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Developmental segregation in the afferent projections to mammalian auditory hair cells

(cochlea/neuronal development/auditory system)

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ABSTRACT **The mammalian ear contains two types of auditory receptors, inner and outer hair cells, that lie in close proximity to each other within the sensory epithelium of the cochlea. In adult mammals, these two classes of auditory hair cells are innervated by separate populations of afferent neurons that differ strikingly in their cellular morphology and their pattern of arborization within the cochlea. At present, it is unclear when or how these distinctive patterns of cochlear innervation emerge and become segregated during development. In the present study, an *in vitro* horseradish peroxidase labeling method was used to examine the formation of individual auditory neuron arbors at the same location within the apex of the developing gerbil cochlea. At birth, most cochlear neurons displayed peripheral arbors that embraced both inner and outer hair cell receptors. During the next 6 days, however, the arbors of individual cochlear afferents become confined to either the inner or outer hair cell zone, and thus there is a complete segregation of afferent innervation. This neural segregation occurs principally through the withdrawal of inappropriate connections to the outer hair cell system and is completed well before hearing commences.**

A central goal of neurobiology is to determine how developing neurons choose their specific synaptic partners. An excellent system for examining this issue is the mammalian auditory endorgan, the cochlea, since its pattern of innervation is among the most precise of any sensory or motor system. The adult cochlea contains two types of auditory receptors, termed inner (IHCs) and outer (OHCs) hair cells, which receive separate and highly distinctive patterns of afferent innervation. Ninety percent of all cochlear afferents project to individual IHCs via thick unbranched radial afferents, each of which terminates in a single synapse. The OHCs, on the other hand, receive a more diffuse innervation from a relatively few thin spiral afferents, each of which projects for hundreds of micrometers along the length of cochlea before branching to contact from 5 to 50 receptors (1–8).

Ultrastructural studies have provided evidence for substantial ontogenic changes in the form, number, and spatial arrangement of synapses between mammalian auditory receptors and cochlear nerve fibers (9–16). To understand the process by which these synaptic patterns are adjusted during development, however, it is necessary to examine the early morphological changes that occur in the formation of individual auditory neuron arbors. Such experiments present several difficulties. (*i*) The developing ear is ensheathed in cartilage and lodged in a cavity of the temporal bone and is, therefore, hard to access for neural tract-tracing studies. (*ii*) More importantly, structural development within the mammalian cochlea proceeds from base to apex (17, 18). Therefore, to avoid confounding spatial with temporal gradients in maturation, it is essential to label auditory nerve fibers at the same location within the developing ear.

Most previous studies have labeled auditory nerve fibers in developing mammals by the Golgi method (19–25), a technique that does not permit labeling of neurons at predefined locations within the cochlea. Consequently, the process of cochlear innervation remains obscure since the few available data on the formation of mammalian auditory neuron arbors have been collected from a variety of cochlear locations and in a variety of species that differ markedly in their rates of cochlear maturation.

In the present investigation, I have employed an *in vitro* horseradish peroxidase (HRP) method that permits labeling of small populations of auditory nerve fibers at any location within the developing cochlea. The Mongolian gerbil was selected for study since this species displays a prolonged period of postnatal cochlear maturation. In addition, the gerbil cochlea, like that of the mouse (26), lengthens <10% after birth, from an average of 10.8 mm in the newborn (unpublished data) to 12.1 mm in the adult (27), which greatly reduces the error in selecting comparable sectors of the cochlea for observations at different postnatal ages. All neurons labeled in these experiments innervated hair cells within the apical-most 1.0 mm of the cochlear spiral, since this location, maturing last, permitted a more extended time period for studying cochlear neuron arbor formation (18). All fiber types depicted could be traced to cell bodies within the spiral ganglion and, therefore, are afferent neurons. Observations reported here are based on reconstructions of 94 afferents from 18 gerbils.

MATERIALS AND METHODS

Breeding gerbils were checked for births twice daily at the hours of 0900 and 1800. At birth all litters were culled to five animals to ensure more homogeneous weight and length distributions among littermates. Whenever possible, pups chosen for a particular developmental series were selected from the same litter.

