

Selective Removal of Lateral Olivocochlear Efferents Increases Vulnerability to Acute Acoustic Injury

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Darrow KN, Maison SF, Liberman MC. Selective removal of lateral olivocochlear efferents increases vulnerability to acute acoustic injury. *J Neurophysiol* 97: 1775–1785, 2007. First published November 29, 2006; doi:10.1152/jn.00955.2006. Cochlear sensory cells and neurons receive efferent feedback from the olivocochlear (OC) system. The myelinated medial component of the OC system and its effects on outer hair cells (OHCs) have been implicated in protection from acoustic injury. The unmyelinated lateral (L)OC fibers target ipsilateral cochlear nerve dendrites and pharmacological studies suggest the LOC's dopaminergic component may protect these dendrites from excitotoxic effects of acoustic overexposure. Here, we explore LOC function in vivo by selective stereotaxic destruction of LOC cell bodies in mouse. Lesion success in removing the LOC, and sparing the medial (M)OC, was assessed by histological analysis of brain stem sections and cochlear whole mounts. Auditory brain stem responses (ABRs), a neural-based metric, and distortion product otoacoustic emissions (DPOAEs), an OHC-based metric, were measured in control and surgical mice. In cases where the LOC was at least partially destroyed, there were increases in suprathreshold neural responses that were frequency- and level-independent and not attributable to OHC-based effects. These interaural response asymmetries were not found in controls or in cases where the lesion missed the LOC. In LOC-lesion cases, after exposure to a traumatic stimulus, temporary threshold shifts were greater in the ipsilateral ear, but only when measured in the neural response; OHC-based measurements were always bilaterally symmetric, suggesting OHC vulnerability was unaffected. Interaural asymmetries in threshold shift were not found in either unlesioned controls or in cases that missed the LOC. These findings suggest that the LOC modulates cochlear nerve excitability and protects the cochlea from neural damage in acute acoustic injury.

INTRODUCTION

Vertebrate hair cell systems, including the cochlea, vestibular organs, and lateral line, are modulated by efferent feedback (for review, see Guinan 1996). In the mammalian cochlea, efferent feedback is provided by the olivocochlear (OC) bundle, which constitutes two components (Fig. 1, A and B). Myelinated medial (M)OC neurons originate in contralateral and ipsilateral periolivary nuclei and innervate outer hair cells (OHCs). Unmyelinated lateral (L)OC neurons originate in and around the ipsilateral lateral superior olive (LSO) and innervate primarily afferent dendrites of the cochlear nerve near their synapses with inner hair cells (IHCs).

MOC terminals on OHCs are cholinergic and, when activated, suppress cochlear responses by decreasing the normal

contribution of OHCs to amplification of cochlear mechanical vibrations (for review, see Guinan 1996). LOC terminals are more complex cytochemically, with immunohistochemical evidence for cholinergic, GABAergic, dopaminergic, and peptidergic transmission (for review, see Eybalin 1993). Morphological studies suggest two LOC subgroups based on cell-body location and extent of peripheral ramification (Brown 1987; Warr et al. 1997). Immunohistochemistry suggests a corresponding cytochemical dichotomy between a cholinergic/GABAergic and a dopaminergic subgroup (Darrow et al. 2006a). Consistent with the observation that LOC activation, by inferior colliculus stimulation, can evoke either slow enhancement or suppression of auditory nerve response (Groff and Liberman 2003), it can be hypothesized that the former effect stems from the cholinergic subgroup and the latter effect from the dopaminergic subgroup.

Many studies have implicated OC feedback in protecting the cochlea from acoustic injury: electrical stimulation of the OC bundle reduces temporary threshold shifts from acoustic overexposure (Rajan 1988; Reiter and Liberman 1995) and chronic section of the OC bundle renders the ear more vulnerable to permanent acoustic injury (Handrock and Zeisberg 1982; Kujawa and Liberman 1997). Although OC contributions to protection are well documented, ambiguity remains with respect to the relative contributions of LOC versus MOC systems, given that: 1) LOC and MOC axons run together in the OC bundle, and thus both electrical stimulation and surgical section experiments could theoretically involve both systems; and 2) both LOC and MOC systems have cholinergic components, and thus the pharmacological blockers used may not be selective.

The most definitive evidence for a protective effect of the cholinergic MOC system is the observation that mice with overexpression of $\alpha 9$ cholinergic receptors in OHCs have enhanced resistance to permanent and temporary acoustic injury (Maison et al. 2002). Although no direct evidence exists for an LOC protective role, it was previously suggested (e.g., Ruel et al. 2001) that one LOC function is controlling what appears to be glutamate excitotoxicity in afferent nerve terminals in the acute stages of acoustic injury (d'Aldin et al. 1995; Liberman and Mulroy 1982; Puel et al. 1998; Robertson 1983): cochleas perfused with dopamine antagonists show vacuolization of cochlear terminals, which mimic that seen after acoustic overexposure (Ruel et al. 2001), and infusion of a dopamine

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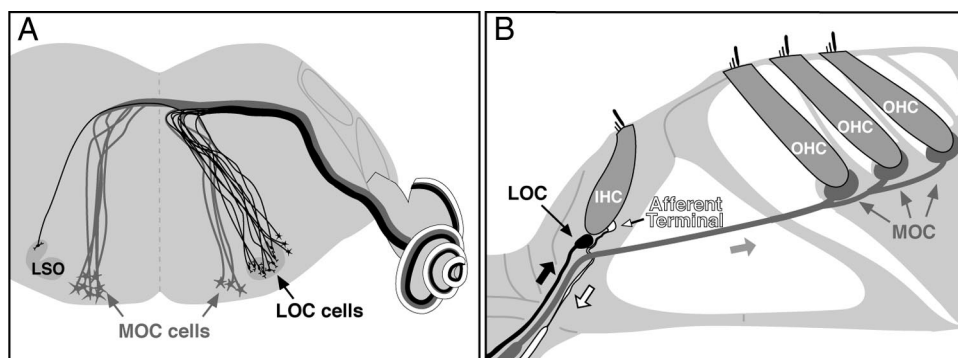


FIG. 1. Anatomical schematics illustrate the central origins (A) and peripheral projections (B) of the medial and lateral components of the olivocochlear (OC) efferent system. Bold arrows in B indicate direction of action potential transmission. Estimates of medial olivocochlear and lateral olivocochlear cell (MOC and LOC, respectively) distribution patterns in mouse, i.e., 75% contralateral and 99% ipsilateral (A) are from Campbell and Henson (1988).

agonist before noise exposure reduced the extent of threshold shift and the amount of neural swelling (d'Aldin et al. 1995).

To directly assess the contribution of the LOC system to protection, we stereotaxically lesioned LOC neurons (Le Prell et al. 2003) in adult mice and compared noise-induced threshold shifts after binaural noise exposure in the ears ipsilateral and contralateral to the lesion. The integrity of the MOC pathway was verified both morphologically (in the brain stem and cochlea) and functionally (with bilateral measurement of MOC-mediated suppression of cochlear responses). In animals with unilateral LOC lesions, the ear ipsilateral to the lesion showed larger threshold shifts than the contralateral ear, but only when measured by the cochlear neural responses [auditory brain stem response (ABR) Wave 1]; threshold shifts measured by OHC-based responses (otoacoustic emissions) remained bilaterally symmetric. Results are consistent with an LOC-mediated protection of the cochlear nerve dendrites during acoustic overexposure.

