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Deletion of EphA4 Enhances Deafferentation-Induced Ipsilateral Sprouting in Auditory Brainstem Projections

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ABSTRACT

Axonal selection of ipsilateral and/or contralateral targets is essential for integrating bilateral sensory information and for coordinated movement. The molecular processes that determine ipsilateral and contralateral target choice are not fully understood. We examined this target selection in the developing auditory brainstem. Ventral cochlear nucleus (VCN) axons normally project to the medial nucleus of the trapezoid body (MNTB) only on the *contralateral* side. However, after unilateral removal of cochlear input in neonates, we found that axons from the unoperated VCN sprout and project to MNTB *bilaterally*. We found that EphA4 is expressed in the mouse auditory brainstem during development and during a sensitive period for ipsilateral sprouting, so we hypothesized that deletion of the Eph receptor EphA4 would impair target selection in these auditory pathways. Lipophilic dyes were used to evaluate quantitatively the brainstem projections in wild-type and EphA4-null mice. VCN-MNTB projections in EphA4-null mice were strictly contralateral, as in wild-type mice. However, after deafferentation, EphA4-null mice had a significant, threefold increase in the proportion of axons from the intact VCN that sprouted into ipsilateral MNTB compared with wild-type mice. Heterozygous mice had a twofold increase in these projections. These results demonstrate that EphA4 influences auditory brainstem circuitry selectively in response to deafferentation. Although this axon guidance molecule is not by itself necessary for appropriate target choice during normal development, it is a strong determinant of ipsilateral vs. contralateral target choice during deafferentation-induced plasticity. J. Comp. Neurol. 504: 508–518, 2007.

Indexing terms: deafferentation; plasticity; cochlear nucleus; Eph receptor; VCN; MNTB

The coordination of crossed and uncrossed neural pathways is necessary for integration of sensory information from the two sides of the body and for coordinated movements. In the auditory brainstem, the integration of binaural inputs is critical for sound localization. An important component of this circuitry is the projection from the ventral cochlear nucleus (VCN) to the medial nucleus of the trapezoid body (MNTB; Irvine, 1986; Sanes, 1990). The VCN receives direct input from the spiral ganglion in the ipsilateral ear (Rubel and Fritzsch, 2002). Globular bushy cells from VCN axons in turn project to MNTB on the contralateral, but not ipsilateral, side of the brainstem, terminating in the calyx of Held (Tolbert et al., 1982; Kuwabara et al., 1991). The lateral superior olive (LSO) receives excitatory input from ipsilateral VCN and inhibitory input from ipsilateral MNTB (Cant and Casseday, 1986; Glendenning et al., 1992). The balance of excitation and inhibition in LSO neurons reflects interaural intensity differences, which are essential for the localization of sound sources. The function of this circuitry depends critically on precision in axon outgrowth to appropriate sides of the brainstem.

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As with many regions in the brain, auditory pathways can be dramatically altered by denervation or injury. After cochlea removal early in development, cell death is induced in the deafferented VCN (Levi-Montalcini, 1949; Trune, 1982; Born and Rubel, 1985; Hashisaki and Rubel, 1989). Most VCN neurons are lost along with their projections, and axons from the intact VCN branch and contact denervated auditory brainstem regions (Kitzes et al., 1995; Russell and Moore, 1995). Globular bushy cell axons sprout and form ipsilateral branches that terminate in calyces in ipsilateral MNTB, so that these projections become bilateral rather than strictly contralateral. Thus the rules for ipsilateral/contralateral target selection are different after injury and during normal development.

The molecular signals governing formation of these induced auditory pathways are incompletely understood. The Eph proteins, including Eph receptor tyrosine kinases and their ephrin ligands, are candidate axon guidance molecules (Klein, 2004; Cramer, 2005; McLaughlin and O'Leary, 2005; Pasquale, 2005). Eph proteins regulate growth of axons at the midline of the spinal cord (Imondi and Kaprielian, 2001; Kadison et al., 2006), the anterior commissure (Henkemeyer et al., 1996), and the optic chiasm (Williams et al., 2003; Mann et al., 2004). Eph protein signaling also influences axonal branching (Davenport et al., 1999; Yates et al., 2001). In the brainstem, Eph proteins control decussation of mouse vestibular projections (Cowan et al., 2000) and growth of developing auditory axons (Cramer et al., 2006). The Eph receptor EphA4 is a particularly interesting candidate insofar as it influences the selection of ipsilateral vs. contralateral targets in the chick auditory brainstem (Cramer et al., 2004) and because mice lacking EphA4 have mirror movements in the limbs resulting from aberrant crossing of corticothalamic projections (Dottori et al., 1998; Coonan et al., 2001; Kullander et al., 2001; Leighton et al., 2001; Yokoyama et al., 2001). We hypothesized that EphA4 inhibits induction of ipsilateral VCN-MNTB projections. We tested this hypothesis by evaluating the ipsilateral and contralateral VCN-MNTB projections in normal and EphA4 null mice, both during normal development and after deafferentation.

MATERIALS AND METHODS

Animals

All procedures were approved by the University of California, Irvine, Institutional Animal Care and Use Committee. EphA4 mutant mice were provided by Dr. Marc Tessier-Lavigne (Genentech, South San Francisco, CA). For PCR genotyping, mice were anesthetized with isofluorane, and DNA was extracted from tails with 100% isopropanol to precipitate DNA after lysis. DNA was resuspended from pellet in 50–100 μl sterile water and stored at 4°C. The PCR was performed in a total volume of 25 μl [100 mg/μl DNA, 20 μM of each primer (PST38-F4-1 5′-GTT TCCGCT CTG AGC TTA TAC TGC-3′, PST38-R4-1 5′-ACA GTG AGT GGA CAA AGA GAC AGG-3′, and GTR2 5′-CGC TCT TAC CAA AGG GCA AAC C-3′), 10 mM PCR Nucleotide Mix (Roche), 1 U/μl Taq polymerase (Sigma) with the appropriate reaction buffer supplied by the manufacturer] and was performed on DNA from each animal. A positive control (a known heterozygote) and a negative control (PCR mix without DNA) were included in every gel. The wild-type band is 639 bp, and the mutant band is 800 bp.

X-gal histochemistry

Animals were perfused transcardially with 0.9% saline followed by 4% paraformaldahyde (PFA) in 0.1 M phosphate-buffered saline (PBS). Brains were removed and postfixed up to 1 hour in 4% PFA, equilibrated in 30% sucrose in PBS, embedded in OCT mounting medium, and cut at 14 μm in the coronal plane on a cryostat (Leica). Slides were rinsed in PBS, then incubated in a solution containing 5 mM K-ferricyanide, 5 mM K-ferrocyanide, 2 mM $MgCl_2$, 1 mg/ml 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside, and PBS (pH 7.4) at 37°C until a blue reaction product appeared, after approximately 1–4 hours. Slides were then rinsed and coverslipped in Glycergel (Dako, Carpinteria, CA).

