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Choline Acetyltransferase Expression During a Putative Developmental Waiting Period

DWAYNE D. SIMMONS,[1,2,3]* CRISTINA BERTOLOTTO,[1,3] JASON KIM,[1] JUMAN RAJI-KUBBA,[1] AND NEIL MANSDORF[1]

[1]Department of Physiological Science, UCLA, Los Angeles, California 90095-1527
[2]Program in Neuroscience, UCLA, Los Angeles, California 90095-1527
[3]Brain Research Institute, UCLA, Los Angeles, California 90095–1527

ABSTRACT

The relationship between the cholinergic expression, morphological development, and target cell innervation of olivocochlear (OC) efferent neurons was investigated in the postnatal hamster. Similar to what was found in previous studies, tracer injections into the contralateral cochlea labeled cells bodies retrogradely in periolivary regions and labeled cell bodies only rarely in the lateral superior olive (LSO). Few morphological differences were found among cell bodies labeled between postnatal day 1 (P1) and P30. Tracer injections into the crossed OC bundles within the brainstem anterogradely labeled terminals below the inner hair cells of the cochlea prior to P5 and labeled terminals below outer hair cells after P5, consistent with a period of transient innervation, as hypothesized previously. Within the superior olive, choline acetyltransferase (ChAT) was expressed differentially. In periolivary regions, ChAT was expressed as early as P0. ChAT-immunoreactive cell bodies in periolivary regions were similar morphologically to retrogradely labeled OC neurons. In contrast, within the LSO, ChAT was not expressed until after P2. Consistent with a medial OC projection to the cochlea at early postnatal ages, ChAT immunoreactivity was detected below inner hair cells as early as P2 but was not detected below outer hair cells until after P6. Our results suggest that medial OC neurons not only provide transient connections to inner hair cells but also may express ChAT when they are below inner hair cells. Furthermore, these results raise the possibility that OC neurons may be capable of acetylcholine synthesis and release prior to or simultaneous with their innervation of the cochlea. J. Comp. Neurol. 397:281–295, 1998.

Indexing terms: olivocochlear neurons; acetylcholine; central and peripheral auditory; cochlea; hearing

The expression of neurotransmitter and cotransmitters as neurons grow into and begin to form synapses with target tissues may coincide with a particular developmental phase (Greif et al., 1991; Messersmith and Redburn, 1993; Haydon and Drapeau, 1995). It has been proposed recently that neurons that synthesize neurotransmitter or neuropeptides as they grow toward a target tissue or cell tend to make many more synaptic connections than are found in the adult. These extrasynaptic connections are believed to undergo some type of competitive elimination. Conversely, neurons that synthesize neurotransmitter or neuropeptides after contact with their target tissue or cell tend to retain their initial connections as they mature. Such a proposal raises questions about the relationship between neurotransmitter expression and synaptogenesis. For example, does the timing of neurotransmitter expression influence whether connections will be transient or permanent during synaptogenesis?

Little is known about the relationship between neurotransmitter expression and synaptogenesis in efferent olivocochlear (OC) neurons as they project to hair cell receptors in the mammalian cochlea. Recent evidence suggests that efferent OC neurons have multiple periods of growth and termination onto their cochlear targets (Robertson et al., 1989; Simmons et al., 1990, 1996a,b; Bruce et al.,

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*Correspondence to: Dr. Dwayne D. Simmons, Center for the Study of the Biology of Hearing, Central Institute for the Deaf, 818 South Euclid Avenue, St. Louis, MO 63110. E-mail: simmons@cid.wustl.edu

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1997). In adult animals, the OC system comprises several populations of neurons that are distinguished on the basis of neurotransmitter, morphology, spatial location, and target cell identity (Rasmussen, 1960; White and Warr, 1983; Lu et al., 1987; Moore and Moore, 1987; Aschoff and Ostwald, 1988; Campbell and Henson, 1988; Vetter et al., 1991; Vetter and Mugnaini, 1992). Medial OC neurons are multipolar cells, contain acetylcholine, are located mostly in the medial portions of the rostral and ventral periolivary regions of the brainstem superior olivary complex, and project via the crossed OC bundles to the contralateral cochlea, where they terminate exclusively on outer hair cells. In contrast, lateral OC neurons are smaller, are fusiform in shape, contain either acetylcholine or γ-aminobutyric acid (GABA), originate from the region in or around the lateral superior olive (LSO), and project to the ipsilateral cochlea, where they terminate mostly on afferent fibers below the inner hair cells (Takeda et al., 1986; Aschoff and Ostwald, 1987; Vetter et al., 1991; Vetter and Mugnaini, 1992). In all studies to date, calcitonin gene-related peptide (CGRP) is colocalized in cholinergic lateral OC neurons, which further differentiates them from the medial OC population (Kawai et al., 1985; Lu et al., 1987; Kruger et al., 1988; Vetter et al., 1991). Recent studies suggest that medial OC neurons make up a unique group of both rostrally and ventrally located cholinergic cells within periolivary regions of the superior olive (Godfrey et al., 1987b; Vetter et al., 1991; Vetter and Mugnaini, 1992). Furthermore, the evidence to date is that cholinergic neurons within periolivary regions project either to the cochlea or to the cochlear nucleus (Godfrey et al., 1987a,b; Vetter et al., 1991; Sherriff and Henderson, 1994).

In postnatal hamsters, we now have evidence for a temporal segregation of arriving efferent terminals: Medial OC neurons project to the cochlea at birth, whereas lateral OC neurons may not innervate the cochlea until the close of the first postnatal week (Simmons et al., 1996a,b). Previous anterograde labeling investigations in our laboratory have hypothesized that a developmental period exists in which medial OC axons may accumulate transiently below inner hair cells during the first postnatal week (Simmons et al., 1996a). However, it is not known whether these early connections are capable of synthesizing and releasing acetylcholine. Because OC neurons may be intermixed with embryonic populations of motor neurons as they migrate after neurogenesis (Fritzsch and Nichols, 1993), it is possible that OC neurons might also demonstrate patterns of synaptogenesis similar to those of motor neurons.

The present study investigates the relationship between medial OC cell bodies found in periolivary regions of the superior olive, their innervation patterns found below cochlear hair cells, and the expression of choline acetyltransferase (ChAT) both in the superior olive and in the cochlea. This study builds on previous studies that have defined the anterograde labeling patterns during postnatal development in the hamster (Simmons et al., 1990, 1996a). Two strategies are used in the present study. First, medial OC cell bodies and axon terminals are labeled by using tracer injections into an in vitro preparation of the brainstem and cochlea, as described previously (Simmons et al., 1996a). Second, the immunocytochemical expression of ChAT is compared developmentally with OC neurons labeled by using tracer injections. The postnatal hamster serves as an excellent model for these kinds of developmental investigations because of its short gestation and its immaturity at birth (Hoffman et al., 1968; Stephens, 1972; Pujol and Abonnenc, 1977; Simmons et al., 1990, 1991). The results of our study give rise to the hypothesis that ChAT expression within OC neurons occurs either prior to or simultaneous with the initial OC connections within the cochlea. Parts of this study have been presented previously in abstract form (Simmons et al., 1994, 1995).

MATERIALS AND METHODS

Data were obtained from a total of 60 hamsters (*Mesocricetus auratus*), 0–30 days old, where the day of birth represents postnatal day 0 (P0). Prior to the experiment, animals were examined to ensure normal (i.e., nonpathological) inner ears. All animal protocols used in this study were approved by the institutional animal care committee.

In vitro labeling

An in vitro brainstem technique developed in this laboratory (Simmons et al., 1996a) was used to trace the development of efferent brainstem neurons and axonal terminals in the cochlea. Thirty-six hamsters were used for tracer studies. All animals were anesthetized with near-lethal injections of sodium pentobarbital (Nembutal; 100 mg/kg, i.p.) prior to intracardial perfusion. Hamsters were perfused intracardially with oxygenated media consisting of sterile-filtered Eagle's minimum essential media (MEM; Sigma Chemical, St. Louis, MO) modified with dextrose (2.7 g/liter) and sodium bicarbonate (2.2 g/liter). After perfusion, hamsters were decapitated, and the dorsal cranium overlying the cerebellum was removed. This preparation was immersed immediately in a constant-temperature (20°C) Radnotti chamber and bathed continuously with fresh-oxygenated media.

In 20 of these animals, the vermis of the cerebellum was ablated to expose the floor of the fourth ventricle. Once the genu of the facial nerve tracts were visualized, an incision was made along the midline extending from the aqueduct of Sylvius rostrally to the facial genu caudally. At this location, an incision 1–2 mm in depth severs the decussation of the olivocochlear bundle and allows both the retrograde and anterograde labeling of olivocochlear cell bodies and axon terminals, respectively. In 12 cases, the otic capsule of the cochlea was removed, and an incision was made into the basal portion of the modiolar nerve, which allows retrograde labeling of the olivocochlear cell bodies. Immediately after either the brainstem incision or the cochlear incision, a micropipette was inserted, and tracer was injected by a nanoliter pump at a rate of 20–50 nl per minutes for 5–15 minutes. The micropipette contained a tracer solution consisting of 2% biocytin (Sigma Chemical) and 15% horseradish peroxidase (Type IV; Sigma Chemical) in 0.1 M Tris buffer, pH 7.4.

Four to six hours after injection, brainstems were blocked and immersed in cold 4% paraformaldehyde-lysine-periodate (PLP) in 0.1 M phosphate buffer (PB), pH 7.4, solution. Cochleae from brainstem injections were isolated, detached, and immersed in a solution of 2.5% glutaraldehyde and 1% paraformaldehyde in 0.1 M PB, pH 7.4, overnight. Cochleae from hamsters P5 or older were also decalcified in 0.1 M EDTA at 10°C. Cochlear tissue was embedded in either a gelatin-agarose or gelatin-albumin mixture and then sectioned on a Vibratome (80–100 μm) perpendicular to the cochlear nerve axis

(modiolus). Brainstems were cyroprotected in 30% sucrose in PB saline, pH 7.4, immersed in optimum cutting temperature (OCT) medium (Miles, Inc., Elkhart, IN), frozen, and then serially sectioned at 20 μm on a cryostat. Sections were processed free floating for histochemical detection of label.