Cochlear neurons were labeled by an *in vitro* HRP procedure. Gerbil pups were anesthetized by an intraperitoneal injection of sodium pentobarbital [48 mg/kg (body weight)] and rapidly perfused through the heart with warm (37°C) Hanks' balanced salt solution (HBSS; GIBCO). The cochleae were rapidly exposed and removed to tissue culture dishes containing ice-cold HBSS. The cartilage overlying the apical membranous cochlear duct was removed with fine forceps. Microhooks fashioned from glass capillary tubing were used to reflect the stria vascularis and Reissner's membrane to thereby expose ≈1.0 mm of the organ of Corti and spiral ganglion. By using visual landmarks, a small deposit of HRP was made into the modiolus, central to the spiral ganglion cell

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Abbreviations: IHC, inner hair cell; OHC, outer hair cell; P0, etc., postnatal day 0, etc.; HRP, horseradish peroxidase.

bodies, using a minute insect pin whose tip (diameter, 30 μm) was coated with an HRP paste. After injection, the tissue was incubated for 1–3 h at 35°C in an oxygenated solution of HBSS containing 0.5% D-glucose and 10 mM Hepes buffer, adjusted to a pH of 7.4, with an osmolarity of 289 milliosmolar. After several rinses in 0.1 M sodium phosphate buffer and overnight fixation [2.5% (vol/vol) glutaraldehyde in 0.1 M sodium phosphate buffer], the cochlear tissue was processed for visualization of the HRP enzyme (28) and prepared as surface preparations consisting of the organ of Corti and the accompanying spiral ganglion. These preparations were viewed with a Nikon Microphot FX microscope fitted with Nomarski optics, a drawing tube, and a Hamamatsu CCD camera (model C2400). Selected images were acquired with a Data Translation DT2255 frame grabber board. These were subsequently contrast-enhanced and montaged by using National Institutes of Health IMAGE software running on an Apple Macintosh IIci computer. IMAGE was also used to perform a quantitative analysis of selected features of cochlear neuron growth, such as ontogenic changes in the width and length of neural arbors.

RESULTS

Examples of individual HRP-filled cochlear afferents obtained from a newborn [postnatal day 0 (P0)] and a 6-day-old (P6) gerbil are provided in Fig. 1. In each panel, the peripheral dendrite is shown as it branches within the sensory epithelium. The dashed line represents the boundary between IHCs (below) and the first row of OHCs (above). The methods employed here cannot ascertain whether such branches make functional contacts with hair cell receptors. However, ultrastructural studies at this cochlear location in newborn gerbils reveal well-developed presynaptic complexes within IHCs and OHCs (unpublished data). Similar findings have also been reported for hamster (12) and mouse (29).

Postnatal changes in the morphology of auditory nerve fibers projecting to IHCs and OHCs within the cochlear apex are depicted in Fig. 2. At each age, dendritic arbors exhibiting

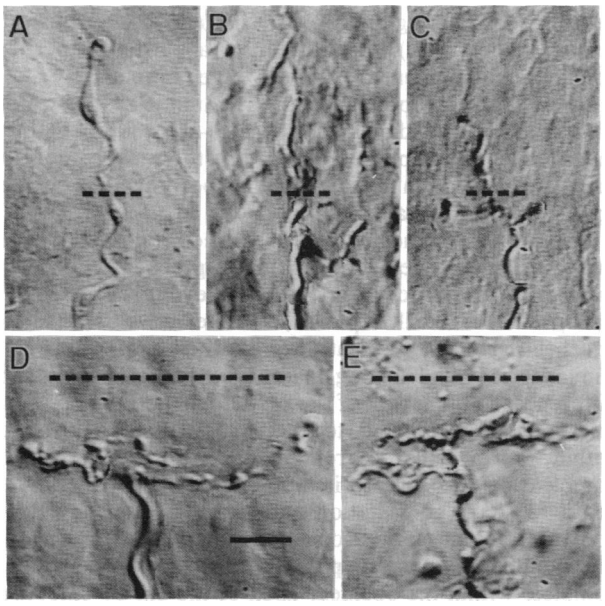

FIG. 1. Video images of HRP-filled afferent neurons from the same location within the apical turn of the gerbil cochlea on the day of birth (*A–C*) and 6 days later (*D* and *E*). The dashed lines in each panel represent the border between the IHCs (below) and the OHCs (above). (*A–E*, bar = 10 μm.) Note that afferents in newborn gerbils span both receptor zones; by P6, however, these arbors are confined to either IHCs (illustrated) or OHCs.