EphA4 immunohistochemistry

Animals were overdosed with isoflurane (Abbot Laboratories, North Chicago, IL) and perfused transcardially with 0.9% saline and then 4% PFA. Brains were removed and postfixed in 4% PFA for 1 hour, embedded in agar, and cut in the coronal plane at 50 μm on a VF-200 microtome (Precisionary Instruments). Floating sections were rinsed in TBST (0.05% Triton in 0.1 M TBS, pH 7.4) and blocked for nonspecific antigens in a blocking solution containing 3% normal goat serum (NGS) in TBST for 30 minutes before incubation in primary antibody (0.5 μg/ml) overnight at 4°C. We used a rabbit polyclonal antibody (provided by Dr. Elena Pasquale) that was generated against a peptide containing the 11 C-terminal amino acids of EphA4 (Soans et al., 1994; Tremblay et al., 2007).

A biotinylated goat anti-rabbit secondary antibody (Vector Laboratories, Burlingame, CA) was applied for 1 hour at room temperature and then sections were rinsed and incubated for 60 minutes in ABC (Vector Laboratories). Sections were reacted with diaminobenzidine and H_2O_2, mounted on slides, air dried overnight, dehydrated, cleared in xylene, and coverslipped with Permount. Every third section was thionin-stained to facilitate identification of anatomical borders of brainstem nuclei. Negative control sections were processed with the above-described protocol except that the primary antibody was omitted. In addition, sections stained with the antibody after preadsorption to the peptide were not labeled. Western blot analysis of the rabbit anti-EphA4 antibody produced a single band corresponding to 110 kD in homogenates from wild-type mice. No band was seen in homogenates from EphA4 mutant mice, indicating that the antibody specifically recognizes the EphA4 protein.

Neuroanatomical labeling

Brainstems (P9–16) were labeled with the carbocyanine dyes DiI (Molecular Probes, Eugene, OR) or NeuroVue Red (PTI Research, Inc., Exton, PA) to assess the projection patterns from VCN to MNTB (Hsieh and Cramer, 2006). The cerebellum was dissected away so that VCN was visible. With fine forceps, a crystal of DiI or a small piece (100–200 μm wide) of NeuroVue Red filter paper was placed in VCN. In animals that underwent cochlea removal, NeuroVue Red was placed in the intact (contralesional) VCN. Labeled tissue was incubated in 4% PFA at 37°C for 2 weeks to allow for dye transport. Coronal vi-

bratome sections were cut at 100 μm, mounted onto slides, and coverslipped with Glycergel.

Cochlea removal

Hypothermia was used to induce and maintain anesthesia in neonatal pups (P2–P5). All procedures were performed with a stereomicroscope and heat-sterilized instruments (Germinator 500; CellPoint Scientific, Gaithersburg, MD). A small incision was made ventral to the pinna, through which the tympanic membrane was exposed. The middle ear mesenchyme and ossicles were aspirated with a sterile pipette. The pipette was then inserted through the oval window, and the contents of the cochlea were aspirated. The skin incision was closed with flexible collodion (Paddock Laboratories, Inc., Minneapolis, MN) or allowed to heal on its own. In sham-operated animals, all steps were included except for the aspiration of the cochlea. Pups were warmed and returned to the cage with their mothers for a survival of 2–7 days.

Analysis

Immunohistochemistry. Sections were examined with a Zeiss Axioskop microscope, and images were collected digitally with an Axiocam camera and Openlab software (Improvision). To produce photomicrographs, we imported these images into Adobe Photoshop 7.0, adjusted them for contrast and brightness, and added labels. Differential interference contrast (DIC) optics and thionin staining of adjacent sections were used to verify the location of the cochlear nucleus and MNTB. To evaluate whether cochlea removal alters EphA4 expression, densitometry measurements of MNTB were performed in animals 2, 3, and 6 days after cochlea removal. Measurements were made by outlining MNTB on the lesioned and unlesioned sides of the brainstem and computing average optical density for each nucleus. A ratio of ipsilesional to contralesional optical density was computed for each brain. Two-tailed Student's *t*-tests were used to evaluate whether ratios in control mice (sham-operated or unoperated) differed from those in operated mice.

Neuroanatomical labeling. We examined the specificity of VCN projections to contralateral MNTB in unoperated wild-type ($EphA4^{+/+}$), heterozygous ($EphA4^{+/-}$), and mutant ($EphA4^{-/-}$) mice. We placed dye in one VCN and examined calyceal terminations in the MNTB ipsilateral to the dye placement (MNTBi) as well as in MNTB contralateral to the dye placement (MNTBc). The nuclear boundaries of VCN and MNTB were ascertained via DIC optics and adjacent thionin-stained sections. Calyces were counted in all sections containing MNTB. All counts were performed by an observer blind to the genotype of the mouse. A ratio of ipsilateral (on the side of the dye placement) to contralateral calyces (I/C ratio) was computed for each brain. This normalizing ratio allowed us to assess the novel ipsilateral projections independently of injection size (Hsieh and Cramer, 2006).

We performed a similar analysis of VCN-MNTB projections in mice after unilateral cochlea removal. Several criteria were set for inclusion of animals. First, we considered the cochlea removal successful if the VCN on the lesioned side was reduced to less than half the size of the intact VCN. Second, we required that labeled calyces be present contralateral to the dye placement side, to confirm effective neuroanatomical tracing. Third, dye placement was restricted to the intact VCN, to preclude contamination of the other VCN-MNTB pathway. Finally, for inclusion in the analysis, a calyceal terminal was considered one that covered at least one-third of the cell surface in MNTB. Labeled calyces were counted with a Zeiss confocal microscope in all sections containing MNTBc and MNTBi. Analysis of variance (ANOVA) and two-tailed Student's *t*-tests were used to test for significance between the I/C ratios for cochlea removal across genotypes.

