNF} and BDNF null mutations (B–D) closely resembles that of wild type (A). Only a reduction of fiber growth to the horizontal (HC), anterior (AC), and posterior (PC) cristae is observed when an endogenous BDNF level is eliminated (B), even in the presence of NT-3 \textsuperscript{tgBDNF} expression (compare A and C and D). At E12.5, transgenic expression of BDNF starts to cause qualitative differences on inner ear innervation of mutant animals relative to that of controls (E–L). BDNF null mutants show no fibers extending to the posterior crista (F, J) even if combined with NT-3 \textsuperscript{tgBDNF} (G, H, K, L). Instead, these fibers are randomly oriented (K) or directed toward the cochlea (K, L, arrow). There is an obvious growth of fibers to the cochlea (G, H) in NT-3 \textsuperscript{tgBDNF} combined with BDNF null mutation. No such fibers are present in wild-type animals at this stage (E). There is an apparent dosage effect on the degree of branch formation of the posterior crista fibers to the basal turn of the cochlea (l, I, arrow), with the furthest extension of fibers in the simple BDNF null mutants. Scale bar, 100 \textmu m.

**Developmental day E11.5**

Analysis of inner ear innervation at E11.5 overall did not reveal striking differences between wild-type or mutant mice with the specific neurotrophin mutation combinations used in this study (Fig. 2A–D). Injections into rhombomere 1 at E11.5 when postmitotic neurons initiate fiber outgrowth showed only one occasional qualitative difference and less fiber outgrowth in the BDNF null mutant mice (Fig. 2B). Combining the BDNF null mutation with NT-3 \textsuperscript{tgBDNF} in heterozygosis or homozygosis resulted only in three of seven ears extending beyond the horizontal cristae toward the posterior crista (data not shown). Even combining absence of BDNF under its own promoter with exclusive expression of BDNF under the NT-3 promoter resulted only in limited alterations in the branching pattern of the fiber growth to the posterior crista (Fig. 2CD). Thus, initial vestibular fiber growth toward the developing sensory epithila is not qualitatively affected by either complete absence of BDNF or misexpression of BDNF under NT-3 promoter control with or without BDNF under its own promoter control (Fig. 2CD). These data suggest that at this stage neurotrophins do not exert a chemo- tropic function on inner ear afferent outgrowth but may, nevertheless, have some limited trophic effects as indicated by the reduced size of the ganglia (Fig. 2, compare A, B).

**Developmental day E12.5**

At E12.5, wild-type and mutant mice with various combinations of transgenic BDNF expression and endogenous BDNF null mutations showed obvious differences (Fig. 2E–L), especially in the innervation of the posterior crista (Fig. 2I–L) and the vestibular fiber growth to the basal turn of the cochlea (Fig. 2E–H). BDNF null mutant mice showed few fibers extending to, but none reaching, the posterior crista (Fig. 2F, J). Most interesting is the more profound outgrowth of fibers to the cochlea in mice with BDNF replacing NT-3 (Fig. 2G, H), a feature never seen in wild-type littermates or in younger transgenic animals (Fig. 2D, E). Brainstem injections showed only a few labeled spiral sensory neurons at this stage in wild-type mice (Fig. 2E) or simple transgenic heterozygotes (data not shown). Wild-type and NT3\textsuperscript{tgBDNF} heterozygous animals have an occasional growth of a few fibers from the posterior canal crista toward the cochlea (Fig. 2I, arrow). However, with the increased expression of the transgenic BDNF and concomitant reduction of the endogenous BDNF, more fibers extend toward the cochlea (Fig. 2K, L, arrow). In BDNF null mice with NT3\textsuperscript{tgBDNF} homozygosis or heterozygosis, no fibers actually reach the posterior crista epithelia (Fig. 2G, H, K, L). Instead, in these mice fibers stall between the basal turn of the cochlea and the posterior crista and either extend in multiple directions or toward the basal turn of the cochlea (Fig. 2K, L). In one animal, all fibers of the posterior crista twig turn to the cochlea (data not shown).

Taken together, these data suggest that initial neurite outgrowth is mainly BDNF independent. However, the topology of the BDNF expression exerts some control over the sites to which fibers are going to project as early as E12.5. Variation of the qualitative pattern in a group of animals with a specific genotype is much less than the variation between different genotypes, suggesting that the variations observed reflect the genotype differences and not random variations not associated with the genotype.

