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Olivocochlear Innervation in the Mouse: Immunocytochemical Maps, Crossed Versus Uncrossed Contributions, and Transmitter Colocalization

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Abstract

To further understand the roles and origins of γ-aminobutyric acid (GABA) and calcitonin gene-related peptide (CGRP) in the efferent innervation of the cochlea, we first produced in the mouse an immunocytochemical map of the efferent terminals that contain acetylcho-line (ACh), CGRP, and GABA. Olivocochlear (OC) terminals in inner and outer hair cell (IHC and OHC) regions were analyzed quantitatively along the cochlear spiral via light-microscopic observation of cochlear wholemounts immunostained with antibodies to glutamic acid decarboxylase (GAD), vesicular acetylcholine transporter (VAT), or the peptide CGRP. Further immunochemical characterization was performed in mice with chronic OC transection at the floor of the fourth ventricle to distinguish crossed from uncrossed contributions and, indirectly, the contributions of lateral versus medial components of the OC system. The results in mouse showed that (1) there are prominent GABAergic, cholinergic, and CGRPergic innervations in the OHC and IHC regions, (2) GABA and CGRP are extensively colocalized with ACh in all OC terminals in the IHC and OHC areas, (3) the longitudinal gradient of OC innervation peaks roughly at the 10-kHz region in the OHC area and is more uniform along the cochlear spiral in the IHC area, (4) in contrast to other mammalian species there is no radial gradient of OC innervation of the OHCs, and (5) all OHC efferent terminals arise from the medial OC system and terminals in the IHC area arise from the lateral OC system.

Keywords

cochlea; efferents; hair cells; acetylcholine; γ-aminobutyric acid; calcitonin gene-related peptide

Olivocochlear (OC) efferent neurons to the cochlea are divided into two main subgroups (for review, see Warr, 1992) according to locations of their cell bodies in the brainstem. A lateral olivocochlear (LOC) system originates from neurons in or around the lateral superior olive and projects predominately to the ipsilateral cochlea via unmyelinated fibers. A medial olivocochlear (MOC) system originates from cells ventral and medial to the medial superior olivary nucleus and projects mostly contralaterally via myelinated fibers (Liberman and Brown, 1986).

The peripheral targets of the LOC and MOC systems are fundamentally complementary: LOC fibers synapse mainly with dendrites of afferent fibers beneath inner hair cells (IHCs), whereas MOC fibers synapse mainly with the outer hair cells (OHCs). Although this summary view is

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undoubtedly correct in major outline, the possibility that the LOC system also projects to the OHCs has remained an open and controversial question. Indeed, the original anterograde tracer experiments suggested minor projections of LOC neurons to the OHC regions and of MOC neurons to the IHC region (Guinan et al., 1983).

Definitive evidence for the projection patterns of MOC neurons came from intracellular labeling experiments in which single neurons were traced from the brainstem to terminals in the organ of Corti. Such intracellularly labeled fibers were always myelinated, always originated from the MOC cell group, always projected to OHCs, and never sent branches to the IHC area (Liberman and Brown, 1986; Brown, 1989). A larger body of data from extracellularly labeled cochlear neurons has supported the view that the myelinated (thus presumably MOC) efferent fiber population never gives rise to branches or en passant endings in the IHC area (Brown, 1987).

In contrast, the idea that some subset of LOC neurons may project to the OHC area, especially in the apical half of the cochlea, has received indirect support from a number of studies. First, immunolabeling for vesicle-associated proteins (e.g., synaptophysin) visualized a significant population of thin, beaded fibers in the apical half of the cat cochlea giving rise to small terminal and en passant swellings (Liberman et al., 1990). Such beaded fiber morphology is characteristic of the LOC system and distinctly different from that associated with classic MOC terminals. Second, a comprehensive immunolabeling study of the OC system in rat showed robust populations of calcitonin gene-related peptidergic (CGRPergic) and γ-aminobutyric acidergic (GABAergic) terminals on OHCs, whereas immunolabeling of brainstem sections showed evidence of GABA or CGRP only in LOC neurons (identified via retrograde tracers in the cochlea; Vetter et al., 1991).

Immunocytochemical studies are in general agreement that OC terminals in the cochlea contain a variety of small molecular-weight neurotransmitters including acetylcho-line (ACh), GABA, and dopamine and several peptidergic transmitters including CGRP and enkephalins (for review, see Fex and Altschuler, 1986; Eybalin, 1993). However, there is no clear consensus as to whether all transmitters are expressed in the IHC and OHC regions, whether cytochemical subgroups exist within the LOC and MOC populations, and, if so, which transmitters are colocalized and which are not. In the rat, Vetter et al. (1991) suggested that LOC cells can be categorized as cholinergic and GABAergic subgroups, with ACh and CGRP colocalized in the GABAergic subgroup and MOC cells exclusively in the cholinergic group. In contrast in the guinea pig, Eybalin and Altschuler (1990), Safieddine and Eybalin (1992a), and Safieddine et al. (1997) agreed that ACh and CGRP are colocalized in LOC neurons but believed that some MOC neurons colocalize ACh and CGRP. They also reported that Met-enkephalin is colocalized with ACh and CGRP in most LOC neurons but never seen in MOC neurons or the OHC region. Conversely, Altschuler et al. (1981, 1984) reported Met-enkephalin positive terminals under OHCs in the apical turns of the cochlea.

The fact that several species (cat, rat, and guinea pig) have been used to investigate MOC and LOC projections and chemical subgroups likely contributes to the lack of clarity on these issues. In recent years, attention has turned to mouse models for study for the OC system because of the increasing availability of transgenic animals in which genes encoding OC transmitters or their receptors have been deleted or overexpressed (Vetter et al., 1999; Zuo et al., 1999). In contrast to the other species mentioned, relatively little is known about the OC system in mice.