RESULTS

Cochlea removal induces novel projections from VCN to MNTBi in the mouse

We investigated deafferentation-induced plasticity in a mouse auditory pathway to test potential molecular pathways in mutant mice. In gerbils and mice, neonatal unilateral cochlea removal results in severe atrophy of the VCN because of deafferentation-induced cell loss. In gerbils, it has been shown that the intact VCN subsequently branches to contact the denervated MNTB, so that axons from the intact VCN make both ipsilateral and contralateral calyceal projections after cochlea removal (Kitzes et al., 1995; Russell and Moore, 1995; Hsieh and Cramer, 2006). To determine whether there is a similar induction of novel, ipsilateral projections in the mouse, we performed cochlea removal in wild-type mice between P2 and P5. Figure 1A shows a darkfield image of a coronal section taken from an animal after cochlea removal surgery. VCN is present on the right, unoperated side but not on the left side of the brain, where it has undergone cell death resulting from deafferentation. A fluorescence image (Fig. 1A′) of the same section shows placement of NueroVue Red dye in the intact VCN and dye transport to brainstem nuclei on both sides of the brainstem. We assessed projections from VCN to MNTBc (contralateral to dye placement) and MNTBi (ipsilateral to dye placement) and found numerous labeled calyceal terminations in MNTB on both sides of the brainstem (Fig. 1B), indicating the induction of novel projections to the denervated MNTB (MNTBi). At higher magnification, calyces in both MNTBs appear to have a similar morphology (Fig. 1C,D, arrows). We calculated the ratio (I/C) of calyces in MNTBi to MNTBc (see Materials and Methods). There were significantly higher I/C ratios (±SEM) in operated animals (0.247 ± 0.052; $n = 5$) than in unoperated control animals (0.003 ± 0.002; $n = 11$; see Fig. 4E; $P < 0.05$, *t*-test), indicating that the intact VCN formed significantly more deafferentation-induced novel projections to MNTBi. In all cases, neonatal cochlea removal resulted in substantial loss of the cochlear nucleus on the lesioned side; little or no residual cochlear nucleus was observed. Thus, the mouse exhibits deafferentation-induced plasticity in the VCN-MNTB pathway at neonatal ages, and the extent is similar to that previously reported for the gerbil.

Expression of EphA4 in the auditory brainstem nuclei during development

To evaluate the role of the candidate molecule EphA4, we examined expression of EphA4 during postnatal ages P3–P10. These ages include the period of formation of the calyx of Held (Kil et al., 1995; Hoffpauir et al., 2006) and the sensitive period when cochlea removal induces cell death in mice (Mostafapour et al., 2000) and

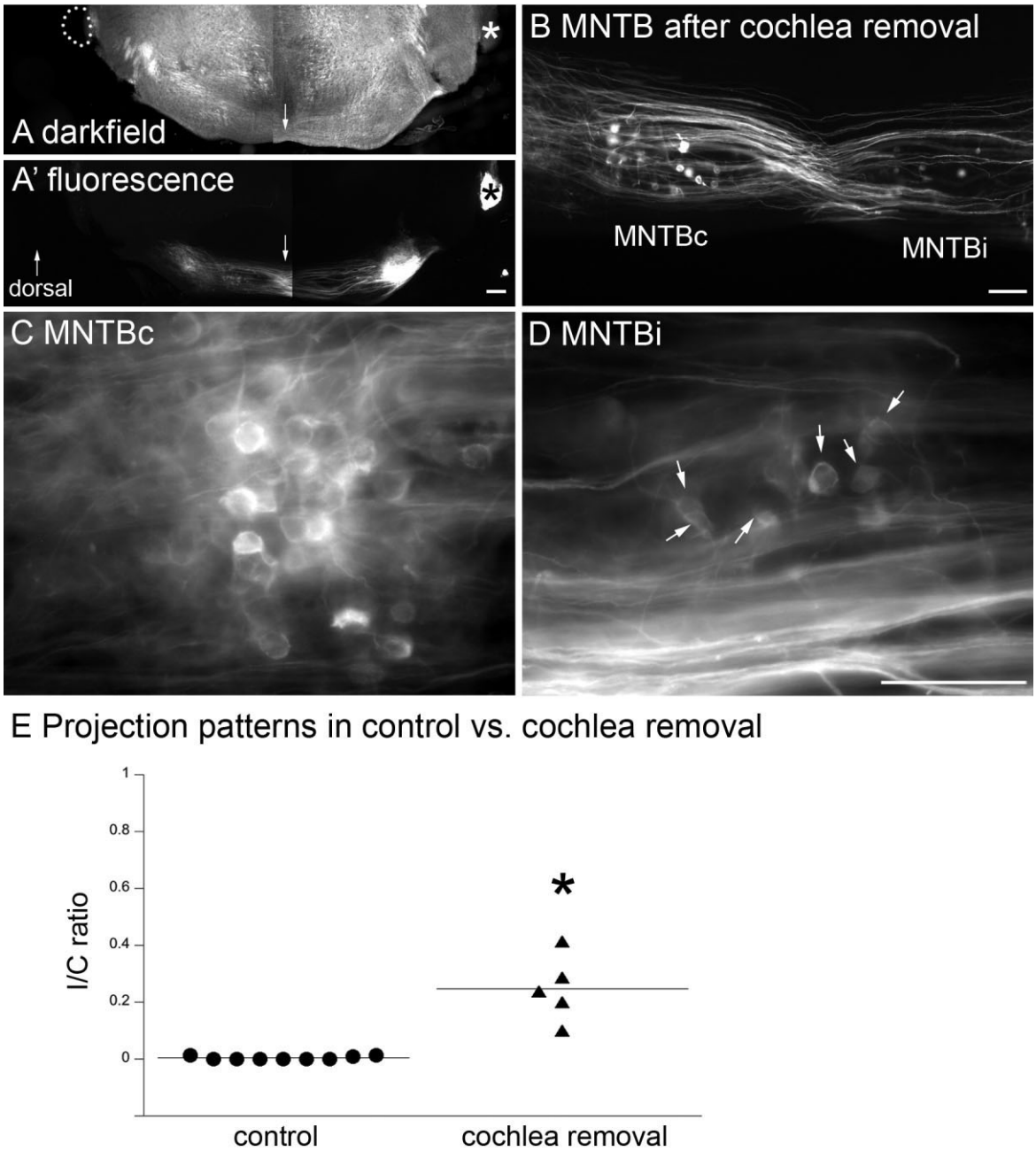


Fig. 1. Deafferentation induces novel, ipsilateral projections in the mouse auditory brainstem. The left cochlea was removed at P2–P5. **A:** Darkfield image of a coronal section indicates the presence of VCN on the right side (asterisk) but not left side (dotted line indicates loss of VCN cells) of the brainstem. **A′:** Fluorescence image of the same section seen in A indicates NeuroVue Red dye placement in the intact VCN (asterisk) and resulting labeling of brainstem nuclei. **B:** NeuroVue Red labeling in MNTBc and MNTBi. Calyceal terminations can be seen in the normal target, MNTBc (**C**), as well as in a novel target, MNTBi (**D**, arrows). Quantification indicates a significantly higher I/C ratio in animals receiving cochlea removal than control animals (**E**, $*P < 0.05$). Scale bars = 200 μm in A′; 100 μm in B,D.

sprouting from the intact VCN in gerbils (Russell and Moore, 1995). Expression of EphA4 was evaluated by using X-gal histochemistry in heterozygous mice and EphA4 immunohistochemistry in wild-type mice. X-gal histochemistry revealed the presence of lacZ, which was inserted downstream of the EphA4 promoter region in the EphA4 mutant mice used in this study and is expressed in the cytoplasm of cell bodies (Leighton et al., 2001).