**Developmental day E13.5**

Injection of tracers into the cerebellum of wild-type animals reveals no fibers to the cochlea but fibers to all five vestibular sensory epithelia (Fig. 3A). BDNF null mutants at E13.5 have only an occasional residual fiber projecting toward the posterior canal crista (Fig. 3D), as described previously (Bianchi et al., 1996). Furthermore, only a single fiber was sometimes found extending from the utricle to the anterior and horizontal cristae (Fig. 3C). Most importantly, when lipophilic tracers were injected into the cerebellum, neither BDNF null mice nor wild-type littermates showed more than an occasional fiber projecting to the cochlea (Fig. 3A, D). In contrast, even NT3\textsuperscript{tgBDNF} heterozygotic embryos showed rerouting of fibers to the basal turn of the cochlea (Fig. 3F), which was even more profound in NT3\textsuperscript{tgBDNF} homozygotic embryos (Fig. 3B). Such rerouting occurred in both homozygous and heterozygous animals and originated predominantly from the saccule (Fig. 3, compare A, B) with fibers also rerouting to the cochlea from the nerve twig to the posterior canal (Fig. 3B, arrow).

When NT3\textsuperscript{tgBDNF} was combined with the BDNF null muta-
tion, more fibers were rerouted from the vestibular epithelia into the cochlea (Fig. 3F, arrowhead). Furthermore, many more fibers could be traced to the anterior and horizontal crista (Fig. 3E) than in the simple BDNF null mutants (Fig. 3C). In addition, fibers to the posterior canal could be traced toward the posterior crista but appeared to stall between the basal turn of the cochlea and the posterior canal, radiating parallel to the cochlea (Fig. 3F, arrow). No projections entered the posterior canal, but a sizable bundle was invariably found to extend toward the basal turn of the cochlea (Fig. 3F, arrow). These data suggest a preferred branch node near the posterior crista at which fibers switch from a non-neurotrophin-dependent mode to a neurotrophin-dependent mode of navigation, neurite survival, or both.

**Inner ear innervation at birth**

We next analyzed the effects of NT-3tgBDNF in the presence or absence of endogenous BDNF on the innervation of the vestibular epithelia at birth (P0) (Fig. 4). Previously, we have shown that NT-3tgBDNF rescues almost completely the innervation of the cochlea basal turn, which is entirely lost in the NT-3 null mutants (Fritzsch et al., 1997). This result had suggested that BDNF and NT-3 are functionally equivalent for the survival of cochlear neurons (Coppola et al., 2001). A more recent study has confirmed our findings on the cochlea by replacing endogenous BDNF with NT-3. This study also suggested that NT-3 cannot rescue vestibular fibers (Agerman et al., 2003). As reported previously (Bianchi et al., 1996), we found that BDNF null mutants have a reduced, although still relatively dense, innervation of the utricle and very few fibers, if any, that extend to the anterior and horizontal crista and none to the posterior crista (Fig. 4F) (data not shown). The anterior and horizontal cristae of NT-3tgBDNF mice are always innervated, independently of the level of endogenous BDNF (wild type, heterozygous, or null) (Fig. 4A, inset). However, all NT-3tgBDNF transgenic mice had projections of fibers outside the sensory epithelium radiating beyond the cristae (Fig. 4A, C, D, arrowheads) or to an area devoid of sensory hair cells near the utriculo-saccular foramen (Figs. 4A–E, arrows). The central projections of these extensions appear to be vestibular like, because these fibers are labeled from the cerebellum injections of dye (Fig. 4G, arrow) despite the fact that they are not in touch with hair cells in the ear. Previous analysis of NT-3 expression with an NT-3-LacZ reporter mouse line showed that there is a faint NT-3 presence in those areas of fiber extension (Farinas et al., 2001). These data suggest that NT-3tgBDNF expression can rescue fibers to the anterior and horizontal crista but not to the posterior crista. This rescue might be related to the close proximity of the anterior and horizontal crista to the utricle, which could provide a source of diffusible BDNF. However, maintenance of posterior crista fibers would require BDNF to diffuse from the cochlea that is a far more distant target. In addition, NT-3tgBDNF causes fiber rerouting into areas that have no sensory hair cells but express limited amounts of NT-3, in the transgenic animals replaced by BDNF. Together, these data support the notion that areas of NT-3 expression in the nonsensory part of the ear will attract and support fibers even in the absence of hair cells, provided they produce BDNF.