METHODS

Stereotaxic surgery

All procedures were approved by the IACUC of the Massachusetts Eye and Ear Infirmary. Experimental animals were CBA/CAJ mice of either sex aged 6–8 wk, weighing between 25 and 30 g. After anesthesia with xylazine [20 mg/kg, administered intraperitoneally (ip)] and ketamine (100 mg/kg, ip), the mouse was held in a Kopf small-animal stereotaxic apparatus by snout clamp and ear bars. The skin overlaying the skull was slit and retracted to reveal the bregma and lambdoidal sutures. Rongeurs were used to make an opening in the skull over the right lambdoidal suture. Using coordinates modified from Franklin and Paxinos (1997), a micropipette filled with a 10-mM solution of the cytotoxin melittin (in saline) was lowered into the brain at a position 0.49 mm caudal and 0.12 mm lateral to the bregma. When a depth of 0.69 mm was reached, 2 μ l of solution was injected by a 10- μ l syringe (Hamilton) coupled to the micropipette. Immediately after injection, the scalp was sutured and the animal was placed in a padded cage with heat lights for about 1 h postsurgery. In all, 18 mice underwent surgery. Animals in which the injection missed the target (see following text) served as surgical controls and eight mice without surgery were included as an additional control.

ABR and DPOAE measurements

Both ABRs and distortion product otoacoustic emissions (DPOAEs) were recorded at 2 and 4 wk postsurgery and again 6 h and 1 wk postacoustic overexposure. For ABRs and DPOAE recordings, mice were anesthetized with ketamine and xylazine (same dose as for surgery) and needle electrodes were inserted at the vertex and pinna. The ABR was evoked with 5-ms tone pips (0.5-ms rise–fall, with a

\cos^2 envelope, at 35/s). The response was amplified (10,000 \times), filtered (0.1–3 kHz), and averaged with a digital input–output board in a PC-based data-acquisition system. Acoustic stimuli were delivered through a closed acoustic coupler housing two electrostatic drivers as sound sources (TDT EC-1, Tucker Davis Technologies) and one electret microphone (Knowles) at the end of a probe tube to measure sound pressure levels (SPLs) in situ. Sound level was raised in 5-dB steps from 0- to 80-dB SPL. At each level, 1,024 responses were averaged (with stimulus polarity alternated) after “artifact rejection.” Threshold was determined by visual inspection. DPOAEs at $2f_1 - f_2$ were recorded in response to primary tones: f_1 and f_2 , with $f_2/f_1 = 1.2$ and f_2 level 10 dB $< f_1$ level. Fast Fourier transforms were computed and averaged over five waveform traces and $2f_1 - f_2$ DPOAE amplitude and surrounding noise floor were extracted. Isoresponse contours were interpolated from plots of amplitude versus sound level, performed in 5-dB steps of primary level. “Threshold” is defined as the primary level (f_2) required to produce a DPOAE at 0-dB SPL.

Acoustic overexposure

Animals were acoustically overexposed at 5 wk postsurgery. The animals were exposed free-field, awake and unrestrained, in a small reverberant chamber. Acoustic trauma consisted of a 15-min exposure to an 8- to 16-kHz-octave band noise presented at 94-dB SPL. Exposure level was measured at four positions inside the cage and varied by < 0.5 dB.

Medial olivocochlear (MOC) assay

MOC assays were performed on six control animals (no surgery and no acoustic exposure) and a randomly chosen subset ($n = 12$) of the experimental animals (≥ 1 wk after the exposure to noise). Animals were anesthetized as for ABR and DPOAE testing and a posterior craniotomy and partial cerebellar aspiration exposed the floor of the IVth ventricle. Shocks (monophasic 150- μ s pulses at 200/s) were applied through silver wires at the midline. Shock threshold for facial twitch was determined, paralysis was induced with α -D-tubocurarine (1.25 mg/kg, ip), and the animal was connected to a respirator. Shock levels were raised 6 dB above twitch threshold. The MOC suppression effects on DPOAEs were then assessed in both cochleas. The f_2 level was set to produce a DPOAE about 10 dB $>$ noise floor. Repeated measures of baseline DPOAE amplitude ($n = 12$) were made before a series of 17 contiguous periods in which DPOAE amplitudes were repeatedly measured during a continuous 70-s shock train to the OC bundle. The shock epoch was followed by a series of DPOAE amplitude measurements ($n = 36$) to observe the extinction of the MOC effect.

Histological preparation

After final testing (about 7 wk postsurgery), animals were perfused intracardially with 10% formalin. The brain stems were extracted,

postfixed overnight, cryoprotected in sucrose, frozen, and cut on a sliding microtome at 80 μm in the transverse plane. Slide-mounted sections were stained for acetylcholinesterase (AChE) activity not only to allow for visualization of the cholinergic OC cells in the brain stem (Osen and Roth 1969), but also to verify that the microelectrode pipette did not sever the OC bundle. Cochleas on both sides were extracted, postfixed overnight, decalcified in EDTA for nearly 48 h, dissected into half-turn segments, and double immunostained for cholinergic and dopaminergic markers: rabbit anti-VAT (vesicular acetylcholine transporter; Sigma catalog No. V5387, at 1:1,000) and sheep anti-TH (tyrosine hydroxylase; Calbiochem catalog No. 657014, at 1:500), respectively. Primaries were followed by a species-appropriate fluorophore-coupled secondary at 1:1,000 (VAT: Alexafluor 568; TH: Alexafluor 488).

Morphometric analysis

BRAIN STEMS. The location, size, and success of the brain stem lesion were quantified by comparing the area of the lateral superior olive (LSO) on both sides of the brain stem. A drawing tube was used to trace the outline of surviving LSO cells in all sections from its caudal to rostral extreme. Tracings were digitized and the areas of both medial and lateral limbs were determined by computerized planimetry.

COCHLEAS. Cochlear location was converted to frequency (Muller et al. 2005) and 10 log-spaced frequency loci were identified in each case. At each locus in each case, an observer blind to the physiology and brain stem analyses separately rated the innervation densities of VAT- and TH-positive terminals in both the inner and outer hair cell areas. A three-point scale was used for VAT-positive terminals in the OHC area: the observer's task was to estimate the fraction of OHCs with at least one VAT-positive terminal: 3 = 100–67%, 2 = 66–34%, and 1 = 33–0%. VAT- and TH-positive terminals in the IHC area were evaluated with a four-point scale: 3 = profuse, 2 = moderate, 1 = sparse, and 0 = none. Each immunostain was separately referenced to its own maximum values: i.e., TH-positive terminals are much rarer than VAT terminals, but maximum density for each would receive a rating "3." To normalize the data, the cochlear spiral dimension was divided into 10 equal-frequency bins; within each bin, ratings from all contralateral ears were averaged for each area (IHC vs. OHC) and for each immunostain (TH vs. VAT). All

values, whether from contra or ipsi ears, were then referenced to the maximum mean value across all frequency bins for that area and for that immunostain. Further details can be found in the caption of Fig. 3.

RESULTS

Histological assessment of completeness and selectivity of the LOC lesion

Brain stems and cochlear whole mounts were evaluated to assess the completeness and selectivity of the lesions, i.e., the extent to which they were successful in eliminating the LOC system and sparing the MOC system and the stapedius muscle reflex.

BRAIN STEMS. When the cytotoxin (melittin) successfully targeted the LSO, there was a clear loss of cholinergic neurons from the LSO area and a clear disruption of the LSO neuropil. The paired micrographs in Fig. 2, *A* and *B* compare surviving portions of the LSO from opposite sides of the brain stem in one injected case (i.e., regions where cholinergic cell bodies remained). Because the LSO is tonotopically organized (Kelly et al. 1998), we separately assessed the areas of the lateral (low-frequency) and medial (high-frequency) limbs. The fractional survival of medial versus lateral limbs (Fig. 3*A*) suggests a clear distinction between LSO *Hit* and LSO *Miss* cases. The data also show that in a subset of cases ($n = 3$), there was greater success in destroying the medial limb than the lateral limb (Fig. 3*A*, arrowheads).