The purpose of the present series of experiments was threefold. First, we wanted to produce a quantitative cytochemical map of the OC innervation in the normal mouse to aid in the interpretation of existing and future studies of mutant strains. To that end, we immunostained mouse cochleae for vesicular acetylcholine transporter (VAT), glutamic acid decarboxylase

(GAD), and CGRP to quantify the cholinergic, GABAergic and CGRPergic innervations in the mouse as a function of cochlear location. Second, we wanted to understand which of these terminals arose from the MOC and which from the LOC systems. To that end, we surgically sectioned the OC bundle at the midline, thereby eliminating 75% of the MOC innervations to each ear while sparing almost 100% of the LOC innervations, and then immunostained and quantified terminal distributions as in phase 1. Third, we wanted to begin to understand the extent to which different chemical subgroups of LOC or MOC neurons exist in the mouse. To that end, we performed a series of colocalization studies involving pairwise double immunolabeling of mouse cochleae.

MATERIALS AND METHODS

Animals and groups

Twelve-week-old CBA/CaJ mice of either sex were used in all experiments. Animals were randomly assigned to a control group or an experimental group that underwent brainstem surgery to cut the OC bundle at the midline. The care and use of the animals reported in this study were approved by the IACUC of the Massachusetts Eye and Ear Infirmary.

Brainstem surgery

To cut the OC bundle, mice were anesthetized with ketamine (100 mg/kg, intraperitoneally) and xylazine (20 mg/kg, intraperitoneally). Skin and muscle over the back of the head were reflected, and an elliptical skull opening was made from near the foramen magnum to the tentorium. The dura was reflected, and the cerebellum was elevated to reveal the floor of the fourth ventricle. A shallow cut was made at the brainstem midline where the OC bundle decussates. After repositioning the cerebellum, muscle and skin layers were sutured. After recovering from anesthesia, mice were returned to their cages for 10 days. To assess the success of the cut, brainstems were fixed in 10% formalin, cryoprotected (in 25% sucrose), and cut on a freezing microtome at 80 μm in the transverse plane. Sections were treated histochemically to reveal acetylcholinesterase (AChE) activity (Adams, 1995), mounted on glass slides, air dried, and coverslipped.

Cochlear immunostaining

After intracardial perfusion with 10% formalin, cochleae were decalcified in ethylene-diamine-tetraacetic acid, and half-turns were dissected with fine forceps and immunostained as wholemounts. For single-stained material, tissue was incubated overnight in primary antisera and then with biotinylated secondary antibodies, a complex consisting of avidin, biotin, and horseradish peroxidase (ABC kit, Vector Laboratories, Burlingame, CA), and 3,3′ diaminobenzidine tetrahydrochloride and H_2O_2, embedded in plastic, and mounted on glass slides. The GAD antiserum was prepared by Oertel et al. (1981) and has been used in many previous cochlear studies (e.g., Whitlon and Sobkowicz, 1989; Merchan-Perez et al., 1990; Vetter et al., 1991). Further, the localization of cochlear GAD-like and GABA-like immunoreactivities was shown to be similar (Fex et al., 1986). Other antisera were commercially available: anti-VAT was from Sigma (St. Louis, MO) and anti-CGRP was from Peninsula (Belmont, CA). Colocalization was assessed by simultaneous incubation in sheep anti-GAD and rabbit anti-VAT. Secondary antisera were biotinylated donkey anti-rabbit and alexafluor-488–labeled chicken anti-goat. The final step included streptavidin-conjugated alexafluor-568- and alexafluor-488–labeled goat anti-chicken. Controls included elimination of each primary antibody without otherwise altering the remaining steps of the procedure.

Quantification of immunopositive terminals

For each cochlea, the entire organ of Corti was dissected into four to five pieces, and each piece was measured by computerized planimetry. Cochlear location was converted to frequency according to Ehret's (1983) map for the mouse. To quantify immunopositive terminals, outlines were traced via a drawing tube using high-numerical aperture objectives (2,000× total magnification), as illustrated in Figure 1C,D. When tracing, fine focus was continually adjusted to optimize imaging of each terminal cluster. Traces were digitized and areas were computed with NIH Image software. In the OHC area, all immunopositive terminals were traced, and values from each row were averaged within bins corresponding to 100 μm of cochlear length. In the IHC area, all immunopositive terminals in the inner spiral bundle were traced in a series of microscopic fields, each corresponding to 100 μm in cochlear length and separated from each other by 260 μm of cochlear length.

RESULTS

Mapping cholinergic, CGRPergic, and GABAergic terminals in the mouse cochlea

Cochlear wholemounts of the CBA/CaJ mouse were immunostained with antibodies against GAD, VAT, and the peptide CGRP to assess the longitudinal and radial gradients of GABAergic, cholinergic, and CGRPergic terminals on cochlear hair cells.

In the inner spiral bundle, beneath the IHCs, light microscopy showed large numbers of GAD-, VAT-, and CGRP-immunoreactive boutons, usually in the form of varicosities of fine (<1 μm) immunostained axons. On rare occasions, terminals on IHCs also were observed (Fig. 1). As shown in Figure 2, the average density of immunopositive terminals (~4–5 $\mu m^2/\mu m$ of cochlear length) showed little variation across cochlear locations for GAD positive and CGRP positive terminals; however, the density of cholinergic terminals decreased markedly toward the apex (~2 $\mu m^2/\mu m$).

In the OHC area, immunopositive terminals were larger (~5 μm^2 on average) for all three markers than those seen in the IHC area. Terminals were most commonly located near the base of the OHC, where they often appeared in clusters (although the individual terminals could be resolved in most cases). Immunopositive terminals were never seen in supranuclear positions on the OHCs. Although the mean GABAergic innervation density appeared greater at peak than that for ACh or CGRP markers, the longitudinal gradient of efferent terminals was similar for the three markers (Fig. 3A). OHC innervation density peaked near the 10-kHz cochlear location, with lower values toward the apex and base (Fig. 3A). In addition, terminal distributions showed no radial gradient across the three OHC rows for any marker (Fig. 3B).

Counts of terminals per OHC for the three markers yielded similar mean values in all cochlear regions, peaking at approximately three per OHC in the middle of the cochlea and falling to approximately one per OHC in the basal and apical extremes (Fig. 4). To estimate the total number of OC efferent terminals per OHC, cochleae were immunostained for SNAP25 (a protein involved in the synapse fusion process), which should stain all vesiculated terminals. The results of these terminal counts (Fig. 4) suggested that all three transmitters were 100% colocalized in all OHC terminals because the total number of SNAP25 positive terminals per OHC was the same in all cochlear regions as that seen with any of the three transmitter markers.