We next examined why the posterior crista fibers could not be rescued. NT-3tgBDNF can rescue the innervation loss of the basal turn of the cochlea caused by NT-3 deletion (Coppola et al., 2001). However, many more fibers project to the cochlea in these animals compared with controls. Surprisingly, cerebellar dye injections label fibers that extend toward the basal turn of the cochlea. These fibers are present in all NT-3tgBDNF animals examined, but their density varies depending on the mouse genotype of the endogenous BDNF (Fig. 5B–D). Only a few fibers can be labeled from the cerebellum in the doubly heterozygous NT-3tgBDNF BDNF animals (Fig. 5A) and none in wild-type animals (Fig. 5, inset). Furthermore, these fibers innervate exclusively the basal turn of the cochlea and arborize near both inner and outer hair cells (Fig. 5A). Careful preparation of the posterior cristae nerve together with the basilar turn of the cochlea shows that many of these fibers derive from the posterior crista nerve (Fig. 5B, arrow). Combining NT-3tgBDNF homozygosity with a reduction (Fig. 5C) or loss (Fig. 5D) of endogenous BDNF shows a dose-dependent effect with NT-3tgBDNF–/–; BDNF–/– mice having the most vestibular fiber projections to the basal turn of the cochlea (Fig. 5B–D). Interestingly, these mice also show a robust projection to both the inner and, in particular, the outer hair cell regions (Fig. 5D). These data suggest that NT-3tgBDNF–/–; BDNF–/– animals lack posterior crista innervation because surviving posterior crista nerve fibers are rerouted into the basal turn of the cochlea. Furthermore, the rerouting suggests that the significant expression of BDNF under the NT-3 promoter control in the basal turn of the cochlea may provide a source of BDNF that
by far exceeds the limited and delayed amounts of endogenous BDNF synthesized in the nonsensory epithelium near the posterior crista in the NT-3$^{+/-}$; BDNF$^{-/-}$. Thus, the fibers rerouted in the transgenic animals appear to follow concentration gradients of BDNF, which are critical for chemoeffecting these fibers to their precise target (Farinas et al., 2001).

Taken together, these data suggest that vestibular fibers of the posterior crista and possibly the saccule are rerouted in NT-3$^{+/-}$ transgenic animals to project to the basal turn of the cochlea. To further prove this point, we next injected one lipophilic tracer into the posterior crista and a differently colored tracer (Fritzsch et al., 2002) into the basal turn of the cochlea. For technical reasons, we used NT-3$^{+/-}$.BDNF$^{-/-}$ mutants combined with BDNF in heterozygosity because these animals have robust basal turn projections as well as a significant innervation to the posterior crista (data not shown). Injections of a red-colored dye into the cochlea and of a green-colored dye into the posterior crista showed an overlapping distribution of vestibular sensory neurons in the inferior vestibular ganglion (Fig. 6A–C) adjacent to the cochlea nerve. The position of the posterior crista neurons in the inferior vestibular ganglion was exactly as described previously (Maklad and Fritzsch, 2002, 2003a), suggesting that indeed all rerouted afferents to the cochlea derive from neurons that project to the posterior crista and the saccule in wild-type animals. Incidentally, a small and variable number of double-labeled vestibular neurons were also found, and their existence was directly demonstrated using single-dye injection into the cochlea and posterior crista, respectively. In three of three animals with a cochlear injection, we found labeled fibers projecting toward the utricle and possibly to the saccule (Fig. 6D,E). Furthermore, injection of a different colored dye into the posterior crista could trace in three of three animals fibers to the basal turn of the cochlea (Fig. 6F). These fibers showed bidirectional projections below outer and inner hair cells. We also examined the brainstem projections in two animals in which we performed simultaneous dye applications into the cochlea and the posterior crista. Whereas control animals showed no fibers entering through the vestibular root, NT-3$^{+/-}$ transgenic animals revealed fibers entering through the vestibular root and projections to the vestibular nuclei that overlap completely with fibers labeled from the posterior crista (Fig. 6G,H, yellow fibers). Some fibers were found to extend from the dorsal cochlear nucleus (Fig. 6H) into the vestibular nuclei. However, such fibers were also occasionally found in control animals (data not shown). In summary, this analysis confirmed that indeed the vestibular fibers of the posterior crista are rerouted to project to the basal turn of the cochlea in NT-3$^{+/-}$ transgenic animals while maintaining their central projection as vestibular fibers.