For histochemical visualization of tracer in the brainstem and cochlea, an avidin-biotin complex peroxidase method (Vectastain Elite ABC kit; Vector Laboratories, Burlingame, CA) was used. Sections were incubated for 2 hours with an avidin-conjugated peroxidase solution containing 0.1 M Tris-buffered saline, 1% bovine serum albumin, and 0.3% Triton X-100, followed by extensive washings (overnight) in Tris-buffered saline. Sections were treated with a diaminobenzidine-glucose oxidase solution (0.025% diaminobenzidine, 1% glucose oxidase, and 0.02% glucose) and a heavy-metal intensification procedure (Adams, 1981).

ChAT immunocytochemistry

To investigate the postnatal development of ChAT within the brainstem and cochlea, 24 hamsters were used for immunocytochemical staining. In these animals, fixation was done by intracardial perfusion of a cold (4°C) solution of PB, pH 7.4, containing 2% dextran followed by either a 10–15 minute perfusion of cold 4% paraformaldehyde and 0.1% glutaraldehyde made in PB or a 30–45 minute perfusion of cold 4% PLP in PB solution. Ten brainstems were dissected and embedded in celloidin, and serial sections were cut on a microtome (20 μm). Ten brainstems were also embedded in a gelatin-agarose mixture and sectioned on a Vibratome (40 μm). Cochleae were dissected, and the otic capsules were removed and decalcified in EDTA if necessary. Roughly half of the cochleae were embedded in a celloidin mixture or in Paraffin Plus (Fisher Scientific, Tustin, CA) and serially sectioned on a microtome (10–20 μm). Sections were taken either parallel to the cochlear nerve axis (modiolus) to achieve cross sections of the organ of Corti or perpendicular to the cochlear nerve modiolus to achieve longitudinal sections of the surface of the organ of Corti.

To minimize nonspecific staining, sections were preincubated in 25% normal serum (from secondary antibody animal) followed by preincubation in 3% H_2O_2. Sections were then incubated overnight in a humidified chamber with primary antisera: anti-ChAT (1:1,000 goat polyclonal; Chemicon, Temecula, CA). Antisera were diluted in a solution containing 0.1 M Tris-buffered saline (TBS), 1% normal serum, and 0.3% Triton X-100 (TBSNT). After appropriate rinses, sections were incubated with peroxidase-labeled secondary antibody (1:500) made up in TBSNT. Both cell bodies in the brainstem and terminals in the cochlea were visualized by using a True blue (tetramethylbenzidine derivative) method (Kirkegaard and Perry Laboratories, Gaithersburg, MD). In our earliest experiments, we used a biotinylated secondary antibody that was visualized histochemically by utilizing an avidin-biotin complex (ABC) method. However, this technique proved to be less sensitive than True blue, which was used in all later processing.

Combined immunocytochemical and in vitro labeling

Four animals (two each at P12 and P20) were prepared for in vitro labeling of OC cell bodies and terminals as well as the assessment of ChAT immunoreactivity. Animals were perfused as described above for in vitro labeling, the brainstems along with attached cochleae were isolated, and injections were made into the crossed OC bundles. After 3–4 hours, the brainstem and cochleae were immersed in 4% PLP fixative in PB. After roughly 24 hours, the brainstems were cryoprotected, immersed in OCT, frozen, and then serially sectioned on a cryostat. Alternative sections were processed free floating for either histochemical or immunocytochemical detection of label, as described above.

Control tests

For all primary antisera used, controls for method specificity were accomplished by replacing the primary antisera with sera that did not contain primary antibody. In all control cases for ChAT, there was no neuronal immunostaining. Immunostaining for ChAT has also been confirmed by cRNA in situ hybridization procedures (Simmons et al., 1997b).

Microscopic analysis

All tissue sections were dehydrated, cleared in xylenes, and coverslipped with either DPX (Aldrich Chemical, Milwaukee, WI) or Permount (Sigma Chemical). The total numbers of labeled cell bodies were counted in the light microscope by using either an X50 APO or an X100 APO (oil immersion) objective lens (Zeiss Aus Jena, Thornwood, NY) for a final magnification of 500× or 1,000×, respectively. All tissue sections were drawn with a drawing tube at 10× magnification for a final magnification of 500×. Drawings of brainstem sections were digitized for computer reconstructions of label maps. Maps from serial sections were superimposed to eliminate any doubly counted cells. Therefore, no correction factors were applied to the counts of the number of positively labeled cell bodies. All morphometric data were obtained by capturing microscopic images with a CCD camera (512 × 512 pixels) and then determining shapes and areas of individual cells with NIH Image software (version 1.59; Wayne Rasband; NIMH, Bethesda, MD). For both measurement and count data, only cells that were diffusely labeled and had at least one primary dendrite were included. Both retrograde labeling and immunocytochemical labeling generally obscured the nucleus and nucleolus, making it difficult to control for measurements taken from tangential sections. Mean values of the data obtained were evaluated by one-way analysis of variance using both the Student's t-test and the Tukey-Kramer HSD test at an alpha (P) level of 0.05.

RESULTS

Table 1 shows that retrograde labeling data were collected from a total of five postnatal ages (P1, P6, P12, and P30). Like in any extracellular labeling study, our data are most probably biased toward those neurons that are most capable of transporting tracer (for example, neurons with larger diameter axons). Observations for the immunocytochemical localization of ChAT came from a total of 12 postnatal ages (P0–P7, P10, P12, P20, and P30). Brainstem nuclei within the superior olivary complex were classified on the basis of previous reports in the rodent (Osen et al., 1984; Schwartz, 1992; Simmons and Raji-Kubba, 1993).

TABLE 1. Brainstem Retrograde Label[1]

Age	Number of experiments with label	Minimum number of labeled cells	Maximum number of labeled cells
P1	3	10	104
P6	5	25	80
P12	6	30	93
P20	2	46	85
P30	2	25	63

[1]Summary of retrograde label experiments. In each instance, a cochlea-attached in vitro brainstem preparation was used to label retrogradely olivocochlear neurons on the contralateral side of the brainstem. Given are the postnatal ages used, the number of experiments with retrogradely labeled cell bodies, and counts of the minimum and maximum number of labeled cells on the contralateral side. P, postnatal day.

Retrogradely labeled OC neurons

To minimize possible contamination and/or ambiguity, investigations of the morphological development of medial OC cell bodies relied almost exclusively on experiments in which OC neurons were labeled retrogradely from the contralateral cochlea (Fig. 1). Neurons could be labeled retrogradely from the contralateral cochlea throughout the postnatal period investigated (Table 1). In our best cases, the highest number of labeled cells varied from 104 at P1 to 63 at P30. On average, 85 ± 19 cells were labeled retrogradely on the contralateral side of the brainstem.

A comparison of labeled medial OC neurons at P1 and P30 shows that few morphological differences existed between the two ages (Fig. 2). In the P1 brainstem, retrogradely labeled cell bodies had multiple, large primary dendrites and fine neuritic extensions found on both the cell bodies and dendrites (Fig. 2A). In the P30 brainstem, retrogradely labeled cell bodies also had multiple, large primary dendrites but lacked the fine neuritic extensions found at P1, suggesting such neuritic extensions may be a developmentally transient feature (Fig. 2B,C). There was a small increase in cell size between P1 and P30, as might be expected. Histograms of cell body areas are plotted at P1 and at P30 (Fig. 3A,B). The increase in the mean cell area was from 113 ± 35 μm^2 (±S.D.; n = 104) at P1 to 150 ± 42 μm^2 (n = 63) at P30. These areas corresponded to long-axis diameters from 8 µm to 10 µm at P1 and from 8 µm to 16 µm at P30. The number of primary dendrites did not show a statistically significant increase between P1 and P30 (Fig. 3C,D). Within the coronal plane of section, the mean number of primary dendrites was 2.7 ± 1.1 (n = 104) at P1 and 3.5 ± 1.7 (n = 63) at P30.

Few differences were also observed in the distribution and location of medial OC neurons across postnatal ages. Table 2 shows that contralateral injections labeled almost exclusively neurons in periolivary regions. Roughly 98% of the total number (n = 425) of all labeled cells across our best cases were found in periolivary regions. Across all five postnatal ages, roughly 7% of cells were located in the rostral periolivary (RPO), 87% of cells were located n the ventral nucleus of the trapezoid body (VNTB), and less than 1% of cells were located in the LSO (Table 2). The remaining cells were distributed in the RPO and the DPO (Table 2). Figure 4 shows a reconstruction of the positions of retrogradely labeled cell bodies in superior olivary sections of a reconstructed P12 brainstem. Of the 93 cells labeled, 89% of these were found in the VNTB. One labeled cell was found in the LSO, and a few labeled cells (roughly four) were located near the border of what should be the LSO capsule or shell, which has been found also in adult animals (Vetter and Mugnaini, 1992). At least in the postnatal hamster, these data support the conclusion that, throughout the postnatal period, medial OC neurons can be labeled retrogradely from the contralateral cochlea and are located almost exclusively in periolivary regions.

