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A disorganized innervation of the inner ear persists in the absence of *ErbB2*

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Abstract

ErbB2 protein is essential for the development of Schwann cells and for the normal fiber growth and myelin formation of peripheral nerves. We have investigated the fate of the otocyst-derived inner ear sensory neurons in the absence of *ErbB2* using *ErbB2* null mutants. Afferent innervation of the ear sensory epithelia shows numerous fibers overshooting the organ of Corti, followed by a reduction of those fibers in near term embryos. This suggests that mature Schwann cells do not play a role in targeting or maintaining the inner ear innervation. Comparable to the overshooting of nerve fibers, sensory neurons migrate beyond their normal locations into unusual positions in the modiolus. They may miss a stop signal provided by the Schwann cells that are absent as revealed with detailed histology. Reduction of overshooting afferents may be enhanced by a reduction of the neurotrophin *Ntf3* transcript to about 25% of wild type. *Ntf3* transcript reductions are comparable to an adult model that uses a dominant negative form of *ErbB4* expressed in the supporting cells and Schwann cells of the organ of Corti. *ErbB2* null mice retain afferents to inner hair cells possibly because of the prominent expression of the neurotrophin *Bdnf* in developing hair cells. Despite the normal presence of *Bdnf* transcript, afferent fibers are disoriented near the organ of Corti. Efferent fibers do not form an intraganglionic spiral bundle in the absence of spiral ganglia and appear reduced and disorganized. This suggests that either *ErbB2* mediated alterations in sensory neurons or the absence of Schwann cells affects efferent fiber growth to the organ of Corti.

Keywords

Ear development; Auditory sensory neuron; Neurotrophin; Pathfinding; Neuronal migration

v-erb-b2 erythroblastic leukemia viral oncogene homolog 2 (*ErbB2*) belongs to a family of 4 ligand activated tyrosine kinase receptors (Schlessinger, 2004; Wang et al., 2004). Among ErbB receptors, *ErbB2* is unique in being an orphan receptor that must heterodimerize with other receptors to signal. Beyond its clinically important function in cancer (Bianco, 2004;

Lemmon, 2003), ErbBs and their ligands, the neuregulins (NRGs) are important in various aspects of survival, proliferation and differentiation of the nervous system. For peripheral nerve development and maintenance, the ligand NRG1 and its receptors, ErbB2 and ErbB3, play key roles in developing neuron-Schwann cell interaction (Adlkofer and Lai, 2000; Michailov et al., 2004). Reduced formation of peripheral nerves, in particular of cranial nerves, has been reported in NRG1 null mutants (Meyer and Birchmeier, 1995; Meyer et al., 1997) and in *ErbB2* null mice (Lee et al., 1995; Morris et al., 1999). NRG1 [Heregulin (HRG) or acetylcholine receptor-inducing activity (ARIA)] is expressed in sensory neurons and motoneurons (Birmingham-McDonogh et al., 1997; Michailov et al., 2004), whereas *ErbB2* and *ErbB3* are expressed in Schwann cells surrounding these fibers in the peripheral nervous system and regulate myelin formation (Corfas et al., 2004; Michailov et al., 2004) and survival of developing Schwann cells (Atanasoski et al., 2006; Lyons et al., 2005; Massa et al., 2005; Young et al., 2005). A dominant negative over-expressor of *ErbB4* using the GFP-promoter that drives *ErbB4* expression in juveniles (Rio et al., 2002) causes progressive sensory loss in adult mice (Chen et al., 2003), presumably acting as a sink for the limited amount of NRG1 and interfering with ErbB2- and ErbB3-mediated signaling (Corfas et al., 2004). While the functional significance of neuregulin ligands and their receptors for nerve-Schwann cell interaction and Schwann cell survival is thus well established in the peripheral nervous system, specific effects of these receptors and ligands as well as their distribution in the developing and adult ear are less clear.

In situ hybridization data suggest the presence of NRG1 in spiral and vestibular sensory neurons (Morley, 1998). However, the NRG1 gene encodes over 15 isoforms of which only some share the EGF-domain (Falls, 2003). Exactly which of these 15 isoforms is expressed in the sensory neurons requires further analysis. Immunocytochemical and in situ studies suggest the presence of ErbB1, 2, 3 and 4 in the adult inner ear with a somewhat differential distribution in hair cells and supporting cells that may vary with the species studied (Hume et al., 2003; Matsunaga et al., 2001; Stankovic et al., 2004; Zhang et al., 2002). Involvement of these ligands and their receptors in proliferation of neonatal vestibular hair cells and supporting cells has been suggested (Hume et al., 2003; Montcouquiol and Corwin, 2001), but no direct test involving ErbB2 or ErbB3 knock down in vitro or in vivo has been performed. In agreement with data from other peripheral nerves, a reciprocal signal between spiral neurons and Schwann cells has been suggested (Hansen et al., 2001), which may relate to the development of acoustic Schwannoma (Hansen et al., 2004). In addition, loss of NRG1 interaction with ErbB2 and ErbB3 has been related to adult sensory neuron death in dominant negative ErbB4 overexpressing transgenic mice (Stankovic et al., 2004), suggesting effects in the ear that closely parallel those in other parts of the peripheral nervous system (Chen et al., 2003; Corfas et al., 2004).

We used *ErbB2* null mutant mice to test whether inner ear innervation depends on Schwann cells. We also wanted to test whether supporting cells have molecular similarities to glial cells (Rio et al., 2002) using the proteolipid protein-enhanced green fluorescent protein (*PLP-eGFP*) mouse (Mallon et al., 2002).

