Deafferentation Induces Novel Axonal Projections in the Auditory Brainstem After Hearing Onset

CANDACE Y. HSIEH AND KARINA S. CRAMER*
[1]Department of Neurobiology and Behavior, University of California, Irvine, Irvine, California 92697-4550

ABSTRACT

Deafferentation of neural tissue can result in cell death, morphological changes, and/or alterations in sources of innervation. These changes often occur during a limited period of development. In the auditory brainstem, the ventral cochlear nucleus (VCN) projects to the contralateral but not ipsilateral medial nucleus of the trapezoid body (MNTB). This pathway is part of a circuit that computes interaural intensity differences used in sound localization. Previous studies have shown that, after the cochlea is removed early in postnatal development, cells in the VCN on the deafferented side die, and the intact VCN innervates MNTB on both sides of the brain. These changes after cochlea removal are limited to an early postnatal period that preceeds hearing onset. In this study, we lesioned the VCN directly to evaluate plasticity in axonal pathways *after* hearing onset. We found that novel projections from the intact VCN to ipsilateral MNTB emerge after lesions performed as late as postnatal day 25. The morphological sequence of events is similar to that seen during the initial development of this pathway. These data suggest that plasticity in the auditory brainstem is possible when pathways are challenged with denervation of target nuclei. The results show that the opportunity for plasticity in auditory brainstem circuitry is more prolonged than previously thought and that novel pathways can form after the normal pathways are fully mature and functional. Moreover, sensitive periods for changes in individual pathways are independently regulated. J. Comp. Neurol. 497:589–599, 2006. © 2006 Wiley-Liss, Inc.

Indexing terms: deafferentation; plasticity; cochlear nucleus; VCN; MNTB; MSO

Nervous system connectivity and function can be altered by changes in innervation, activity, or experience (Wiesel and Hubel, 1963a,b; Merzenich et al., 1983; Weinberger, 1995). Although neural plasticity is observed throughout adulthood, the modifiability of some pathways is restricted to a limited developmental time period. This sensitive period is often early in development and may correspond to changes in expression of molecules involved in developmental plasticity, including neurotransmitters, neurotransmitter receptors, signal transduction proteins, and proteins that regulate apoptosis (Hensch, 2004; Harris et al., 2005).

In the mammalian auditory brainstem, cell survival, connectivity, and physiology can be modified by manipulation of afferent input (Levi-Montalcini, 1949; Born and Rubel, 1985; for review see Syka, 2002). Structures in the mammalian auditory brainstem contain distinct cell types with precise projection patterns to targets on both sides of the brain. An important organizing feature of connectivity in the auditory system is that targets of axon branches generally differ on the two sides (Cant and Casseday, 1986). The organization of these pathways allows for computation of interaural time and intensity differences used in sound localization. In the brainstem, globular bushy cells in the ventral cochlear nucleus (VCN) project to the contralateral but not ipsilateral medial nucleus of the trapezoid body (MNTB) through large-diameter axons that terminate in calyces of Held on MNTB cell bodies (Kuwabara et al., 1991). MNTB cells send inhibitory pro-

Grant sponsor: National Institutes of Health; Grant number: DC005771; Grant number: DC007538; Grant sponsor: National Organization for Hearing Research Foundation.

*Correspondence to: Karina S. Cramer, Department of Neurobiology and Behavior, University of California, Irvine, Irvine, CA 92697-4550. E-mail: cramerk@uci.edu

Received 28 October 2005; Revised 18 January 2006; Accepted 17 February 2006

DOI 10.1002/cne.21002

Published online in Wiley InterScience (www.interscience.wiley.com).

© 2006 WILEY-LISS, INC.

WILEY InterScience® DISCOVER SOMETHING GREAT

jections ipsilaterally to cells in the lateral superior olive (LSO). LSO cells also receive excitatory projections from the ipsilateral spherical bushy cells of VCN. The balance of excitation and inhibition within the LSO is the neural substrate underlying detection of interaural intensity differences used in sound localization (Irvine, 1986; Sanes, 1990). In addition, spherical bushy cells from VCN project bilaterally to the medial superior olive (MSO; Cant and Casseday, 1986). In this projection, ipsilateral axons terminate on lateral MSO dendrites, whereas contralateral axons terminate on medial MSO dendrites. This circuitry is used to detect interaural time differences in sound localization for low frequencies (Cant and Casseday, 1986; Smith et al., 1993).

Organization of the auditory brainstem is susceptible to alterations in input resulting from injury, especially at young ages. For example, unilateral cochlea removal performed during the first postnatal week results in robust changes in cell size and number in VCN in both gerbils and mice, whereas deafferentation after postnatal day (P) 7 has little or no effect (Trune, 1982; Nordeen et al., 1983b; Hashisaki and Rubel, 1989; Tierney et al., 1997; Mostafapour et al., 2000). Deafferentation can also dramatically alter connectivity (Nordeen et al., 1983b; Kitzes et al., 1995; Russell and Moore, 1995). Cochlea removal at P2 induces an additional, novel projection from the intact VCN to MNTB on the ipsilateral side of the brain. These novel projections are induced within 24 hours of lesion and persist in the adult. The ectopic ipsilateral projections appear to arise as branches emanating from decussating axons destined for the contralateral MNTB. Deafferentation-induced calyceal terminations are typically present 3 days after cochlea removal. Cochlea removal also expands projections from the intact VCN to contralateral LSO as well as to the portion of the MSO denervated by the lesion. This reorganization of projections was not observed in animals subjected to cochlea removal after P10 (Russell and Moore, 1995). Cochlea removal at these ages results in comparatively little cell death in VCN but reduces excitatory input to MNTB. It is thus difficult to discern whether plasticity of projections from the intact VCN declines at P10, because the projections from the VCN remain after cochlea removal.

In the present study, we ablated VCN at P10 and later, ages at which cochlea removal does not induce significant cell death in VCN. Cochlear nucleus ablation results not only in reduced activity in MNTB but also in the removal of axonal projections to MNTB. We found significant projections from VCN to the ipsilateral MNTB. The morphogenesis of the ectopic calyceal projections was similar to that seen in P2 cochlea removal studies. Projections from VCN to the ipsilateral MNTB could be induced until at least P25. However, plasticity induced by cochlear nucleus removal occurs in some but not all brainstem pathways; it was not observed after P10 in the projection from VCN to MSO.

MATERIALS AND METHODS

Animals

Mongolian gerbils (*Meriones unguiculatus*) were obtained from Charles River and were bred in our colony. Animals were used at P2, P4, P10, P15, and P25. All procedures have been approved by the University of California, Irvine, Institutional Animal Care and Use Committee.

Cochlea removal

Hypothermia was used to induce and maintain anesthesia in young pups (P2–P4). For older postnatal animals (P10–P15), we used ketamine (75–85 mg/kg, i.m.) and xylazine (0.1–0.5 mg/kg, i.m.). A small incision was made ventral to the pinna, exposing the tympanic membrane. 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), and animals were given 0.5 ml of warmed lactated Ringer's subcutaneously immediately after surgery. They were then returned to their home cage for a survival of 6 days.

Cochlear nucleus removal

The cochlear nucleus was removed in gerbils under ketamine/xylazine anesthesia at P10, P15, and P25 as described above. Procedures for ablating the cochlear nucleus are similar to those for cochlea removal, except that, after the cochlea was removed, the pipette was advanced a few millimeters beyond the regions for cochlea removal, and the cochlear nucleus was aspirated. Animals recovered for 1–6 days after surgery.

Neuroanatomical tracing

After the appropriate survival time, animals were killed with isofluorane and perfused transcardially with 0.9% saline, followed by 4% paraformaldehyde (PFA). Brains were removed and examined for the presence of the cochlear nucleus on the operated side. The brainstem and cerebellum were dissected and postfixed for ≥24 hours in 4% PFA at 4°C. For cochlea removal surgery in animals P10 or older, we verified that both cochlear nuclei were intact. For cochlear nucleus removal and cochlea removal in animals younger than P10, we confirmed the absence of VCN in coronal brainstem sections.

The intact VCN-MNTB pathway was labeled with the carbocyanine dyes DiI or NeuroVue red (PTI Research, Inc., Exton, PA; Fritzsch et al., 2005). The cerebellum was carefully dissected away so that VCN was clearly visible. By using a fine dissecting needle and forceps, a small crystal of DiI or piece of NeuroVue red filter was placed in the intact (contralesional) VCN. NeuroVue red dye is embedded in filter paper that was cut to an appropriate size (100–200 μm^2) before placement. For both dyes, labeled tissue was incubated in 4% PFA at 37°C for 2–3 weeks. Coronal vibratome sections were cut at a thickness of 100 μm, mounted onto slides, and coverslipped with Glycergel (Dako, Carpinteria, CA).