Brain sections from EphA4$^{+/-}$ mice were stained with X-gal at P3, P7, P9, and P10 (n = 3–5 at each age). At P3, there was light lacZ expression in VCN (Fig. 2A) and more pronounced expression in MNTB (Fig. 2B). At P10, the staining in VCN (Fig. 2C) appeared to be more intense

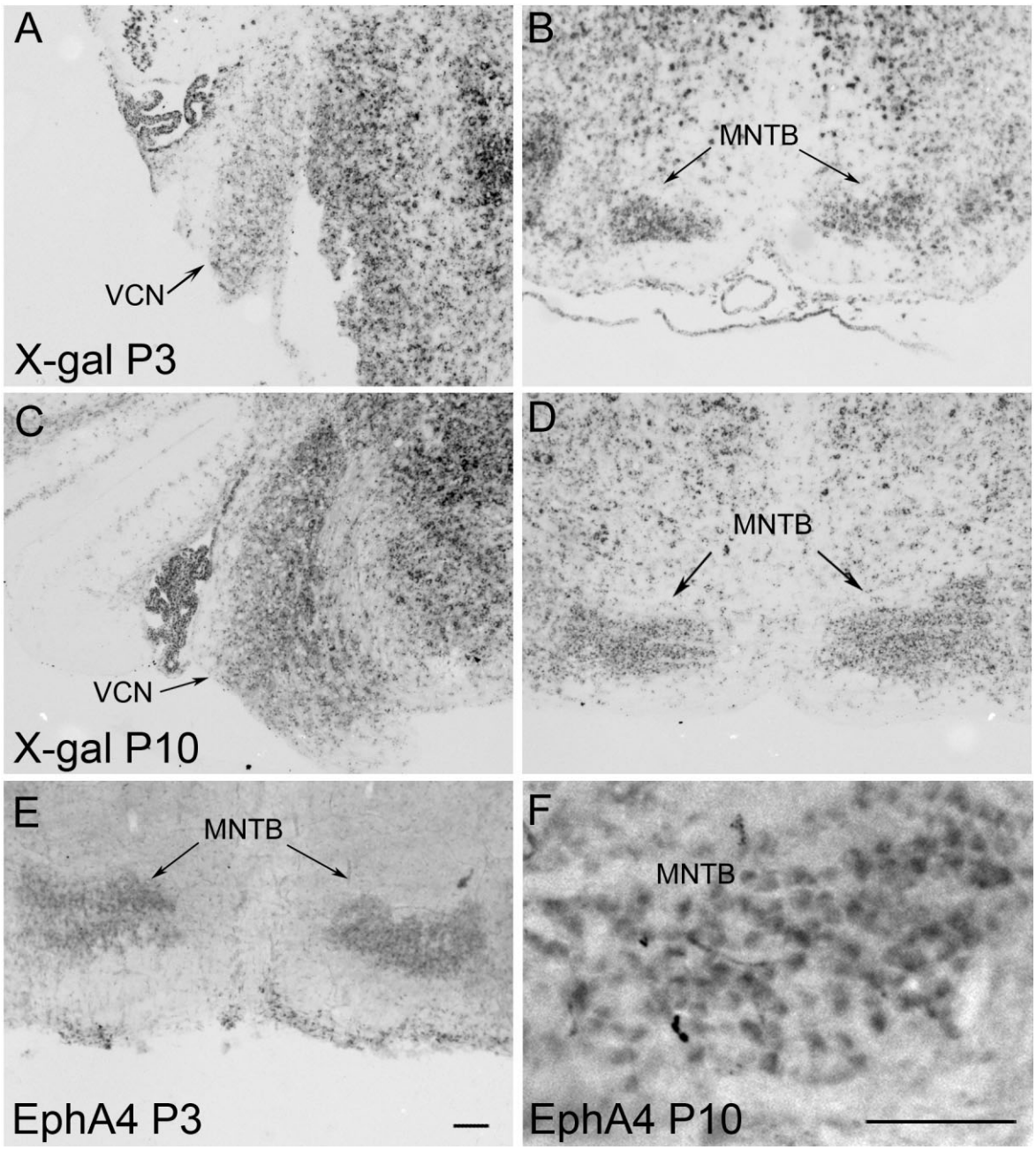


Fig. 2. EphA4 expression in the auditory brainstem during development. **A:** X-gal histochemistry in P3 heterozygous EphA4 mice reported expression in the ventral cochlear nucleus (VCN). **B:** EphA4 is also expressed in MNTB at P3. **C:** X-gal histochemistry at P10 reported higher levels of EphA4 in VCN than at P3. **D:** At P10 X-gal histochemistry showed continued expression of EphA4 in MNTB. **E:** EphA4 immunohistochemistry revealed expression throughout MNTB at P3. **F:** EphA4 expression remains in MNTB at P10. Scale bars = 100 μm in E (applies to A–E); 100 μm in F.

than at younger ages, suggesting that expression levels increase during this developmental period. Expression in MNTB remained high at this age (Fig. 2D).

Wild-type mice were used for EphA4 immunohistochemistry at P3 (n = 3) and P10 (n = 3; Fig. 2). Results from immunohistochemistry supported those obtained with X-gal histochemistry. At P3, EphA4 was lightly expressed in the cochlear nucleus (not shown) and distinctly expressed throughout MNTB (Fig. 2E). At P10, immunolabeling remained throughout MNTB (Fig. 2F). The labeling appeared perisomatic, but we could not determine whether there was expression specifically in cell bodies or calyces. Taken together, these labeling methods show that EphA4 is expressed in the cochlear nucleus and MNTB during the maturation and deafferentation-induced plasticity of VCN-MNTB projections.