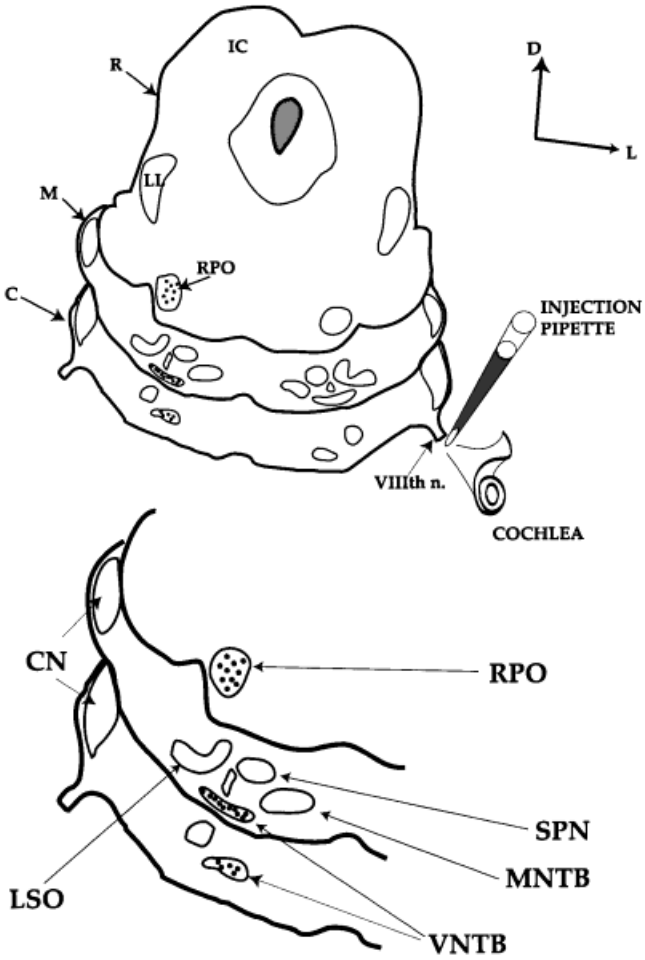


Fig. 1. A semischematic representation of rostral (R), middle (M), and caudal (C) brainstem sections illustrating the retrograde labeling of neuronal cell bodies (solid circles) in the superior olivary complex. These coronal sections are shown with the rostralmost section on top. Injections were made into the contralateral cochlear nerve, as illustrated. The following nonolivary structures are labeled: inferior colliculus (IC), nucleus of the lateral lemniscus (LL), cochlear nucleus (CN), and the eighth cranial nerve (VIIIth n.). The following olivary structures are identified: lateral superior olive (LSO), rostral periolivary (RPO), superior paraolivary nucleus (SPN), ventral periolivary (VNTB), and medial nucleus of the trapezoid body (MNTB). In the upper diagram, the darkly filled, center area represents the rostral portion of the fourth ventrical. Dorsal (D) and lateral (L) directions are as indicated.

ChAT immunoreactivity in periolivary regions

The distribution of ChAT-immunoreactive cell bodies was studied within the superior olive at 12 different postnatal ages. Table 3 summarizes the postnatal distribution of ChAT expression at P0, P4, P12, P20, and P30. Cell bodies in RPO and rostral VNTB regions may be the first to express ChAT immunoreactivity in the superior olivary complex. Similar to previous studies in adult animals,

ChAT$^+$ cells were found only in the LSO, VNTB, RPO, and DPO regions of the superior olive (Altschuler et al., 1985; Vetter et al., 1991; Sherriff and Henderson, 1994). The number of ChAT$^+$ cells at P0 was only half as great as the maximum number of retrogradely cells at P1. In all four P0 animals, the greatest number of ChAT$^+$ cells was present in RPO regions. Motor neurons, such as those in the facial and trigeminal motor nuclei, had a diffuse ChAT immunostaining that ranged from light to dark.

Figure 5A shows that the early ChAT expression in the superior olivary complex sometimes demonstrated an unusual staining pattern. The perikarya of some cells were darkly outlined, and the cytoplasm was stained very lightly. The dark outlines of the perikarya extended into the dendrites as well. These cells were difficult to identify at first, because they resembled fiber plexi and puncta rather than immunostained cell bodies. However, due to the thickness (20 μm) of our brainstem sections, focusing the microscope objective through the tissue section made it possible to distinguish these as immunoreactive perikarya and not fiber plexi. Whether this unusual staining pattern was strictly membrane associated can be assessed further only by study using, for example, electron microscopy. This type of ChAT immunoreactivity was not unique to periolivary regions and was observed later in the initial immunostaining that was found within the LSO (see Fig. 8B).

By P2, the RPO regions contained all darkly labeled cell bodies (Fig. 5B). By P3, ChAT-immunoreactive cell bodies were also labeled darkly and diffusely throughout the entire VNTB. In all cases from P0 through P3, ChAT-immunoreactive cell bodies in these periolivary regions had small, multipolar shapes. These ChAT-immunoreactive cells in both RPO and VNTB were similar in shape and appearance to those found at later ages, as shown in Figure 5C. ChAT$^+$ cell bodies in the RPO and the VNTB, as described previously for retrogradely labeled medial OC neurons, had multipolar shapes with three to four primary dendrites. Rostral periolivary cells were stellate-shaped, whereas VNTB cells had their dendrites aligned parallel to the ventral surface. Although the areas of ChAT$^+$ cell bodies in RPO and VNTB were similar to retrogradely labeled cells, there was no significant change in cell body size until after P12. At P4, P12, and P20, immunoreactive periolivary cells had average cell body areas of 114 ± 41 μm^2 (n = 49), 114 ± 50 μm^2 (n = 48), and 143 ± 53 μm^2 (n = 67), respectively (Fig. 6). Similar to retrogradely labeled cells, the average number of dendrites did not change significantly, as shown in Figure 6. Periolivary cells that were positive for ChAT had an average of 3.5 primary dendrites at P0, 3.2 at P12, and 3.5 at P20. Although the orientation of their dendrites differed, there were no systematic differences in the number of primary dendrites between the RPO and the VNTB.

The correspondence between retrogradely labeled cells and ChAT$^+$ cells was also investigated. Although direct double-labeling studies were not performed, alternate sections cut at 20 μm were processed either for retrograde label or for ChAT immunoreactivity. At P12 and P20, retrogradely labeled cell bodies in periolivary regions had

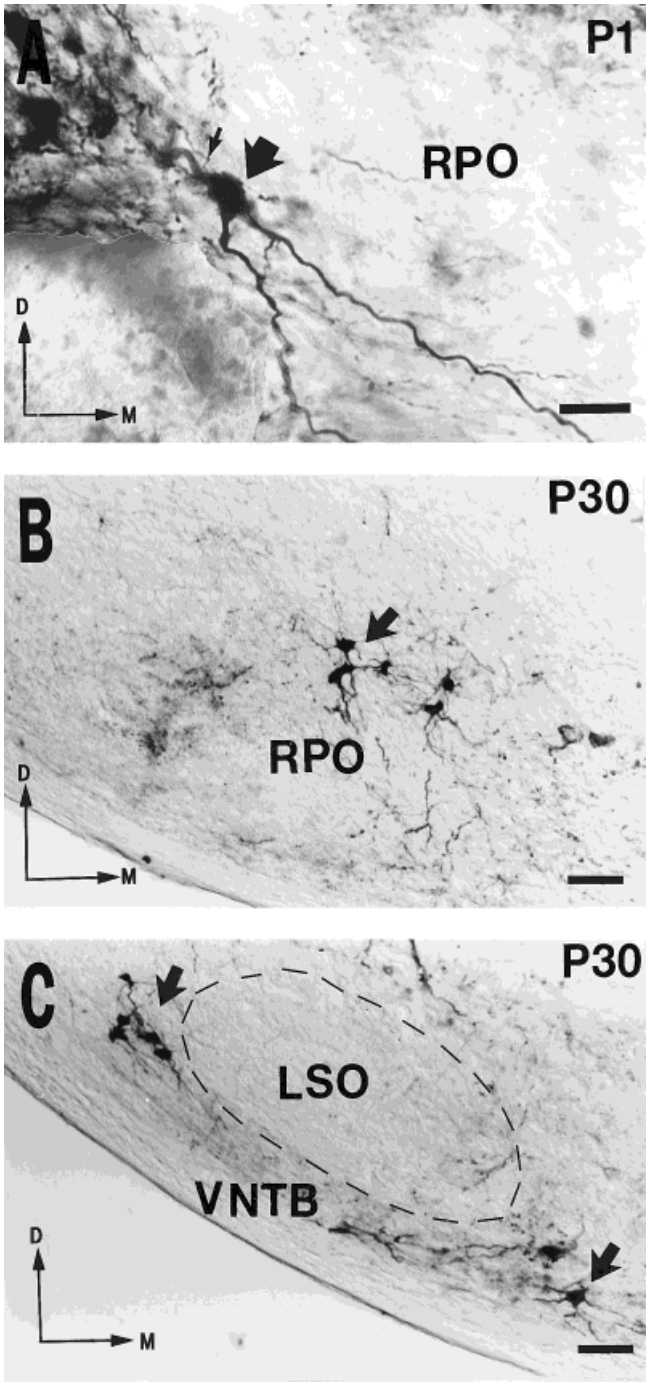


Fig. 2. Photomicrographs of retrogradely labeled cells in periolivary regions of the superior olivary complex. Neurons were labeled retrogradely from tracer injections made into the contralateral cochlea from an in vitro preparation. **A:** Retrogradely labeled neuron (large arrow) in the postnatal day 1 (P1) brainstem. This neuron resides in the rostral periolivary (RPO) region of the superior olivary complex, and it has several large primary dendrites. Fine neuritic extensions (small arrow) are found along the cell body and dendrites. **B:** Retrogradely labeled neurons in the RPO of a P30 brainstem. These retrogradely labeled neurons have multiple, large primary dendrites, and they lack the fine neuritic extensions that are found on cell bodies at P1. **C:** Retrogradely labeled neurons in the ventral periolivary (VNTB) regions. These neurons are taken from a P30 brainstem. Similar to RPO neurons, VNTB neurons have multiple, large primary dendrites but lack any neuritic extensions. The lateral superior olive (LSO) is identified by the dashed outline and is devoid of any retrogradely labeled cell bodies. Scale bars = 20 μm in A, 50 μm in B,C.