Colocalization of VAT and GAD in IHC and OHC areas

The suggestion that GABA and ACh are colocalized in the same population of OC neurons was directly investigated via sequential immunostaining with antibodies to VAT and GAD. As illustrated by the micrographs in Figure 5, virtually all VAT positive terminals in IHC and OHC efferents were also GAD positive, and vice versa. A careful screening of the double-

stained cochleae (n = 2) failed to reveal any significant differences in staining patterns longitudinally or radially. In one cochlea, the entire organ of Corti, from the base to the apex, in IHC and OHC areas was photographed, first with a 20× objective and then with a 100× objective, to produce a set of paired fluorescent images such as those shown in Figure 5. Careful pairwise examination of all these images failed to reveal any significant disparity between GAD positive and VAT positive terminals. A series of negative controls was performed in which one of the secondary antibodies (directed against the sheep anti-GAD or the rabbit anti-VAT) was eliminated, and the appropriate fluorescent signal disappeared in each case.

MOC versus LOC projections to IHC and OHC areas

Retrograde labeling studies in mice have shown that, as for other mammalian species investigated, LOC neurons project almost exclusively to the ipsilateral cochlea, whereas 75% of MOC neurons project to the contralateral ear (Campbell and Henson, 1988; Brown, 1993). Thus, as shown in Figure 6A, a midline cut through the OC bundle at the floor of the fourth ventricle should remove three-fourths of the MOC innervation to each ear and spare virtually all LOC innervations to both ears.

We exploited this anatomic complementarity to determine the central origins of GABAergic, CGRPergic, and cholinergic innervations in the mouse cochlea. In a series of mice, we cut the crossed OC bundle (COCB) at the floor of the fourth ventricle and immunostained the organ of Corti 10 days later. A transverse section through the brainstem from one such animal, stained for AChE, shows a successful section of the COCB (Fig. 6B). Comparison of the photomicrograph of an anti-GAD–immunostained organ of Corti from a successful cut (Fig. 6C) with an identically stained cochlea from a control ear (Fig. 1) shows no obvious change in the innervation density in the IHC area, but a major loss of OHC terminals.

For each of the three antibodies (VAT, GGRP, and GAD), three cochleae from successful COCB cuts were immunostained and analyzed as previously done in controls. Mean values for the innervation density in IHC and OHC regions after successful COCB cuts are shown in Figure 7. The data from control ears are reproduced for comparison. In the IHC area, VAT, CGRP, and GAD labelings were essentially unaffected by the COCB section: the differences between data from control and COCB-sectioned ears were not significant by analysis of variance. In contrast, in the OHC area, mean innervation densities were reduced to approximately 25% of control values. The lack of effect of COCB section on immunostaining in the IHC area was consistent with the idea that these projections arise entirely from LOC neurons. The dramatic effect of the COCB section in the OHC area suggested that all OC terminals on OHCs, whether cholinergic, GABAergic, or CGRPergic, arise from the MOC system.

DISCUSSION

OC innervation in the mouse: similarities to and differences from other mammals

In the IHC area, our results showed that the OC system in the mouse cochlea is fundamentally similar to that in the two best studied species, guinea pig and rat. In these three species, there is rich plexus of nerve fibers and terminals in the inner spiral bundle that stains for cholinergic and GABAergic markers and for CGRP (e.g., Fex and Altschuler, 1984, 1986; Fex et al., 1986; Eybalin et al., 1988; Vetter et al., 1991; Safieddine et al., 1997). As observed for the rat, total terminal areas in the mouse inner spiral bundle for the three immunomarkers were similar to each other, and all three extended throughout the length of the cochlea (Vetter et al., 1991). Others have reported a less extensive distribution (Fex and Altschuler, 1984, 1986; Kitajiri et al., 1985); however, the lack of quantification in previous studies makes meaningful comparisons difficult.

At the OHC level, the mouse shares with other mammalian species studied a clear longitudinal gradient of OC innervation density. In cat and guinea pig, the distribution of vesiculated terminals on OHCs peaks in the upper basal turn, with significantly lower innervation densities, toward the basal and apical extremes. In the mouse, the longitudinal gradient of immunopositive terminals for all three markers studied peaked in the middle of the cochlear spiral. Such a longitudinal distribution was consistent with electrophysiologic studies of the MOC system in the mouse, where cochlear suppressive effects occurred due to electrical stimulation of the MOC peak near the 10-kHz region (Fig. 8). These cochlear suppressive effects were cholinergic in origin: they disappeared in mice with targeted deletion of the α9 nicotinic cholinergic receptor on the OHCs (Vetter et al., 1999). Thus, the distribution of cholinergic terminals is particularly relevant to MOC functional effects, and the correlation between this distribution and the frequency distribution of MOC effects is striking (Fig. 8).

The longitudinal gradients in the OHC region for GABAergic and CGRPergic terminals appear to be variable across mammalian species, and there have been discrepancies within a species across investigators. In cat, there do not appear to be GABAergic or CGRPergic terminals in the OHC area (Adams, unpublished observations). In guinea pig (Fex et al., 1986; Eybalin et al., 1988; Eybalin and Altschuler, 1990), the GABAergic innervation is restricted to the apical half of the cochlea. Some investigators have described a similar pattern in rat (Merchan-Perez et al., 1990; Vetter et al., 1991); however, one study suggested that a GABAergic innervation of OHCs is present throughout the cochlea (Dannhof et al., 1991). In squirrel monkey, GABAergic terminals are distributed throughout the entire length of the organ of Corti (Thompson et al., 1986). In mouse, Whitlon and Sobkowicz (1989) reported GABA positive terminals on OHCs only in mid and apical turns, whereas our study indicated GABAergic and CGRPergic terminals on OHCs throughout the cochlea. In considering this apparent intraspecies discrepancy, one must remember that we used GAD, the final enzyme in the GABA synthetic pathway, rather than GABA as the immunologic marker and that the previous mouse study indicated that the lack of GABA staining in the base arises from “difficulties in stainability of this turn” (Whitlon and Sobkowicz, 1989).