When estimating the extent of lesion success on the LOC cell bodies, it is also important to assess, in both the *Hit* and *Miss* cases, which other structures were damaged. In particular the extent of MOC involvement is key to interpreting the results. As schematized in Fig. 4, the cholinergic MOC cells form an elongated cluster, the caudal end of which is ventromedial to the LSO, and the rostral end that extends well beyond the rostral tip of the LSO (Brown 1993; Campbell and Henson 1988). In each LSO *Hit* case (Fig. 4*A*, $n = 10$), the injection destroyed part of the LSO; in only one case (arrowhead in

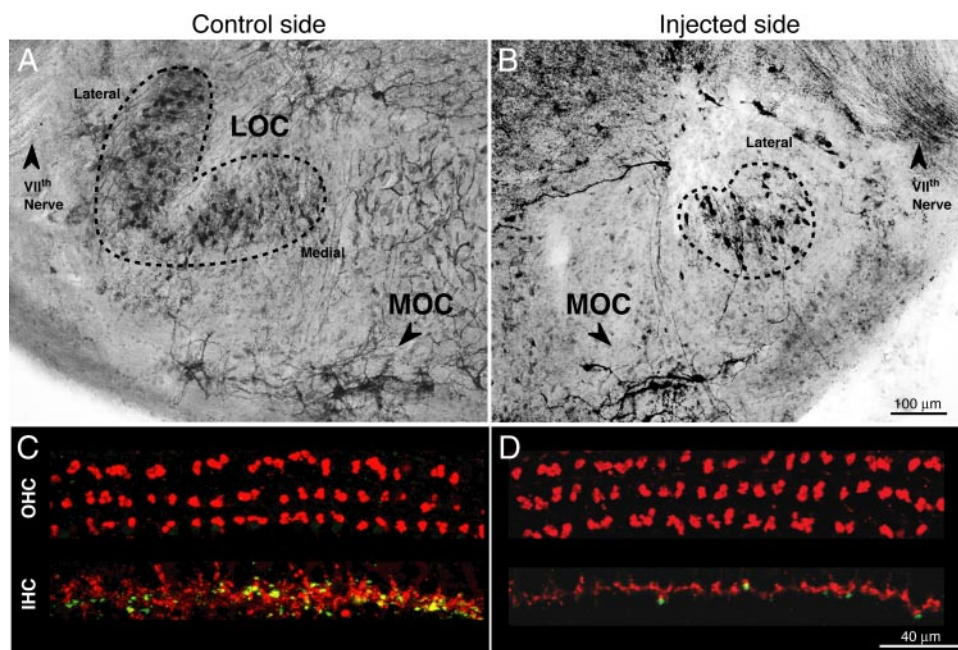


FIG. 2. Histological verification of LOC lesions, as seen in acetylcholinesterase (AChE)-stained brain stem sections (*A*, *B*) or in cochlear whole mounts double-immunostained for a cholinergic marker (red) and a dopaminergic marker (green) (*C*, *D*). *A* and *B*: brain stems are from opposite sides of one LOC *Hit* case. Dashed lines indicate the outline of the surviving lateral superior olive (LSO) in each section: on the control side, lateral and medial limbs are indicated. AChE-positive fibers of the VIIth nerve, visible in each section, were used to identify comparable rostrocaudal locations on the two sides. Scale in *B* also applies to *A*. *C* and *D*: images are from the 22.6-kHz region of opposite sides of a LOC *Hit* case (different from the one shown in *A* and *B*). Scale in *D* also applies to *C*.

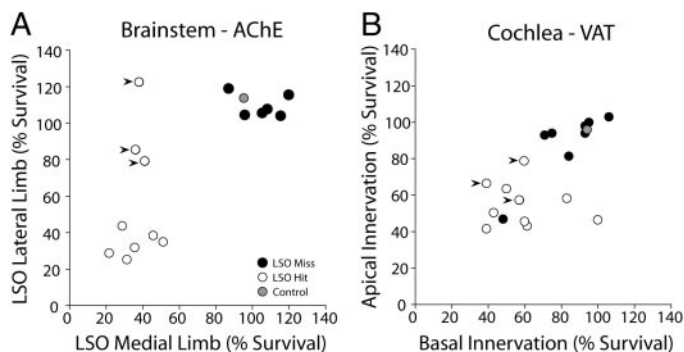


FIG. 3. Analysis of lesion success based on (A) the fractional survival of the LSO, as seen in AChE-stained brain stem sections, and (B) the fractional survival of cholinergic terminals in the inner hair cell (IHC) area, as seen in immunostained cochlear whole mounts. Filled arrowheads in B are the 3 cases in A (filled arrowheads) where the lesion affected the medial limb only. A: fractional survival is the total surviving area (as depicted in Fig. 2, A and B) of each LSO limb from its rostral to caudal extent (the LSO spans about 480 μm in the rostrocaudal plane), normalized to the mean area of control sides. B: fractional survival of cholinergic [vesicular acetylcholine transporter (VAT)-positive] terminals in the IHC area is calculated by dividing the cochlear spiral into 2 bins (apical vs. basal to the midpoint), and averaging the semiquantitative estimates of fractional survival in each bin for each case. See METHODS for further details.

Sections 11 and 13) did the lesion appear to significantly impinge on a portion of the MOC cell cluster, near its rostral extent. In half of the LSO *Miss* cases ($n = 4$), there was no sign of injection, suggesting that the pipet tip may have clogged. In the remaining LSO *Miss* cases ($n = 4$), the lesion was always rostral to the LSO (Fig. 4B). In one LSO *Miss* case, the lesion impinged on MOC cell bodies, rostral to the LSO (Fig. 4B, arrowheads in Sections 11 and 13).

In addition to the MOC system, the innervation of the stapedius muscle also needs to be assessed, given that the stapedius reflex was also previously implicated in cochlear protection (Henderson et al. 1994; Ryan et al. 1994). Retrograde labeling of stapedius motoneurons in rat (Shibayama et al. 1990) shows a majority population of neurons in the ipsilateral brain stem ventromedial to the facial nucleus and a minority population (roughly 6%) scattered more rostrally near the facial nerve exit from the brain stem. Assuming that stapedius motoneurons are similarly located in mouse, the lesion in some hit cases may have included some stapedius motoneurons near the facial nerve exit (Fig. 4A, Sections 5 and 7).

COCHLEAS. Consistent with cochlear projections and laterality of LOC and MOC systems (Fig. 1A), successful and selective LSO *Hit* cases showed: 1) reduction of OC terminals in the IHC area, and not the OHC area, of the ipsilateral ear; and 2) no change in OC terminal density of the IHC and OHC areas in the contralateral ear (see Fig. 2, C and D). Because the LOC innervation of the IHC area in mice is composed of both cholinergic and dopaminergic terminals (Darrow et al. 2006a), each cochlea was double stained with VAT and TH, respectively. Innervation densities in the IHC and OHC areas were semiquantitatively evaluated by an observer blind to both brain stem histology and cochlear physiology. As shown in Fig. 5, in cases classified as LSO *Hit* based on the brain stem histology (Fig. 3A), there was, on average, roughly 50% reduction in the density of both cholinergic and dopaminergic terminals in the ipsilateral IHC area (Fig. 5, A and B, respectively), without

obvious reductions in the OHC area (Fig. 5C). There was no significant change of ipsilateral innervation of IHC or OHC areas in LSO *Miss* cases; nor was there any loss of IHC innervation contralaterally in either surgical group (Fig. 5, A–C). In the contralateral OHC area, a modest, but clear-cut loss of efferent innervation was noted in two cases (Fig. 5D).