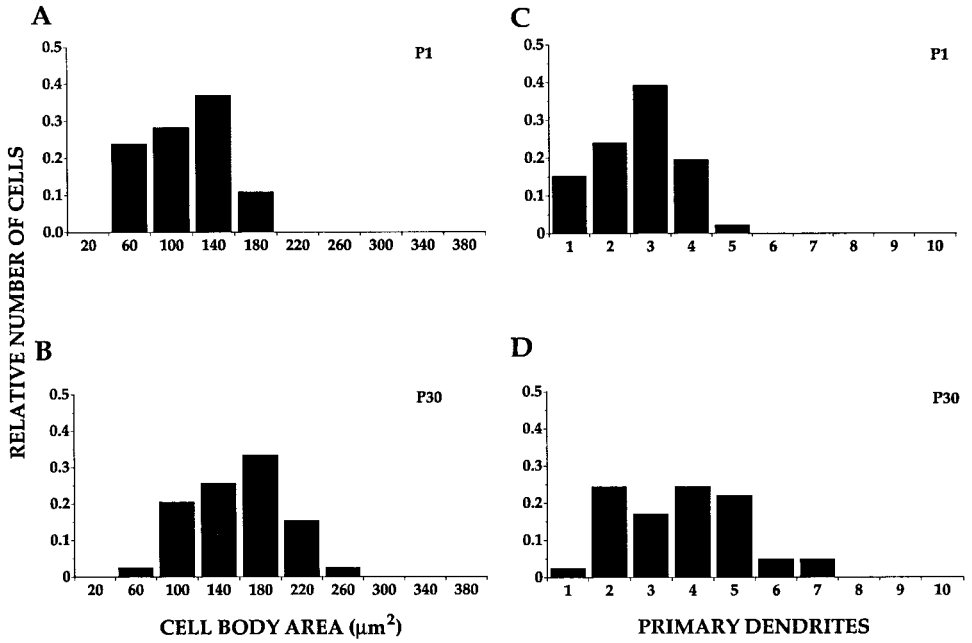


Fig. 3. Histograms of the cell body area and number of primary dendrites at P1 and P30. Images of labeled cells were captured with a CCD camera at 500× magnification. The maximum cell body area was outlined with a cursor on a computer monitor and computed by using NIH Image software (version 1.5x). Only labeled cells with at least one dendrite were measured. For each labeled cell area, the number of primary dendrites was also counted. **A:** Histogram of the relative fraction of cell body areas taken from measurements of retrogradely labeled cell bodies in a P1 brainstem. In total, 104 cell bodies were measured. **B:** Histogram of the relative fraction of cell body areas taken from measurements of retrogradely labeled cell bodies in a P30 brainstem. In total, 63 cell bodies were measured. **C:** Histogram of the relative fraction of primary dendrites at P1. **D:** Histogram of the relative fraction of primary dendrites at P30.

TABLE 2. Distribution of Retrograde Label in the Superior Olivary Complex[1]

Age	Number of labeled cells	% RPO	% VNTB	% LSO
P1	104	5	86	0
P6	80	2	91	1
P12	93	9	89	2
P20	85	8	82	1
P30	63	10	87	0

[1]Summary of the percent distribution of retrogradely labeled cells within the superior olivary complex at the specified postnatal ages. The data given are the cases at each postnatal age that had the highest number of retrogradely labeled cells contralateral to the cochlear injection side. Labeled cells were counted in the rostral periolivary (RPO), the ventral periolivary (VNTB) and the lateral superior olive (LSO). The percent distribution only sums to 100% at postnatal day 12 (P12). At the other postnatal ages, there were always a few cells (ranging from 3% to 9%) found in other periolivary regions. The table summarizes data obtained from 425 retrogradely labeled cells from the best cases across five postnatal ages: P1 (n = 104), P6 (n = 80), P12 (n = 93), P20 (n = 85), and P30 (n = 63).

clearly overlapping distributions with ChAT-immunoreactive cell bodies, and, in several instances, retrogradely labeled and ChAT-immunoreactive cell bodies overlapped directly. Figure 7 illustrates two serial sections that were processed for retrograde label and ChAT immunoreactivity at P12. The overlapping distributions, similar sizes, and similar morphology found within these consecutively processed sections gave us reasonable confidence that the retrogradely labeled neurons were a subset of the total number of ChAT$^+$ cell bodies.

ChAT immunoreactive cells in the LSO

To compare medial and lateral OC development, ChAT immunoreactivity was investigated within the LSO. In all adult animals studied to date, the lateral OC system is composed, in part, of cholinergic neurons that coexpress CGRP (Vetter et al., 1991). Cells that were positive for ChAT were found either within the LSO or closely associated with the LSO. Figure 8 shows that ChAT$^+$ cells within the LSO were consistently smaller and had fewer primary dendrites than ChAT$^+$ cells within periolivary regions. Although there was a statistically significant increase in cell size from P4 (56 $\pm$ 21 μm^2) to P12 (77 $\pm$ 26 μm^2) and from P12 to P20 (102 $\pm$ 42 μm^2), the mean number of primary dendrites remained constant (roughly 2.3) during the same period.

Significantly, ChAT immunoreactivity was not expressed within the LSO prior to P3 (Fig. 8A). Similar to the earlier appearance of some ChAT-immunoreactive cells in periolivary regions at P0 (see Fig. 6A), a number of ChAT$^+$ LSO cells had dark perikaryal outlines rather than the more typical diffuse label (Fig. 8B). After P6, ChAT-immunoreactive cells within the LSO were all diffusely labeled and had bipolar-to-fusiform shapes characteristic of immunolabeled cells in the adult LSO (Fig. 8C).

OC efferent terminals in the cochlea

To investigate the relationship between OC development in the brainstem and OC innervation in the cochlea, we took advantage of the crossed OC pathway that runs from cell bodies in periolivary regions of the superior olive to the cochlea (Rasmussen, 1960; Robertson et al., 1989; Simmons et al., 1996a). Tracer injections were made into the crossed OC bundles (see Materials and Methods), and the morphology of anterogradely labeled OC terminals was studied along with the expression of ChAT immunoreactivity. Similar to previous reports (Simmons et al.,

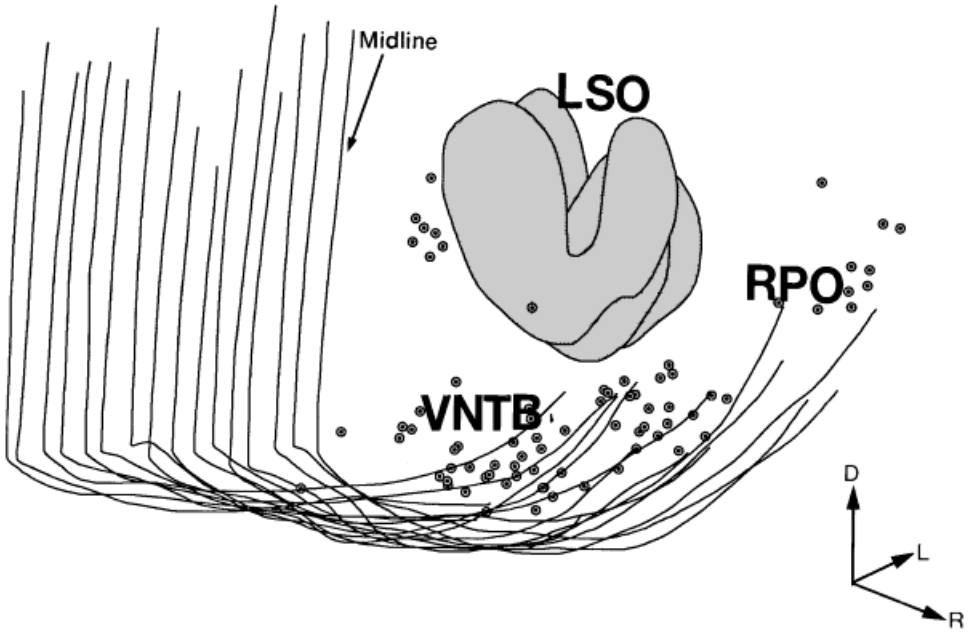


Fig. 4. Computer reconstruction (rostral to caudal) of retrogradely labeled cells in the superior olivary complex. In total, 17 serial brainstem sections were reconstructed. Sections are spaced 50 μm apart. The lateral superior olive (LSO) has been reconstructed only partially. Spheres identify the positions of retrogradely labeled cells and are found mostly in ventral periolivary (VNTB) and rostral periolivary (RPO) regions. The sections are from a P12 animal, with a total of 80 retrogradely labeled cells from an injection placed in the contralateral cochlea. The midline of each section is shown. Lateral (L), dorsal (D), and rostral (R) directions are as indicated (magnification, roughly 100×).

TABLE 3. Choline Acetyltransferase Immunoreactivity in the Postnatal Superior Olivary Complex[1]

Age	Total number of cells (mean ± S.D.)	% RPO (mean ± S.D.)	% VNTB (mean ± S.D.)	% LSO (mean ± S.D.)
P0	44 ± 6	65 ± 7	35 ± 5	0
P4	96 ± 31	9 ± 8	26 ± 5	58 ± 10
P12	164 ± 12	12 ± 3	23 ± 6	68 ± 3
P20	112 ± 56	8 ± 5	25 ± 8	68 ± 4
P30	181 ± 10	4 ± 2	16 ± 1	79 ± 5

[1]Summary of the percent distribution of choline acetyltransferase (ChAT)-positive cell bodies in various superior olivary regions at the specified postnatal ages. Only the best case at each age was analyzed. Best cases were those that could be reconstructed completely and had the highest signal-to-background labeling. The numbers given represent the mean ± standard deviation of both sides of the brainstem. For abbreviations, see Table 2.

1996a), efferent terminals were anterogradely labeled as early as P1 from injections into the crossed OC bundles. Such injections resulted in labeled efferent terminals in the cochlea and cell bodies in periolivary regions of the superior olivary complex, as noted previously (Simmons et al., 1996a). During the period from P1 through P5, anterogradely labeled terminals formed a dense plexus of fibers spiraling underneath inner hair cells, as shown in Figure 9A. These fibers, as they spiraled below inner hair cells, gave rise to periodic en passant swellings as well as terminal swellings. However, no growth cones were observed. Anterogradely labeled fibers terminated on outer hair cells by P6. Figure in 9B shows that, by P20, injections into the crossed OC bundle exclusively labeled fibers that terminated on outer hair cells.

The earliest demonstration of ChAT immunoreactivity in the cochlea was in one animal at P2. By P3, ChAT immunoreactivity was present routinely in basal regions of the cochlea in all animals. This immunoreactivity was confined initially to the area below inner hair cells through P6 (Fig. 10A). In cross sections of the organ of Corti, ChAT immunoreactivity was typically seen as very diffuse label, although discrete terminals were visible occasionally. The earliest demonstration of ChAT immunoreactivity below the outer hair cells occurred around P7 and was present routinely by P10 in all animals. Between P10 and P20, there were no obvious changes in ChAT expression: Immunoreactivity was visible as more or less diffuse labeling below inner hair cells and discretely labeled terminals on the outer hair cells (Fig. 10B,C). The pattern of anterograde labeling and immunoreactivity is summarized in Table 4. Although it is delayed by 1–2 days, the chronological sequence of ChAT expression in the cochlea parallels its expression in the brainstem superior olive.