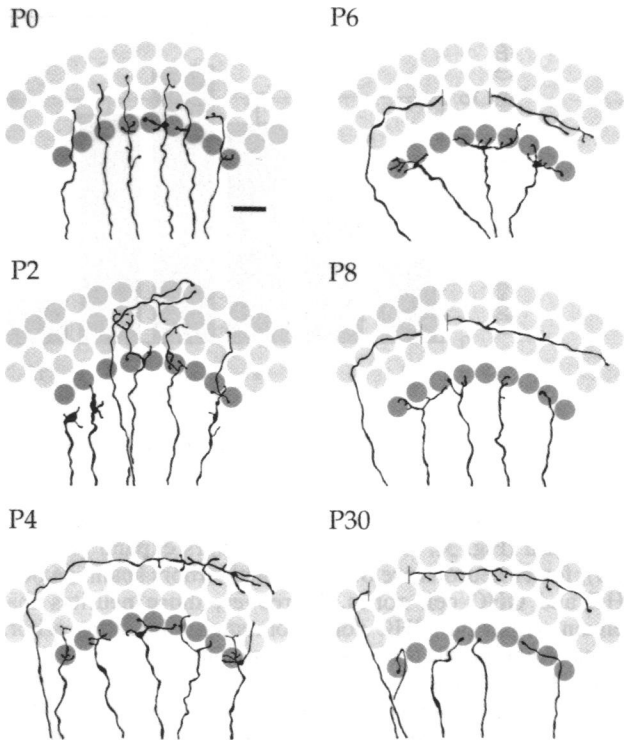

FIG. 2. Progressive changes in the morphologies of IHC and OHC afferents within the gerbil cochlea during early postnatal life. Dark shaded circles represent IHCs and light shaded circles depict OHCs within the same relative segment of the organ of Corti. The center of each plot is ≈0.4 mm from the very apex of the cochlea, which would be to the left in all illustrations. All afferents are drawn to the same scale. (Bar = 20 μm.) At later stages of development, the OHC afferents have been foreshortened to display their branching terminal arbors. This was done by removing unbranched segments equaling 195 μm for P6, 141 μm for P8, and 172 μm for P30. *P0–P30* indicate postnatal day of development shown.

the simplest and most complex morphologies are included to illustrate the range of variation observed. Note also that the tunnel of Corti, which, in the adult cochlea, is interposed between the IHCs and the first row of OHCs, does not open at this location until ≈P5; consequently, prior to this age, these receptors lie closer together.

On the day of birth (P0), afferent neurons project radially within the osseous spiral lamina and span both IHC and OHC receptor zones. Two types of morphology are observed. Most prevalent are neurons with short (5–10 μm) arbors contacting small groups of both IHCs and OHCs (Figs. 1 *B* and *C* and 2). In addition, a few fibers are observed close to the basilar membrane that lack branches entirely and terminate, in a punctate fashion, well below the bases of the adjacent OHCs (Figs. 1*A* and 2). During the first few days of postnatal life, some cochlear afferents at this location are still growing within the osseous spiral lamina and have not yet reached their target hair cells. These neurons are capped by growth cones that become increasingly more complex in their morphology as they near that portion of the organ of Corti that contains the auditory receptors (see Fig. 2*P2*).

By P2, afferents associated exclusively with OHC receptors are recognizable for the first time. The percentage of these neurons within a given preparation cannot be reliably quantified based upon HRP labeling; however, qualitatively, they always represent <20% of the total fibers labeled. The dendrites of these neurons bypass the IHCs to penetrate the OHC zone, after which they invariably turn abruptly and spiral toward the base of the cochlea prior to branching. Initially, these afferents extend only 20–40 μm along the