VCN-MNTB pathway is normal in EphA4 mutant mice

To evaluate the role of EphA4 in development, we tested whether deletion of this protein alters normal development of VCN axons, which terminate on contralateral but not ipsilateral MNTB. Dye was placed in VCN on one side of EphA4$^{+/+}$, EphA4$^{+/-}$, and EphA4$^{-/-}$ mice, and the resulting distribution of calyces in MNTBc (contralateral to dye placement) and MNTBi (ispilateral to dye placement) was assessed (Fig. 3). Figure 3A shows that, as expected, dye placement resulted in labeled calyces in MNTBc, but not in MNTBi, in wild-type mice (EphA4$^{+/+}$). Higher magnification of these calyces is shown in Figure 3A′ (arrows). Dye labeling in brains from EphA4$^{+/-}$ mice (Fig. 3B,B′) and EphA4$^{-/-}$ mice (Fig. 3C,C′) shows that, as in wild-type brains, in both of these groups there were labeled terminations in MNTBc and not in MNTBi. Higher magnification shows details of the terminal morphology, consistent with the observation that these terminations are calyceal (Fig. 3B′,C′, arrows). We counted labeled calyces in MNTBc and MNTBi. The I/C ratio was used to compare specificity for contralateral vs. ipsilateral targets across genotype groups. The I/C ratios for wild type (0.003 ± 0.002; n = 11), heterozygous (0.002 ± 0.002; n = 8), and mutant (0.011 ± 0.006; n = 5) mice were very small, indicating that all groups had extremely few projections from VCN to MNTBi (Fig. 3D). Moreover, Student's *t*-tests indicated no differences between groups ($P > 0.5$). These results suggest that deletion of EphA4 does not alter projection patterns during initial development.

EphA4 regulates ipsilateral sprouting after cochlea removal

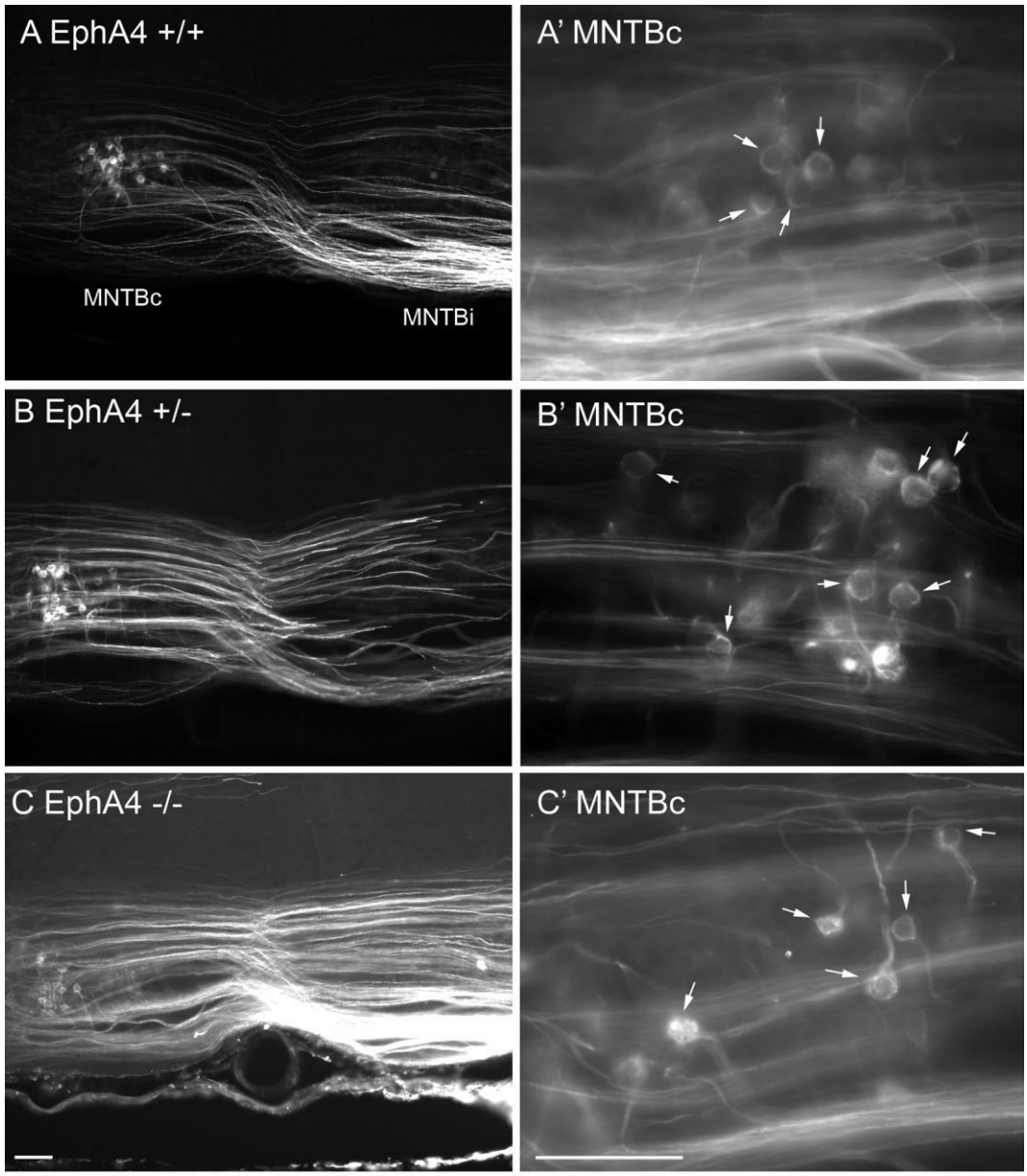
We next examined the role of EphA4 in ipsilateral/contralateral target selection during deafferentation-induced plasticity. We removed the cochlea unilaterally in EphA4$^{+/-}$ and EphA4$^{-/-}$ mice between P2 and P5. After a 6–7-day survival, animals were perfused and dye was placed in the intact VCN. After cochlea removal, VCN cells were nearly completely lost in mice from all genotypes, indicating that mice from all three genotypes undergo similar extensive cell death in the cochlear nucleus. Although all genotype groups formed novel ipsilateral VCN-MNTB projections in response to cochlea removal, the extent of this reorganization was significantly greater in EphA4$^{-/-}$ mice (Fig. 4). EphA4$^{+/+}$ mice are shown in Figure 4A,A′. In EphA4$^{+/-}$ (Fig. 4B) and EphA4$^{-/-}$ (Fig. 4C) mice, the intact VCN projected bilaterally to MNTB after deafferentation. We counted calyceal terminations on both sides and calculated I/C ratios for all genotypes. The mean I/C ratios for homozygous null mice (0.783 ± 0.110; n = 3) and heterozygous mice (0.412 ± 0.096; n = 6) were significantly greater than those for wild-type mice (0.247 ± 0.052; n = 5; Fig. 4D). ANOVA showed a significant difference between groups, and Student's *t*-tests revealed significantly higher I/C ratios in mutant mice (Fig. 4D, asterisk) than in wild-type mice ($P < 0.005$) or heterozygous mice ($P < 0.05$). Heterozygotes had values that were intermediate between those of wild-type and mutant mice. I/C ratios were dependent on genotype, in that results from Spearman's ρ analysis suggest that the quantity of EphA4 expression predicted by gene dosage is negatively correlated with I/C ratios ($\rho = 0.71$, $P < 0.005$). Thus, deletion of EphA4 resulted in a significant, threefold enhancement of induced ipsilateral projections in the VCN-MNTB pathway. Moreover, this difference was not accounted for by variability in effectiveness of dye labeling, because the total number of dye-labeled calyces did not differ between groups (Ps > 0.1). These results suggest that EphA4 regulates target selection during deafferentation-induced ipsilateral sprouting but not during normal development.