DISCUSSION

In the postnatal hamster, the present study suggests that medial (periolivary) OC neurons may express ChAT either prior to or simultaneously with their axons accumulating below inner hair cells. As early as P1, medial OC cell bodies are found in rostral and ventral periolivary regions of the superior olive, and efferent terminals are found below inner hair cells. Very different from adult innervation patterns, these results support the hypothesis that medial OC neurons provide a perinatal innervation to the inner hair cell area (Simmons et al., 1996a). It is also clear from the data presented that periolivary neurons are among the first cells within the superior olivary complex to express ChAT immunoreactivity. The expression of ChAT within the LSO occurs after ChAT expression within periolivary regions. The strong correspondences observed between retrogradely labeled cell bodies and ChAT-

immunoreactive cells further support the notion that the medial OC system is responsible for an early expression of ChAT, whereas the lateral OC system is responsible for a later expression of ChAT. Such correspondences raise questions about the relationship between contact with peripheral targets, the expression of ChAT, and the potential release of acetylcholine prior to synapse formation.

If the above notion of the ontological expression of ChAT in the OC system is true, then it leads to several predictions. The first prediction is that similar expression patterns should be found within the cochlea. This prediction was tested in the present study. The pattern of expression found in the cochlea did parallel that found in the brainstem: ChAT is expressed initially below inner hair cells followed by ChAT expression below outer hair cells. A hypothesized relationship between cochlear innervation and the neurochemical expression of ChAT is shown schematically in Figure 11. Although the expression pattern in the cochlea parallels the pattern found in the brainstem, there was a delay between periolivary onset and cochlear onset. Several scenarios can be suggested for the observed delay between ChAT expression in the brainstem and in the cochlea. First, it is possible that the delay is an artifact of our immunologic technique. For example, although the antibody technique used is sensitive enough for detecting early expression on brainstem sections, it may not be sensitive enough to detect the presumably lower concentrations that would be found in the cochlear terminals. Second, it is possible that the delay is a developmental phenomenon. For example, ChAT could be produced first in cell bodies but not transported down axons for several days. Alternatively, it is possible that ChAT may undergo some type of unmasking: that is, a developmental modification in epitope. Third, it is possible that the first cells to express ChAT in rostral periolivary regions may project instead to some other region, such as the cochlear nucleus. Extensive investigations in adult animals suggest that the majority, if not all, of the cholinergic neurons found in the superior olivary complex send descending projections to either the cochlea or the cochlear nucleus (Osen et al., 1984; Godfrey et al., 1987a,b). Specifically, large, darkly labeled, ChAT$^+$ neurons located in the VNTB and RPO regions are suggested to project to the cochlea (Vetter and Mugnaini, 1990, 1992; Vetter et al., 1991). However, this suggestion has never been studied developmentally. Medial OC neurons, which express ChAT in VNTB at P2, may be responsible for the early ChAT expression found below inner hair cells in the cochlea also at P2. Nonetheless, our results make it less likely that the earliest ChAT expression found in the cochlea is due to lateral OC neurons, because the earliest expression in the LSO was not until P3, at the time when ChAT expression in the RPO and VNTB was mature. Studies of ChAT gene expression using in situ hybridization techniques may shed additional light on the issue of the onset of ChAT expression in the various regions of the superior olive.

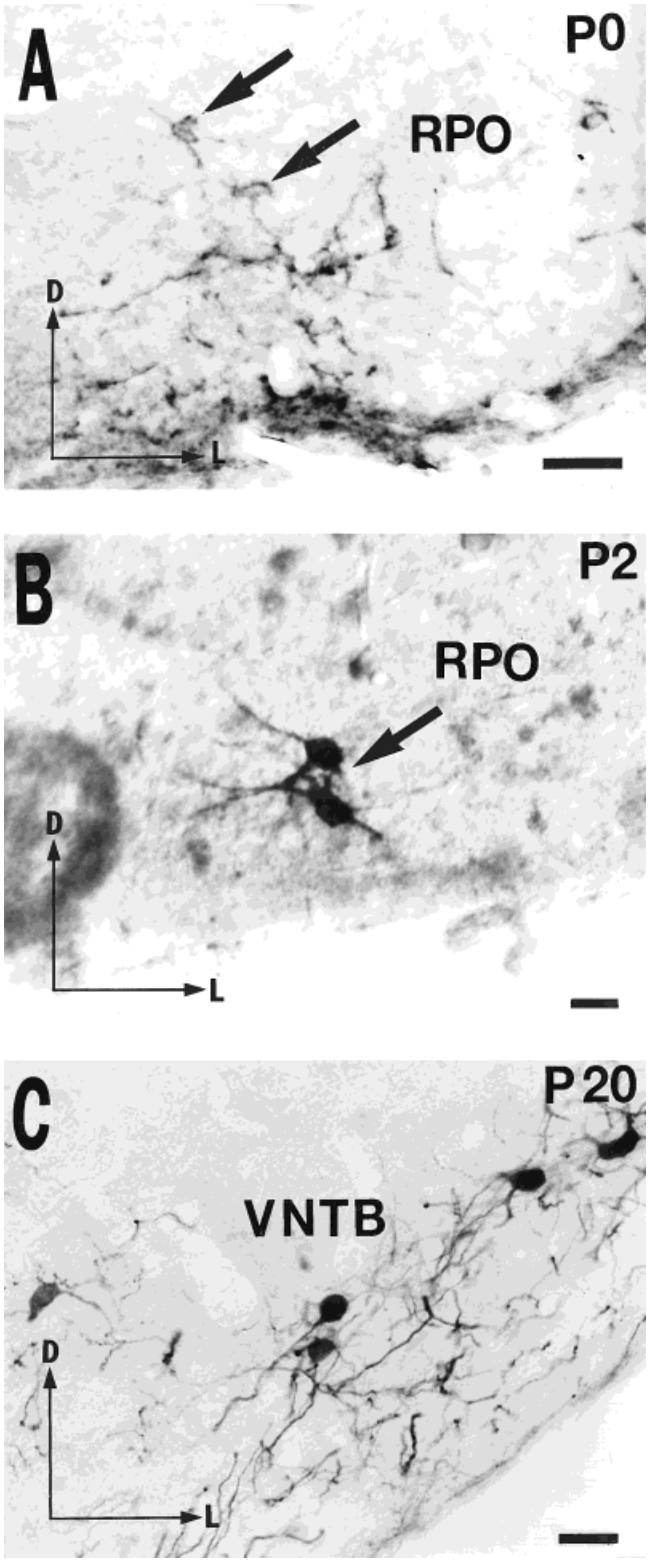


Fig. 5. Photomicrographs of choline acetyltransferase-positive (ChAT$^+$) cells in periolivary regions of the superior olivary complex at various postnatal ages. **A:** Photomicrograph of ChAT$^+$ cells (arrows) taken from a P0 animal in rostral periolivary (RPO) regions. These cells have darkly outlined perikarya and lightly immunostaining cytoplasm. They are clearly identified as labeled cells by focusing through the section. **B:** Photomicrograph of ChAT$^+$ cells (arrow) taken from a P2 brainstem section. The labeled cells are located in the RPO. All labeled cells in the RPO at P2 are diffusely labeled with darkly immunostaining cytoplasm. **C:** Photomicrograph of ChAT$^+$ cells taken from a P20 brainstem section. The cells are located in the ventral periolivary (VNTB) region. Lateral (L) and dorsal (D) directions are as indicated. Scale bars = 30 μm in A, 10 μm in B, 40 μm in C.