Animals were included in the analysis only if the lesioned VCN was reduced to less than half the size of the intact VCN. For studies of projections to MNTB, appropriate dye placement and successful dye transport were confirmed by the presence of labeled axons originating in the intact VCN, coursing along the ventral region of the midline, and terminating in calyces in contralateral MNTB. Our criterion for a calyceal termination was that it covered at least one-fourth of the cell surface in MNTB. For studies of projections to MSO, we included tissue with labeled axons originating in intact VCN and ending in

terminal arbors at MSO. Brains with dye placed outside of the intact VCN were not studied further.

MAP2 immunohistochemistry

We used MAP2 immunohistochemistry to obtain data on the morphology of auditory neurons in response to manipulations of afferent input. After sections were viewed and photographed, coverslips were removed and floating 100-μm sections rinsed in phosphate-buffered saline (PBS), pH 7.4. Sections were then incubated in blocking solution containing 0.5% Triton X-100 and 4% goat serum (Vector Laboratories, Burglingame, CA) in PBS for 1 hour, then incubated overnight in MAP2a,b,c mouse monoclonal antibody (AP18; NeoMarkers, Fremont, CA) diluted 1:250 in blocking solution. This antibody binds specifically to phosphorylated forms of MAP2a,b,c, and, according to the manufacturer, immunoblotting yields bands at 280 kDa and ~70 kDa. The antibody resulted in robust labeling of dendrites and somata. Sections were rinsed in blocking solution, then incubated for 2 hours in Alexa Fluor 488 goat anti-mouse secondary antibody (Molecular Probes, Eugene, OR) diluted 1:1,000 in blocking solution. Sections were rinsed in PBS and coverslipped with Glycergel.

Analysis

Brainstems from gerbils after cochlear nucleus removal or cochlea removal were examined for the presence of projections from VCN to both contralateral MNTB (MNTBc; contralateral to intact, labeled VCN) and MNTBi (ipsilateral to intact, labeled VCN) and to either side of both MSOs. Nuclear boundaries of VCN, MNTB, and MSO were determined by using differential interference contrast and darkfield microscopy. We focused our quantitative analysis on the projections to MNTB in which large calyceal terminations can be readily counted. Labeled calyces were counted in all sections containing MNTBc and MNTBi. A ratio of the number of calyces in MNTBi to the number of calyces in MNTBc was computed for each brain. This ratio, the I/C ratio, was used rather than absolute numbers to account for variability in the effectiveness of dye transport. *t*-Tests were used to test for significance between the ratios in different treatment groups.

Images were obtained with either a Zeiss Axiocam digital camera and acquired in Openlab software (Improvision, Lexington, MA) or a Zeiss LSM 5 Pascal Laser Scanning Microscope and acquired in LSM 5 Pascal 3.0 software. Images were imported into Adobe Photoshop 7.0 to optimize contrast before being imported into Adobe InDesign 3.0.1 for figure layout (Adobe Systems, San Jose, CA).

RESULTS

Cochlear nucleus removal but not cochlea removal results in ectopic projections from VCN to ipsilateral MNTB after P10

We performed cochlea removal on young (P2–P4; n = 6) and old (P10–P15; n = 8) postnatal gerbils to confirm that, in our hands, cochlea removal before P10 results in deafferentation-induced cell death in VCN and ectopic projections in the brainstem, whereas cochlea removal after P10 does not result in either phenomenon (previously described by Kitzes et al., 1995; Russell and Moore, 1995). The left cochlea was ablated and gerbils survived for 6 days before perfusion and dye placement (NeuroVue red or DiI) in the right VCN. Figure 1A–D illustrates the effects of cochlea removal on a P4 gerbil. VCN on the lesioned side (Fig. 1A, asterisk) is substantially smaller than VCN on the nonlesioned side of the same section (Fig. 1B), indicating strong deafferentation-induced cell death in VCN. Additionally, cochlea removal at P4 resulted in normal projections to MNTBc (Fig. 1C, arrowheads) and induced calyceal terminations in MNTBi (Fig. 1D, arrowheads). As expected, cochlea removal on gerbils at P10 (Fig. 1E–H) failed to induce deafferentation-induced cell death in VCN (Fig. 1E vs. F). Cochlea removal at P10 also failed to induce ectopic projections from VCN to MNTBi but did not disrupt the projections to MNTBc (Fig. 1G,H).

The lack of induced projections in older postnatal animals could be due to reduced plasticity of VCN on the nonablated side or to persistence of projections from VCN on the ablated side. To distinguish between these possibilities, we investigated VCN-MNTB projections after cochlear nucleus removal at later ages. Figure 2A shows a dorsal view of a P21 brain perfused 6 days after P15 cochlear nucleus removal. Dotted lines indicate approximate boundaries of the cochlear nuclei. The surgical procedure resulted in removal of the cochlear nucleus (asterisk), including anterior and posterior divisions of VCN and regions of the dorsal cochlear nucleus, whereas the cochlear nucleus on the unoperated side is intact. The changes in MNTB and MSO (described below) further support the interpretation that large regions of VCN were removed. Coronal sections of the brainstem demonstrate the extent to which VCN was lesioned (Fig. 2B,C). The size of VCN is severely reduced on the operated side (Fig. 2B, asterisk) compared with the unoperated side (Fig. 2C). Neuroanatomical labeling revealed that VCN neurons on the intact side of the brainstem send ectopic projections to the MNTBi (Fig. 2D, arrowheads) as well as to the normally appropriate target, MNTBc (not shown).

To determine whether a significant number of calyceal terminations had been induced by cochlear nucleus removal, a ratio of calyces in ipsilateral (on the side of the dye placement) to contralateral (I/C) MNTB was computed for each brain. Analysis revealed a significant difference in these I/C ratios between gerbils receiving P15 cochlear nucleus removal [0.374 ± 0.03 (SEM), n = 6] vs. P15 cochlea removal (0.073 ± 0.02, n = 5; $P < 0.0001$, Student's *t*-test), indicating that significantly more calyces in MNTBi were induced after cochlear nucleus removal than cochlea removal. The I/C ratio after P15 cochlear nucleus removal was not significantly different from the I/C ratio after P2–P4 cochlea removal (P15 cochlear nucleus removal 0.374 ± 0.03 vs. P2–P4 cochlea removal 0.477 ± 0.07, n = 6; $P > 0.05$), suggesting that similar proportions of ectopic projections were induced after cochlea removal in young gerbils and cochlear nucleus removal in P15 gerbils.

Formation of ectopic projections

To characterize the emergence of induced ectopic projections from VCN to MNTBi after cochlear nucleus removal, P15 gerbils were allowed to survive for 1, 2, 3, 4, or 5 days after surgery before perfusion and dye placement (Fig. 3). One day after cochlear nucleus removal, neuro-