The most striking difference between the OC innervation pattern in the mouse and that of other mammalian species studies is in the relative innervation density on the three OHC rows. In cat and guinea pig, where the best quantitative information is available, there is a dramatic radial gradient in the distribution of vesiculated terminals on the OHCs. In cat, for example, at the cochlear region of peak OHC innervation density, the mean OC terminal area for first row OHCs was more than four times greater than that for the third row (Liberman et al., 1990). In contrast, the mouse data showed no radial gradient in innervation density across the three rows of OHCs for any of the three markers. The preponderance of OC efferents on first-row OHCs may reflect a putative dominance of the first-row cells in the mechanical contributions of OHCs to cochlear mechanics (Yoshida and Liberman, 1999).

LOC versus MOC projections

The original anterograde tracer studies, based on injection of radioactively labeled tracers into the superior oli-vary complex, showed that the bulk of LOC peripheral projections are to the IHC region, whereas the bulk of the MOC projects to the OHC region (Warr and Guinan, 1979; Guinan et al., 1983). However, these original studies also noted some evidence for a minor projection from LOC to OHCs and from MOC to IHCs. Since those classic studies, many workers in the field have ignored the possibility of minor projections and simply equated IHC area terminals with the LOC system and OHC area terminals with the MOC system. Nevertheless, three other strategies have been employed for explicitly investigating the issue of LOC versus MOC projections to the cochlea, and a number of studies have presented evidence suggesting that, in particular, the LOC system sends projections to the OHC area.

One technique to explicitly investigate LOC and MOC projections involves intracellular labeling of single OC neurons, which allows direct tracing from cell body to cochlear terminals. This approach, applicable only to myelinated fibers, has shown that MOC cells always project solely to the OHC area (Robertson and Gummer, 1985; Liberman and Brown, 1986; Brown, 1989; Wilson et al., 1991). All such studies have concluded that fibers destined for the OHC area never send branches to, or make en passant swellings in, the IHC area, although they may spiral for short distances in the inner spiral bundle.

Another approach, pioneered by Ryan and Schwartz (1986) and Ryan et al. (1992), involved the cochlear per-fusion of radiolabeled amino acids and their analogs followed by autoradiographic analysis of cochlear terminals and olivary regions of the brainstem. They showed in the gerbil that perfusion of labeled nipecotic acid results in labeling of 50–60% of terminals on afferent dendrites in the IHC area and all the terminals under OHCs. Examination of the brainstem showed radiolabeling of only MOC neurons, suggesting that MOC neurons in the gerbil project to IHC and OHC areas.

A different approach involves comparison of the immunochemical profiles of MOC and LOC cell bodies in the superior olivary complex with those of terminals in the IHC and OHC areas of the cochlea. When done rigorously, the OC cells must first be identified from the multitude of neighboring cells in the olivary complex with similar gross morphology via retrograde transport of tracers injected into the cochlea. Only one rigorous study of this type has been performed: In the rat, Vetter et al. (1991) reported that all LOC and MOC cells are positive for a cholinergic marker (VAT), but that only LOC cells are immunopositive for a GABAergic marker (GAD) or CGRP. Given that the rat cochlea showed robust innervation of OHCs by GABAergic and CGRPergic terminals (as seen in the present study), Vetter et al. suggested that the GABAergic and CGRPergic innervation of OHCs must arise form the LOC system. Similarly, a number of studies have suggested that some OHC terminals are positive for opioid peptides such as Met-enkephalin (Altschuler et al., 1981, 1984; Scholtz et al., 1998), yet studies of the olivary complex have suggested that only the LOC neurons show immunoreactivity to Met-enkephalin (Altschuler et al., 1983; Saffiedine and Eybalin, 1992a). Other studies of the olivary complex have suggested that some MOC neurons are immunopositive for GABAergic markers or CGRP (Fex and Altschuler, 1986; Eybalin and Altschuler, 1990; Saffiedine and Eybalin, 1992a); however, the lack of definitive identification of MOC neurons via retrograde transport casts some doubt on such claims, especially given the diffuse distribution of relatively rare MOC neurons among the large number of other large non-OC cells in the same olivary regions.

In the present study, we introduced a fourth approach to tackle the question of MOC versus LOC projections. It exploited the observation that, in all species studied to date including monkey, cat, gerbil, guinea pig, rat, mouse, and bat, LOC neurons project predominantly to the ipsi-lateral side, whereas MOC neurons project to the contralateral side of their origin (Warr, 1992). Specifically, in the mouse, 75% of MOC neurons project contralaterally and 99% of LOC neurons project ipsilaterally (Campbell and Henson, 1988; Brown, 1993). In our study, midline OC bundle section reduced cholinergic, CGRPergic, and GABAergic innervations of the OHC area by 75% without affecting the innervation of the IHC area. Together these results provide strong evidence that virtually all OC innervations of OHCs arise from the MOC system, including cholinergic, GABAergic, and CGRPergic projections; whereas IHC area endings arise almost exclusively from the LOC system.

The discrepancy between the present results and those of Vetter et al. (1991) concerning the source of the GABAergic and CGRPergic innervation of OHCs may be due to species differences between rat and mouse. However, the lack of immunostaining for GAD or CGRP among identified MOC neurons might have arisen from a false-negative result due to the

technical challenges involved in superimposing immunohistochemical procedures on top of the histochemical processing necessary for identification of retrogradely transported horseradish peroxidase and having sufficient immunohistochemical sensitivity to detect enzymes in the cell body that may be present only transiently on their way to the terminals to synthesize the transmitter. Indeed, in an in situ hybridization study, Saffiedine and Eybalin (1992b) identified putative MOC neurons that had a significantly weaker signal for CGRP mRNA than that seen in putative LOC cells. The lack of explicit identification of the OC nature of these cells leaves some doubt as to their conclusions, but it remains a reasonable hypothesis that the small number of GAD and CGRP molecules in MOC cell bodies is too weak a signal to detect immunohistochemically.