We next analyzed in more detail the terminations of the vestibular fibers in the cochlea by using whole mounted and sectioned ears. Fibers labeled in the cochlea from dye placed in the cerebellum (Fig. 7) showed terminal morphologies unknown from either afferent or efferent fibers at this stage of development (Bruce et al., 1997; Fritzsch et al., 1997). In fact, individual fibers formed multiple branches near the outer or inner hair cells or extended for some distance along the inner or outer hair cells (Fig. 7A–C). These data suggest that BDNF, in addition to its chemoattractant role on these sensory fibers, can also influence their branching pattern and morphology of the terminals.

To further investigate how the altered BDNF expression can affect the pattern of innervation of inner and outer cochlear hair cells, we first looked at the effects caused by the simple BDNF and NT-3 deletions. As reported previously (Bianchi et al., 1996), we
found that BDNF null mutants have abundant afferent fibers projecting to inner hair cells in the basal turn, whereas they have a reduced presence of fibers to several rows of the outer hair cells (Fig. 7G) resembling closely the basal turn innervation in wild-type littermates (Fig. 7E). In contrast, NT-3 null mutant mice retain some innervation of inner hair cells with only occasional fibers to one row of the outer hair cells (Fig. 7D). These data confirm our previous findings that outer hair cell innervation in the base depends predominantly on NT-3 with some minor effects mediated by BDNF (Fig. 7D, G). Filling afferent fibers from the cerebellum of BDNF mutants combined with the NT-3 heterozygous (-/-) mutation showed a more prominent projection of fibers to the outer hair cells (Fig. 7F) despite the fact that no NT-3 is present in these mice. Surprisingly, most of the vestibular fibers from the cerebellum do not reach the level of the cochlear hair cells. Rather, they terminate underneath the cochlear hair cells just below the basilar membrane near the spiral vessels (Fig. 7F).

We next analyzed the fiber organization in sectioned material. Radial sections through the cochlea show unique fibers around the habenula perforate that do project only rarely toward the hair cells of the organ of Corti (Figs. 7H, I). These data suggest that the habenula perforate is more than just a hole through which spiral ganglion afferents enter to the organ of Corti. In fact, the apparent inability of vestibular fibers to enter the organ of Corti suggests that the habenula perforate may serve as an additional filter to allow spiral ganglion neuron afferents to enter the organ of Corti.

In summary, our data provide compelling evidence that BDNF expressed under the control of the NT-3 promoter can rescue not only cochlear (Coppola et al., 2001), but also vestibular, neurons in BDNF null mutant mice. However, vestibular fibers, in addition to being rescued, are also rerouted to unusual targets near the vestibular epithelia and to the cochlea, where BDNF is expressed under NT-3 promoter control.

Discussion

Mammalian neurotrophins and their receptors are crucial for sensory neuron survival (Mocchetti, 2001). In contrast to this function, much less is known about their suggested role in pathfinding (Ming et al., 2002). Recently, using beads soaked with neurotrophins, fiber outgrowth toward the ectopic neurotrophin site has been reported in vivo (Tucker et al., 2001). However, while providing support for an axon guidance role for neurotrophin, these data do not distinguish between a neurotrophic and a neurotropic function of neurotrophins. By using the inner ear as a model system and a gene replacement strategy, we have provided for the first time conclusive evidence suggesting that neurotrophins can exert both roles on the same neuron in vivo. Sensory neurons of the inner ear have an overlapping distribution of the neurotrophin receptors TrkB and TrkC (Ylikoski et al., 1993; Farinas et al., 2001) that allows to support their survival with either BDNF or NT-3 (Coppola et al., 2001; Agerman et al., 2003). By replacing NT-3 with BDNF in combination with different endogenous levels of BDNF, we have shown that the initial afferent fiber outgrowth in the ear is independent of neurotrophins because peripheral dendritic growth is unperturbed by any genetic manipulation of neurotrophin expression. In a second phase of fiber outgrowth, starting at E12.5, neurotrophins not only support neuronal survival but exert also a role in dendrite guidance because mis-expression of BDNF causes rerouting of vestibular neuron dendrites to the cochlea.