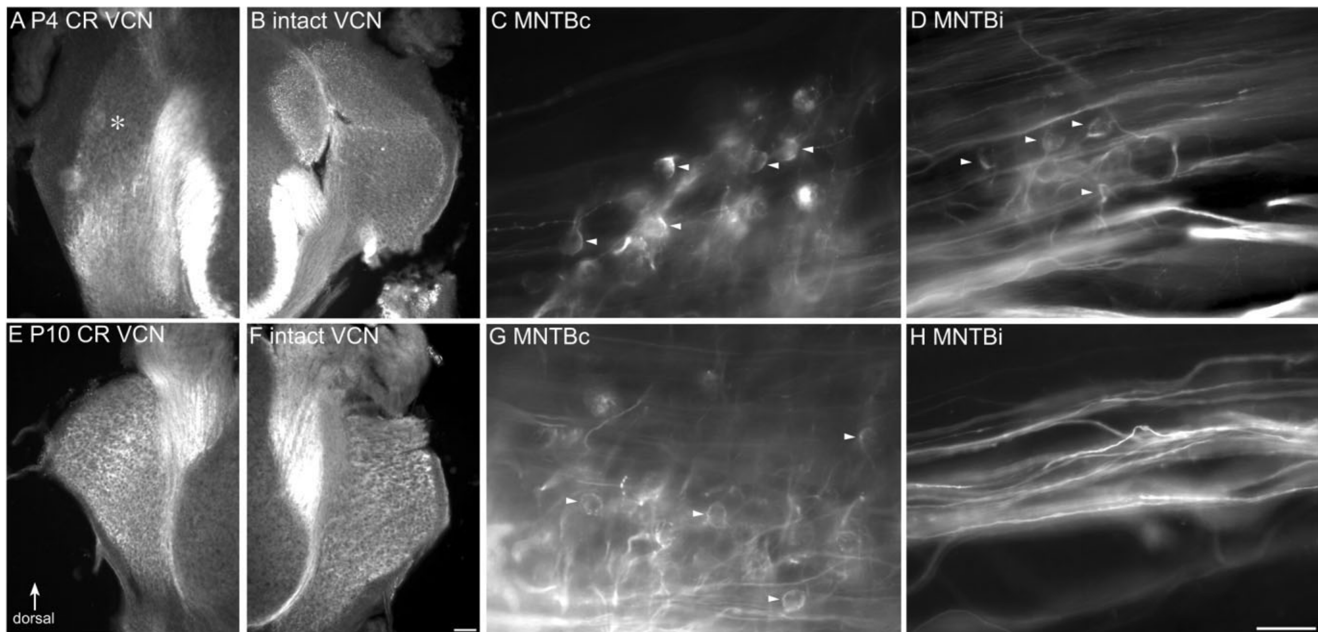

Fig. 1. Cochlea removal at P4 vs. P10. **A–D:** P4 cochlea removal results in deafferentation-induced cell death in VCN (A, asterisk indicates atrophied VCN). Note the size difference between the cochlea removal (CR) side and the intact VCN (B). Additionally, cochlea removal at P4 results in normal projections to MNTBc (C) and induces ectopic projections in MNTBi (D). **E–H:** P10 cochlea removal does not result in deafferentation-induced cell death in VCN (E), and both VCNs are of similar size (E,F). Additionally, cochlea removal at P10 results in normal projections to MNTBc (G) but does not induce any ectopic projections in MNTBi (F). Arrowheads indicate calyceal terminations. Scale bar = 50 μm in H (applies to C,D,G,H); 100 μm for A,B,E,F.

anatomical labeling revealed perpendicular fibers emerging from axons traveling ventral to MNTBi (Fig. 3A, arrowheads). At 2–3 days after cochlear nucleus removal (Fig. 3B), growth cones (arrow) were present on the ends of these perpendicular fibers (arrowheads). At 4–5 days after cochlear nucleus removal (Fig. 3C), thin, immature calyceal terminals were present in MNTB (arrowheads), and some terminals had short filopodia-like extensions (arrows). Six days after cochlear nucleus removal (Fig. 3D), the calyceal terminations adopted a more mature appearance, with digit-like processes appearing to envelop MNTBi neurons. The I/C ratio for 4- and 5-day survivals was similar to that of the 6-day survival group described above (0.395 ± 0.17; n = 3 vs. 0.374 ± 0.03; n = 6; $P > 0.5$), indicating that, even though the calyces appeared immature at that survival interval, their numbers were similar to cases in which calyces appeared mature.

Sensitive period of cochlear nucleus removal-induced plasticity

We next performed cochlear nucleus removal at P25 to determine the developmental period over which cochlear nucleus removal can induce reorganization of the VCN-MNTB projection. In animals subjected to cochlear nucleus ablation at P25, neuroanatomical labeling revealed numerous induced projections from VCN to MNTBi (Fig. 4A, arrowheads). Similarly to the case in animals whose cochlear nucleus was removed at P15, the induced ipsilateral projections had calyceal terminations 6 days after cochlear nucleus removal (Fig. 4B). However, the I/C ratio after P25 cochlear nucleus removal was significantly lower than that obtained after P15 cochlear nucleus removal (0.223 ± 0.02; n = 3 vs. 0.374 ± 0.03; $P < 0.05$). Thus, although ipsilateral projections can be induced at P25, the extent of this reorganization declines with age.

A summary graph illustrating the I/C ratios in all surgical manipulation groups is shown in Figure 4C. I/C ratios for P2 cochlea removal and P10–P15 and P25 cochlear nucleus removal were significantly greater than those obtained after P10 cochlea removal. There was no significant difference between I/C ratios for P2 cochlea removal and P10–P15 cochlear nucleus removal ($P > 0.05$), demonstrating that the potential for reorganization is as great at these ages as in neonates. P25 animals show a significant decline in the ability of VCN on the nonablated side to send a projection to MNTBi.

Cochlear nucleus removal at P15 does not induce ectopic projections to MSO

We next examined projections from the intact VCN to MSO to determine whether cochlear nucleus removal induces sprouting to both sides of MSO at later ages, when cochlea removal alone has no effect. In normal gerbils, cells in VCN project to the lateral dendrites in the ipsilateral MSO (MSOi) and to the medial dendrites in the contralateral MSO (MSOc). We found that cochlea removal at P4 induced projections from VCN to both the medial and lateral dendrites of both MSOs. These results are similar to those reported for cochlea removal at P2 (Kitzes et al., 1995). MSOc from an animal lesioned at P4 is shown in Figure 5A. The arrowheads indicate the row of MSO cell bodies and arrows illustrate inappropriate projections traveling over the dorsal portion of MSO to reach the lateral dendrites. In contrast, cochlear nucleus removal at

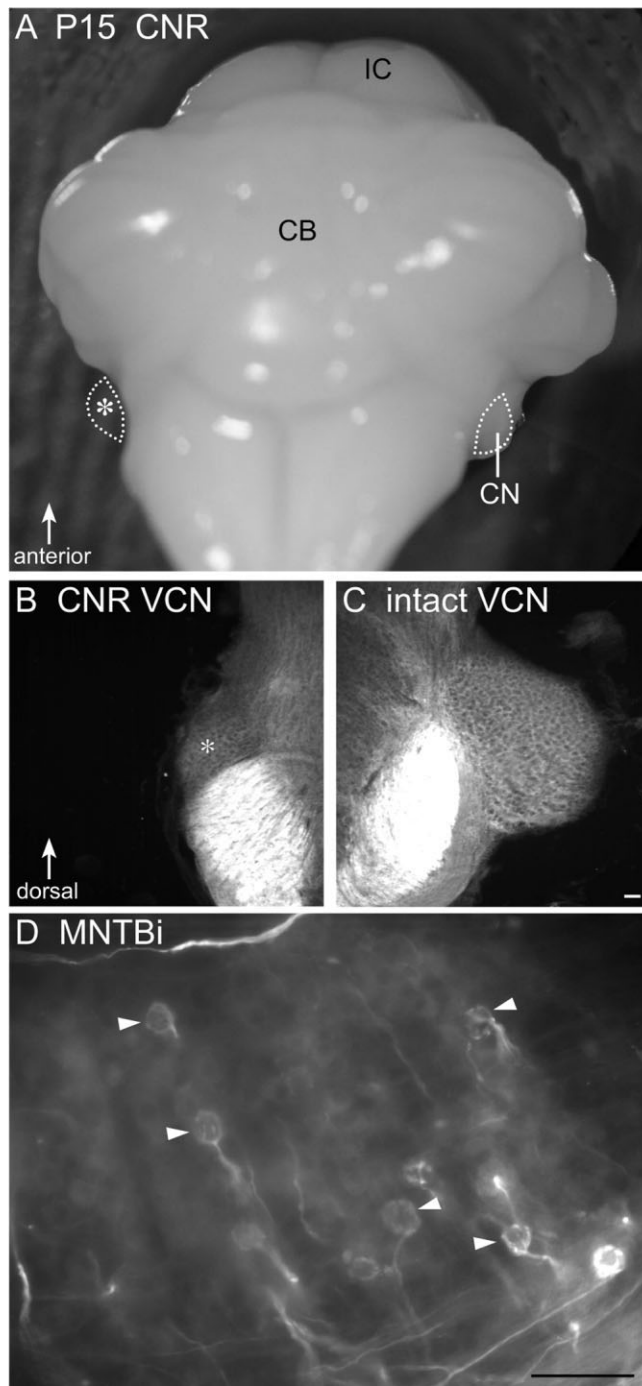

Fig. 2. Cochlear nucleus removal at P15. **A:** Dorsal view of a perfused brain after left-side cochlear nucleus removal at P15. The intact cochlear nucleus (CN) can be seen on the right side (dotted line approximates CN boundary), whereas the CN on the operated side is absent (asterisk). **B:** Coronal section showing the operated side after cochlear nucleus removal (CNR). Asterisk indicates the severely atrophied VCN. **C:** The same section shows intact VCN on the unoperated side. **D:** CNR-induced calyceal projections in MNTBi. Arrowheads indicate calyceal terminations. IC, inferior colliculus, CB, cerebellum. Scale bars = 50 μm.