Cytochemical subgroups within LOC or MOC

The idea that cytochemical subgroups might exist within the MOC or LOC subsystem has long been an intriguing possibility, given the multitude of neurotransmitters and neuromodulators that appear to be expressed in the cochlear terminals of these fibers systems. Despite the importance of these question to understanding OC function, relatively few direct studies concerning these question have been published, because the experiments are technically challenging. To address the question rigorously in the brainstem, retrograde transport from the cochlea (to identify the small number of OC neurons from the large number of other neurons in the olivary complex and periolivary nuclei) must be coupled with double immunostaining. Only one published study (in rat) has taken this rigorous approach: they concluded that MOC neurons comprise a homogeneous group of cholinergic neurons, whereas LOC neurons could be split into a cholinergic group that coexpressed CGRP and a separate and distinct GABAergic group (Vetter et al., 1991). The only other explicit colocalization study of putative OC neurons relied on brainstem location in rats and guinea pigs and immunostaining for cholinergic markers to identify the MOC and LOC cells. Those researchers concluded that there was only one population of LOC neurons, with more than 80% of cholinergic LOC cells coexpressing CGRP and Metenkephalin; no GABAergic markers were investigated (Saffiedine and Eybalin, 1992a). As for putative MOC cells, there was evidence that a small subpopulation of the cholinergic cells coexpressed CGRP, whereas none expressed Met-enkephalins.

Our double-immunostaining results clearly suggested complete congruence of GABAergic and cholinergic markers in the IHC and OHC areas. The conclusion that cyto-chemical subgroups do not exist, at least for cholinergic and GABAergic populations in mouse OHCs, was strongly supported by the observations that the numbers of terminals per OHC were identical for cholinergic, GABAergic, and CGRPergic markers applied individually and 2) that these in turn were identical to the number of terminals revealed with staining for SNAP25, a synaptic protein that should label all vesiculated terminals. The morphometry in the IHC area provided more equivocal support, given that measures of VAT positive terminal areas in the apical half of the cochlea were smaller than those for GAD or CGRP (Fig. 2). A possible explanation of the discrepancy is that the immunostaining intensity was lower for VAT than for GAD (fluorescent images shown in Fig. 5 were balanced to equalize brightness and highlight the similarity of the labeled terminal outlines). This intensity difference, when tracing outlines of immunolabeled terminals directly from the microscope, could translate into a systematically smaller measured areas for VAT-labeled terminals.

The differences between our study in mouse and the work of Vetter et al. (1991) in rat could be due to species differences. However, the apparently discrete subgroups seen in rat might have arisen from an immunoreactivity detection threshold coupled with differential longitudinal gradients for GABAergic and cholinergic markers. In the apical IHC area, the cholinergic labeling in our mouse cochleae was weaker than the GABAergic labeling. Perhaps the GABAergic LOC neurons identified by Vetter et al. (1991) correspond to those innervating

the apical turns, in which the expression of cholinergic enzymes was too low to be captured by immunolabeling at the cell body. Further evidence for a fundamental similarity between rat and mouse is the immunoelectron microscopic study of Dannhof et al. (1991), which provided strong evidence for choline acetyltransferase and GAD colocalization in efferent terminals on OHCs throughout the cochlea.

The idea that GABA and ACh are colocalized within the same terminal, although not commonly reported in the nervous systems, is not without precedent. For example, Kosaka et al. (1988) reported the coexistence of GAD and choline acetyltransferase in some cell types of the retina, cerebral cortex, basal forebrain, and spinal cord. Iwasa et al. (1999) showed that GABA immunoreactivity is colocalized with AChE in nerve fibers of the mouse adrenal gland, and N'Guyen and Grzywacz (2000) found GABA and GAD immunoreactivities in the cholinergic amacrine cells of the adult turtle retina.

Functional implications

The only known cochlear effects of activation of the OC bundle are the suppression of cochlear responses such as compound action potentials (CAPs) or distortion product otoacoustic emissions (DPOAEs). These classic OC effects have long been thought to arise from cholinergic effects of the MOC synapses on OHCs (for reviews, see Eybalin, 1993; Sewell, 1996). This has been convincingly shown by the recent demonstration that all such effects of OC stimulation on CAPs and DPOAEs are absent in mice lacking the α9 nicotinic cholinergic receptors expressed by OHCs (Vetter et al., 1999).

The functional role of GABA and CGRP in OC terminals is poorly understood. In vitro, patch-clamp recordings from apical OHCs in guinea pig have shown that GABA induces a dose-dependent hyperpolarization, which appears to be mediated via the $GABA_A$ receptor (Gitter and Zenner, 1992). This receptor has been localized by in situ hybridization of its α1 subunit, predominantly in the OHC region but also in the IHC area, with a much weaker signal (Kempf et al., 1994). Previous in vivo work suggested that GABAergic efferents mediate the stereotyped, slow alteration in the amplitude of the $f_2–f_1$ DPOAE seen as a function of time after stimulus onset (Kirk and Johnstone, 1993). The evidence for this claim was that such slow alterations could be blocked by perfusion of bicuculline, a classic GABAergic antagonist. However, subsequent work by Kujawa et al. (1995) showed that the phenomenon is not eliminated by tetrodotoxin or by complete OC section, thus casting significant doubt on the original claim. The most compelling work on the functional role of CGRP in hair cell systems comes from Bailey and Sewell (2000) who showed, with lateral line preparations, that in vitro perfusion of the neuromast with CGRP suppresses mechanical responsiveness as it increases spontaneous discharge rate in sensory fibers.

In the mammalian cochlea, in vivo data from the α9 knockout mouse suggested that, in the absence of cholinergic actions, the effects of GABA and CGRP are difficult to discern. In this knockout model, the OC synapses on OHCs develop in normal numbers, and the OC innervation of OHCs continues to be immunopositive for cholinergic markers (Vetter et al., 1999). Immunolabeling for GABAergic and CGRPergic markers have not been performed in this knockout mouse. However, if we assume that these transmitters are still present, the functional data showed that, without the α9 receptor, electrically evoked release of GABA and/or CGRP from these MOC terminals (if it occurs) has no measurable effect on CAP or DPOAE at $2f_1–f_2$. Of course, the electrical stimulation of the OC bundle probably would not activate the LOC system (Gifford and Guinan, 1987); thus, the possible effects of GABA and CGRP in the IHC area might not be evoked by such procedures.

It remains a viable hypothesis that GABA and CGRP are colocalized in the OHC terminals with ACh and act to modulate the strength of cholinergic effects, as has been suggested for

CGRP at the neuromuscular junction (Dezaki et al., 1996) and in isolated chick hair cells, where preincubation with CGRP was reported to potentiate the amplitude of ACh-induced changes in calcium concentration (Shigemoto and Ohmori, 1990). Future work with knockout mice lacking component transmitters or receptors of the OC system will shed more light on the functional significance of each chemical component of this heterogeneous pathway.