1. Results

1.1. E13.5 ErbB2 null mice show subtle alterations in afferent projections

Previous investigations have shown that the absence of neurotrophins will result in innervation alteration of both afferents (sensory neuron fibers) and efferents (modified facial branchial motoneuron fibers; Karis et al., 2001) from this age onwards (Fritzsch et al., 2004), a stage when all sensory neurons are postmitotic (Matei et al., 2005; Ruben et al., 1967).

We started our investigation of the effects of *ErbB2* abrogation at E13.5 embryos by injecting lipophilic tracers into the brainstem second order auditory (cochlear) and vestibular nuclei. Such injections resulted in labeled fibers extending to all six sensory epithelia of the ear in wild type and *ErbB2* null mice (Fig. 1A, B). Closer examination of the vestibular projection showed a somewhat less precise targeting as more fibers seemed to diverge away from the vestibular epithelia. Comparison of the cochlear projection indicated two differences: (1) spiral ganglia were not found close to the basal turn of the organ of Corti in *ErbB2* null mice, and (2) spiraling fibers, present in wild type littermates, were absent in *ErbB2* mutants (Fig. 1A, B). Interestingly, the cochlear projecting (spiral) neurons were in the center of the cochlear modiolus and not near the organ of Corti.

1.2. Afferent and efferent fibers show considerable alterations in *ErbB2* null mice at E14.5

From this stage onward, we could label selectively the afferent fibers to the cochlea and vestibular sensory epithelia with injections into the cochlea and vestibular nuclei (labeled in red in all figures). Simultaneous injections into the medial vestibular nucleus near the internal facial genu labeled efferent fibers to the cochlea and vestibular epithelia as well as vestibular afferents with dye (labeled green in Fig. 1). These injections revealed the following differences between *ErbB2* null mutants and wild type littermates (Fig. 1C–F).

Wild type littermates showed only afferent and efferent fibers in the modiolus and an assembly of spiral ganglion neurons in a spiral ganglion that paralleled the organ of Corti (Fig. 1D). Only short radial fibers emanated from the spiral ganglion to the organ of Corti (arrow in Fig. 1D). In contrast, spiral ganglion cells were not found in the position of the spiral ganglion in *erbB2* null mice but apparently filled the much enlarged center of the modiolus (Fig. 1C). Long radial fibers emanated from these sensory neurons located in the modiolus. Despite the altered position of the spiral neurons and the increased length of radial fibers in *ErbB2* null mice, numerous afferent fibers extended to the organ of Corti (Fig. 1C, D).

Analysis of efferent fibers (Fig. 1E, F) also showed differences. Wild type efferent fibers extended along the spiral ganglion cells and started to form the intraganglionic spiral bundle within which single efferent fibers spiral from the base of the organ of Corti toward the apex (Fig. 1F). In contrast, no intraganglionic spiral bundle was found in *ErbB2* null mice (Fig. 1E). Instead efferent fibers extended along and in parallel to the elongated afferent radial fibers from the modiolus to the organ of Corti (Fig. 1C, E).

1.3. *ErbB2* null mice show overshooting fibers at E16.5

E16.5 *ErbB2* null mice showed an abundance of fibers to the cochlea and beyond (Fig. 2B). Indeed, *ErbB2* null mice had more fibers reaching the level of the organ of Corti than 2 day older wild type animals in which we found only a limited growth of fibers to the outer hair cells (Fig. 2A). Focusing through the confocal stacks of images at different focal planes showed that the vast majority of fibers did not actually enter the organ of Corti but stayed below the basilar membrane. These fibers could be traced to the lateral wall where they coalesced into large bundles that spiraled toward the apex (LW in Fig. 2B). These data show that afferent fiber growth is more profound to the endorgans in *ErbB2* null mice than in littermates (Fig. 2A, B). However, the normal pattern of innervation is disrupted and fibers are extending outside the organ of Corti.

1.4. Defects of afferent and efferent innervation persist in *ErbB2* null mice at E18.5

All six sensory epithelia were found to be innervated by both afferent and efferent nerves at E18.5 in wild type and *ErbB2* null mice. Although these epithelia were innervated, the pattern of innervation was altered, with the cochlea epithelia having the most pronounced

alteration in the pattern of innervation. As already observed at E14.5, the spiral ganglion was no longer present in its usual position (Fig. 3B, C), but resided instead in an expanded central part of the modiolus in *ErbB2* null mice (Fig. 3A, E). As a consequence of the unusual position of these neurons, much elongated radial fibers extended from the modiolus to the organ of Corti in *ErbB2* null mice (Fig. 3A, E) compared to short radial fibers from the prominent spiral ganglion to the organ of Corti in wild type mice (Fig. 3B, F). Closer examination at a higher magnification showed that the radial fibers in the *ErbB2* null mice were less organized as demonstrated by several fibers crossing between radial bundles (Fig. 4A, B). Overall, the *ErbB2* null mutants showed an apparent reduction of radial fiber density to the organ of Corti (Fig. 3A, B, E, F). These data suggest that in *ErbB2* null mice the spiral ganglion neurons have migrated beyond their normal location of the spiral ganglion near the osseous spiral lamina and are now in the center of the modiolus.