P15 did not result in inappropriate projections to either MSO (n = 6). Figure 5B shows MSOc after cochlear nucleus removal at P15. Axons from VCN terminate on the medial but not lateral dendrites in MSOc (arrowheads indicate row of MSO cell bodies). Normal segregation of inputs was similarly observed when the cochlear nucleus was ablated at P25 (n = 3, data not shown). Thus, cochlear nucleus removal at older postnatal ages does not induce ectopic projections from VCN to MSO. Importantly, these brains, in which cochlear nucleus removal failed to induce ectopic projection from VCN to MSO, clearly showed induced ectopic projections from VCN to MNTB. These results suggest that deafferentation-induced changes in axonal pathways of different cell types (e.g., globular vs. spherical bushy cells) in VCN are subject to different sensitive periods.

Effects of deafferentation on MSO dendrites

To evaluate the effects of cochlear nucleus removal on the growth of novel projections to denervated regions of MSO, we examined the expression of MAP2, a marker for cell bodies and dendrites (Fig. 6). In tissue from operated animals, dye-labeled sections were viewed and photographed before immunolabeling for MAP2. Figure 6A shows MSOi (ipsilateral to intact, labeled VCN) from an animal that had undergone cochlear nucleus removal at P10. There is a clear vertical row of labeled cell bodies, and MAP2-positive dendrites were seen only on the lateral side of MSOi, which receives innervation from the intact VCN. In some cases, a pair of labeled dendrites was observed emerging from the soma at slightly different angles (Fig. 6A, arrows). Figure 6B shows a higher power image of MAP2-positive MSOi with a pronounced asymmetry in dendrites (asterisks indicate cells lacking medial dendrites). These results demonstrate significant dendritic atrophy on the denervated side of MSOi and provide further evidence that globular bushy cells in VCN were removed. In animals subjected to cochlea removal but not cochlear nucleus removal at P10, MAP2-positive dendrites were present on both sides of MSOi (Fig. 6C and at higher power in 6D). These results suggest that, at P10, dendritic atrophy is produced when the cochlear nucleus is removed but not when the cochlea alone is removed.

DISCUSSION

In the present study, we examined the susceptibility of auditory brainstem circuits to deafferentation-induced reorganization. Previous studies showed that cochlea removal induces changes in axonal projections, but only when performed at very early postnatal ages (Nordeen et al., 1983b; Kitzes et al., 1995; Russell and Moore, 1995). Here we evaluated projection patterns in the gerbil auditory brainstem following unilateral removal of the cochlear nucleus. This experimental approach allowed us to challenge VCN projections at later ages by directly removing inputs to a target of these axons, the MNTB.

The main findings of our study are shown schematically in Figure 7. Normal auditory pathways (Fig. 7A) include both ipsilateral and contralateral projections. Cochlea removal at early ages induces an extensive set of novel projections, shown in red in Figure 7B. In addition, we

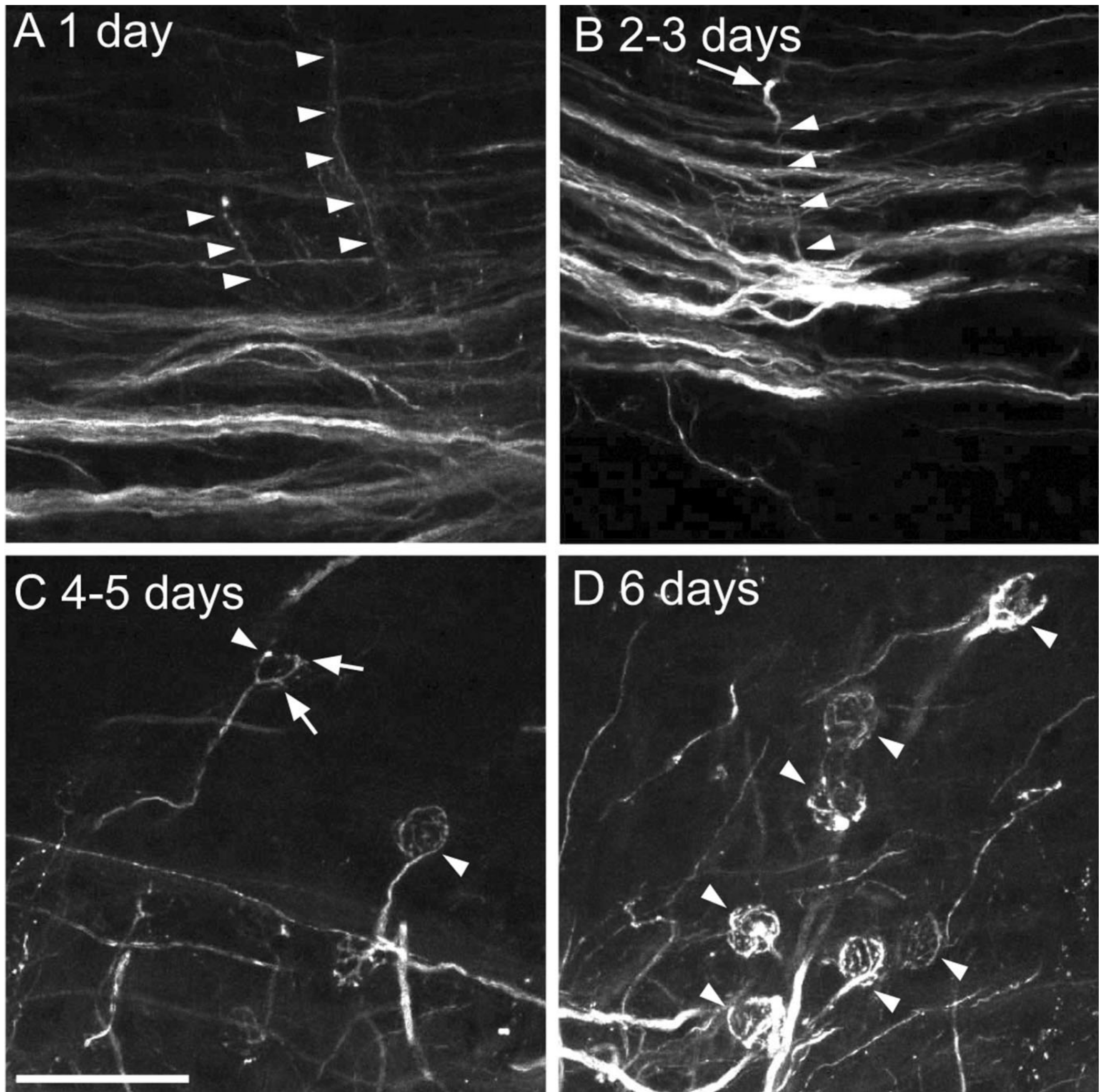

Fig. 3. Formation of ectopic projections after P15 cochlear nucleus removal. **A:** At 1 day after cochlear nucleus removal, fibers are present emerging from axons ventral to MNTBi (arrowheads indicate fibers). **B:** At 2–3 days after cochlear nucleus removal, growth cones can be seen at the ends of the fibers (arrowheads indicate fiber, and arrow indicates growth cone). **C:** At 4–5 days after cochlear nucleus removal, thin, immature calyces with short filopodia-like radiations appear in MNTBi (arrowheads indicate calyces, and arrows indicate filopodia). **D:** At 6 days after cochlear nucleus removal, near-mature calyces with digit-like processes wrapping around MNTBi cells appear (arrowheads indicate calyces). Scale bar = 25 μm.

have shown the following. 1) Whereas cochlea removal at later ages (Fig. 7C) does not result in novel projections, cochlear nucleus removal at P10 to at least P25 induces ectopic projections from the intact VCN to MNTBi (Fig. 7D). 2) The morphological sequence of events is similar to that seen during initial development of the VCN projection and during early reorganization, but the time course is slightly longer. The formation of ectopic ipsilateral calyces takes about 2–3 days longer at P10 than at P2 (Kitzes et al., 1995; Russell and Moore, 1995; Fig. 3). This time does not increase further at P25. 3) Ectopic projections from VCN to denervated regions of MSO are not induced at older postnatal ages after cochlear nucleus removal, and MSO dendrites atrophy (Fig. 7D). Taken