Thus, after an initial phase of normal fiber outgrowth, which is neurotrophin independent, the precise spatiotemporal expression of neurotrophins is indeed crucial to avoid developmental rerouting of vestibular fibers to the cochlea of the ear. These data provide evidence that support the existence of two distinct phases in molecular guidance in the developmental pattern of innervation of the inner ear. Similar support of BDNF for dendritic
The role of neurotrophins in fiber guidance

We have previously shown that undifferentiated hair cells (Xiang et al., 2003) or even complete elimination of the sensory epithelium, a site of neurotrophin expression (Pauley et al., 2003), is compatible with directed fiber growth. Recently, it has been reported that semaphorins may, at least in part, provide molecular guidance at this stage because manipulating the neuropilin-1 receptor causes fiber misguidance (Gu et al., 2003). The profound effect caused by manipulations in the neurotrophin system, after the initial growth toward the sensory epithelia has occurred, indicates that neurotrophins mediate survival and routing of fibers only in a second phase. It is tempting to refer to this redirection as a neurotrophic fiber guidance because it coincides with the survival of sensory neurons.

We have found that in NT-3^tgBDNF mice fibers project to both vestibular sensory epithelia as well as to nonsensory areas known to express NT-3 in wild-type mice (Farinas et al., 2001). For example, the exogenous BDNF can support fibers innervating the anterior and horizontal crista as well as small nonsensory patches near the utricle. Such fibers form both in the presence or absence of endogenous BDNF (Fig. 4). These data suggest that BDNF per se is a potent chemotaxant that support fibers at least as well as hair cells and may be the major attractor of fiber growth to hair cells because it is prominently expressed in all developing hair cells (Pirvola et al., 1992; Farinas et al., 2001).

The pattern of experimentally rerouted fibers resembles patterns in nonmammalian vertebrates

In nonmammalian vertebrates, fibers can project to the basilar papilla (cochlea) along two different pathways (Fritzsch, 1987, 2002; Fritzsch and Wake, 1988). Interestingly, in those vertebrates, fibers to the cochlea that come off the nerve twig to the posterior crista seem to branch off at or near the branch point of fibers to the papilla neglecta, similarly to what we observed here in the NT-3^tgBDNF mice (Figs. 3F, 8). The effect caused by neurotrophin replacement on these fibers when they reach this area suggest that, at this position, they may shift to a different mode of guidance (e.g., from semaphorins to neurotrophins).

Mammals seem to be the only vertebrates in which the ear expresses an additional neurotrophin, NT-3, in significant amounts (Pirvola et al., 1997; Hallbook et al., 1998). Our previous data have shown that expression of NT-3 in the basal, high-frequency turn of the cochlea of mammals is followed by a delayed upregulation of BDNF in this turn (Farinas et al., 2001). This seems to provide a unique solution to the rerouting problem of fibers from the posterior crista nerve twig found in some nonmammalian vertebrates. However, premature expression of BDNF under NT-3 promotor control in the basal turn of the NT-3^tgBDNF transgenic mice experimentally nullifies this solution, causing fibers to reroute in a pattern similar to that of some nonmammalian vertebrates (Fritzsch and Wake, 1988). More data on the relative timing of hair cell proliferation and branching and maintenance, but not initial growth, has been reported for forebrain pyramidal neuron dendrites (Gorski et al., 2003b) and supports the involvement of BDNF and its receptor in neuronal plasticity (Minichiello et al., 1999; Gorski et al., 2003a) as well as in pathological learning deficits (Chen et al., 2003; Martinowich et al., 2003).
expression of BDNF in nonmammalian vertebrates is needed to critically test this hypothesis.