In the inner ear of the wild type E18.5 embryos, the efferent fibers turn into the intraganglionic spiral bundle (IGSB) inside the spiral ganglion (Figs. 3D and 4D). The IGSB gives rise to the densely spaced, short radial fiber bundles (Fig. 4D). In the *ErbB2* null embryos, fibers project as disorganized radial bundles of efferent fibers directly to the organ of Corti without formation of an IGSB (Figs. 3C and 4C). This disorganization may be due to the abnormal position of the spiral ganglion which is normally present closer to the organ of Corti (Fig. 4A, B). Additionally, fewer and more disorganized efferent fibers can be labeled in the *ErbB2* null mutant embryos compared to the control fibers (Figs. 3C, D and 4C, D). Previous studies demonstrated that the efferent fibers rely on the afferent fibers for guidance to the organ of Corti (Farinas et al., 2001; Tessarollo et al., 2004). Therefore, it appears that the improper migration of the spiral ganglion and aberrant afferent projections results in disorganization in the efferent fibers (Fig. 4A–D) comparable to the afferent fibers.

1.5. PLP-eGFP distribution at E18.5

Afferent fibers interact with surrounding Schwann cells to regulate myelin formation and certain aspects of pathfinding (Corfas et al., 2004; Michailov et al., 2004; Morris et al., 1999). Afferent fiber bundles to the sensory epithelia are surrounded by Schwann cells, which start to form myelin sheaths in the mouse ear as early as E18.5 (Fig. 5). Previous work using *PLP* in situ hybridization suggested that the adult Schwann cells of the ear are unique in that they do not express *PLP* except for the ganglion close to the proximal VIIIth nerve root (Knipper et al., 1998). We used a recently available *PLP*-eGFP expressing mouse line (Mallon et al., 2002) in which eGFP is expressed under the control of the mouse *PLP* promoter and contain an additional mRNA stability region that is found in the 3' upstream region of the endogenous mouse *PLP* gene. In these *PLP*-eGFP transgenic mice, all Schwann cells of the ear were strongly *PLP*-eGFP positive, except for a small population of cells near the VIIIth nerve root, which were negative (Fig. 5A–C). All sensory epithelia contained eGFP positive cells (Fig. 4D–F) in wild type embryos. Specifically, in the cochlea, only supporting cells that surrounded the inner and outer hair cells were positive for eGFP. However, some of these supporting cells were not positive after fixation and a patchy distribution with various intensities of label or even apparent absence at E18.5 was observed (Fig. 5D). Lipophilic tracers were used to label the nerve fibers (Fig. 5B, E). These data demonstrated that Schwann cells were found around all vestibular and spiral ganglion neurons as well as along all nerve fibers (Fig. 5C, F). Schwann cell process accompanied nerve fibers to the habenula perforate, the entering holes into the organ of Corti (Fig. 5D–F, inserts). In the organ of Corti, fibers extended between eGFP positive supporting cells surrounding inner and outer hair cells (Fig. 5E, F). These data demonstrated that Schwann cells and eGFP positive supporting cells of the sensory epithelia surround the afferent and efferent fibers at E18.5 or earlier. Such cells can provide a cellular basis for afferent and

afferent fibers to grow along and be supported by trophic factors in addition to those known to be released from hair cells (Farinas et al., 2001).

1.6. Schwann cells are absent in *ErbB2* null mice

In mice null for *ErbB2* and *ErbB3*, Schwann cells are absent in the peripheral nervous system. This absence of Schwann cells suggests an important role for these receptors in Schwann cell development (Morris et al., 1999) in addition to maturation (Corfas et al., 2004; Lyons et al., 2005; Michailov et al., 2004) but not in adults (Atanasoski et al., 2006). These results are consistent with our data as we could not detect *PLP-eGFP* cells along the peripheral nerves of the inner ear of *ErbB2* null embryos. The only eGFP positive cells identified were scattered in the center of the spiral modiolus (Fig. 6A, C) and the supporting cells of the sensory epithelia. Nevertheless, nerve fibers reached all sensory epithelia and clearly reached the hair cells in both the cochlea and the vestibular epithelia, albeit in a disorganized manner (Fig. 6A, C).

Comparison between wild type and *ErbB2* null embryos demonstrated fewer fibers in the cochlea and vestibular sensory epithelia of *ErbB2* null mice, many of which with aberrant projections (Figs. 6 and 7). For example, many fibers did not enter the cochlea but stayed on the scala tympani side of the basilar membrane (Fig. 7A, arrow). Other fibers extended beyond the hair cell region of the cochlea and reached the lateral wall which also displayed some *PLP-eGFP* positive cells (Fig. 6A). Overall, fibers did not form a regular lattice of fiber projections to the cochlea in distinct radial bundles but rather showed fibers which crossed paths, particularly in the region adjacent to the organ of Corti (Fig. 7A) where all fibers enter the cochlea in *ErbB2* wild type mice (Fig. 7B). In addition, *ErbB2* null mice had expression of *PLP-eGFP* in almost all supporting cells of the cochlear and vestibular region, with particularly high levels of eGFP expression in the border cells of the organ of Corti, leaving very few cells unlabeled (Fig. 7C, D).

Given the disorganization of afferent fibers in the ear, we wanted to know whether central projections of vestibular and cochlear fibers are at least grossly normal. NeuroVue dye tracing revealed grossly normal projections to cochlear and vestibular nuclei (Fig. 6E, F). However, the labeled fiber density of central projections was markedly reduced compared to wild type animals, proportional to the apparent reduction at the periphery. To analyze further these effects and separate ear-specific from, brain-specific effects will require generation of ear-specific *ErbB2* conditional null mice.