Correlating cochleas and brain stems

Considered individually, each case classified as LSO *Hit* based on the brain stem sections (Fig. 3A) showed a reduction of OC terminals in its ipsilateral cochlea (Fig. 3B); these nine cases are unambiguously classified as “LOC *Hits*.” In one injected case, the ipsilateral cochlea showed obvious loss of OC terminals, although the cholinergic cells in the LSO appeared intact. It is possible that this exceptional case arose because the pipette or the toxin destroyed OC axons without affecting the cell bodies. Nevertheless, this case is considered ambiguous and was removed from further consideration. There was no obvious correlation between the apical–basal gradient of cochlear deafferentation and the destruction of medial versus lateral limb of the LSO: in the three brain stems with a “medial limb only” lesion (Fig. 3A, arrowheads), there was a decrease in LOC terminals in both the apex and base of the cochlea (arrowheads in Fig. 4B indicate the same three cases). This discrepancy may also reflect the fact that some of the LOC cell bodies survived, although their peripheral projections, or other interneurons in the local neural circuitry driving them, had degenerated.

On a case-by case-basis, there was a good correlation between the brain stem and cochlear data with respect to MOC involvement. As shown in Fig. 5D, the cochlear analysis identified only two cases as having a decrease in efferent terminals in the OHC area of the contralateral ear. Correspondingly, these two cases—one LOC *Hit* and one LOC *Miss*—are the only cases for which the brain stem analysis suggested that the lesion had impinged on MOC cell bodies (Fig. 4). The contralateral effect of the loss is expected based on the largely contralateral projection of the MOC system (Fig. 1A).

Functional integrity of the MOC system

In addition to assessing the morphological integrity of the MOC system, we assessed MOC function by electrically stimulating the OC bundle at the midline and while recording suppression of DPOAEs in both ears. DPOAEs are distortions produced and amplified by normal healthy OHCs, which are propagated as mechanical vibrations back through the middle ear to the ear canal, where they can be measured in the sound-pressure waveform. Because activation of the MOC feedback system essentially turns down the gain of the OHC electromechanical amplifier (Cooper and Guinan 2003; Murugasu and Russell 1996), the suppression of DPOAEs provides a rapid and reliable assay of MOC activation level. The maximum effect of MOC stimulation on DPOAE amplitude is for primary tones near 22.6 kHz (Maison et al. 2002, 2003); thus we show data for only 22.6 kHz (Fig. 6), although results at other test frequencies were similar.

The bilateral symmetry of MOC effect size did not differ significantly in control versus injected cases (Fig. 6), suggesting that: 1) MOC function is relatively symmetric in animals with normal OC innervation and 2) our lesions did

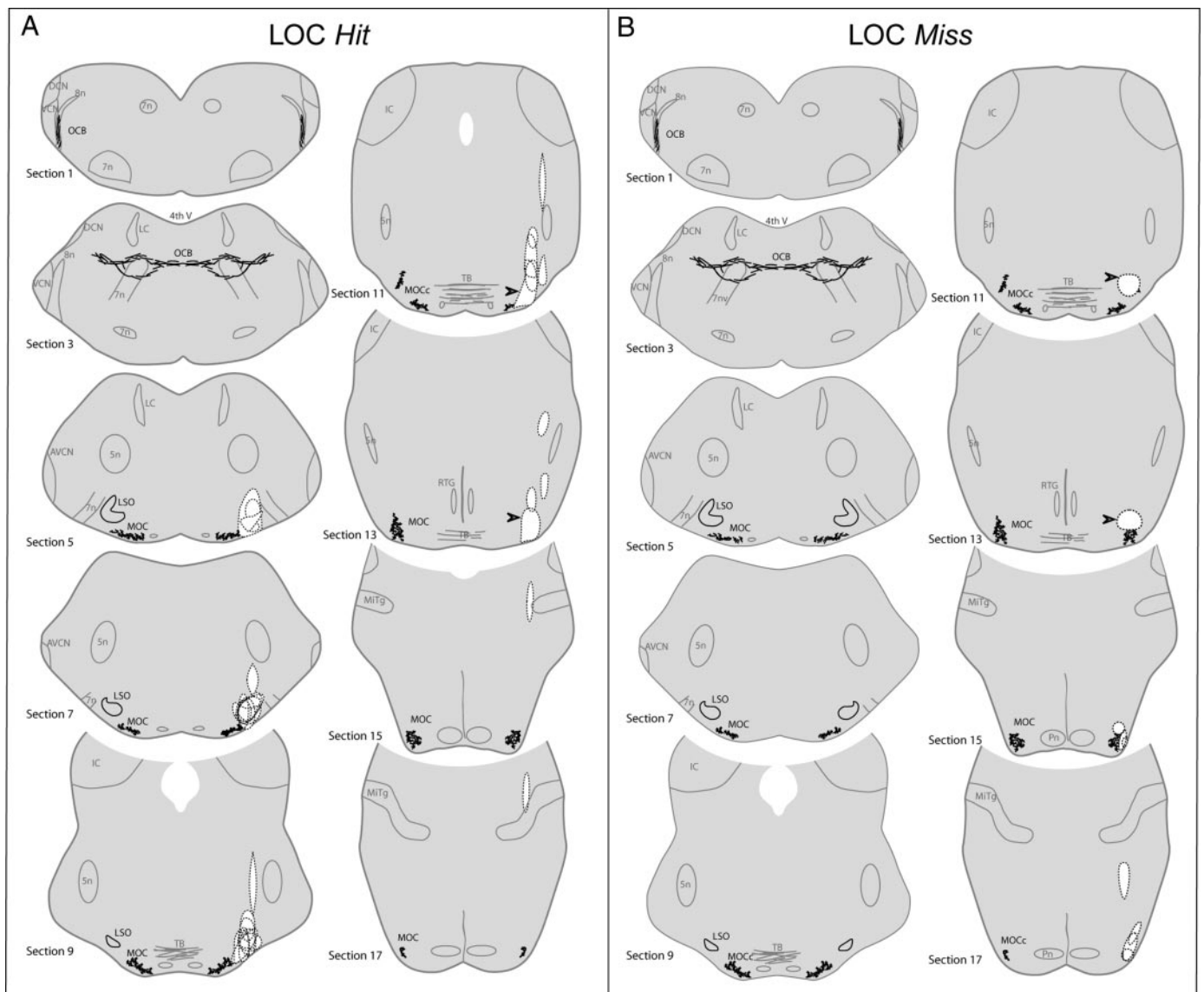


FIG. 4. Lesion locations in all *LOC Hit* cases (A) and *LOC Miss* cases (B). In each case, the lesion is outlined (dashed lines, white centers) and superimposed on “atlas” sections (derived from an AChE-stained control mouse). Alternate 80- μ m sections are shown: *Section 1* is the most caudal and *Section 17* the most rostral to include the OC system. One case from each group with partial damage to the MOC system is indicated by arrowheads in *Sections 11* and *13*. Abbreviations: 4th V, 4th ventricle; 5n, trigeminal nerve or nucleus; 7n, facial nerve or nucleus; 8n, cochlear nerve; AVCN, anteroventral cochlear nucleus; DCN, dorsal cochlear nucleus; IC, inferior colliculus; LC, locus coeruleus; LSO, lateral superior olive; MiTg, microcellular tegmental nucleus; MOC, medial olivocochlear cells; OCB, olivocochlear bundle; Pn, pontine nuclei; RTG, reticulotegmental nucleus; TB, trapezoid body.

not significantly perturb MOC function. Interestingly, the two injected cases for which there was histological evidence of MOC involvement (Figs. 4 and 5D) showed MOC effects roughly half as large in the contralateral versus the ipsilateral ear (Fig. 6B, arrowheads). The paired reduction of contralateral MOC effects and contralateral OHC terminals agrees with the known projection patterns of the MOC system (Fig. 1).

Effect of LOC lesion on cochlear threshold and suprathreshold responses

Cochlear thresholds, as measured by either ABRs or DPOAEs, were unaffected by the LOC lesions: mean values were not significantly different between ears or lesion-success groups (Fig. 7, A–D) or compared with nonsurgical control

mice (data not shown). However, significant binaural asymmetries of suprathreshold neural responses (ABRs; Fig. 8, A and B) were observed in *LOC Hit* cases, without corresponding changes in DPOAEs (Fig. 8, C and D). The ABR represents the summed activity of auditory neurons along the ascending afferent pathway and the earliest wave, Wave 1, represents the activity of the cochlear nerve. The DPOAEs reflect events “upstream” of the ABR, in the sense that the contribution of OHCs to cochlear amplification is a necessary but not sufficient component of a normal ABR response. The latter also relies on the integrity of synaptic transmission between the IHCs and the cochlear nerve and of the cochlear nerve fibers themselves. Thus selective changes in ABR amplitudes without accompanying shifts in DPOAE responses are consistent with expected LOC-based effects on neural activity only.