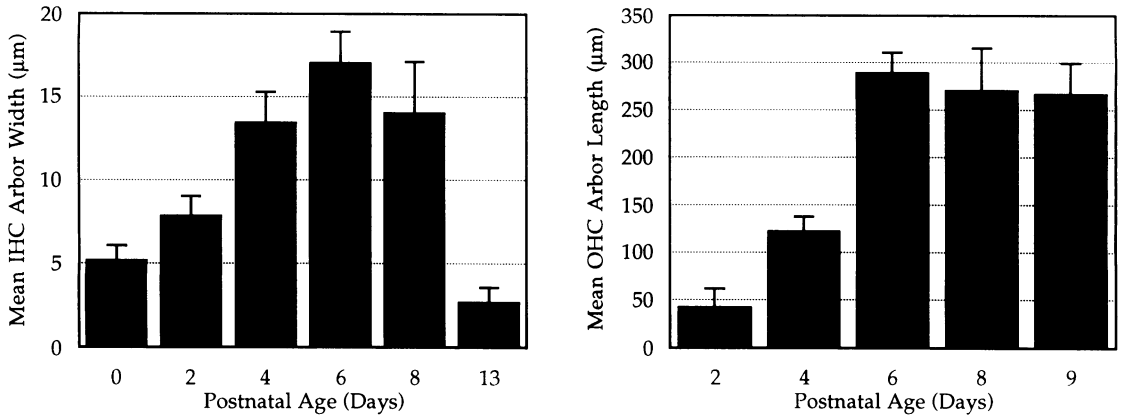

FIG. 3. Ontogenic changes in mean IHC arbor width and mean OHC arbor length. The error bars represent the SEM.

length of the cochlea but by P4 they have more than doubled in length. By P6, they have achieved mature lengths in excess of 300 μm (Fig. 3). As these spiral arbors expand (see Fig. 2*P4*), fewer neurons are observed with arbors spanning both IHC and OHC cell zones. Moreover, those neurites that remain within the OHC zone from such bifurcating arbors appear to be in the process of retraction.

By P6, afferent projections to the two classes of auditory hair cells are completely segregated within the apical turn of the cochlea. Terminal arbors are restricted to either the IHC or OHC zone. Afferent neurons contacting IHCs, however, still have widespread arbors within the inner spiral plexus that contact between two and five receptors (Figs. 1 *D* and *E* and 2 *P6* and *P8*), with the largest arbors found most apically within the cochlear spiral. During the second postnatal week, IHC arbors undergo extensive pruning, resulting in the punctate bouton endings that typify adult mammalian IHC afferents (Fig. 3); a representative example is illustrated from a 1-month-old animal (Fig. 2*P30*).

DISCUSSION

An *in vitro* HRP labeling method has been used to examine postnatal modifications in the neural projections to two classes of mammalian auditory hair cells within the apex of the developing cochlea. The results obtained confirm and extend earlier observations that the neural arbors associated with IHCs and OHCs differ fundamentally in their patterns of growth (Fig. 3). Neural arbors associated with IHCs initially expand to contact several receptors but then contract to produce punctate bouton endings associated with an individual hair cell, whereas arbors to OHCs grow progressively in length and contact an increasing number of receptors until reaching adult proportions (19–25, 30, 31).

The data presented here, however, further suggest that cochlear afferents may differ in their initial specificity for IHCs and OHCs (Fig. 2). At birth, most afferents within the apex of the gerbil cochlea possess arbors that contact both types of receptors. A few, however, are associated exclusively with OHCs from the onset of arbor formation. The early appearance, radial projection, and numerical superiority of the first group of fibers strongly suggest that these are nascent IHC afferents that have sprouted branches to contact adjacent OHC receptors. In contrast, the fibers in the second group lie close to the basilar membrane, arborize solely within the OHC zone, and spiral toward the base of the cochlea, all features that are unique to OHC afferents (4, 7, 8, 21).

There have been few previous observations of immature afferent neurons contacting both IHCs and OHCs. Moreover, when reported, such neurons differ from those described here in the following two ways: (*i*) they are generally observed later in development, shortly before hearing onset and (*ii*) they have the appearance of well-developed spiral afferent fibers with small branches to one or more IHCs. Two such fibers have been reported within the cochlear apex of a newborn cat (24), another is evident in a Golgi preparation of the cochlea from an 8-day-old mouse (22), and several are illustrated in cochlear material from a human fetus at 6 months of gestation (23). In the present study, this form of cochlear afferent innervation was also rare; only one spiral afferent fiber with a branch to a single IHC was observed within the apical cochlea of a 6-day-old gerbil.