Expression of EphA4 in MNTB after cochlea removal

Because EphA4 expression influences the extent of projections induced by cochlea removal, we next determined whether cochlea removal affects EphA4 expression levels in MNTB. We performed unilateral cochlea removal on P3–P4 wild-type mice, then examined brainstem sections for EphA4 immunolabeling 2 days (n = 3), 3 days (n = 4), and 6 days (n = 3) postoperatively. These survival times span the period of formation of ipsilateral calyces after deafferentation in gerbils (Kitzes et al., 1995; Hsieh and Cramer, 2006). Figure 5 shows expression levels of EphA4 in ipsilesional and contralesional MNTB in animals 2 days (Fig. 5A), 3 days (Fig. 5B), and 6 days (Fig. 5C) after cochlea removal and after sham surgery (Fig. 5D). In all conditions, EphA4 was expressed in MNTB on both sides of the brain, and the size of the two MNTBs was similar. The ratio of ipsilesional to contralesional MNTB optical density did not differ from sham control for any of the time points examined (Ps > 0.5; Fig. 5E). These data suggest that relative EphA4 expression levels do not change in MNTB as a consequence of unilateral cochlea removal.

DISCUSSION

Auditory brainstem pathways underlying sound localization are precisely established during development (Friauf and Lohmann, 1999; Kandler and Gillespie, 2005; Hoffpauir et al., 2006). After unilateral deafferentation, axons sprout into denervated targets in an orderly manner (Kitzes et al., 1995; Russell and Moore, 1995). Eph receptors significantly influence axon guidance during development, but the relationship between this function and the role of these proteins in deafferentation-induced plasticity has not been determined. In this study, we evaluated the role of EphA4 in development and plasticity of VCN-MNTB projections in vivo. We demonstrated that, after unilateral cochlea removal in wild-type mice, VCN-MNTB projections from the unoperated side branch and form ipsilateral calyces. We found that EphA4 is expressed in VCN and MNTB during the formation of both normal and induced projections. In mice lacking EphA4, VCN-MNTB projections are strictly *contralateral*, as in wild-type animals. However, the *ipsilateral* projection induced after deafferentation is significantly more extensive in mice lacking EphA4 than in wild-type mice. This effect likely is due to increased ipsilateral sprouting from the intact VCN and not to differential effects on VCN cell survival, because cell loss was similar in mutant and wild-type mice and included most or all VCN cells. Thus, in this projection, EphA4 mutant mice develop normally but respond more strongly to deafferentation. This observation suggests that EphA4 has an inhibitory role during deafferentation-induced plasticity.



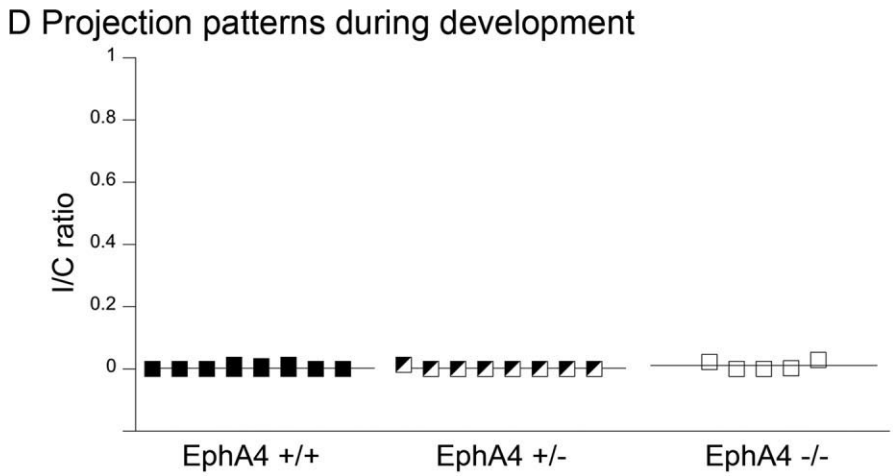


Fig. 3. Projection patterns from VCN to MNTB in EphA4 mice. Neuroanatomical labeling of the VCN-MNTB pathway was performed in EphA4$^{+/+}$, EphA4$^{+/-}$, and EphA4$^{-/-}$ mice between P9 and P14. VCN projects to MNTBc but not MNTBi in EphA4$^{+/+}$ (**A**), EphA4$^{+/-}$ (**B**), and EphA4$^{-/-}$ (**C**). Higher magnification of calyces in MNTBc is shown for the three genotypes (**A′–C′**). Quantification of these projections is expressed as I/C ratios (**D**) and indicates no significant difference between groups ($P > 0.05$). Scale bars = 100 μm in C (applies to A–C); in C′ (applies to A′–C′).