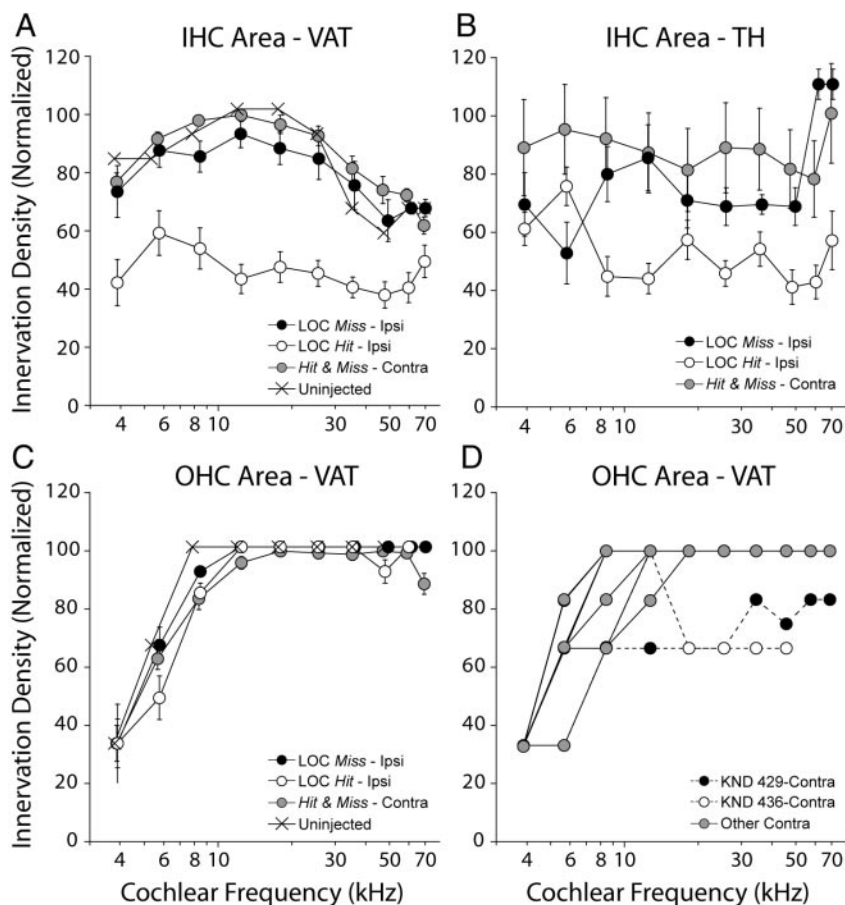


FIG. 5. Semiquantitative analysis of cholinergic (VAT: *A, C, D*) and dopaminergic [tyrosine hydroxylase (TH): *B*] markers in the IHC (*A, B*) and outer hair cell (OHC; *C, D*) areas. In each cochlea, innervation density was estimated at 10 locations along the cochlear spiral (apical and basal halves of each of 5 dissected pieces). In *A–C*, the mean (\pm SE) innervation density in ipsilateral ears from LOC *Hit* (open circles; $n = 10$) and LOC *Miss* (dark-filled circles; $n = 8$) are compared with all contralateral ears from LOC *Hit* (open circles; $n = 10$) and LOC *Miss* (dark-filled circles; $n = 8$) are compared with all contralateral ears from LOC *Hit* (open circles; $n = 10$) and LOC *Miss* (dark-filled circles; $n = 8$) are compared with all contralateral ears (gray-filled circles; $n = 19$) and uninjected controls ($n = 2$). Key in *A* also applies to *B* and *C*. *D*: individual contralateral ears from *C* are shown to indicate the 2 cases (dashed lines) with decreased terminal density in the OHC area. See METHODS for description of analysis procedures.

In LOC *Hit* cases, mean ABR amplitudes were enhanced in the ipsilateral ear (Fig. 8A): the difference between the two ears was statistically significant in the *Hit* cases (e.g., at 22.6 kHz: $P = 0.019$, $F = 6.007$, by two-way ANOVA) and were not in the *Miss* cases (e.g., at 22.6 kHz: $P = 0.387$, $F = 0.762$, by

two-way ANOVA).¹ This enhancement of ABR amplitude was roughly a constant percentage as the tone level increased (data not shown). Thus to summarize changes across frequency, the mean interaural amplitude difference (expressed as a percentage) across the higher sound levels (50- to 80-dB SPL) was computed for each frequency, for each case, and then averaged across cases within each group. When viewed in this way, ABR enhancements are seen across all test frequencies (Fig. 8B) and not in DPOAEs (Fig. 8D). In those cases where the brain stem lesion appeared to spare the lateral (low-frequency) limb (Fig. 3A, arrowheads) there was no obvious difference in ABR enhancements between low- and high-frequency regions, thus mirroring the lack of a base–apex gradient in the cochlear efferent innervation in these same cases.

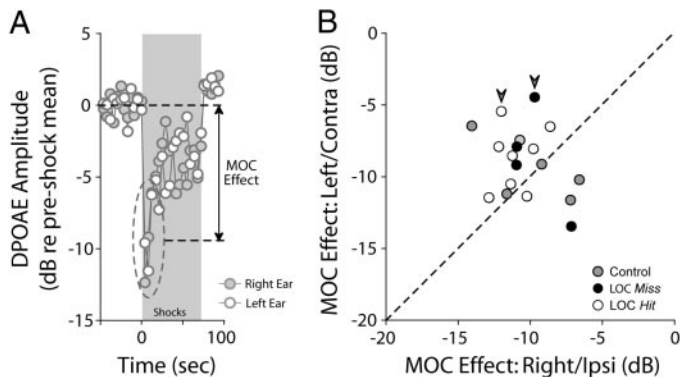


FIG. 6. Bilateral suppression of distortion product otoacoustic emissions (DPOAEs), elicited by midline electrical stimulation of MOC fibers, suggests that MOC function is minimally affected by the lesions. *A*: one run of the MOC assay in a control case demonstrates symmetrical DPOAE suppression in right and left ears. “MOC effect” is defined as the difference in decibels (dB) between mean DPOAE amplitude in the first 3 measures after shock onset, compared with the pre-shock baseline. *B*: MOC effects in right and left ears of control, LOC *Hit* and LOC *Miss* cases. Large arrowheads indicate the cases with histological evidence of MOC lesions (Figs. 3, 4, and 5D). For data shown, f_2 was 22.6 kHz.

Effect of LOC lesion on vulnerability to acoustic injury

Dendritic vacuolization in the IHC area—the morphological sign of glutamate excitotoxicity—is seen only in the acute stages (<24 h) of acoustic injury (Liberian and Mulroy 1982; Puel et al. 1998; Robertson 1983); thus we designed an exposure stimulus (94 dB at 8–16 kHz for 15 min) to create a

¹ In a larger sample of cases, summarized in a study of the LOC role in balancing bilateral neural excitability (Darrow et al. 2006b), a complementary reduction was seen in the ABR amplitudes in the contralateral ears of LOC *Hit* cases. We hypothesize that the lack of contralateral effects in the smaller subset of animals used in these acoustic injury experiments reflects (chance) differences in the degree to which the crossing ascending projections to the contralateral LSO were interrupted by the lesions.