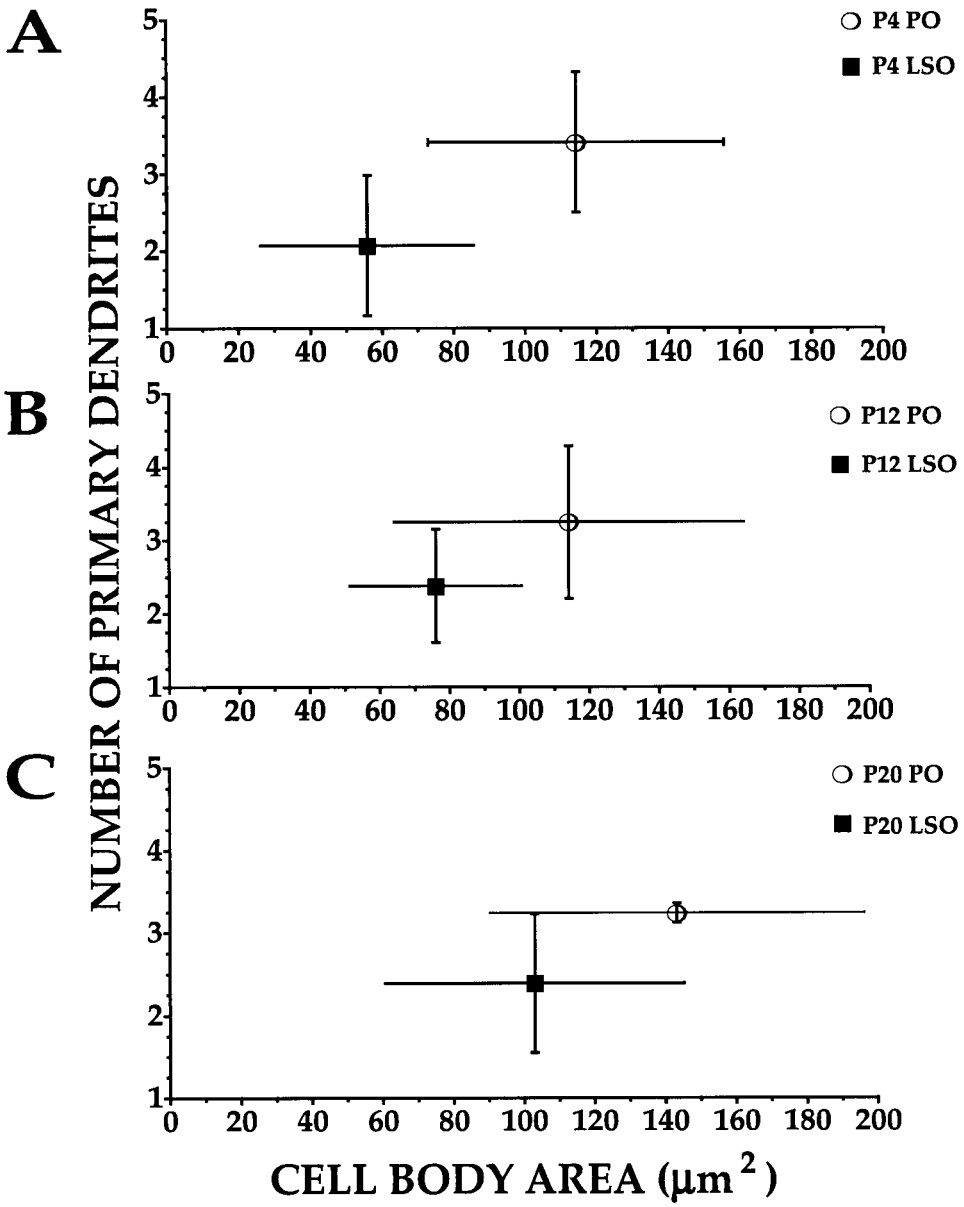


Fig. 6. The mean number of primary dendritic branches is plotted against the mean cell body area for choline acetyltransferase-positive ($ChAT^+$) cells in periolivary (PO) and lateral superior olive (LSO) regions at different postnatal ages. Only labeled cells that had at least one visible dendrite were analyzed. Error bars represent the standard deviation. **A:** $ChAT^+$ cells at P4 within the PO region (n = 49) and the LSO (n = 88). **B:** $ChAT^+$ cells at P12 are plotted from the PO region (n = 48) and the LSO (n = 125). **C:** $ChAT^+$ cells at P20 are plotted from the PO region (n = 67) and the LSO (n = 123).

Although this study is one of the first investigations of ChAT expression during postnatal development within the auditory brainstem, the onset of transmitter substances associated with cholinergic terminals in the cochlea has been investigated previously in the mouse and rat. In mice, Sobkowicz and coworkers (Sobkowicz and Emmerling, 1989; Emmerling et al., 1990) examined the enzyme activity and morphological differentiation of ChAT and acetylcholine esterase (AChE) at various postnatal ages. At birth, there was 20% activity of AChE and 50% activity of ChAT compared with adult values. The activity of ChAT rose to adult levels by P15 and remained unchanged afterward. The activity of AChE rose to adult levels by P25. Furthermore, those investigators were able to demonstrate that efferent fibers positive for either ChAT or AChE were present under inner hair cells before birth, crossed the tunnel of Corti after birth, and were present in all outer hair cell rows before the first vesiculated endings were morphologically discernible in the electron microscope. Similar to the pattern and sequence found in hamsters, these investigations suggests that the neurochemical maturation of efferent fibers either precedes or roughly coincides with morphological differentiation in the neonatal cochlea.

In the rat, there have been conflicting reports regarding the onset of ChAT expression. In one of the earliest

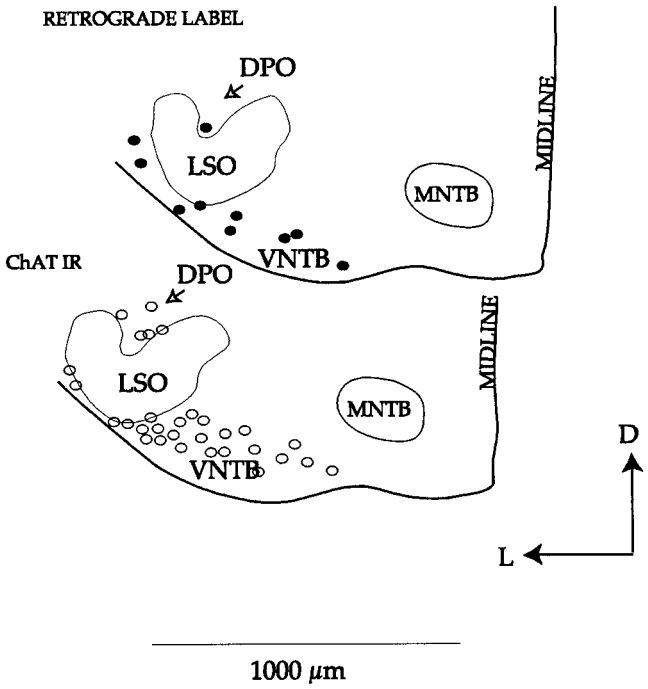


Fig. 7. Semischematic representation of two consecutive brainstem sections at P12 illustrating the correspondence between retrogradely labeled cells (solid circles) and choline acetyltransferase (ChAT)-immunoreactive cells (open circles) in periolivary regions. After a contralateral cochlear injection of tracer solution, alternate sections were treated either histochemically to visualize retrograde label or immunocytochemically to visualize ChAT$^+$ cells. Although ChAT immunoreactivity was also found in the lateral superior olive (LSO) at this postnatal age, this computer reconstruction of consecutive sections shows only ChAT immunoreactivity in periolivary regions for direct comparison purposes. The regions identified within the superior olivary complex are the LSO, the medial nucleus of the trapezoid body (MNTB), the ventral periolivary (VNTB) region, and the dorsal periolivary (DPO) region.

investigations of ChAT expression in neonatal rats, Roth et al. (1991) could not detect ChAT immunoreactivity in surface preparations of the rat cochlea until P20. However, a more recent investigation suggests that ChAT immunoreactivity is present at birth. By using cyrostat sections of the cochlea, Merchan-Perez et al. (1994) found ChAT immunoreactivity below the inner hair cells at birth and below the outer hair cells by P3. The study by Merchan-Perez et al. is very consistent with the developmental pattern and sequence found to date in mice and now in hamsters. The discrepancies between Roth et al. and Merchan-Perez et al. are likely due to methodological variables, such as fixation, antibody, and tissue preparation.

Efferent waiting period

Both the ChAT expression patterns and our anterograde labeling patterns are consistent with the idea that effer-

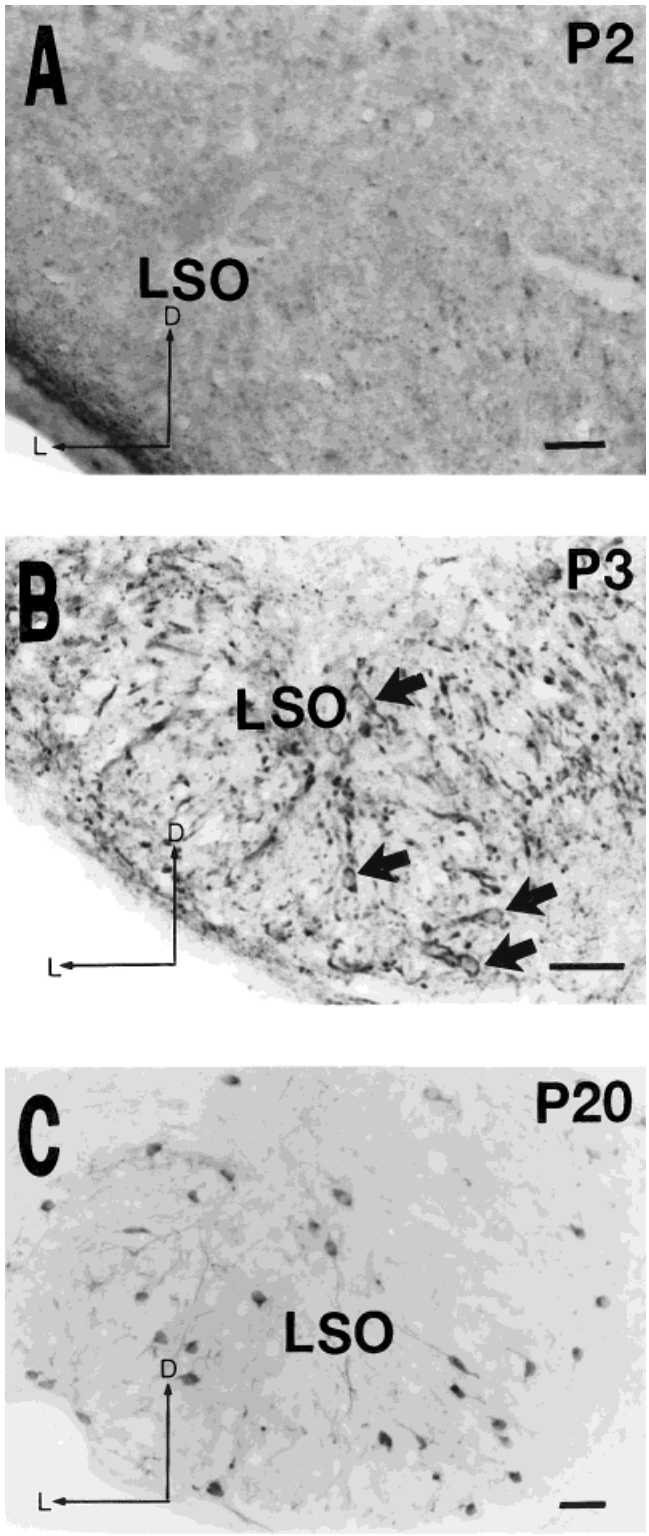


Fig. 8. Photomicrographs of choline acetyltransferase (ChAT)-immunoreactive cells in the lateral superior olive (LSO) at various postnatal ages. **A:** Photomicrograph of the LSO taken from a P2 brainstem section. Although there were darkly labeled ChAT$^+$ cells in periolivary regions, none was visible within the LSO. **B:** Photomicrograph of ChAT$^+$ cells taken from a P3 brainstem section. The labeled cells are located in the LSO. These cells have darkly outlined perikarya and lightly immunostaining cytoplasm. They are clearly identified as labeled cells by focusing through the sections. **C:** Photomicrograph of ChAT$^+$ cells taken from a P20 brainstem section. All labeled cells in the middle LSO are diffusely labeled with darkly immunostaining cytoplasm. Lateral (L) and dorsal (D) directions are as indicated. Scale bars = 25 μm in A, 30 μm in B, 50 μm in C.