We next examined the histology of the organ of Corti to verify that development of the sensory epithelia is grossly normal and that Schwann cells are indeed absent as suggested by the virtually complete lack of *PLP-eGFP* positive cells along the nerve fibers of *ErbB2* null mice (Fig. 7A, B). Epoxy resin sections showed that the spiral ganglion cells were indeed further away from the organ of Corti in the *ErbB2* null mice (Fig. 8A, B). Higher magnification of the spiral ganglion showed both neurons and Schwann cells in the E16.5 wild type (Fig. 8D) but only neurons in the *ErbB2* null mice (Fig. 8C, E). Moreover, large empty spaces were present in the spiral ganglion of *ErbB2* null mice and many apoptotic cells were distributed throughout the ganglion (Fig. 8C, E). Whether these apoptotic cells are neurons or Schwann cells remains unknown.

1.7. *ErbB2* null effects on neurotrophin expression

Previous work has shown that inner ear innervation is critically dependent in the embryo on two neurotrophins, *Bdnf* and *Ntf3* (Fritzsch et al., 2004). Of these two neurotrophins, *Ntf3* is predominantly expressed in E13–18 embryos in supporting cells of the cochlea and some vestibular organs (utricle and saccule; Farinas et al., 2001). In contrast, *Bdnf* is

predominantly expressed in hair cells in E13–18 embryos with only a limited expression in supporting cells, particularly near the apex of the cochlea (Fritzsche et al., 2005a). Recent experiments have shown that over-expression of a dominant negative ErbB4 in juvenile supporting cells can reduce *Ntf3* to the extent that spiral neurons die in adult mice whereas *Bdnf* is only mildly affected (Stankovic et al., 2004). In order to determine if *Bdnf* or *Ntf3* expression was altered in the *ErbB2* null embryos, the presence of *Bdnf* and *Ntf3* in the ear of wild type and *ErbB2* null embryos at E18.5 was determined using RT-PCR analysis. No significant reduction of *Bdnf* mRNA was observed, while *Ntf3* was significantly reduced to 25% compared to wild type embryos (Fig. 9). Overall, these effects are similar to observations in adult mice overexpressing a dominant-negative *ErbB4* (Stankovic et al., 2004) suggesting that similar molecular changes occurred at the level of the supporting cells of the inner ear sensory epithelia in *ErbB2* null embryos.

In summary, our data demonstrate that *ErbB2* null mutants have aberrant projections of afferents, and aberrant migration of spiral neurons; both effects are possibly related to the absence of Schwann cells that may provide a stop and guidance signal. *ErbB2* null mice display a reduction and disorganization of both afferent and efferent fibers in the much longer trajectory from the center part of the modiolus to the organ of Corti. In contrast, wild type mice have much shorter and straight trajectories of afferent and efferent fibers coming off the spiral ganglion and IGSB, respectively, that are adjacent and parallel to the organ of Corti. However, despite the absence of Schwann cells, there is an obvious innervation of all sensory epithelia in the *ErbB2* null mice.

2. Discussion

The inner ear sensory neurons of the vestibular and spiral ganglion develop from the inner ear and require the bHLH gene *Neurogenin1* for their formation (Ma et al., 2000; Rubel and Fritzsche, 2002) and the bHLH gene *Neurod1* for proper migration and maturation (Kim et al., 2001) comparable to the neural crest-derived cranial ganglia. However, despite this molecular similarity in early development, the inner ear sensory neurons differ from all other cranial neurons in that they send a dendrite back to their area of delamination, the ear (Pauley et al., 2005). Inner ear sensory neurons are also unique in that they express two neurotrophin receptors, *trkB* and *trkC* (Farinas et al., 2001), whereas other peripheral sensory neurons require only a single trk receptor (Huang and Reichardt, 2001). Here we demonstrate a role for the *ErbB2* gene in proper migration and pathfinding of spiral sensory neurons and show that Schwann cells are not necessary for initial targeting of afferent fibers, although efferent fibers are reduced and disorganized in *ErbB2* null mice. Inner ear sensory neurons differ in this respect from other cranial ganglion neurons (Morris et al., 1999).

2.1. Effects of ErbB2 null mutation on Schwann cells and supporting cells of the ear

Schwann cells in the PNS start myelin formation around birth. Our data show that these cells express the *PLP*-eGFP transgene at this stage. In contrast, myelin in the CNS does not express *PLP* prior to 10 days postnatal in the rat (Milner et al., 1985), consistent with our observation that no *PLP*-eGFP positive cells are found in the proximal part of the vestibular or cochlear nerve in embryos (Fig. 5). This seemingly contrasts with a report that shows strong expression of *PLP* only in the proximal part of the vestibular–cochlear nerve in adult rats (Knipper et al., 1998). However, it is possible that *PLP* expression is both spatially and temporally dynamic and the apparent differences in distribution reported here as compared to the previous study (Knipper et al., 1998) may reflect differential changes of *PLP* expression in postnatal mice rather than real expression differences.