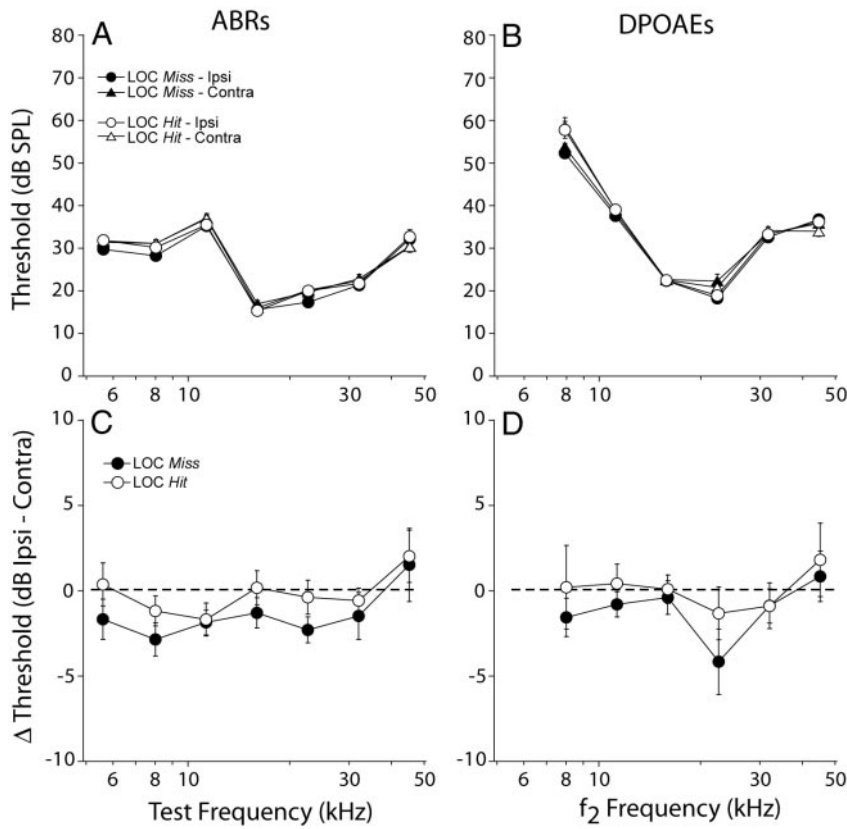


FIG. 7. Mean cochlear thresholds (\pm SE), as measured by auditory brain stem response (ABR, *A*) and DPOAE (*B*) were not affected by a successful LOC lesion, nor were interaural threshold differences as measured by either ABR (*C*) or DPOAE (*D*). Keys in *A* and *C* also apply to *B* and *D*, respectively.

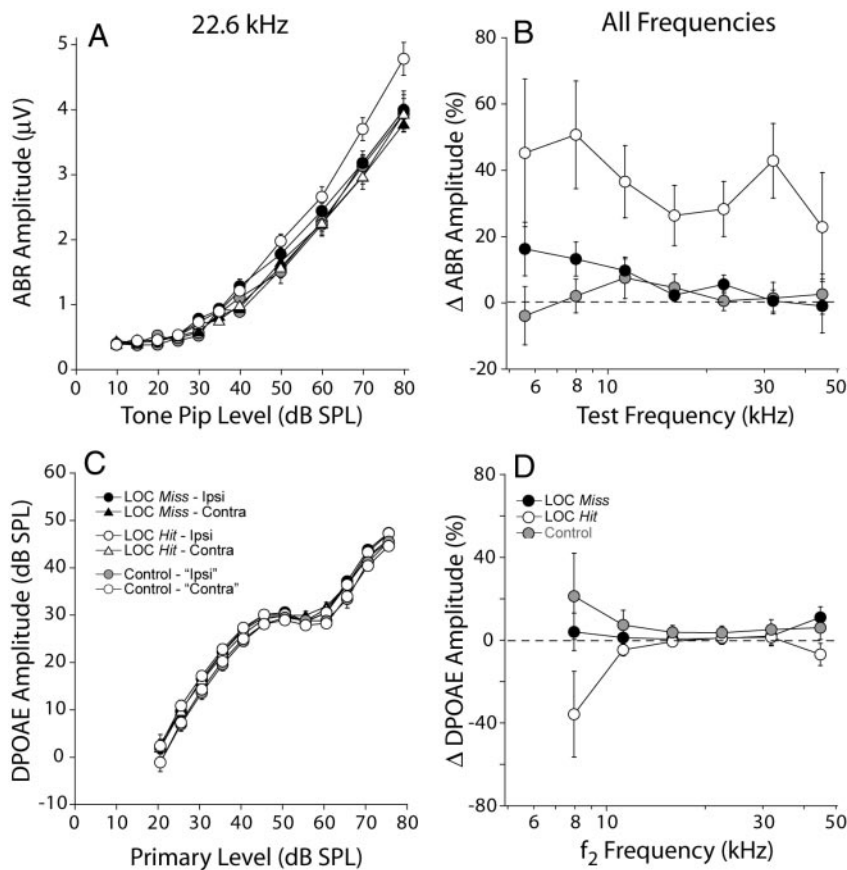


FIG. 8. ABR amplitudes (*A*, *B*) are enhanced in the ipsilateral LOC *Hit* ears and not in LOC *Miss* ears, whereas DPOAE amplitudes (*C*, *D*) are unaffected in all groups. *A* and *C*: mean (\pm SE) amplitude vs. level functions for ABR and DPOAE, respectively, for responses at 22.6 kHz; key in *C* applies to *A*. *B* and *D*: mean interaural discrepancies in ABR and DPOAE amplitudes, respectively. Key in *D* applies to *B*. See text for further details.