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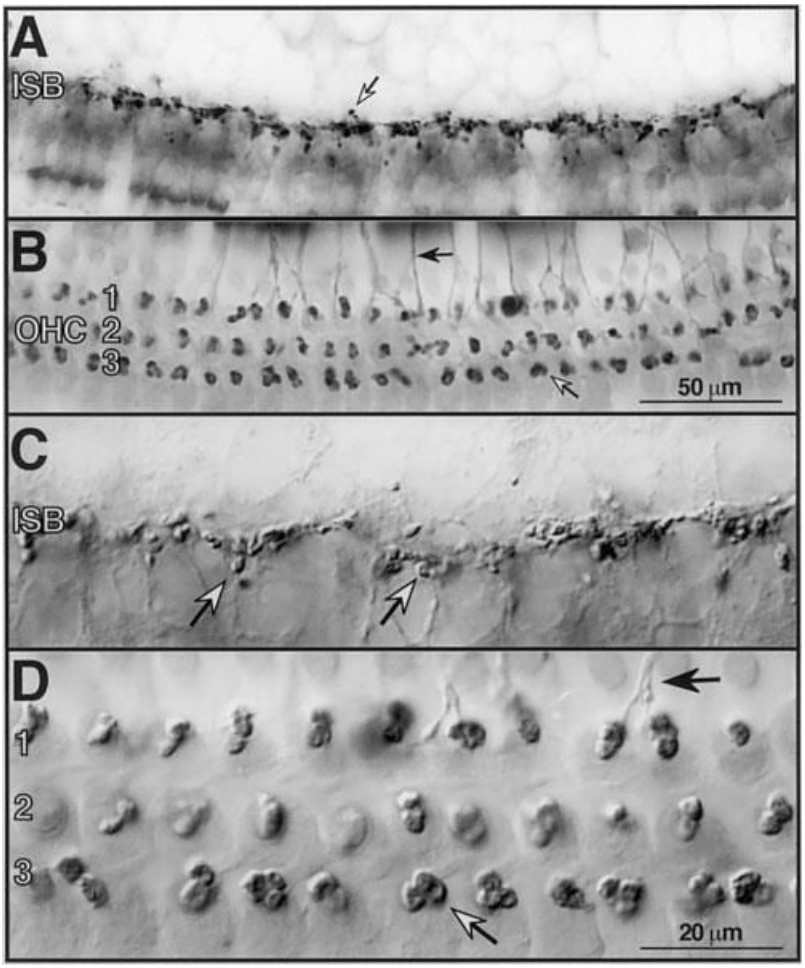

Fig. 1.

Glutamic acid decarboxylase (GAD) immunostaining in a cochlear wholemount from the 8-kHz region. A: Brightfield micrograph of the inner spiral bundle (ISB) shows the plexus of GAD positive terminals (e.g., at arrow) beneath inner hair cells in a control mouse. **B:** Brightfield micrograph of the same cochlear region shown in A demonstrates multiple immunostained terminals beneath almost every outer hair cell (e.g., white arrow). Fascicles of efferent fibers can be seen crossing the tunnel of Corti (black arrow). OHC rows are numbered. **C, D:** High power Nomarski micrographs of inner and outer hair cell areas, respectively, represent the kind of images used for manual tracing of immunostained terminals and subsequent morphometry. When using objectives with high numerical apertures, the focal plane is thin, and a single micrograph can capture only a small subset of terminals in crisp focus (e.g., white arrows in C and D). When tracing outlines, the focus is rolled to capture each terminal cluster (black arrow in D) at optimal focus. Scale bars in B and D apply to A and C, respectively.

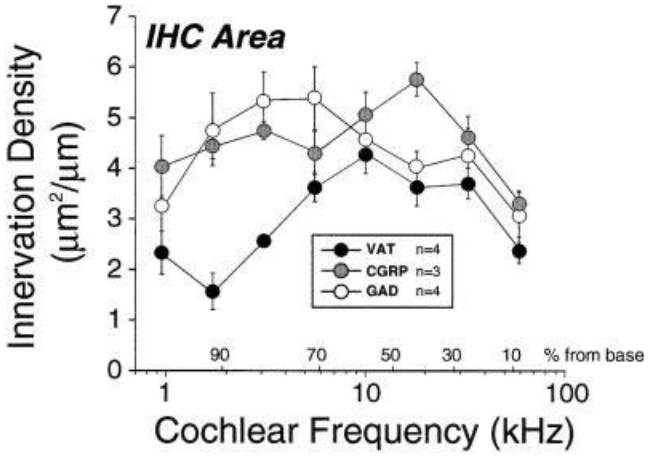

Fig. 2.

Innervation densities for terminals positive for vesicular acetylcholine transporter (VAT), calcitonin gene-related peptide (CGRP), and glutamic acid decarboxylase (GAD) in the inner hair cell (IHC) area as a function of cochlear location. For each antibody (see key), the longitudinal gradient of immunopositive terminals in the IHC area was determined for three to four cochleae. Mean values (± standard error of the mean) were obtained by averaging data from similar locations. Innervation densities are expressed as total silhouette area per micrometer of cochlear length. Cochlear position is expressed as a percentage of distance from the base (upper scale) and the frequency correlate, as estimated by a cochlear frequency map (Ehret, 1983).