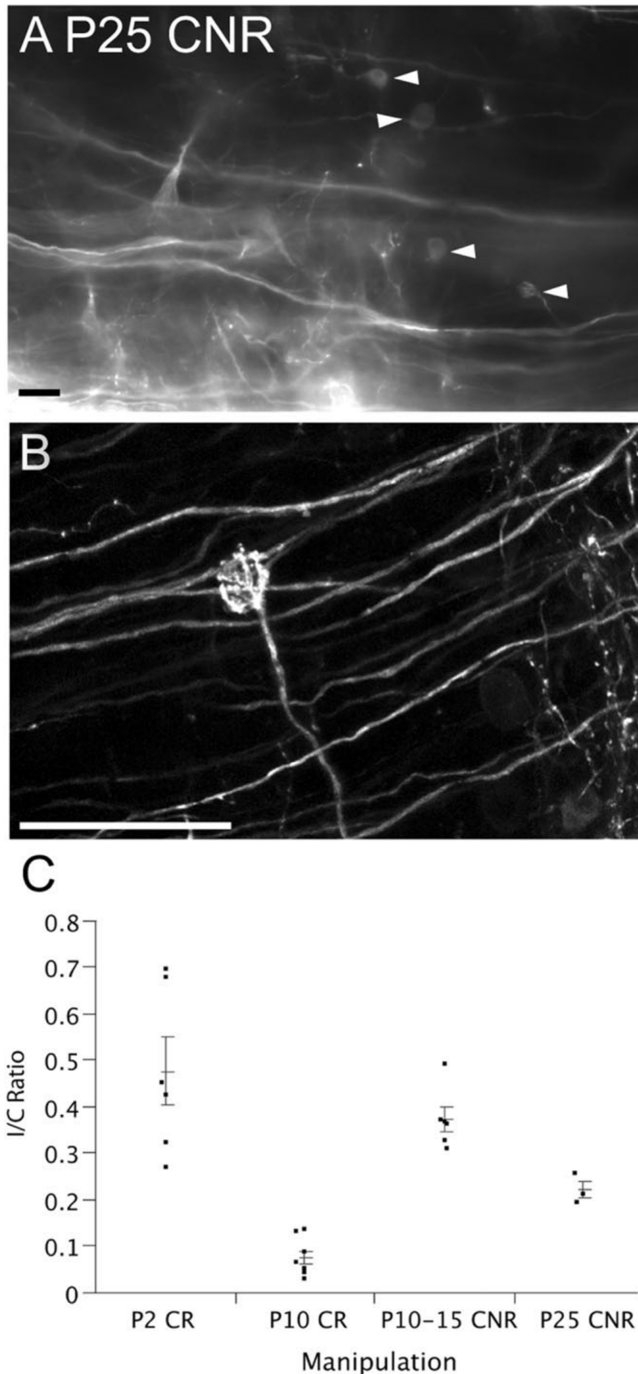

Fig. 4. Plasticity after P25 cochlear nucleus removal. **A:** P25 cochlear nucleus removal induces terminations in MNTBi (arrowheads indicate terminations). **B:** Higher magnification reveals a representative calyceal termination in MNTBi. **C:** Summary graph of results from all experimental groups. Scale bars = 25 μm.

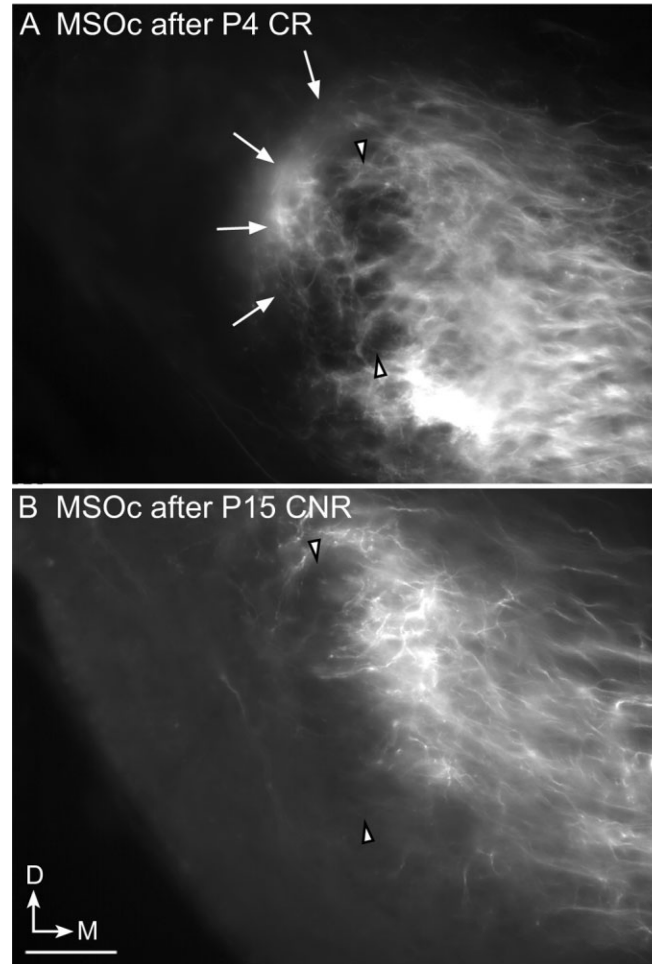

Fig. 5. Differential plasticity in MSO. **A:** P4 cochlea removal induces ectopic projections to the lateral dendrites in MSOc. Arrowheads indicate the row of cell bodies in MSO, and arrows indicate ectopic projections to the inappropriate side of MSO. **B:** P15 cochlear nucleus removal does not result in novel projections from VCN to MSOc (arrowheads indicate row of MSO cell bodies). Axes indicate dorsal (D) and medial (M). Scale bar = 100 μm.

together, the data indicate that the VCN is capable of producing new connections beyond the end of a previously defined sensitive period (P10). Moreover, individual pathways emanating from VCN have distinct windows of plasticity during the postnatal period.

Formation of novel projections at late ages

Cochlear nucleus removal initiates a substantial novel projection in gerbils at late ages, after hearing onset and when cochlea removal alone no longer induces these projections. Several studies have examined the responses of the auditory brainstem to early cochlea removal. One of the most dramatic responses is the loss of neurons in the cochlear nucleus, which occurs when the cochlea is removed during the first postnatal week in gerbils (Nordeen et al., 1983a; Hashisaki and Rubel, 1989; Tierney et al., 1997) or mice (Mostafapour et al., 2000). The developmental limitation in deafferentation-induced cell loss after cochlea removal results in part from changes in expression of genes in cell death pathways (Mostafapour et al., 2002; Harris et al., 2005). Temporal limits on the induction of novel projections have also been described (Kitzes et al., 1995; Russell and Moore, 1995). Cochlea removal at young postnatal ages results in loss of VCN neurons and their