We show histologically as well as by the absence of *PLP*-eGFP positive cells that all Schwann cells are absent in *ErbB2* null ears, consistent with previous reports on other

peripheral nerves (Lyons et al., 2005; Massa et al., 2005; Morris et al., 1999). Interestingly, *PLP* expression in the inner ear sensory epithelia supporting cells is not only present but appears to be upregulated (Figs. 6 and 7). Most importantly, whereas only some supporting cells of the organ of Corti of *ErbB2* wild type littermates show expression of *PLP-eGFP* transgene, all cells are strongly positive for *PLP-eGFP* in *ErbB2* null littermates (Figs. 6 and 7). These data suggest that absence of *ErbB2* alters the maturation of these cells with respect to *PLP* expression. Consistent with these effects of *ErbB2* on gene regulation in supporting cells, our data on *Ntf3* expression suggest that expression of this neurotrophin, known to be expressed in supporting cells of the ear in embryos (Farinas et al., 2001), is affected (Fig. 9). Despite all these cellular and molecular deficiencies, afferent and efferent fibers nevertheless reach the sensory epithelia in large numbers. However, there is an apparently transient massive overshooting of afferents fibers (Fig. 2) that may be corrected by the concurrent wave of apoptosis in the ganglia (Fig. 8).

2.2. Migration deficits of spiral ganglion neurons

During ear development, spiral and vestibular neurons delaminate from the ear and migrate to the vestibular and spiral ganglia, respectively, and project back with their dendrites (Fritzsche, 2003; Pauley et al., 2005; Rubel and Fritzsche, 2002), in part via attraction to hair cells and the neurotrophin they release (Fritzsche et al., 2004; Tessarollo et al., 2004). The spiral ganglia migrate to the peripheral part of the modiolus inside the ear whereas the vestibular ganglia migrate outside the ear to form the vestibular ganglion between the ear and brainstem. Vestibular sensory neuron migration depends in part on *Neurod1* (Kim et al., 2001). Previous work has already indicated that ErbB receptors and their ligands are involved in neuronal migration (Rio et al., 1997) and migration of breast tumor cells (Bianco, 2004; Lemmon, 2003) and our present data support this idea for the ear spiral neurons. In the absence of ErbB2-mediated signaling, spiral neurons migrate further into the center of the modiolus and toward the vestibular ganglion, behaving much like vestibular ganglion sensory neurons. This phenotype implies that some interaction with the substrate, possibly mediated by the neural crest-derived Schwann cells, provides a stop signal for spiral neurons to stay at the periphery of the modiolus in Rosenthal's canal. In the absence of such a stop signal, spiral neurons continue their migration deeper into the center of the modiolus. The formation of longer radial fibers emanating from the modiolar center in *ErbB2* null mice (Figs. 3 and 4) is a consequence of the altered position of spiral ganglion neurons in these mutants. Conditional ear-specific *ErbB2* null mutations are needed to analyze whether the additional migration is mediated by signals intrinsic to the neurons or is mediated by the neural crest-derived Schwann cells. Ear-specific conditional *ErbB2* null mice should not affect Schwann cell development and thus could help narrow down the cellular basis for this effect.

2.3. Spiral ganglion neurons survive but have projection deficits

Our data suggest that a profound fiber outgrowth that over-shoots the organ of Corti in *ErbB2* mutants (Fig. 2) is followed by a near complete loss of these overshooting fibers (Fig. 7), possibly through apoptosis (Fig. 8). But even at E18.5 some disorganized afferent fibers that overshoot the organ of Corti remain (Figs. 6A and 7A). Other studies have found unusual afferent fibers that pass below the organ of Corti when *Ntf3* is replaced by *Bdnf* (Tessarollo et al., 2004). As in these transgenic mice, we found that the fibers that extended beyond the organ of Corti and toward the striola had failed to enter the organ of Corti through the habenula perforata. The habenula perforata is near the border cells that are strongly positive for *PLP-eGFP* in *ErbB2* wild type and null littermates (Figs. 6A, B and 7A, B). It is possible that the overall reduction in *Ntf3* expression (Fig. 9) as well as the likely specific reduction of *Ntf3* in these cells does not allow proper navigation of spiral neuron processes into the organ of Corti.

Recent work has shown that expression of a dominant negative form of *ErbB4* under the control of a GFAP promoter reduces *Ntf3* expression to about 25% of the control values and causes severe loss of approximately 80% of spiral ganglion neurons in juvenile mice (Stankovic et al., 2004). GFAP is expressed in the supporting cells of the ear (Rio et al., 2002), the same cells in which we report here *PLP-eGFP* expression (Fig. 7). Interestingly, we find a comparable reduction in *Ntf3* expression levels to 25% and also show an apparent reduction in fiber density, likely related to spiral neuron loss through apoptosis (Fig. 8). Owing to the unusual position of spiral ganglion neurons, we need to label them distinctly from vestibular neurons using a marker such as *Gata3* (Karis et al., 2001; Lawoko-Kerali et al., 2002), to count them independent of vestibular ganglion neurons. In addition, quantification requires newborn mice as we find numerous apoptotic cells in the spiral and vestibular ganglion of *ErbB2* null mice. This suggests that cell death may continue in newborn mice, possibly eliminating all overshooting fibers.

The overall reduction in fiber density and disorganization of afferents is also obvious in all vestibular epithelia (Fig. 7). However, no specific losses of fibers to distinct epithelia are apparent, thus indicating that the phenotype is different from a *Bdnf* null phenotype and more closely resembles the *Ntf3* null phenotype. Closer examination of inputs from the cerebellum in postnatal animals (Maklad and Fritzsche, 2003) is needed to clarify in more detail the degree of disorganization. We are currently raising conditional mutants with a targeted deletion of *ErbB2* in the ear to conduct such studies.