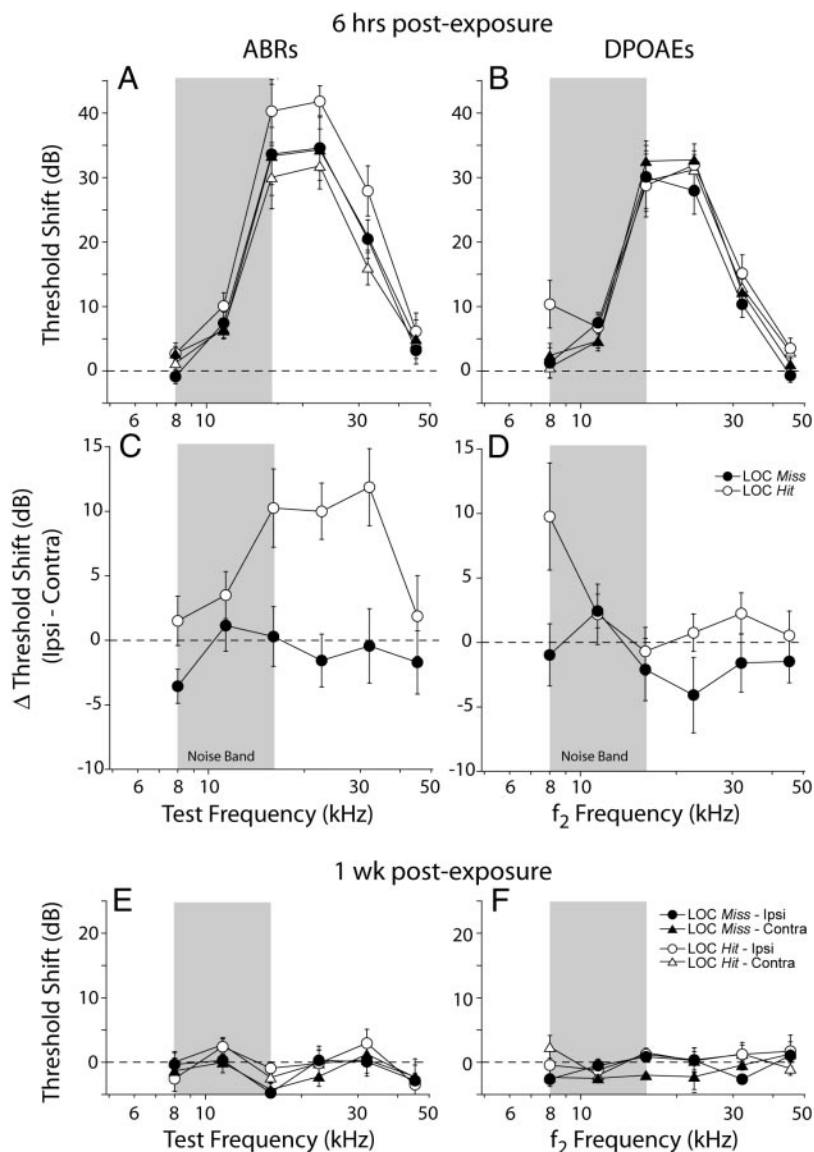


FIG. 9. Mean ABR threshold shifts in LOC *Hit* mice, 6 h after acoustic overexposure, were 10–15 dB higher in the ipsilateral ear; this asymmetry was not present in LOC *Miss* cases (A, C) or in the mean DPOAE data from either *Hit* or *Miss* groups (B, D). A and B: threshold shift is defined as the difference from mean preexposure values for the same group. C and D: interaural threshold-shift difference defined as thresholds in ipsilateral minus contralateral ears of each case. At 1 wk postexposure, mean ABR (E) and DPOAE (F) threshold shifts returned to preexposure levels. Key in F also applies to A, B, and E. Key in D also applies to C. Error bars in all panels indicate \pm SEs. Gray box indicates noise exposure bandwidth.

moderate (about 35 dB) threshold shift when tested 6 h post-exposure (Fig. 9, A and B), and to recover when tested at 1 wk postexposure (Fig. 9, E and F).

After exposure, threshold shifts in the LOC *Miss* cases were bilaterally symmetrical at 6 h postexposure, whether measured by ABRs (Fig. 9, A and C) or DPOAEs (Fig. 9, B and D). Furthermore, threshold shifts in the LOC *Miss* cases were of similar magnitude when measured by ABRs (Fig. 9A) or DPOAEs (Fig. 9B), suggesting that the functionally important changes are occurring at, or “upstream” of, the OHCs.

In contrast, in the LOC *Hit* cases, the ABR threshold shifts were significantly larger in the ipsilateral ear compared with either the contralateral ear or either of the LOC *Miss* ears (Fig. 9, A and C). Differences between the two ears of LOC *Hit* mice were highly significant ($P = 0.001$, $F = 17.385$, by two-way ANOVA). Although it appears that the mean threshold shifts of the contralateral ears in the LOC *Hit* group were slightly lower than those from the LOC *Miss* cases, the differences were not statistically significant ($P < 0.525$, $F = 0.426$, by two-way ANOVA). Importantly, the interaural asymmetries

in ABR threshold shift were not mirrored in the DPOAE data (Fig. 9, B and D), indicating the additional vulnerability arising from the loss of the LOC system involves additional damage to IHCs or neural elements, not to OHC function. The interaural symmetry of the DPOAE-based threshold shifts and the lack of differences between *Hit* and *Miss* cases also argue strongly against the possibility that differences in stapedius reflex strength underlie the differences in vulnerability.

DISCUSSION

Peripheral effects of the LOC system in modulating neural excitability

Peripheral effects of the LOC system were previously studied by electrical stimulation, pharmacological or genetic manipulation of transmitter/receptor combinations, and surgical lesion (see following text). In these studies, LOC effects are characterized by level-independent and frequency-independent changes in cochlear neural-evoked potentials without corre-

sponding alterations in OHC-based responses such as cochlear microphonics or DPOAEs (e.g., Groff and Liberman 2003; Le Prell et al. 2003). A similar constellation of effects is reported here after selective LOC lesion (e.g., Fig. 8).

The complex cytochemistry and pharmacology of the LOC system make it difficult to predict the effects of its removal. Activating the LOC pathway by electrical stimulation of the inferior colliculus suggests there are two functional subsystems capable of eliciting slow suppressive or excitatory effects on cochlear nerve activity (Groff and Liberman 2003). Correspondingly, the LOC system is cytochemically heterogeneous: co-localization studies suggest that a majority population of cholinergic cells also contain γ -aminobutyric acid (GABA), whereas a minority population is dopaminergic (Darrow et al. 2006a). There is also immunohistochemical evidence for peptide transmitters [urocortin, calcitonin gene-related peptide (CGRP), and opioids] in LOC terminals: although evidence is incomplete, these transmitters may also co-localize in the cholinergic terminals (for review, see Eybalin 1993). Pharmacological studies with agonists and antagonists of putative LOC neurotransmitters suggested both excitatory and inhibitory effects on cochlear nerve activity. Cochlear perfusion of certain opioid agonists enhances cochlear nerve gross potentials (Sahley et al. 1991). Acetylcholine (ACh) perfusion increased both spontaneous and glutamate-induced spiking (Felix and Ehrenberger 1992). In contrast, GABA did not affect spontaneous activity, but decreased glutamate-induced and ACh-induced activity (Arnold et al. 1998; Felix and Ehrenberger 1992), whereas dopamine decreased both spontaneous and sound-driven activity (d'Aldin et al. 1995; Oestreicher 1997; Ruel et al. 2001). CGRP application increased spontaneous rates and decreased mechanically driven rates of primary afferents in the lateral line (Bailey and Sewell 2000). Conversely, targeted gene deletion of CGRP in mice decreased suprathreshold neural responses, without corresponding changes in DPOAE amplitudes (Maison et al. 2003).

Previous lesion studies have taken four approaches to studying LOC function: 1) cut the entire OC bundle, thereby interrupting both MOC and LOC fibers (e.g., Liberman 1990; Zheng et al. 1999); 2) cut only the crossed OC bundle, thereby interrupting two thirds of the MOC while sparing the LOC (e.g., Kujawa and Liberman 1997); 3) stereotaxically lesion the LSO/LOC system (Le Prell et al. 2003); or 4) perfuse the cochlea with agents designed to selectively destroy one class of LOC neurons (LePrell et al. 2005). Data from the first two approaches suggest that the LOC can modulate spontaneous and sound-evoked discharge rates in cochlear nerve fibers without appreciable effects on threshold or tuning (Liberman 1990; Walsh et al. 1998). In chinchillas, complete deafferentation (approach 1) increased sound-driven discharge rates (Zheng et al. 1999), suggesting that resting tone in the LOC pathway tends to suppress sound-evoked responses, consistent with the postlesion enhancement of ABR amplitudes seen here (Fig. 8). In contrast, in guinea pig, LOC lesion (approach 3) decreased the amplitudes of cochlear neural response (Le Prell et al. 2003), suggesting that resting LOC tone tends to enhance auditory nerve responses. Destruction of dopaminergic neurons (approach 4) also led to decreases in neural responses (Le Prell et al. 2005), which is contrary to expectations, given that dopamine is inhibitory (Ruel et al. 2001). The authors suggested that other co-localized transmitters were destroyed,

However, it is possible that the neurotoxin also killed many nondopaminergic neurons because the authors report a large-scale loss of efferents in the IHC area; yet other reports confirm that in guinea pig, as in mouse, the dopaminergic neurons constitute a small subset of the LOC efferent population (Darrow et al. 2006; Mulders and Robertson 2004).

The fact that LOC destruction in some experiments can increase, and in others decrease, neural excitability may simply reflect the existence of multiple LOC subgroups with both excitatory and inhibitory effects on cochlear nerve response. If the balance between the resting activation levels, or relative sizes or degrees of co-localization, of different cytochemical subgroups is different in different species, or is differentially affected by different anesthetics, one might expect such qualitatively different effects of LOC destruction, especially when the destruction is subtotal, as in all the relevant experiments. Correspondingly, the possibility must be considered that there are species differences in the protective effects we have now documented in mouse.