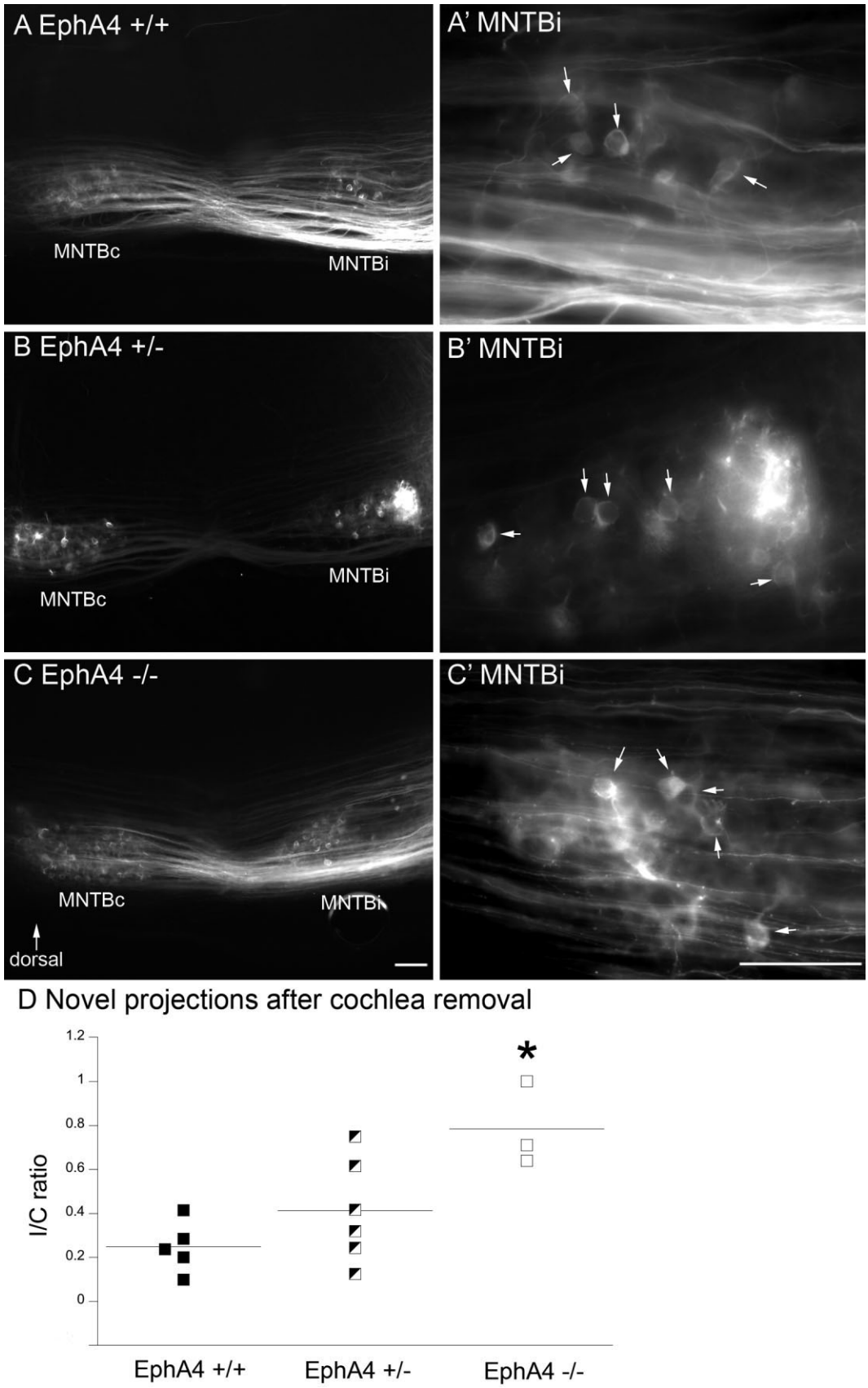


Fig. 4. EphA4 regulates the induction of deafferentation-induced novel projections in the mouse. Cochleas were removed unilaterally in EphA4$^{+/+}$, EphA4$^{+/-}$, and EphA4$^{-/-}$ mice, and dye was placed in the intact VCN. In EphA4$^{+/+}$ mice, novel projections were present in MNTBi as well as the normal target, MNTBc (**A**). Higher magnification of the novel projections in MNTBi (**A′**, arrows indicate calyces). EphA4 $^{+/-}$ and $^{-/-}$ mice showed induction of novel projections (**B,C** and **B′,C′**, arrows), and the proportion of ipsilateral calyces was greater in EphA4 $^{-/-}$ mice. Quantification indicates a significantly higher I/C ratio in EphA4 $^{-/-}$ mice than in EphA4 $^{+/+}$ or EphA4 $^{+/-}$ mice (**D**, $*P < 0.05$). Scale bars = 100 μm in C (applies to A–C); in C′ (applies to A′–C′).

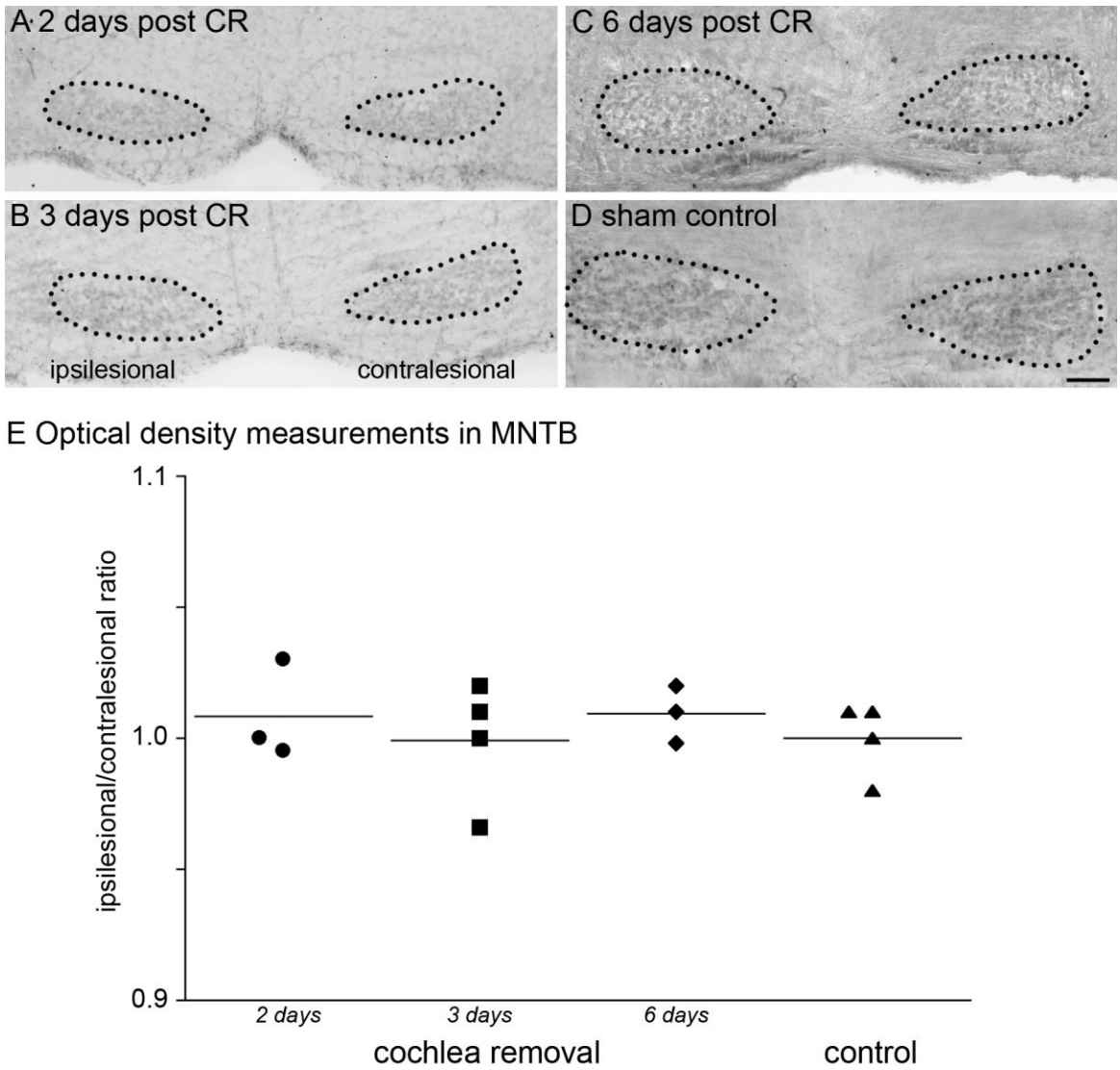


Fig. 5. EphA4 protein expression levels are not altered by cochlea removal. Animals underwent cochlea removal, and EphA4 immunohistochemistry was performed 2 days (**A**), 3 days (**B**), and 6 days (**C**) postoperatively. EphA4 immunohistochemistry was also performed in sham controls (**D**). Optical density was measured in MNTB on both sides and the ratio of ipsilesional/contralesional levels were compared between groups. **E:** Expression levels did not differ between cochlea removal under any survival condition vs. sham control (Ps > 0.5). Scale bar = 100 μm.