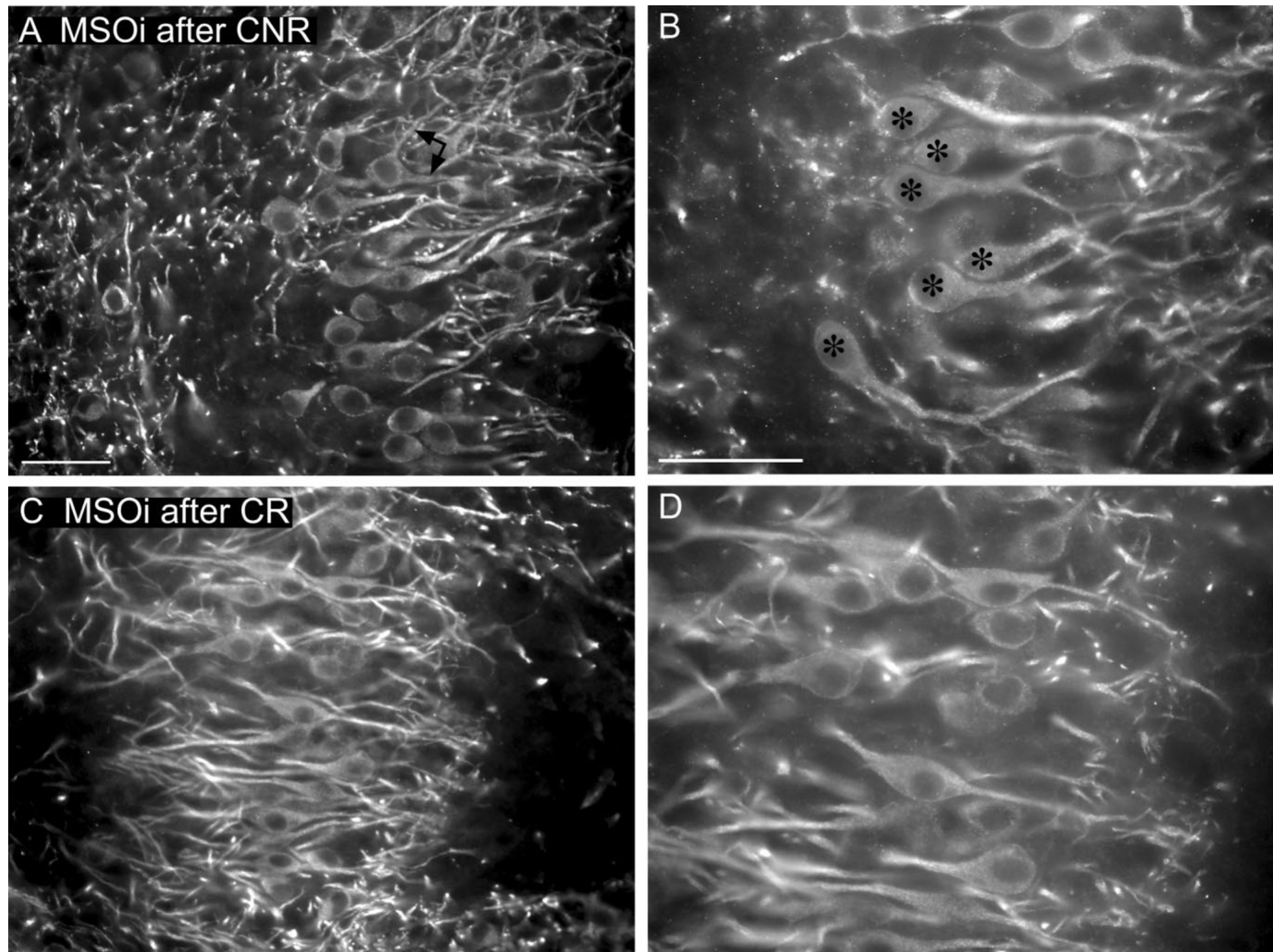

Fig. 6. MAP2 expression in MSO after CNR and CR. **A:** MAP2-positive cells in MSOi after P15 CNR have dendrites only on the lateral side. In some cases, pairs of labeled dendrites were present (indicated by arrows). **B:** Higher power image of MSOi indicates a clear loss of medial dendrites (asterisks indicate cells lacking dendrites on the medial side). **C:** MAP2-positive cells in MSOi after P15 CR have symmetrical dendrites. **D:** Higher power image of MSOi shows clear medial and lateral dendrites. Scale bars = 50 μm in A (applies to A,C); 50 μm in B (applies to B,D).

projections, and the denervated targets of VCN axons attract novel inputs from the intact side. This reorganization is no longer seen after P10 (Russell and Moore, 1995). These authors also described cases in which the cochlear nucleus was removed at P10 and no VCN-MNTBi projections were observed. A possible explanation for the discrepancy between that study and our results is that, in the previous study, the survival time was only 4 days, which we show here is insufficient to induce mature ipsilateral calyceal projections. The results suggest that the previously defined limitation on the formation of ipsilateral MNTB projections is a consequence of the limitation on cochlea removal-induced VCN cell death. Although cochlea removal reduces activity in the MNTB contralateral to the lesion, the change is insufficient to induce novel projections at later postnatal ages. In contrast, direct removal of VCN-MNTB projections has a pronounced effect. Thus, when sufficiently challenged, the pathway demonstrates an extended period of plasticity.

Induced projections from VCN to MNTB were observed through P25, at which time the new calyces were significantly less numerous than at P10–P15. At these ages, the auditory brainstem pathways are mature in that calyces have formed, and animals can hear (Woolf and Ryan, 1984). This maturity contrasts with that seen at P2, when VCN axons have not yet formed calyceal terminal arbors in MNTB (Morest, 1968; Kandler and Friauf, 1993; Kil et al., 1995). Despite these differences in maturity, the growth of VCN axons toward MNTBi after P10 nonetheless resembles the emergence of both the early induced projection to MNTBi and the normal growth of connections to MNTBc.

Factors that promote the formation of novel projections to MNTB seem to remain stable over a long developmental period. Although the axon branches project to the wrong side of the brain, there is nonetheless selectivity in the choice of MNTB as a target, as well as the morphological specialization of the nerve ending. Although the time

The Journal of Comparative Neurology. DOI 10.1002/cne

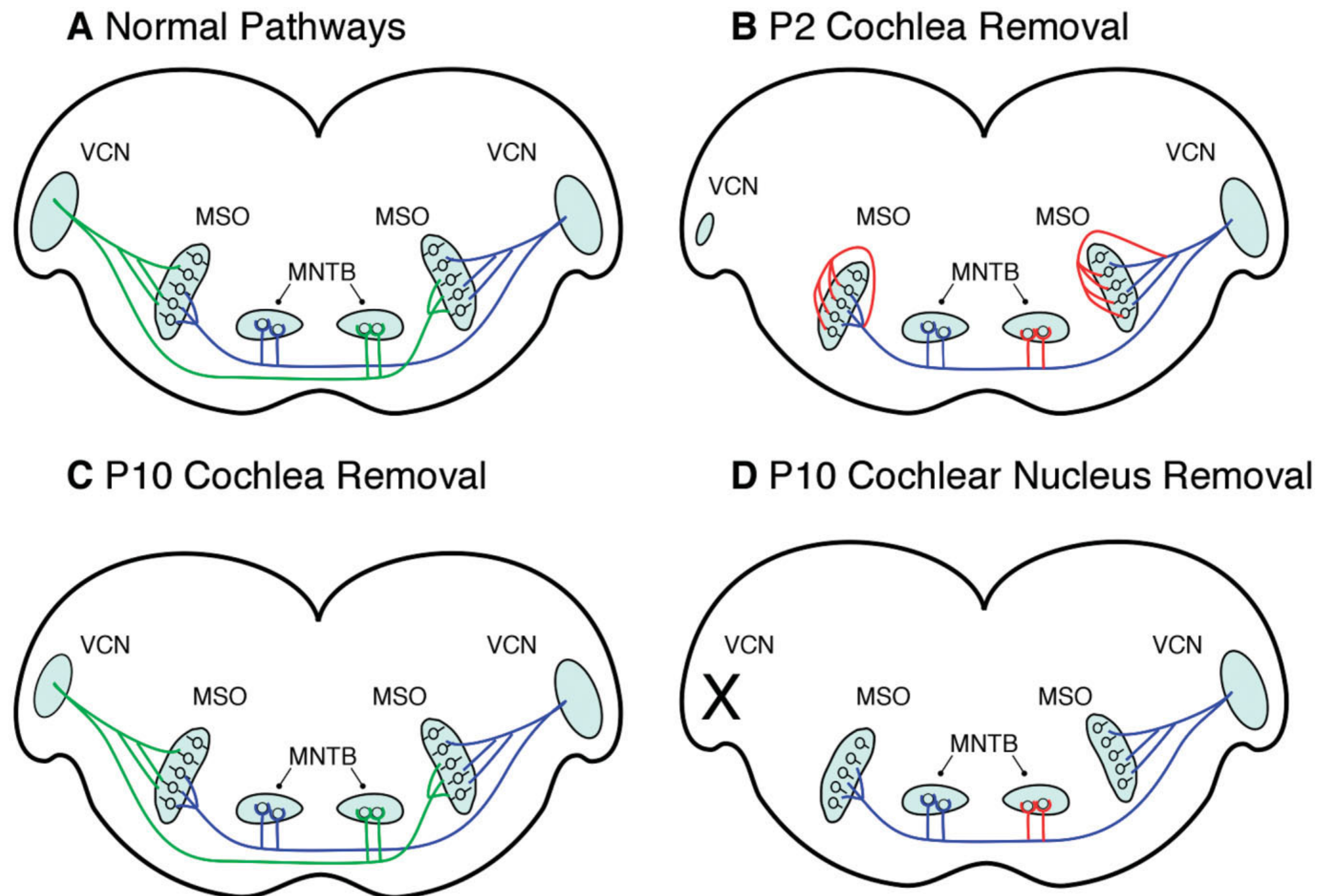

Fig. 7. Summary of gerbil auditory brainstem reorganization. **A:** Normal organization of the mammalian auditory brainstem pathways examined in this study. Cells in the ventral cochlear nucleus (VCN) project to the lateral superior olive (not shown), the medial superior olive (MSO), and the medial nucleus of the trapezoid body (MNTB). Projections from the right VCN are shown in blue, and projections from the left VCN are shown in green. Although distinct sets of VCN neurons project to different targets, they are shown here as a single pathway for simplicity. These pathways allow for determination of interaural time differences and interaural intensity differences. Adapted with permission from Cramer (2005). **B–D:** Schematic diagrams illustrating the changes observed after the manipulations performed in this study. Novel, ectopic projections are shown in red. B: Cochlea removal at young ages (P2–P4) results in deafferentation induced-cell death in VCN and ectopic projections from the intact VCN to MNTBi and to both sides of MSO, in which dendrites did not appear altered. C: Cochlea removal at older postnatal ages does not result in deafferentation-induced cell death in VCN or ectopic projections to MNTBi or MSO. D: Cochlear nucleus removal at older postnatal ages (P10–P25) results in ectopic projections to MNTBi but not MSO. Deafferented dendrites in MSO on both sides of the brain show distinct atrophy.

course for the maturation of calyceal terminations is slightly longer, the sequence of events leading to the ipsilateral calyx is similar to that reported for the normal pathway during early development (Morest, 1968; Kandler and Friauf, 1993; Kil et al., 1995). In both processes, thin, sinuous axon branches emerge from the trapezoid body, grow toward MNTB, and form thin, cup-like contacts with MNTB neurons, with filopodial extensions disappearing slightly later. The precision and morphological sequence of events in the formation of this novel projection suggest that the molecular signals that promote formation of calyces of Held are active throughout the sensitive period.