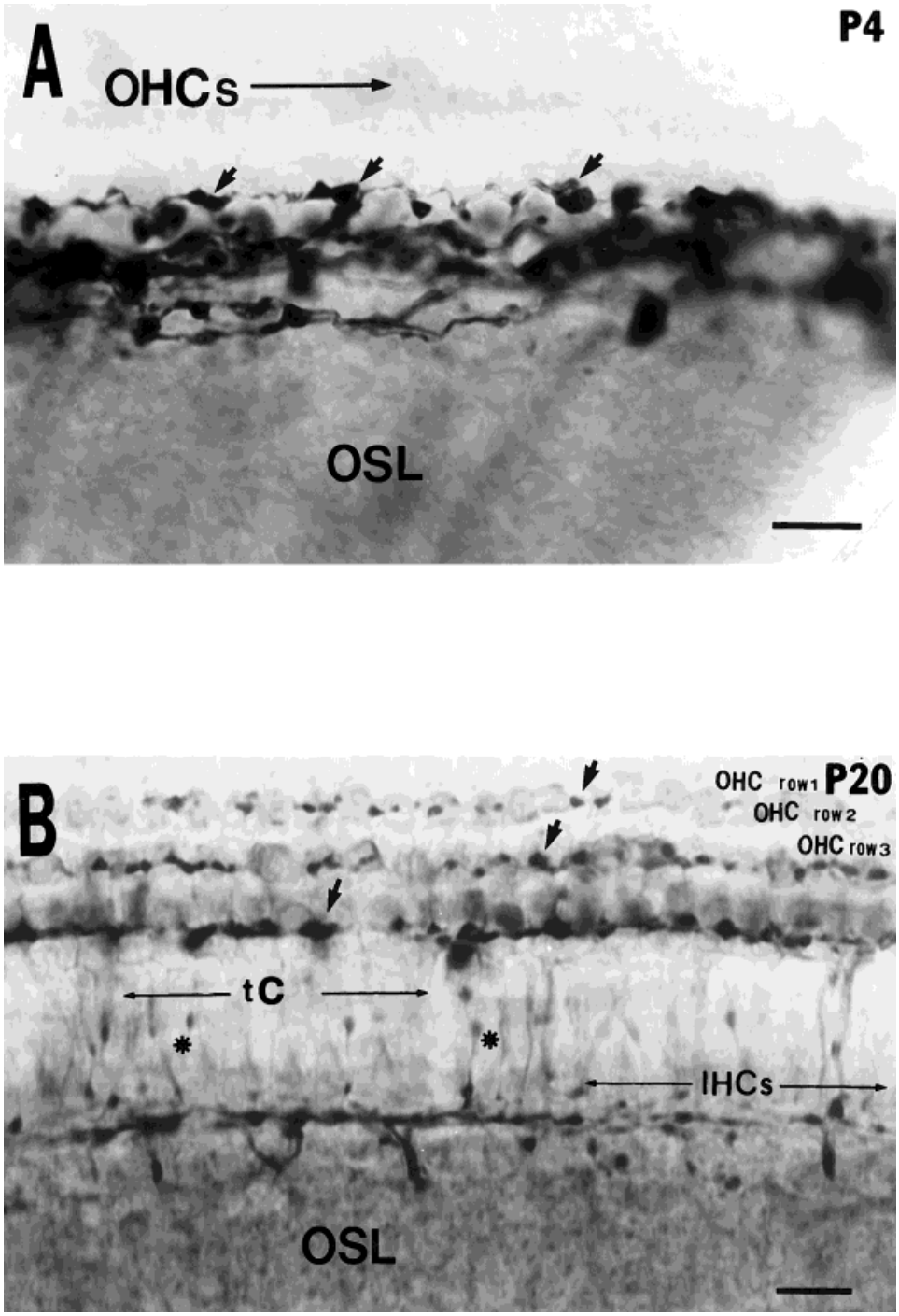


Fig. 9. Photomicrographs taken of anterogradely labeled terminals in the cochlea. At the postnatal ages indicated, tracer injections were made into the crossed olivocochlear (OC) bundles at the floor of the fourth ventricle in an in vitro preparation of the brainstem (see Materials and Methods). **A:** Photomicrograph of a surface view taken from a P4 cochlea in the basal region. Labeled fibers spiraled some distance below inner hair cells (IHCs) before terminating. Short arrows indicate terminal swellings and en passant swellings below IHCs. No labeled fibers or terminals were found in the outer hair cell (OHC) region. The osseous spiral lamina (OSL) is also identified. **B:** Photomicrograph of a surface view from a P20 cochlea taken from the basal region. Labeled fibers spiraled some distance below IHCs without any obvious terminations and then crossed the tunnel of Corti (tC; asterisks) to terminate on OHCs. Short arrows indicate terminal swellings on or below OHCs. There were no terminal swellings visible on or below the IHCs. Scale bars = 20 μm.

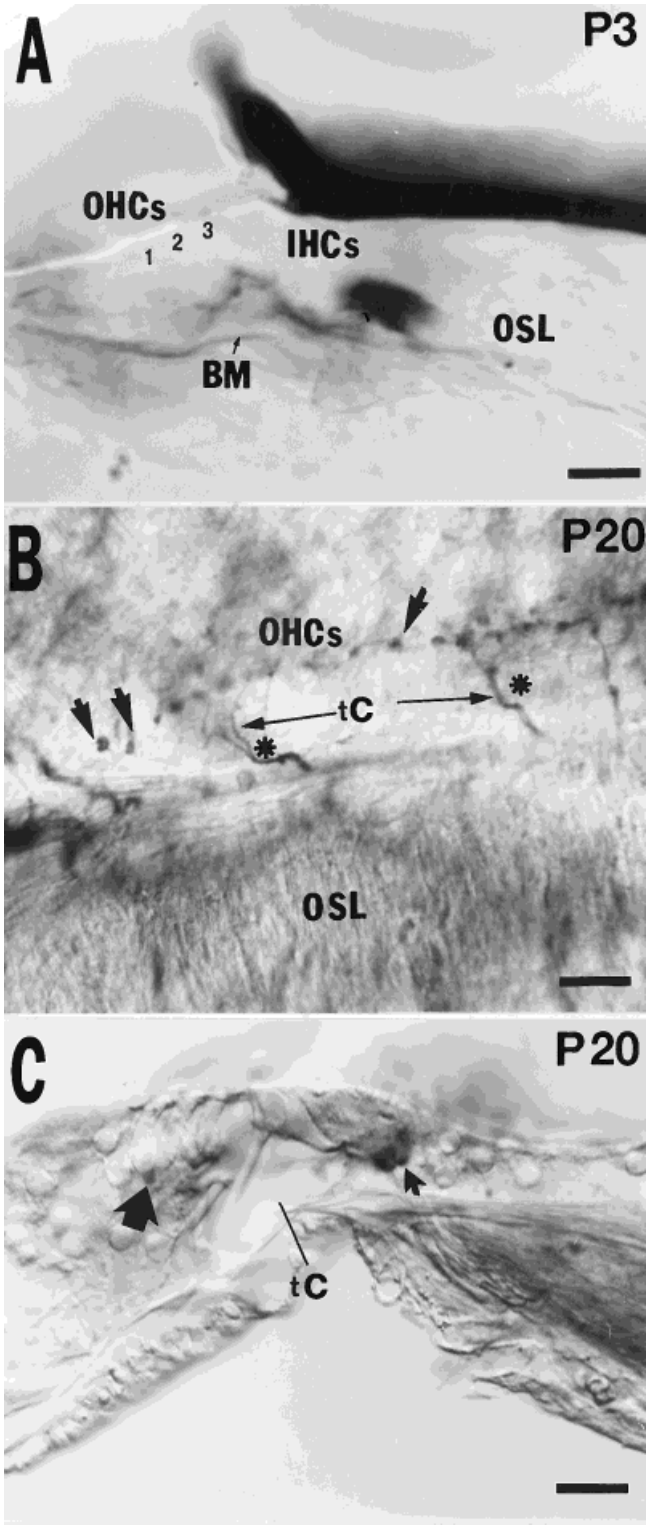
ents exhibit a developmental waiting period below inner hair cells (Simmons et al., 1996a). By P2, ChAT is expressed in the brainstem only by periolivary cells, and, in the cochlea, it is expressed only below inner hair cells. In our anterograde labeling studies, we observe that efferent fibers and their terminals transiently accumulate below

inner hair cells through P5 and then accumulate below outer hair cells from P5 through P10. That this transient accumulation of efferent fibers and terminals below inner hair cells is from medial OC neurons can be inferred from 1) the retrograde and anterograde labeling pattern of OC cell bodies and axons and 2) the coincidence of ChAT expression patterns in the superior olive and the region immediately below inner hair cells. These results lead us to hypothesize that medial OC neurons send terminals to the inner hair cell region and begin expressing ChAT well before they terminate on outer hair cells (see Fig. 11). Because ChAT expression by LSO cells is delayed compared with periolivary expression, lateral OC synaptogenesis in the cochlea may also be similarly delayed. However, our data do not exclude the possibility that medial and lateral OC axons may have overlapping projections to the inner hair cell region during this period.

TABLE 4. Efferent Anterograde Label and Immunoreactivity[1]

Age	Inner hair cell terminals	Outer hair cell terminals	Inner hair cell choline acetyltransferase	Outer hair cell choline acetyltransferase
P0	+	—	—	—
P1	+	—	—	—
P3	+++	—	+	—
P4	+++	—	++	—
P5	+++	+	++	—
P6	++	+	++	—
P7	+	++	+++	+
P10	+	+++	+++	++
P20	—	+++	+++	++

[1]Summary of anterograde labeling and choline acetyltransferase immunoreactivity below inner and outer hair cells at the postnatal ages specified. Four categories indicate the relative amount of label for each of the labeling conditions: not present (—), weak (+), moderate (++), or strong (+++).