Acoustic injury and olivocochlear feedback

OHC dysfunction plays a major role in the genesis of both temporary threshold shifts (TTSs) and permanent threshold shifts (PTSs): e.g., slow OHC depolarizations are well correlated with TTS magnitude (Cody and Russell 1985) and the loss of OHCs and/or damage to their stereocilia are well correlated with PTS magnitude (Liberman and Dodds 1984). A long-standing theory of MOC function is that it protects the cochlea from both TTS and PTS by its actions on OHCs (Handrock and Zeisberg 1982; Kujawa and Liberman 1997; Maison and Liberman 2000; Maison et al. 2002; Rajan 1988; Reiter and Liberman 1995). The idea has been supported by four types of experimental findings: 1) the degree of TTS is decreased when the OC bundle is electrically stimulated simultaneously with the acoustic overexposure (Rajan 1988); 2) in animals with chronic OC bundle section, including both MOC and LOC components, deafferented ears are more vulnerable to both TTS and PTS (Handrock and Zeisberg 1982; Kujawa and Liberman 1997); 3) the strength of the MOC reflex (measured as an acoustically driven suppression of DPOAE amplitude) is strongly correlated with vulnerability (Maison and Liberman 2000); and, most definitively, 4) transgenic mice with overexpression of the ACh receptor in OHCs show enhanced MOC effects coupled with enhanced resistance to both TTS and PTS (Maison et al. 2002).

Although MOC-mediated cholinergic effects on OHCs can clearly reduce acoustic vulnerability, there are hints in previous lesion studies that some of the increased vulnerability seen after complete deafferentation is attributable to loss of the LOC system. For example, when acoustic vulnerability was assessed in totally deafferented guinea pigs, neurally derived threshold shifts were 10–15 dB higher than OHC-derived shifts (a disparity not present in normal noise-exposed animals), and vulnerability was not significantly affected by the midline lesion, which interrupts two thirds of the MOC while sparing the LOC (Kujawa and Liberman 1997). However, this and other prior evidences linking the LOC system to protection from acoustic injury are indirect.

Neuronal damage appears to play an important role in the genesis of noise-induced TTSs (Liberman and Mulroy 1982; Puel et al. 1998; Robertson 1983) and the targeting of cochlear

neurons by the LOC system makes LOC-mediated protection from acoustic injury an attractive hypothesis. A common cochlear pathology seen in the first 24 h after acoustic overexposure is swelling of cochlear nerve dendrites in the IHC area (Liberman and Mulroy 1982; Puel et al. 1998; Robertson 1983). This type of dendritic swelling can also be observed in cochleas perfused with glutamate agonists, but without noise exposure (Puel et al. 1994). Furthermore, when noise is presented with simultaneous intracochlear perfusion of a glutamate antagonist, there is less threshold shift (after washout of the glutamate antagonist) and fewer vacuoles (Puel et al. 1998). These observations suggest that dendritic swelling is a type of excitotoxicity brought on by excessive release of glutamate from the IHC. The LOC's dopaminergic component is hypothesized to mediate this protective effect: 1) dendritic vacuolizations in the IHC area have been observed in cochleas perfused with a dopamine antagonist (without exposure to intense noise; Ruel et al. 2001) and 2) cochlear perfusion of dopamine agonists before noise exposure reduced the amount of noise-induced threshold shift and ischemia-induced neuronal swelling (d'Aldin et al. 1995). Because the LOC system targets both IHCs and auditory nerve dendrites (Liberman et al. 1990), this LOC-mediated protection could be mediated presynaptically (e.g., by reducing glutamate release from the IHC) or it could result from postsynaptic modulation of the Ca^{2+} entry known to mediate excitotoxicity in other systems.

Previous work in our laboratory documented the presence of IHC area vacuoles in mice with 40 dB of TTS, i.e., similar in magnitude to that produced in this study (Wang et al. 2002). The dramatic nature of dendritic swelling suggests that it should be accompanied by a loss of synaptic transmission between the IHC and cochlear nerve: electron microscopic images show loss of intracellular components from, and membrane rupture of, the postsynaptic afferent terminal (Le Prell et al. 2004; Puel et al. 1998; Robertson 1983; Ruel et al. 2001). It follows that loss of synaptic transmission should contribute to the acute threshold shift and that TTS should be larger in neural measures (e.g., ABRs) than in OHC-based measures (e.g., DPOAEs). In the present study, LOC lesions resulted in enhanced ABR threshold shifts with respect to DPOAE threshold shifts, consistent with a significant component of "additional" TTS attributable to a dysfunction at the level of the inner hair cell or cochlear nerve. In contrast, mice with normal LOC innervation showed almost identical degrees of ABR and DPOAE shift (Fig. 9), suggesting that, with an intact LOC system, all the functionally important changes underlying the TTS were present at the level of OHC-dominated active cochlear mechanics.

If dendritic vacuolization is a functionally relevant contributor to TTS, why do ears with an intact LOC system show comparable ABR and DPOAE shifts (Kujawa and Liberman 2006; Maison et al. 2003; Mills 2003; Wang et al. 2002)? In comparing ABR- and DPOAE-based threshold shifts it is important to consider the relative insensitivity of neural-based sound-evoked gross potentials such as ABRs to a distributed loss of neural elements. In adult chinchillas with selective IHC loss, which is functionally similar to primary neuronal degeneration with respect to ABR generation, a distributed loss of 50% of the responding neurons in a particular cochlear region resulted in a neural-based gross potential shift of <6 dB (Liberman et al. 1997). This can be understood by considering that a sound level increase of 6 dB can double the discharge

rate of individual cochlear neurons near threshold (e.g., Winter et al. 1990) and thereby compensate for the loss of half the responsive neural elements. This line of argument resolves the apparent paradox that significant numbers of dysfunctional-looking cochlear nerve dendrites can be observed in normal animals with TTS, without a significant degree of "additional" threshold shift in ABRs with respect to DPOAEs.

The insensitivity of neural-based metrics to distributed IHC loss, and subsequently to neural degeneration, also implies that the 10–15 dB of "additional" ABR shift observed in LOC-lesioned ears (Fig. 9) must correspond to a very large number of dysfunctional auditory nerve fibers. Furthermore, considering that the LOC lesions in this report were incomplete, an average of roughly 50% destruction from base to apex (Fig. 5), the data also suggest a very strong antiexcitotoxic effect of an intact LOC system. Given the existing pharmacological data implicating dopaminergic transmission in blocking dendritic swelling (Ruel et al. 2001), and given the recent report that the mouse LOC system consists of two functional subsystems (one cholinergic and one dopaminergic; Darrow et al. 2006a), it is tempting to speculate that the loss of the dopaminergic component is responsible for the antiexcitotoxic effects described here. On the other hand, there is also a growing literature, in other systems, on the neuroprotective and antiexcitotoxic effects of cholinergic transmission through nicotinic ACh receptors (Dajas-Bailador and Wonnacott 2004), both by postsynaptic effects (eliciting changes in expression of Ca^{2+} -buffering proteins) and by presynaptic receptors (reducing glutamate release). RT-PCR studies suggest expression of a variety of nicotinic ACh receptors in spiral ganglion cells (Bao et al. 2005), the major LOC target.

It was previously suggested that OC-mediated protection is an epiphenomenon rather than a functional role that evolved because it confers selective advantage, given that the traumatic acoustic exposures used in "protection" experiments are well above levels that ever existed in the preindustrial age (Kirk and Smith 2003). The present study uses sound pressures that are significantly lower than those used in previous studies of MOC protection (94- vs. ≥ 105 -dB SPL), thus making it more plausible that antiexcitotoxicity is one of the functional roles of the LOC system, rather than an epiphenomenon. Furthermore, the incompleteness of the present lesions and the magnitude of the protective effect they reveal make it likely that profoundly protective effects may be present for exposures at even lower sound pressures.

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