Differences between VCN pathways

We found a substantial difference between pathways in the extent to which novel projections formed in the auditory brainstem after cochlear nucleus removal. Whereas the VCN-MNTBi pathway was robust at late ages after cochlear nucleus removal, we did not observe reorganization of the projection from VCN to MSO. Because both pathways are reorganized after early cochlea removal (Kitzes et al., 1995; Russell and Moore, 1995), our findings suggest that the two pathways exhibit distinct sensitive periods. The two pathways differ in architecture so that unilateral cochlear nucleus removal results in the loss of most or all excitatory input to MNTB neurons, but a loss of about half of the excitatory input to MSO neurons. The resulting differences in the levels of activity in the two nuclei may underlie differences in the extent to which the denervated cells attract novel inputs. The extent to which inhibitory inputs regulate axonal sprouting or dendritic retraction at different ages is not known. Future studies of plasticity in LSO may provide an opportunity to examine this question, in that, at early ages, sprouting occurs after

cochlea removal (Kitzes et al., 1995), although the nucleus retains inhibitory input from MNTB.

Several studies have demonstrated that reduction or removal of cochlear input results in changes in the size of the denervated dendrites in MSO (Perkins, 1973; Feng and Rogowski, 1980; Russell and Moore, 1999). Although changes in the symmetry were demonstrated, dendrites remain present on both sides. In our study, cochlear nucleus removal at later ages resulted in the loss of dendrites on the denervated side of both ipsilateral and contralateral MSO, as assessed with MAP2 immunohistochemistry. Early cochlea removal, however, did not result in degeneration of dendrites in previous studies (Kitzes et al., 1995). This difference in dendritic responses may account for differences between early and late deafferentation and may depend in turn on the response of axons.

An investigation of plasticity in MSO may be informed by experiments on the avian analogue of MSO, nucleus laminaris (NL). Dorsal NL dendrites receive input from ipsilateral nucleus magnocellularis (NM), the avian homologue of VCN, whereas ventral NL dendrites receive input from contralateral NM (Parks and Rubel, 1975). Transection of the dorsal cochlear tract, through which contralateral NM axons pass, results in dramatic reduction in the size of the denervated dendrites of NL (Benes et al., 1977; Deitch and Rubel, 1984, 1989a,b). These changes are rapid and depend on levels of synaptic input (Sorensen and Rubel, 2006). Evidence in the chick pathway (Rubel et al., 1981) suggests that the formation of a projection to the inappropriate side of NL after lesion results from the integration of factors that influence axonal sprouting and dendritic regeneration. Here we show evidence that similar factors operate in the mammalian pathway. Thus, at early ages, dendritic degeneration in MSO may be prevented by rapid and extensive sprouting of VCN axons (Kitzes et al., 1995). At later ages, sprouting may be very weakly or slowly induced, allowing dendritic degeneration to outweigh growth of axons to both sides of MSO.

These studies suggest that there are developmental changes in the strength of factors that promote dendritic degeneration or axonal sprouting in MSO. The mechanisms responsible for these developmental differences are unknown. They might include differences in the lengths of pathways, differences in cellular responses to loss of input, or developmental regulation of molecules that influence axon growth or dendritic morphology. Moreover, there may be common mechanisms that influence both dendritic degeneration and axonal sprouting. Developmentally restricted reorganization of inputs has also been reported in projections to the inferior colliculus (IC). The gerbil IC receives projections from several auditory brainstem nuclei on both sides of the brain (Nordeen et al., 1983a), and neonatal cochlea removal results in a substantial increase in the number of ipsilateral projections from the cochlear nucleus (Nordeen et al., 1983b; Kitzes and Semple, 1985; Moore and Kitzes, 1985). Cochlear nucleus removal at later ages did not show this effect (Moore and Kitzes, 1986), but, because these animals were operated after 2 months or later, it is not known whether changes in this pathway occurred at shorter postoperative times.

Limitations on auditory plasticity may arise in part from myelination. Axonal sprouting in the central nervous system is limited by myelination and myelin-associated proteins (Filbin, 2003; Schwab, 2004). Furthermore, deletion of the gene for the Nogo-66 receptor, which binds the myelin-derived Nogo protein, allows for an extended period of ocular dominance plasticity in mice (McGee et al., 2005). Regeneration after both axotomy and changes in experience are thus negatively regulated by myelin, and a reduced ability to form novel projections in the VCN pathway may be associated with maturation of myelin. However, studies in rats show that myelination begins at P8 in axons projecting to MNTB and is significant by P13 (Leao et al., 2005). We show that at this age and later ages in gerbils, VCN-MNTBi projections after cochlear nucleus removal are as numerous as at P2, suggesting that induction of these projections is possible despite the presence of myelin. It is nonetheless possible that continued maturation of the myelin sheath has a role in the subsequent reduction of novel projections after P25 cochlear nucleus ablation. The role of myelination in this process thus remains an important consideration.

Sensitive periods in auditory development

The experiments reported here provide evidence for distinct sensitive periods for two auditory pathways. Moreover, the results emphasize that sensitive periods can be extended when a pathway is sufficiently challenged. Our results are consistent with previous studies on the importance of auditory experience in birds. For example, for the barn owl, it was widely believed that the auditory sound localization pathway is adaptive only in the juvenile animal (Knudsen, 1999). However, recently, a capacity for plasticity in adult owls was revealed with incremental training (Linkenhoker and Knudsen, 2002). Similarly, for Bengalese finches, it was believed that song learning could only occur during adolescence (Marler and Tamura, 1964). However, Woolley and Rubel (2002) demonstrated that induction of hair cell loss resulted in initial degradation of song but was followed by restoration of the original song or new song learned at later ages, as hair cells regenerated (for review see Brainard and Doupe, 2002; Woolley, 2004).

In the present study, we have reexamined the sensitive period of deafferentation-induced plasticity of auditory brainstem projections. Cochlea removal ceases to be an effective challenge after P10, most likely because the sensitive period for deafferentation-induced cell death in VCN declines by P10. However, cochlear nucleus removal experiments demonstrate that, in at least one pathway, extensive changes in brainstem projections form at late ages when the circuitry in the auditory brainstem is mature.

ACKNOWLEDGMENTS

The authors are grateful to Cindy Hong and Shazia Siddiqui for technical assistance, to Dr. Susana Cohen-Cory for assistance with confocal microscopy, and to Dr. Len Kitzes and Dr. Ilona Miko for helpful discussions and comments on the manuscript. We thank Brian Gray (PTI Research) for the gift of NeuroVue dyes.

LITERATURE CITED

Benes FM, Parks TN, Rubel EW. 1977. Rapid dendritic atrophy following deafferentation: an EM morphometric analysis. Brain Res 122:1–13.

Born DE, Rubel EW. 1985. Afferent influences on brain stem auditory nuclei of the chicken: neuron number and size following cochlea removal. J Comp Neurol 231:435–445.

Brainard MS, Doupe AJ. 2002. What songbirds teach us about learning. Nature 417:351–358.

Cant NB, Casseday JH. 1986. Projections from the anteroventral cochlear nucleus to the lateral and medial superior olivary nuclei. J Comp Neurol 247:457–476.

Cramer KS. 2005. Eph proteins and the assembly of auditory circuits. Hear Res 206:42–51.