Similar anterograde labeling patterns have been obtained previously in both the hamster and the rat (Simmons et al., 1990, 1996a; Cole and Robertson, 1992). For example, in a semiquantitative study of efferent innervation patterns in neonatal hamsters, we first suggested a developmental waiting period after injections into the crossed OC bundle gave rise to terminals on or below inner hair cells between P1 and P4 but failed to demonstrate any terminals on outer hair cells prior to P5 (Simmons et al., 1996a). Importantly, this study provided ultrastructural evidence that labeled efferent terminals contacted inner hair cells prior to P5. Similar findings were obtained in a study conducted in neonatal rats. Cole and Robertson (1992) applied 1,1′dioctadecyl-3,3,3′-tetramethylindocarbocyanine perchlorate (DiI) to the cut central ends of crossed OC bundle axons and anterogradely labeled their terminations in the organ of Corti. Cole and Robertson found that efferent fibers had massive projections to inner hair cells first (P1–P2) and then, at later postnatal ages (P4–P6), projected to outer hair cells. Recently in mice, an embryonic efferent projection to inner hair cells has been described that is consistent with studies in neonatal rats

Fig. 10. Photomicrographs of choline acetyltransferase (ChAT)-immunoreactivity in the cochlea. **A:** Cross section from the basal region of a P3 cochlea showing diffuse ChAT label below the inner hair cells (IHCs). The outer hair cell (OHC) region did not show any label. In this particular case, a dark reaction seen in the tectorial membrane was due to the nonspecific staining of the secondary antibody. However, the osseous spiral lamina (OSL) remains unstained except in the nerve tract. The tunnel of Corti does not open until after P5. Arrow identifies the basilar membrane (BM). **B:** Photomicrograph of a surface view of the organ of Corti taken from the basal region of a P20 cochlea. $ChAT^+$ fibers can be seen traversing the OSL, crossing below the IHCs, crossing the tunnel of Corti (tC; asterisks), and terminating (short arrows) on OHCs. **C:** Photomicrograph taken of a cross section from the basal region of a P20 cochlea showing $ChAT^+$ puncta below the OHCs (thick arrow) and diffuse label below the IHCs (thin arrow). The tunnel of Corti (tC) is also identified. Scale bars = 25 μm.

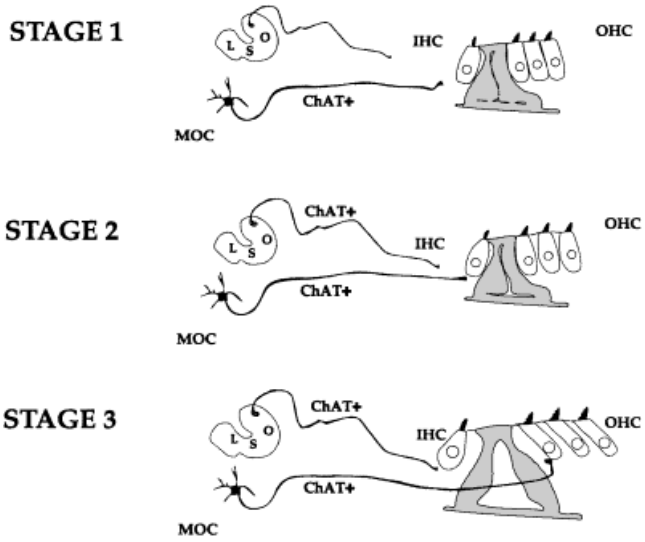


Fig. 11. Schematic of a simplified relationship between choline acetyltransferase (ChAT) expression and cochlear innervation patterns by olivocochlear (OC) neurons. During stage 1, medial OC neurons begin their expression of ChAT as they innervate the inner hair cell (IHC) region of the organ of Corti, whereas lateral OC neurons are negative for ChAT. During stage 2, there is increased ChAT expression in medial OC neurons and the initiation of ChAT expression within lateral OC neurons. During stage 3, medial OC axons begin to terminate below outer hair cells (OHCs), during which time ChAT expression also occurs below OHCs. After stage 3, both medial and lateral OC neurons appear adult-like in their neurochemical expression and morphology.

and hamsters (Bruce et al., 1997). These studies, combined with the present results, strongly support the notion that medial OC fibers via the crossed OC bundle stop for a period of time near inner hair cells before going on to terminate on outer hair cells.

Previous studies have also reported transient developmental features attributed to efferent axons and their terminals as they innervate the cochlea. For example, Golgi studies show efferent endings directly on inner hair cells as well as efferents sending branches to both inner and outer hair cells (Perkins and Morest, 1975; Ginzberg and Morest, 1983). These features rarely persisted in the mature cochlea and were considered transient. However, most developmental studies presume that the early efferent endings on or below inner hair cells derive from lateral OC neurons, which, in adults, give rise to the fine spiral terminals of the inner spiral bundles (Shnerson et al., 1982a,b; Despres et al., 1988; Gil-Loyzaga and Pujol, 1988; Emmerling et al., 1990; Merchan-Perez et al., 1990, 1993). This assumption is based almost entirely on electron microscopic studies and has not been tested until recently (Kikuchi and Hilding, 1965; Pujol et al., 1979; Pujol and Carlier, 1982; Ginzberg and Morest, 1984; Pujol and Lavigne-Rebillard, 1985). For our data to be consistent with this interpretation would imply that both our immunocytochemical and our tracer methods are capable of labeling lateral OC axons but not their cell bodies.

Several physiological studies (Carlier and Pujol, 1976; Walsh and McGee, 1988, 1997; Walsh and Romand, 1992) have suggested that the OC efferent system is active prior to synapse formation on outer hair cells. In kittens, it is possible to elicit a cochlear potential response with intense sound just prior to when OC efferents contact outer hair cells (Carlier and Pujol, 1976; Pujol et al., 1979; Gil-Loyzaga and Pujol, 1988). Stimulation of the crossed OC bundle in the brainstem increases this response (Carlier and Pujol, 1976). This result was originally interpreted as a possible lateral OC effect on inner hair cells. However, in light of our recent data, the response may be due at least in part to the contribution of the transient medial OC innervation below inner hair cells. Furthermore, other recent studies have suggested that OC efferents may be responsible for the maturation of afferent responses (Walsh and Romand, 1992; Walsh and McGee, 1997). For example, sectioning of the crossed OC bundles gives rise to increases in asynchronous responses of afferent nerve fibers in neonatal kittens. Although these physiological studies have been performed in neonatal kittens, there is every reason to believe that the efferent system in kittens undergoes a maturation similar to that found in rodents; that is, efferent terminals are found on or below inner hair cells prior to their synaptogenesis on outer hair cells (Pujol et al., 1978, 1979; Ginzberg and Morest, 1984). Taken together, the physiological and anatomical data raise the possibility that the proposed developmental waiting period may play an important role in the maturation of cochlear afferent responses.

Consistent with a role in cochlear physiology, another possibility is that efferent axons wait below inner hair cells until outer hair cells have matured either structurally or physiologically. Elsewhere, developmental waiting periods have been correlated with a temporal mismatch between incoming axonal projections and the maturation of the final targets (Shatz et al., 1990). Developmental waiting periods have been best characterized between the cortical neurogenesis and incoming thalamic projections. Not only do thalamic axons accumulate within the intermediate zone immediately below the immature cortical plate, but they also make temporary synaptic connections on subplate neurons. During cochlear development, the maturation of outer hair cells lags behind inner hair cells, and several maturational changes occur within outer hair cells coincident with efferent synaptogenesis (Pujol and Abonnenc, 1977; Pujol et al., 1978, 1979, 1980; Pujol and Carlier, 1982). In addition to changes in shape, ultrastructure, innervation, and relation to supporting cells, outer hair cells begin having motile responses at the time of efferent innervation (Pujol et al., 1980, 1991). Because medial OC efferents are believed to modulate outer hair cell motility, it is possible that medial OC axons wait below inner hair cells until the cellular components of this motility are in place.

Cholinergic synaptogenesis

In cultured motor neurons, the ability to synthesize acetylcholine and, thus, the expression of ChAT precedes the morphological maturity of synapses. Such studies show that growth cones release acetylcholine prior to any synapse formation (McManaman et al., 1988; Rabinovsky et al., 1992). Although the expression of immunoreactivity does not necessarily imply activity, the first expression of ChAT within nerve terminals is consistent with the ability to synthesize and release acetylcholine (Haydon and Drapeau, 1995). It is conceivable that, as OC neurons innervate their cochlear targets, they could be exerting some neurotrophic effect on cochlear hair cells similar to the manner in which motor neurons affect muscle cells.

Knowing the developmental profile of ChAT activity within OC neurons in the hamster would provide some resolution to the question of whether ChAT is capable of synthesizing acetylcholine. In addition, knowing when OC terminals express proteins associated with presynaptic mechanisms of release would provide further clues about the capacity for acetylcholine release.

In the hamster cochlea, growth associated protein (GAP-43), a marker for growing axon terminals, has been detected below inner hair cells as early as P2, whereas synaptophysin, which is a marker for mature synapses, has been detected below inner hair cells as early as P4 (Simmons et al., 1996b). These markers are typically associated with efferent terminals in the cochlea (Merchan-Perez et al., 1993; Simmons et al., 1996b). Thus, the initial period of efferent accumulation below inner hair cells has characteristics of growing axons, whereas the later period of accumulation below inner hair cells has characteristics consistent with mature synapses. The presence of synaptophysin provides further evidence that the nerve terminals below inner hair cells have synapse-like characteristics and may be capable of neurotransmitter release. If synapses are present on the inner hair cells, then do inner hair cells express appropriate postsynaptic receptors, and are these receptors capable of generating some type of postsynaptic response? Evidence for acetylcholine release would further raise the possibility that there are either neurotrophic or neurotransmitter receptors present on hair cells.

Our observations raise several questions about the extrinsic or intrinsic factors that influence ChAT expression and the level of enzyme activity once ChAT is expressed. Is it possible that the signals that induce efferent fibers to grow toward outer hair cells also induce ChAT activity once these fibers arrive in the cochlea? With the possible exception of nerve growth factor receptors, very little information is known about the factors involved in the efferent innervation of the cochlea (Despres et al., 1991; Fritzsch, 1996). Because OC neurons may be derived from the same embryological source as facial motor neurons, the same or similar factors that support motor neurons developmentally might be involved with OC neurons (Fritzsch, 1996). Studies involving the colocalization of cholinergic markers, synaptic markers, and/or growth factors and receptors within crossed OC bundle terminals need to be carried out in order to address what factors play a role in the onset of cholinergic function and innervation.

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