The variability in the morphology, time of first occurrence, and number of these nonspecific cochlear afferent neurons is most likely related to differences in the rate of cochlear maturation and also to the variety of cochlear locations sampled in these species. The time period between conception and hearing onset varies widely for different mammalian species. In precocial mammals, which have relatively long gestational periods, such as primates, cats, and guinea pigs, hearing begins in the late prenatal stages or at birth (32). Whereas for altricial species, such as mouse, hamster, rat, ferret, and gerbil, the gestational period is relatively short and hearing does not begin until at least 10 days after birth (for review, see ref. 18). Consequently, a particular event in cochlear development may occur either prenatally or postnatally depending upon the species examined. Furthermore, since the cochlea displays a base-to-apex gradient in its maturation, it is especially important to compare developmental events within the same relative portion of the cochlear spiral in different mammals.

Recently, a scheme has been proposed for normalizing key events in auditory development among precocial and altricial mammals. If a particular maturational event is expressed as a proportion of the silent period, the time between conception and hearing onset for that species, then a high degree of similarity is found among mammals, despite considerable differences in the length of the gestational period (33). When this concept is applied to the data presented here, the withdrawal of radial afferent branches to apical OHCs is complete by ≈P6, which is 80% of the silent period in the gerbil (25 days of gestation and hearing onset at P12). If cochlear afferent segregation occurs at a similar proportion of the silent period in other species then this process should be complete within the cochlear apex of the cat by embryonic day 53 (65 days of gestation and hearing onset at P0) and in the rat by P4 (22 days of gestation and hearing onset at P10). Since much of our knowledge concerning the formation of cochlear neuron arbors is based upon material from newborn kittens (24, 25) and from 4- to 7-day-old rats (22, 24), this might explain the relatively small number of nonspecific cochlear afferents observed in previous reports.

The data presented here support the hypothesis that the supernumerary afferent innervation to mammalian OHC receptors, commonly observed during cochlear development (11–15, 29, 30), arises from a brief period during which both radial and spiral afferent neurons make contact with the OHC (16). An understanding of the mechanisms by which these afferent connections are constructed will require additional study. However, it is of interest to note that fibronectin-like protein, a substrate adhesion molecule known to be important in neurite guidance (34), is transiently expressed within the extracellular spaces below gerbil IHCs and OHCs from birth to P4 (35), the time period during which these nonspecific afferent arbors are forming.

Further studies will also be necessary to discern how these superfluous afferents are pruned during normal cochlear maturation. One suggestion is that they may compete with and eventually be displaced by late-developing efferent fibers that grow into the cochlea from the auditory brainstem (14). Although the efferent innervation to OHCs may develop earlier than previously thought (36, 37), it seems unlikely, for several reasons, that competition from such neurons underlies the remodeling of afferent arbors observed in this study. (*i*) OHC receptors within the apex of the cochlea, the region studied here, receive only a sparse efferent innervation (38). (*ii*) Neural-tract tracing experiments have shown that the ingrowth of cochlear efferents to the OHC zone, although earlier than suspected, does not begin until after P5 (39, 40), by which time the afferent projections to apical cochlear cells have already become segregated according to receptor type.

An alternative interpretation for the results reported here is that IHC and OHC afferents compete with each other during cochlear development. In this scenario, the first afferents to invade the cochlear sensory epithelium are presumed to be radial fibers associated principally with IHC receptors. At first, many of these neurons also have the capacity to form transitory connections with nearby OHCs. Shortly thereafter, spiral afferents appear that grow past the IHC zone to form arbors associated exclusively with OHCs. As these arbors elongate, superfluous projections to the OHC region from immature radial afferents are eliminated, either through selective pruning of inappropriate sprouts or entire neurons, resulting in a progressive confinement of individual cochlear neuron arbors to either the IHC or OHC region. Collateral support for the role of afferent interactions in cochlear neural pattern formation comes from observations of developing organotypic cultures of the cochlea that, although devoid of efferent innervation, nevertheless exhibit a specific postnatal reduction in the afferent innervation to OHCs, which is quantitatively similar to that observed *in vivo* (29, 41).

The sequence of events proposed above could be tested experimentally, at a variety of cochlear locations, by observing the growth of afferents, in real time, within cochlea, cultures. Regardless of the mechanism by which auditory nerve fibers become segregated within the gerbil cochlea, however, it is clear that this process occurs well before the onset of hearing on P12 (42) and the stabilization of cochlear tonotopy on P17 (43). This implies that sound-induced activity within developing gerbil auditory nerve fibers cannot play a major role in their initial remodeling.

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