Deitch JS, Rubel EW. 1984. Afferent influences on brain stem auditory nuclei of the chicken: time course and specificity of dendritic atrophy following deafferentation. J Comp Neurol 229:66–79.

Deitch JS, Rubel EW. 1989a. Changes in neuronal cell bodies in N. laminaris during deafferentation-induced dendritic atrophy. J Comp Neurol 281:259–268.

Deitch JS, Rubel EW. 1989b. Rapid changes in ultrastructure during deafferentation-induced dendritic atrophy. J Comp Neurol 281:234–258.

Feng AS, Rogowski BA. 1980. Effects of monaural and binaural occlusion on the morphology of neurons in the medial superior olivary nucleus of the rat. Brain Res 189:530–534.

Filbin MT. 2003. Myelin-associated inhibitors of axonal regeneration in the adult mammalian CNS. Nat Rev Neurosci 4:703–713.

Fritzsch B, Muirhead KA, Feng F, Gray BD, Ohlsson-Wilhelm BM. 2005. Diffusion and imaging properties of three new lipophilic tracers, NeuroVue maroon, NeuroVue red and NeuroVue green and their use for double and triple labeling of neuronal profile. Brain Res Bull 66:249–258.

Harris JA, Hardie NA, Bermingham-McDonogh O, Rubel EW. 2005. Gene expression differences over a critical period of afferent-dependent neuron survival in the mouse auditory brainstem. J Comp Neurol 493:460–474.

Hashisaki GT, Rubel EW. 1989. Effects of unilateral cochlea removal on anteroventral cochlear nucleus neurons in developing gerbils. J Comp Neurol 283:5–73.

Hensch TK. 2004. Critical period regulation. Annu Rev Neurosci 27:549–579.

Irvine DRF. 1986. A review of the structure and function of auditory brainstem processing mechanisms. In: Ottoson D, editor. Sensory physiology. Berlin: Springer Verlag. p 1–279.

Kandler K, Friauf E. 1993. Pre- and postnatal development of efferent connections of the cochlear nucleus in the rat. J Comp Neurol 328:161–184.

Kil J, Kageyama GH, Semple MN, Kitzes LM. 1995. Development of ventral cochlear nucleus projections to the superior olivary complex in gerbil. J Comp Neurol 353:317–340.

Kitzes LM, Semple MN. 1985. Single-unit responses in the inferior colliculus: effects of neonatal unilateral cochlear ablation. J Neurophysiol 53:1483–1500.

Kitzes LM, Kageyama GH, Semple MN, Kil J. 1995. Development of ectopic projections from the ventral cochlear nucleus to the superior olivary complex induced by neonatal ablation of the contralateral cochlea. J Comp Neurol 353:341–363.

Knudsen EI. 1999. Mechanisms of experience-dependent plasticity in the auditory localization pathway of the barn owl. J Comp Physiol A185: 305–321.

Kuwabara N, DiCaprio RA, Zook JM. 1991. Afferents to the medial nucleus of the trapezoid body and their collateral projections. J Comp Neurol 314:684–706.

Leao RM, Kushmerick C, Pinaud R, Renden R, Li GL, Taschenberger H, Spirou G, Levinson SR, von Gersdorff H. 2005. Presynaptic Na^{+} channels: locus, development, and recovery from inactivation at a high-fidelity synapse. J Neurosci 25:3724–3738.

Levi-Montalcini R. 1949. The develoment of the acoustico-vestibular centers in the chick embryo in the absence of the afferent root fibers and of descending fiber tracts. J Comp Neurol 91:209–242.

Linkenhoker BA, Knudsen EI. 2002. Incremental training increases the plasticity of the auditory space map in adult barn owls. Nature 419: 293–296.

Marler P, Tamura M. 1964. Culturally transmitted patterns of vocal behavior in sparrows. Science 146:1483–1486.

McGee AW, Yang Y, Fischer QS, Daw NW, Strittmatter SM. 2005. Experience-driven plasticity of visual cortex limited by myelin and Nogo receptor. Science 309:2222–2226.

Merzenich MM, Kaas JH, Wall J, Nelson RJ, Sur M, Felleman D. 1983. Topographic reorganization of somatosensory cortical areas 3b and 1 in adult monkeys following restricted deafferentation. Neuroscience 8:33–55.

Moore DR, Kitzes LM. 1985. Projections from the cochlear nucleus to the inferior colliculus in normal and neonatally cochlea-ablated gerbils. J Comp Neurol 240:180–195.

Moore DR, Kitzes LM. 1986. Cochlear nucleus lesions in the adult gerbil: effects on neurone responses in the contralateral inferior colliculus. Brain Res 373:268–274.

Morest DK. 1968. The growth of synaptic endings in the mammalian brain: a study of the calyces of the trapezoid body. Z Anat Entwicklungsgesch 127:201–220.

Mostafapour SP, Cochran SL, Del Puerto NM, Rubel EW. 2000. Patterns of cell death in mouse anteroventral cochlear nucleus neurons after unilateral cochlea removal. J Comp Neurol 426:561–571.

Mostafapour SP, Del Puerto NM, Rubel EW. 2002. bcl-2 Overexpression eliminates deprivation-induced cell death of brainstem auditory neurons. J Neurosci 22:4670–4674.

Nordeen KW, Killackey HP, Kitzes LM. 1983a. Ascending auditory projections to the inferior colliculus in the adult gerbil, *Meriones unguiculatus*. J Comp Neurol 214:131–143.

Nordeen KW, Killackey HP, Kitzes LM. 1983b. Ascending projections to the inferior colliculus following unilateral cochlear ablation in the neonatal gerbil, *Meriones unguiculatus*. J Comp Neurol 214:144–153.

Parks TN, Rubel EW. 1975. Organization and development of brain stem auditory nuclei of the chicken: organization of projections from n. magnocellularis to n. laminaris. J Comp Neurol 164:435–448.

Perkins RE. 1973. An electron microscopic study of synaptic organization in the medial superior olive of normal and experimental chinchillas. J Comp Neurol 148:387–415.

Rubel EW, Smith ZD, Steward O. 1981. Sprouting in the avian brainstem auditory pathway: dependence on dendritic integrity. J Comp Neurol 202:397–414.

Russell FA, Moore DR. 1995. Afferent reorganisation within the superior olivary complex of the gerbil: development and induction by neonatal, unilateral cochlear removal. J Comp Neurol 352:607–625.

Russell FA, Moore DR. 1999. Effects of unilateral cochlear removal on dendrites in the gerbil medial superior olivary nucleus. Eur J Neurosci 11:1379–1390.

Sanes DH. 1990. An in vitro analysis of sound localization mechanisms in the gerbil lateral superior olive. J Neurosci 10:3494–3506.

Schwab ME. 2004. Nogo and axon regeneration. Curr Opin Neurobiol 14:118–124.

Smith PH, Joris PX, Yin TC. 1993. Projections of physiologically characterized spherical bushy cell axons from the cochlear nucleus of the cat: evidence for delay lines to the medial superior olive. J Comp Neurol 331:245–260.

Sorensen SA, Rubel EW. 2006. The level and integrity of synaptic input regulates dendrite structure. J Neurosci 26:1539–1550.

Syka J. 2002. Plastic changes in the central auditory system after hearing loss, restoration of function, and during learning. Physiol Rev 82:601–636.

Tierney TS, Russell FA, Moore DR. 1997. Susceptibility of developing cochlear nucleus neurons to deafferentation-induced death abruptly ends just before the onset of hearing. J Comp Neurol 378:295–306.

Trune DR. 1982. Influence of neonatal cochlear removal on the development of mouse cochlear nucleus: I. Number, size, and density of its neurons. J Comp Neurol 209:409–424.

Weinberger NM. 1995. Dynamic regulation of receptive fields and maps in the adult sensory cortex. Annu Rev Neurosci 18:129–158.

Wiesel TN, Hubel DH. 1963a. Effects of visual deprivation on morphology and physiology of cells in the cats lateral geniculate body. J Neurophysiol 26:978–993.

Wiesel TN, Hubel DH. 1963b. Single-cell responses in striate cortex of kittens deprived of vision in one eye. J Neurophysiol 26:1003–1017.

Woolf NK, Ryan AF. 1984. The development of auditory function in the cochlea of the mongolian gerbil. Hear Res 13:277–283.

Woolley SM. 2004. Auditory experience and adult song plasticity. Ann N Y Acad Sci 1016:208–221.

Woolley SM, Rubel EW. 2002. Vocal memory and learning in adult Bengalese Finches with regenerated hair cells. J Neurosci 22:7774–7787.