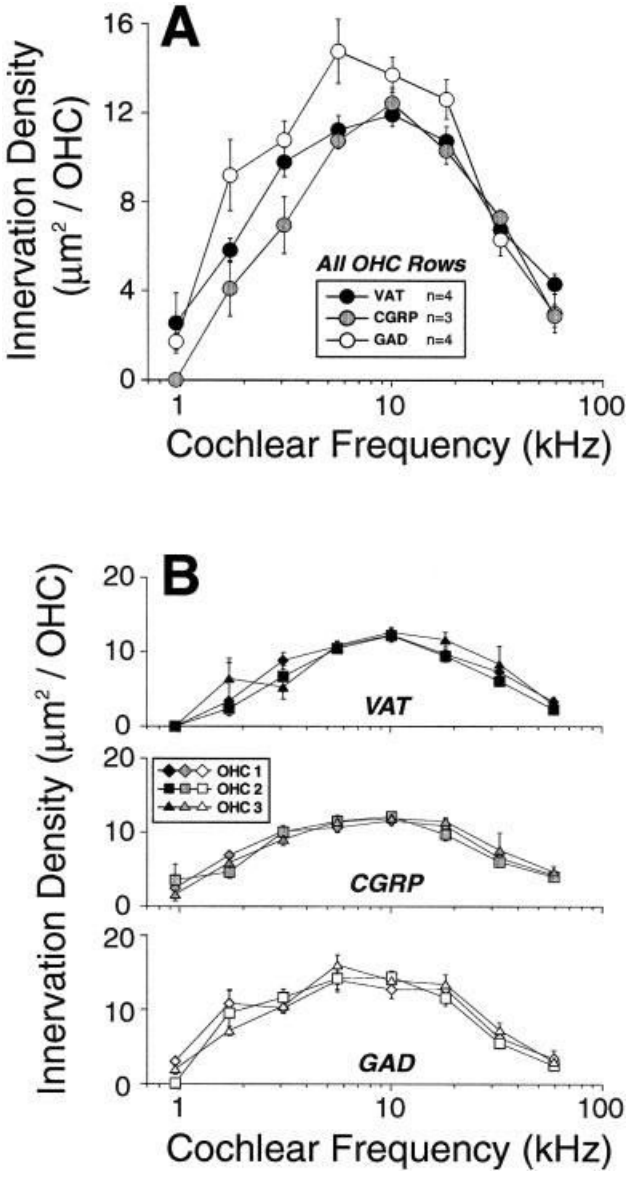

Fig. 3.

Innervation densities for terminals positive for vesicular acetylcholine transporter (VAT), calcitonin gene-related peptide (CGRP), and glutamic acid decarboxylase (GAD) in the outer hair cell (OHC) area as a function of cochlear location. **A:** Data summed across all three OHC rows. **B:** Data for each row computed separately. For each antibody (see key), the longitudinal and radial gradients of immunopositive terminals in the OHC area were determined for three to four cochleae. Mean values (± standard error of the mean) were obtained by averaging data from similar locations. Innervation densities are expressed as total silhouette area per OHC.

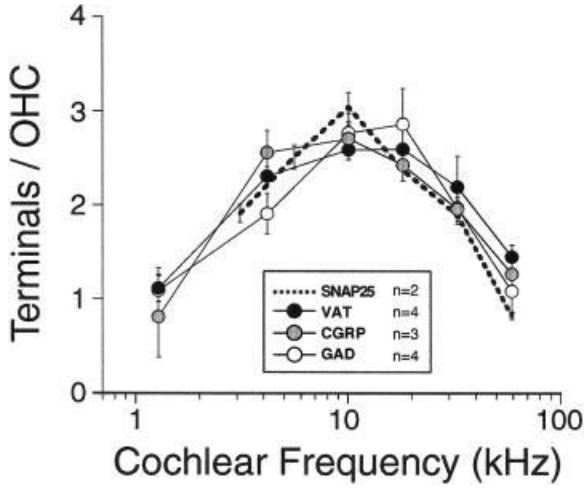

Fig. 4.

Numbers of terminals positive for vesicular acetylcholine transporter (VAT), calcitonin gene-related peptide (CGRP), and glutamic acid decarboxylase (GAD) per outer hair cell (OHC) as a function of cochlear location. Counts of terminals per OHC for the γ-aminobutyric acid, acetylcholine, and CGRP markers were performed at six different cochlear regions in three to four control cochleae. Two additional cochlea were labeled with anti-SNAP25 to estimate the total number of vesiculated (i.e., olivocochlear efferent) terminals per OHC. Values reflect the mean count (± standard error of the mean) of immunopositive terminals for each of the four markers.

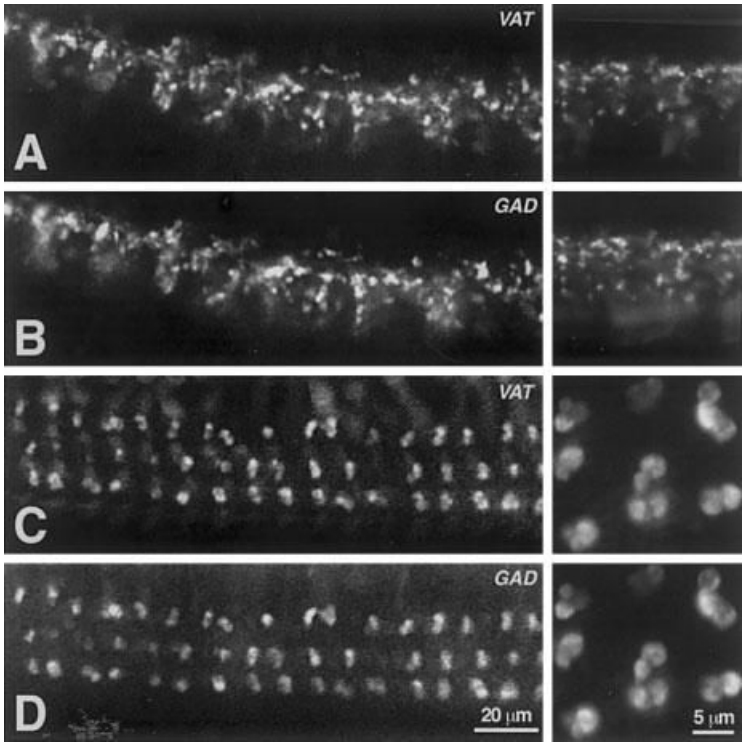

Fig. 5.

Colocalization of glutamic acid decarboxylase (GAD) and vesicular acetylcholine transporter (VAT) in efferent terminals. Paired images from a cochlea double immunostained with anti-GAD and anti-VAT coupled with fluorescent markers. High and low power micrographs of inner (A, B) and outer (C, D) hair cells are presented. Each pair of micrographs was acquired at the same focal plane, with only the filter set changed. Image intensity was adjusted (the VAT signals were weaker) to facilitate comparison of terminal shapes and sizes.