2.4. Deficits in efferent fiber projections

Previous work has demonstrated that efferent fibers largely follow afferents to their sensory epithelia no matter what the molecular basis of the deficit (Farinas et al., 2001; Fritzsche et al., 2004). We show here that efferent fibers do indeed home toward the sensory epithelia in *ErbB2* null mice but do so in apparently reduced numbers (Figs. 1, 3, and 4). Most important is the clear absence of the intraganglionic spiral bundle, a well known feature of efferent trajectories to the cochlea (Figs. 1, 3, and 4). Nevertheless, fibers reach the organ of Corti, albeit disorganized and reduced. These data suggest that the absence of Schwann cells affects the fiber growth of efferents as much as that of afferents. We suggest that the reduction of *Ntf3* levels to only 25% of wild type alters afferents and reduces their ability to act as a substrate for efferents to grow along. However, it cannot be ruled that low levels of expression of neurotrophin receptors exist in efferent neurons thus mediating a more direct effect of the reported *Ntf3* reduction. Targeted deletion of *erbB2* only in the ear, maintaining the neural crest-derived Schwann cells, is on its way to clarify this issue.

3. Experimental procedures

3.1. Animals

Breeding and genotyping of *ErbB2* null mice and *PLP-eGFP* mice followed previously established procedures (Mallon et al., 2002; Morris et al., 1999). *ErbB2* heterozygous mice carrying the gene to rescue the heart phenotype were crossed with mice carrying the *PLP-eGFP* reporter gene to allow direct visualization of the effects of *ErbB2* absence on the degree of differentiation of Schwann cells and other cells that express *PLP*. Timed mating was used to determine the date of conception by the appearance of a vaginal plug as embryonic day 0.5 (E0.5). Embryos were collected from the mother at embryonic days 13.5, 14.5, E16.5 or 18.5. E13.5, E14.5 and E16.5 embryos were fixed by immersion in 4% paraformaldehyde in 0.1 M Sorenson's buffer (pH 7.4). E18.5 embryos were fixed using perfusion with the same fixative. A section of the tail was obtained for PCR analysis of genomic DNA to establish genotype using primers previously described (Morris et al., 1999).

3.2. Neural tracing and imaging

Ears from E13.5 ($N = 8$), E14.5 ($N = 6$), E16.5 ($N = 6$) and 18.5 ($N = 12$) *ErbB2* mutants and a similar number of wild type and heterozygous littermates were successfully analyzed using lipophilic tracers in fixed tissue. Briefly, different colored NeuroVue tracers (Fritzsche et al., 2005b) were injected to directly image the inner ear afferents and efferents as well as expression of *eGFP* under control of the *PLP* promoter. Fixed heads were bisected. Filter strips with dye were inserted into the bisected brains near the crossing of the olivo-cochlear bundle to label efferents selectively. For afferents, dye was inserted into the cerebellum (nodulus and uvula) or the cochlear nuclei to label auditory and/or vestibular afferents. After appropriate diffusion times (Fritzsche et al., 2005b; Tessarollo et al., 2004), ears were dissected in cold buffer and mounted flat in glycerol. Ears were viewed in a Nikon E800 using a BioRad 2000 confocal system or a Zeiss LSM 510 confocal system.

We also traced the connections from the ear to the brain using lipophilic tracers as previously described (Fritzsche et al., 2005b; Maklad and Fritzsche, 2003). We inserted Neurovue maroon into the saccule and Neurovue red into the posterior canal in E18.5 fixed embryos. After a diffusion time of approximately 10 days, the brains were dissected, embedded in gelatine and sectioned at 100 μm thickness, as previously described (Gurung and Fritzsche, 2004). Stacks of images were taken with a Zeiss LSM 510 confocal microscope using the proper excitation and emission filters. Images were combined to create plates using CorelDraw software.

3.3. Immunocytochemistry for acetylated tubulin

In order to confirm our findings with the lipophilic tracers, we also used immunocytochemistry to image nerve fibers to the ear (Farinas et al., 2001). To achieve this, we dissected the sensory epithelia of E16.5 and E18.5 *ErbB2* null and littermate control mice fixed in 4% PFA. Sensory epithelia preparations with attached nerves were defatted, blocked and incubated in primary antibody for tubulin (Sigma) followed by an Alexa 647 conjugated secondary antibody. Preparations were mounted whole in glycerol and imaged using the z axis drive of the Zeiss LSM 510 confocal system to obtain stacks of images. These stacks were collapsed into single images to render the distribution of fibers in a single focal plane.

3.4. Histology

We investigated further aberrations in development, in particular the location of sensory neurons and presence or absence of Schwann cells, using thin plastic sections. For this technique, E16.5 and E18.5 ears were fixed first in 4% PFA, followed by fixation in 0.5% OsO₄ in 0.1 M Sorensen phosphate buffer. Ears were dehydrated, embedded in epoxy resin, sectioned at 1 μm thickness using glass knives and lightly counterstained. Coverslipped sections were imaged using a Nikon E800 microscope, high-resolution cameras and Metamorph software.