Plasticity in auditory brainstem pathways

Deafferentation-induced ipsilateral projections have been demonstrated previously in gerbils (Kitzes et al., 1995; Russell and Moore, 1995; Hsieh and Cramer, 2006). Here we have demonstrated a similar form of plasticity in mice. The ages we used are within the sensitive period for cochlea removal-induced ipsilateral projections in gerbils (Russell and Moore, 1995), which is similar to the ages when cochlea removal induces cell death in VCN in both gerbils (Tierney et al., 1997) and mice (Mostafapour et al., 2000). The VCN-MNTB pathway terminates in large calyces of Held, which can be analyzed quantitatively with a light microscope. Moreover, because the ipsilateral projection originates in VCN neurons that also project contralaterally, the ratio of ipsilateral/contralateral calyces provides an accurate assessment of the degree of plasticity. The lack of aberrant ipsilateral projections during the course of normal development in EphA4 mutant mice suggests that EphA4 is not necessary for the selection of contralateral targets. Alternatively, compensatory changes in the expression of other Eph family proteins could in principle obscure a role for EphA4 during development. However, during developmental plasticity produced by deafferentation, removal of EphA4 is sufficient to produce a large, significant increase in the proportion of induced ipsilateral projections. Thus EphA4 may be necessary to prevent or limit the formation of ipsilateral calyces particularly during plasticity after injury but not during normal development. These findings demonstrate that distinct molecular mechanisms regulate

ipsilateral vs. contralateral target choice in the VCN-MNTB pathway under different conditions.

Eph proteins in development and plasticity

Eph proteins have well-documented roles in nervous system development. Their interactions, elicited by cell–cell contact, mediate repulsive or attractive axon guidance, selection of targets, and formation of topographic maps (McLaughlin and O'Leary, 2005). Eph signaling has several important functions during auditory system development. These proteins are necessary for axon guidance in the auditory brainstem of the chick (Cramer et al., 2004, 2006; Huffman and Cramer, 2007); in addition, they may be necessary for forming tonotopic projections in the mammalian auditory brainstem (I. Miko, C. Hsieh, and K. Cramer, unpublished observations). Eph proteins thus have a broad range of functions within neural circuits. Some of these functions overlap during normal development and in response to injury. Although protein levels are generally down-regulated at later ages, expression of Eph proteins in adult brain regulates synaptic plasticity and regeneration of axonal pathways (Purcell and Carew, 2003; Yamaguchi and Pasquale, 2004; Liu et al., 2006).

Ephrins have also been implicated in developmental plasticity in projections of retinal ganglion cells. In mice with experimentally induced projections from the retina to the auditory thalamus, deletion of ephrin-A2 and ephrin-A5 enhanced cross-modal projections (Lyckman et al., 2001). Unoperated mutant mice did not display these cross-modal targeting errors. Here we have shown that EphA4 has a function after deafferentation distinct from that during normal development in the choice of ipsilateral vs. contralateral target. Together, these studies support an inhibitory function for Eph signaling in deafferentation-induced plasticity.

In this study, we did not observe changes in expression of EphA4 protein as a consequence of the experimental lesion. It remains possible that a change in expression levels occurs very briefly (Cruz-Orengo et al., 2006), represents a change in distribution, or is too small to be detected by immunohistochemistry. However, another possibility is that deafferentation alters the expression or activity of one or more proteins that interact with EphA4. The Eph family of proteins is large, and differences in other Eph proteins, including ephrins, may arise in MNTB following the cochlear lesion. The change in neuronal input to MNTB is likely to elicit a large set of changes in gene expression; subsequent changes in levels of proteins that interact with EphA4 may selectively alter the growth of VCN axons to MNTB. Thus, although EphA4 levels remain constant after cochlea removal, other changes in gene expression may shift the balance of molecular influences on VCN axons so that EphA4 has an inhibitory role after deafferentation but not during normal development.

Ipsilateral vs. contralateral targets

An interesting aspect of our findings is that the novel target in the auditory brainstem is identical to the normal target, except that it is on the opposite side of the brain. Although precise ipsilateral vs. contralateral connectivity is essential for auditory processing, the emergence of novel, homotypic ipsilateral projections represents a significant response to brain injury in early development (Staudt et al., 2002) and in adults (Napieralski et al., 1996; Chen et al., 2002; Allred and Jones, 2004; Luke et al., 2004). Generation of ipsilateral projections may provide compensation for some brain functions but may also occur at the expense of other functions. In the auditory brainstem, bilateral VCN-MNTB projections may provide higher auditory areas with a relatively full complement of inputs after deafferentation, when binaural cues can no longer be used for sound source localization.

Ipsilateral sprouting is variable and depends on lesion type and activity changes (Carmichael and Chesselet, 2002), but the molecular determinants of sprouting are not known. Because this process represents a significant response to brain injury, the identification of underlying molecular processes would have broad clinical implications. Here we have shown that EphA4 may regulate calyx formation and sprouting after deafferentation. Additional studies are needed to evaluate whether this protein has a similar role in other forms of lesion-induced plasticity.

The assembly of neural circuits requires integration of several signals that converge on growing axons. As removal of EphA4 differentially affects deafferentation-induced plasticity in the auditory pathway, deafferentation may shift the balance of axon guidance signals converging on VCN axons. This protein family might thus be linked with activity-dependent processes. Eph/ephrin signaling has been shown to regulate localization of N-methyl-D-aspartate receptors (Dalva et al., 2000) and AMPA receptors (Kayser et al., 2006) and to have demonstrated roles in several forms of hippocampal synaptic plasticity (for review see Calo et al., 2006). Because Eph proteins have important roles both in development and in adult brain, an interesting possibility is that relative levels of Eph proteins influence the sensitive period during which deafferentation can cause dramatic changes in circuitry. An understanding of the conditions under which Eph proteins permit or inhibit regeneration of pathways will be of value in determining how the brain responds to injury.

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