The role of neurotrophins in intraepithelial innervation
Although these data settle the functional role for neurotrophins in interepithelial connection specification, a second role in intraepithelial connection specification is still controversial. It has been suggested that inner hair cell innervation of the cochlea is lost in NT-3 and TrkC null mutants, whereas outer hair cell innervation is lost in BDNF and TrkB null mutants (Erfors et al., 1995; Schimmang et al., 1995). This idea is consistent with the fact that the number of spiral sensory neurons lost in a given neurotrophin null mutant closely matches the number of a given type of spiral sensory neurons (Fritzsch et al., 2001; Agerman et al., 2003). However, additional detailed analysis of NT-3-/- mice (Fritzsch et al., 1997) showed that inner hair cells, and only few outer hair cells, (Fig. 7D) are innervated. The BDNF null mutant mice studied here have afferent fibers innervating several rows of outer hair cells (Fig. 7G), as previously reported for other mutant lines (Bianchi et al., 1996; Agerman et al., 2003). The profound growth of fibers to outer hair cells in the NT-3tgBDNF mice is not compatible with a cell-specific attraction of different spiral afferents.

BDNF mediates spatiotemporal distribution and direction of cochlear fibers
The situation is more complex for the fibers innervating the cochlea. For one thing, NT-3 expression progresses along the cochlea (from base to apex), across the cochlea (from lesser to greater epithelial ridge), and changes cell types (from Deiters’ cells to inner hair cells (Farinas et al., 2001). In contrast to some vestibular epithelia, fibers enter the cochlea in both TrkB and BDNF null mice (Fritzsch et al., 1995; Schimmang et al., 1995; Bianchi et al., 1996; Agerman et al., 2003). However, replacement of NT-3 with BDNF, while rerouting vestibular fibers to the cochlea, is not sufficient to allow these fibers to cross the habenula perforata. Only occasional branches can reach the inner hair cells (Fig. 7). It is possible that the habenula perforata may actually provide a selective and attractive entry point for spiral ganglion neuron dendrites into the cochlea and that the limited diffusion of BDNF from its expression in supporting cells may provide neurotrophic support for these fibers. Alternatively, cochlear hair cells may release a non-neurotrophin factor that acts as a specific attractor for spiral sensory afferents and to which vestibular sensory afferents cannot respond. Previous work has shown that cochlear innervation to the apex is rather normal in mice with limited differentiation of hair cells (Xiang et al., 2003).

In summary, we present here data on afferent development of the ear using the recently reported transgenic expression of BDNF under NT-3 promotor control (Coppola et al., 2001). Previous work has shown that BDNF and NT-3 can functionally replace each other in the cochlea (Coppola et al., 2001; Agerman et al., 2003). By combining the NT-3tgBDNF mice with BDNF null mutation, we show that the initial fiber growth is not determined by neurotrophins (Fig. 8). Additional fiber growth occurs during the cell death phase in such mutants (E12.5–E15.5), leading to a massive rerouting of vestibular sensory neuron afferents to the cochlea. This rerouting is mediated by BDNF, suggesting that...
Figure 8. Schematic representation of the changes in the developmental pattern of vestibular innervation of the inner ear caused by the altered expression of BDNF and NT-3. BDNF and NT-3 expression are shown, respectively, in green and red; NT-3 tgbDNF is shown in blue. At E11.5, the initial fiber projection is predominantly to the anterior (AC), horizontal (HC), and posterior (PC) crista and overall is identical in all genotypes. At E12.5, fibers to the crista organs are reduced in BDNF null mutants, and by E13.5, there is a complete loss of projections to the posterior crista (top row). The late and limited expression of NT-3 next to the crista sensory epithelia at E13.5 (top right) seems to be unable to rescue those fibers. Starting at developmental day E12.5 there is a significant growth of fibers to the utricle (U) and saccul (S) in all genotypes. At this stage, homozygous NT-3 tgbDNF animals show a progressive development of novel fiber tracts (shown in blue), which extend either from the vestibular ganglion directly to the cochlea (Co) or emanate from the posterior crista twig (bottom right pictures). More of these fibers are found if the transgenic BDNF is combined with the BDNF null mutation (blue arrows in the two bottom rows). In addition, some fibers that do not reach or innervate the posterior crista reroute to the basal turn of the cochlea (bottom right).

neurite growth is primarily regulated in the ear by BDNF, whereas NT-3 acts predominantly as a survival factor (Fig. 8). This finding suggests that growth of afferents to regenerating hair cells (Kawamoto et al., 2003) may be primarily mediated by BDNF expression in differentiating hair cells.

References


J. Neurosci., March 10, 2004 • 24(10):2575–2584 • 2583
Lewis ER, Leverenz EL, Bialek WS (1985) The vertebrate inner ear. Boca Raton, FL: CRC.