3.5. mRNA analyses

Since our data suggested the presence and overgrowth of sensory fibers, we investigated the expression of *Ntf3* and *Bdnf* using Q-PCR to understand whether fiber growth is disregulated as a function of neurotrophin expression changes. E18.5 *ErbB2* null and littermate control mice were euthanized, and the inner ears immediately removed, frozen in liquid nitrogen and ground to a powder using a mortar and pestle. Total RNA was extracted using a PowerGen 700 tissue homogenizer (Fisher Scientific) and Trizol reagent (Invitrogen) according to the manufacturer's protocol. The RNA was further purified using RNeasy Midi Columns (Qiagen). The quantity and purity of the RNA were determined by obtaining the

OD260 and OD260/OD280 ratio, and by an RNA 6000 Pico Assay (Agilent) using a Bioanalyzer (Agilent). Real-time PCR was performed with MGB-labeled probes and primers created using Assays-by-Design (Applied Biosystems) and samples were run in quadruplicate. Sequences spanning exons were selected preferentially for probes and primers by Assays-by-Design (Applied Biosystems, Foster City, CA). Real-time PCR was performed using the TaqMan One-Step RT-PCR Kit per manufacturer's instructions (Applied Biosystems) on an ABI Prism 7000. The relative efficiency of amplification of the selected gene versus the control GAPDH was plotted to ensure that the slope of total RNA versus ΔC_T was less than 0.05. The $2^{-\Delta\Delta C_T}$ was used to determine relative gene expression.

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Fig. 1.

The inner ear projections of E13.5 (A, B) and E14.5 (C–F) wild type (B, D, F) and *ErbB2* null mice (A, C, E) are shown. Afferent projections were transganglionically labeled after injection of NeuroVue red into the cochlear/vestibular nuclei of the brainstem (red) and efferent fibers were labeled with an injection of NeuroVue maroon into the olivo-cochlear bundle near the facial genu (green). In E13.5 wild type animals, afferent fibers extend to the cochlea with a few fibers extending toward the apex (B) whereas afferent fibers are more disperse in the *ErbB2* null mice (A). At E14.5, the spiral ganglion cells (arrow in panel D) are in their adult position at the lateral margin of the modiolus in wild type animals (D) and connect to the brain via a thin fiber bundle of the cochlear nerve in the center of the modiolus. In contrast to this, *ErbB2* null mice have no spiral ganglion cells in the typical spiral ganglion position (C). Instead, many cells are in the greatly enlarged modiolus. Efferent fibers project to the spiral ganglion of wild type animals (F) where they begin to form the intraganglionic spiral bundle (IGSB) that extends from the base toward the apex with virtually all fibers pointing toward the apex (F). In contrast, in *ErbB2* mutants, radial efferent fibers extend from the middle turn toward the base, in parallel to the afferents, largely pointing toward the base (E). Note that the basal turn innervation (base) is less dense in *ErbB2* mutants, in particular in efferents. PC, posterior crista; OC, organ of Corti; Vest ggl, vestibular ganglion; IGSB, intraganglionic spiral bundle. Scale bar indicates 1 mm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Fig. 2.

Immunocytochemistry for acetylated tubulin shows differences in fiber organization of wild type (A) and *ErbB2* null embryos (B). In older wild type embryos, radial fibers (RF) extend to the organ of Corti (OC) with a few fibers projecting beyond inner hair cells to reach outer hair cells (A). In contrast, *ErbB2* null mice show a profound overshooting of many fibers that reach to the lateral wall (LW) where they form spiral fibers to the apex (B). Dotted line indicates the approximate position of the organ of Corti. Scale bar indicates 100 μm.

Fig. 3.

These E18.5 images show the projection of afferents in dissected and whole mounted cochlea after injection of lipophilic tracers into the cochlear/vestibular nuclei of the brainstem (red) and of efferents after injection into the olivo-cochlear bundle (green). The spiral ganglion neurons show an incorrect position and are near the center of the modiolus in *ErbB2* null mice (A, E) rather than between the organ of Corti (OC) and the center of the modiolus as in wild type mice (B, F). These incorrect locations require that the spiral ganglion neurons extend elongated radial fibers to the organ of Corti (OC) where most fibers end around the inner hair cells (IHC). In contrast, in wild type mice, spiral ganglion neurons have considerably shorter radial fibers to the organ of Corti (B, F). Efferent fiber distribution shows the presence of an intraganglionic spiral bundle (IGSB) in the wild type (D). In contrast, *ErbB2* have fewer and disorganized efferent fibers and have no IGSB (C). Dotted squares indicate the approximate positions of Fig. 4. Scale bar indicates 100 μ m. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Fig. 4.

These images illustrate in detail the differences in afferent (red, A, B) and efferent (green, C, D) fiber organization in *ErbB2* null mice (A, C) compared to wild type littermates (B, D) in the areas indicated by dotted lines in Fig. 3. The spiral ganglion (B) or the IGSB (D) of wild type mice are located approximately half way between the center of the modiolus and the organ of Corti (OC). Only short radial fiber bundles extend to the organ of Corti. In contrast, spiral ganglion neurons are close to the center of the modiolus in the *ErbB2* mutant except for an occasional neuron in the right position of the wild type spiral ganglion and form longer radial fibers that are disorganized (A). The efferent fibers of *ErbB2* mutants also show fewer and much longer radial fibers that are as disorganized as the afferent fibers (C). In contrast to wild type littermates (D), *ErbB2* mutants (C) lack an intraganglionic spiral bundle (IGSB). Even though disorganized, afferents and efferents nevertheless reach in most cases the hair cells of the organ of Corti, in particular the inner hair cells (IHC) near the radial fibers and extend to the outer hair cells with an orientation toward the base (arrows in panels A–D). Scale bar indicates 10 μm.

Fig. 5.