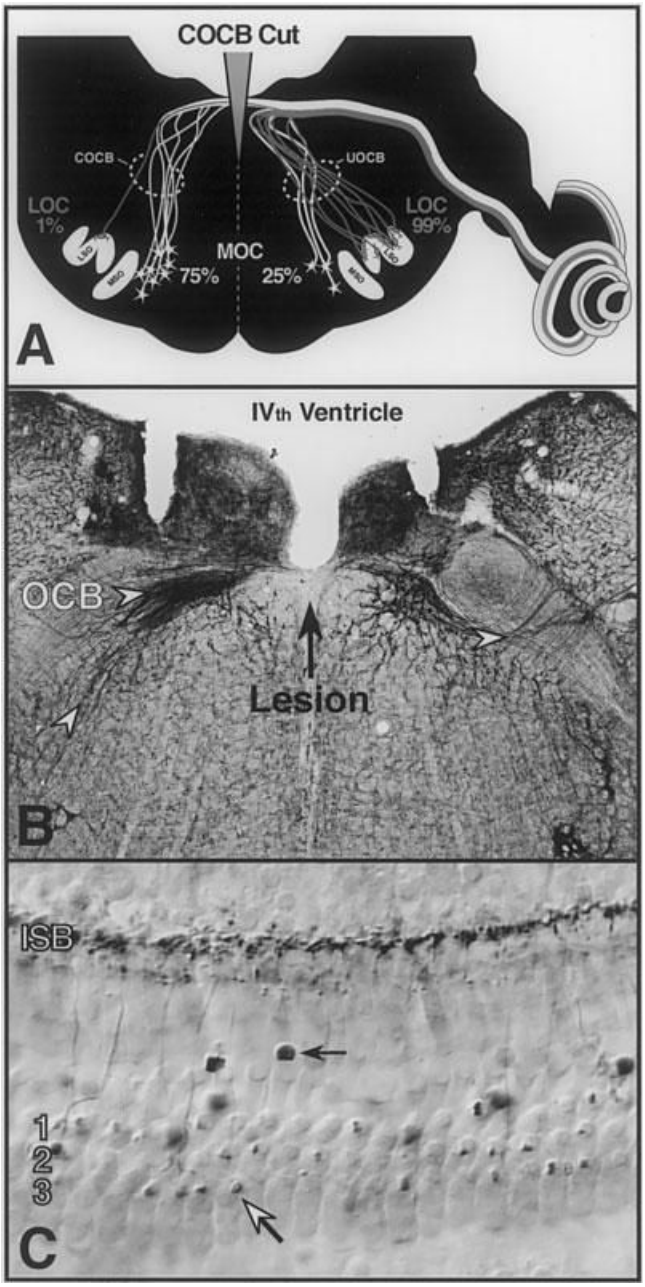

Fig. 6.

Midline section of the olivocochlear (OC) bundle to differentiate medial (MOC) from lateral (LOC) OC projections. **A:** Schematic cross-section through the brainstem shows locations of LOC and MOC cells, approximate pathways for OC axons from the olivary complex to the cochlea, published values for ipsilateral and contralateral projections of LOC versus MOC systems (Campbell and Henson, 1988; Brown, 1993), and positions of the surgical cuts through the crossed OC bundle (COCB) in the present experiments. UOCB, un-crossed OC bundle. **B:** Transverse section of an mouse brainstem stained with acetylcholinesterase 10 days after the COCB cut. The black arrow points to a shallow cut made at the floor of the fourth (IVth) ventricle, where the OCB (white arrowhead) decussates. **C:** Glutamic acid decarboxylase (GAD) immunostaining in a cochlear wholemount from the 8-kHz region 10 days after COCB section. In the inner spiral bundle (ISB), GAD positive terminals form a plexus beneath inner

hair cells (black arrow) comparable to that in controls (Fig. 1). However, loss of terminals beneath outer hair cells (OHCs) is obvious: the white arrow points to one of the few remaining immunopositive terminals. A few blood cells appear in the image (black arrow), which stain due endogenous peroxidases. OHC rows are numbered for reference.

Fig. 7.

Innervation densities for terminals positive for vesicular acetylcholine transporter (VAT), calcitonin gene-related peptide (CGRP), and glutamic acid decarboxylase (GAD) in outer (OHC) and inner (IHC) hair cell areas after sectioning the crossed olivocochlear bundle (OCB). For each antibody (VAT, CGRP, and GAD), three cochleae were immunostained and analyzed as in controls after a successful crossed OCB section, as determined histologically (see Fig. 6B). The data from control ears (solid symbols) are reproduced for comparison (see Figs. 2-3). The contribution of the crossed OCB (dashed lines) was computed by subtracting the post-cut measures (open symbols) from the control values. Top: Mean (± standard error of the mean) innervation density measured in the IHC region, expressed as total silhouette area per micrometer of cochlear length. Bottom: Mean (± standard error of the mean) innervation density in the OHC region, expressed as total silhouette area per OHC.

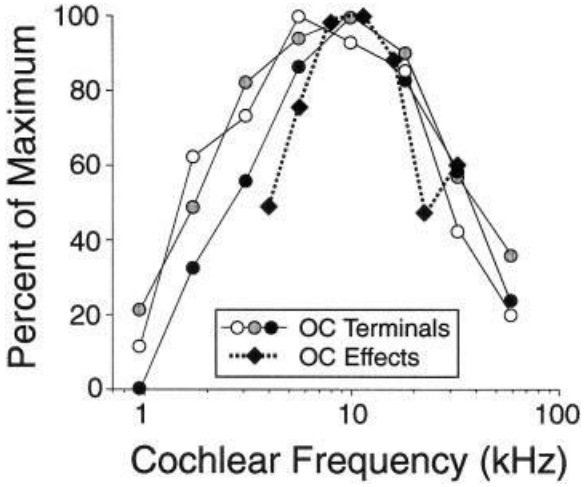

Fig. 8.

Comparison of anatomic and functional data for the medial olivocochlear (MOC) system. The longitudinal gradients of OC terminals on all three OHC rows (from Fig. 3) were normalized to peak value for each marker. These data were compared with a metric of classic MOC effects on cochlear suppression in the mouse, i.e., the average suppression of compound action potential, expressed as “effective attenuation” in decibels during electrical stimulation of the OC bundle (from Vetter et al., 1999).