Wild type embryos that express eGFP transgene under the control of the *PLP* promoter were injected with NeuroVue red dye to label afferent vestibular (A–C) and spiral (D–F) sensory neurons. Panels C and F are overlays of panels A, B and D, E, respectively. In these embryos, the *PLP-eGFP* expression is found in all Schwann cells surrounding nerve fibers and ganglia, as well as in the supporting cells (Deiters, Border) of the organ of Corti. However, the most proximal part of the vestibular and cochlear nerves near the brain is negative for *PLP-eGFP* positive cells (A–C). In the cochlea, a distinct gap separates the *PLP-eGFP* Schwann cells from the *PLP-eGFP* positive border cells surrounding the inner hair cells (dashed white line in panels D–F). In the organ of Corti, mainly inner hair cells (IHC) are innervated (E, F), while few fibers (Type II) extend to the outer hair cells (E, F). Higher magnification shows that Schwann cell (SC) processes extend to the habenula perforate (HB) where afferent fibers enter the organ of Corti near border cells (Compare inserts in panels D–F). Scale bar indicates 100 μm .

Fig. 6.

Pattern of afferent innervation (NeuroVue red) and distribution of *PLP*-eGFP (green) is compared in wild type (B, D) and *ErbB2* null mice (A, C) and central projections are shown as revealed by dye injections into the posterior canal crista (red, E, F) and saccule (green, E, F) in these overlays. Schwann cells are completely absent along the radial fibers of the cochlea (A) as well as toward the crista organs (AC, HC in panel C) in the *ErbB2* null mice. A few *PLP*-eGFP positive cells are present in the modiolus (A) of the *ErbB2* null mice and the stria vascularis (stria) indicating that *PLP*-eGFP transgene expression is not restricted to Schwann cells and sensory epithelia supporting cells. The Deiters and border cells of the organ of Corti appear more uniformly eGFP positive in the *ErbB2* mutant with fewer cells being negative (A, B). As previously shown (Figs. 1–4), afferent fibers to the sensory epithelia appear disorganized in *ErbB2* null mice compared to the wild type. Sections of the brainstem near the VIIIth nerve root show that PC afferents (red, E, F) project in either wild type (F) or *ErbB2* null mice (E) in the more ventral part of the medial vestibular nucleus (MVN). In contrast, the saccule projects more dorsal as well as to the cell group Y (E, F). Note that the basal turn of the cochlea was also labeled in the mutant, resulting in a topographically restricted projection to the ventral cochlear nucleus (VCN). In addition, efferent fibers (EFF) are visible in this section of *ErbB2* null mice (E). Note that the overall labeling of fibers is more sparing in *ErbB2* null mutants, both in endorgans and in the brain. AC, anterior crista; HC, horizontal crista; MVN, medial vestibular nucleus; Spiral ggl, spiral ganglion; Stria, stria vascularis; Y, cell group Y. Scale bar indicates 100 μ m.

Fig. 7.

NeuroVue red labels bundled afferent fibers that extend to the base of the organ of Corti in wild type littermates and reach the (unlabeled) inner hair cells next to the border cells and pillar cells (B) in these overlays. Some type II fibers extend in a coordinated fashion between the *PLP-eGFP* (green) positive Deiters cells toward the (unlabeled) outer hair cells. In contrast, *ErbB2*-deficient mice show fewer radial fibers toward the basal turn organ of Corti (A) that are less obviously bundled and appear disorganized as they approach the border cells. Nevertheless, some afferent fibers form a spiral bundle near the border cells and some afferent fibers extend toward Deiters cells and beyond (A). The fibers that extend beyond the *PLP-eGFP* positive cells Deiters cells do not actually reach into the organ of Corti. Rather, these z axis collapses show fibers in the tympanic duct underneath the organ of Corti, where some of them extend along the spiral vessel (arrow in panel A). Extracting the *PLP-eGFP* (green) signal alone shows that the Deiters, pillar and border cells of the organ of Corti are more uniformly eGFP positive in the *ErbB2* mutant with fewer cells being negative (A, B) and that the border cells display a more profound expression of *PLP-eGFP* in *ErbB2* mutants and wild type animals alike (C, D). Fibers to the anterior crista (AC) and utricle are reduced and disorganized within the epithelia (E, G) but remain, like in wild type littermates (F, H) confined to the epithelia. AC, anterior crista; CE, cruciate eminence; P, pillar cells; SC, Schwann cells. Scale bar indicates 100 μ m.

Fig. 8.

These 1-μm thick epoxy resin sections show the histological differences between E16.5 *ErbB2* null mice (A, C, E) and wild type littermates (B, D). *ErbB2* null mice and wild type littermates (A, B) have an apparently normal development of the organ of Corti (OC) and greater epithelial ridge (GER). Note that the distance of IHC and spiral ganglion is large in the *ErbB2* null mice compared to wild types (double arrow in panels A, B). Closer examination of the spiral ganglion shows both neurons and Schwann cells (S) in an approximately 1:6 ratio in wild type mice (D). In contrast, no Schwann cell is found in *ErbB2* null mice (C, E). Instead, numerous apoptotic cells (arrows, C, E) are interspersed between spiral neurons and larger empty spaces are apparent. Scale bar indicates 100 μm (A, B) and 50 μm (C-E).

Fig. 9.

RNA extracted from the inner ears of E18.5 *ErbB2* null and littermate control embryos was analyzed using real-time RT-PCR to determine the expression of *Bdnf* and *Ntf3*. The level of expression of *Bdnf* (left) and *Ntf3* (right) relative to GAPDH indicated that *Ntf3* is significantly decreased in *ErbB2* null mice, while *Bdnf* shows only an insignificant alteration. $N = 3$ mice. * Indicates that the mean is significantly different from the corresponding wild type control using a Student's t test, where $P \leq 0.05$.