

Novel Afferent Terminal Structure in the Crista Ampullaris of the Goldfish, *Carassius auratus*

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ABSTRACT

Using transmission electron microscopy, we have identified a new type of afferent terminal structure in the crista ampullaris of the goldfish *Carassius auratus*. In addition to the bouton-type afferent terminals previously described in the ear of this species, the crista also contained enlarged afferent terminals that enveloped a portion of the basolateral hair cell membrane. The hair cell membrane was evaginated and protruded into the afferent terminal in a glove-and-finger configuration. The membranes of the two cells were regularly aligned in the protruded region of the contact and had a distinct symmetrical electron density. The electron-dense profiles of these contacts were easily identified and were present in every crista sampled. In some cases, efferent terminals synapsed onto the afferents at a point where the hair cell protruded into the terminal.

The ultrastructural similarities of the goldfish crista afferents to calyx afferents found in amniotes (birds, reptiles, and mammals) are discussed. The results of the study support the hypothesis that structural variation in the vertebrate inner ear may have evolved much earlier in evolution than previously supposed. © 1996 Wiley-Liss, Inc.

Indexing terms: ear, vestibular, synapse, morphology, electron microscopy

The processing of complex sensory information is accomplished, in part, through a diversity of structural and functional components in the sensory periphery. For example, auditory and vestibular input are transduced by a variety of morphologically and physiologically distinct sensory hair cells in the vertebrate inner ear (Lewis et al., 1985). In the vestibular endorgans of amniotes (reptiles, birds, and mammals), two types of sensory hair cells are present, type I and type II. Type I hair cells have a distinct flask-shaped cell body that is almost entirely enveloped by a large afferent terminal, known as a chalice or calyx ending (Wersäll, 1954, 1956; Engström, 1960; Hamilton, 1968). Type II hair cells are cylindrical and innervated by small boutonlike afferent terminals. The distinctive morphology of the calyx afferent is the primary means of identifying type I versus type II hair cells. Differences are also present, however, in the ultrastructure of the hair cells themselves.

Distinguishing multiple hair cell types in the ears of amniotes (amphibians, bony fishes, elasmobranchs, and agnathans) has been more difficult. Early studies have indicated that the inner ear of amniotes contains a single type of sensory hair cell, i.e., type II, because calyx afferents were not observed in these species (Wersäll, 1960; Flock, 1964; Wersäll et al., 1965; Wersäll and Bagger-Sjöbäck, 1974). Recently, however, we have demonstrated the presence of distinct hair cell types in the otic endorgans of a

cichlid fish, *Astronotus ocellatus* (the oscar). At least two hair cell types can be distinguished by patterns of innervation and immunoreactivity. In addition, the ultrastructural features of these hair cells vary in a way that is similar to those of amniote type I and type II hair cells (Chang et al., 1992; Popper et al., 1993; Saidel et al., 1995). In the ear of the goldfish *Carassius auratus*, different types of hair cells have been identified in the rostral versus caudal ends of the auditory epithelium, the saccule (Lanford and Popper, 1993; Saidel et al., 1995). These variations in hair cell morphology and cytochemistry are similar to those found in the oscar. Interestingly, the variation of hair cell morphology in the goldfish saccule correlates to known differences in hair cell and afferent physiology (Furukawa and Ishii, 1967; Sugihara and Furukawa, 1989; Furukawa and Sugihara, 1990). Based on these and other studies, we have hypothesized that diversity of hair cell structure and function may be a feature common to all vertebrates and may have evolved much earlier than previously thought (Chang et al., 1992; Popper et al., 1993; Saidel et al., 1995).

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The present study uses transmission electron microscopy (TEM) to examine structural diversity further in the inner ear of teleosts. We describe a new type of afferent terminal in the crista ampullaris of the goldfish that is structurally distinct from the bouton-type afferents that are found throughout the goldfish ear (Hama, 1969; Nakajima and Wang, 1974; Lanford and Popper, 1993; Saidel et al., 1995). We examine the ultrastructural characteristics of the contact and describe the fine structure of the hair cell and afferent membranes, the position of afferent synapses, and the presence and position of efferent terminals.

Some characteristics of these specialized contacts are similar to those of enlarged afferent terminals identified in other anamniotes (Lowenstein et al., 1968; Wegner, 1981; Sans and Highstein, 1984); however, it is not clear how far these similarities extend. Two of those studies did not include electron micrographs of the enlarged endings (Lowenstein et al., 1968; Sans and Highstein, 1984), and one showed only a single low-magnification electron micrograph (Wegner, 1981). Our study demonstrates specific ultrastructural features of the specialized terminals in the goldfish crista that are similar to those of the calyx endings found in amniotes.

METHODS

Animals were housed in tanks of recirculated water (26°C) under a 12-hour light/dark illumination cycle. All animal use in these studies was supervised by a veterinarian and approved by the Animal Care and Use Committee of the University of Maryland, College Park.

Ten semicircular canal cristae from eight different goldfish were examined: five from the horizontal canal, three from the anterior canal, and two from the posterior canal. Animals ranging from 5 to 7 cm in standard length were deeply anesthetized with MS-222 (3-aminobenzoic acid ethyl ester, methanesulfonate salt; Sigma) and decapitated. The cranium was opened ventrally, and the cristae were removed. All tissues were fixed in 2% paraformaldehyde and 3% glutaraldehyde in 0.10 M phosphate buffer (pH 7.4) for at least 3 hours and then rinsed in buffer and postfixed in 1% osmium tetroxide. Tissues were dehydrated to 100% ethanol and infiltrated with propylene oxide and epoxy resin (EmBed 812, Electron Microscopy Sciences), and the epoxy was hardened in a 60°C oven for 48 hours. At least 15 thin sections were taken at one area of each crista with an AO Reichart Ultramicrotome. The position of sectioning along the length of the epithelium was not standardized except to provide the best possible alignment to the long axis of the hair cell body. Sections were placed onto formvar-coated slot grids and stained in methanolic uranyl acetate and aqueous lead citrate. All samples were viewed and photographed on a Zeiss 10AC transmission electron microscope.

The linear distance of afferent-to-hair cell contact was measured from single sections of 17 hair cells by using a line-length measurement program on a Hewlett-Packard digitizing system. Each contact profile was measured three times and average of the three taken. A fraction of the contact demarcated by a symmetrical electron density on the cell membranes (see below) was also measured and the percentage of the whole calculated. Finally, the distance between membrane outer leaflets was measured manually from TEM negatives (original magnification 24,100 \times) projected by an enlarger to a total magnification of 96,400 \times .

RESULTS

Morphology of the afferent terminal

In all sections of the cristae sampled, we found hair cells that had a specialized structural relationship with postsynaptic afferent neurons. The afferent terminal was enlarged and contacted a portion of the basolateral surface of the hair cell. In one area of the contact, the hair cell membrane was evaginated and protruded into the afferent cell (Fig. 1A,B). This portion of the hair cell membrane was thus enveloped by the surrounding terminal. The protrusion often extended several microns in the form of a fingerlike structure (Fig. 1B) or was broader and cup shaped (Fig. 2). The variation in contact morphology may have been the result of differences in the plane of section because some cup-shaped contacts taper into smaller-diameter structures (Fig. 2, arrow).

The afferent and hair cell membranes in the protrusion area of the contact were regularly aligned at 8–12 nm apart (Fig. 3A). No contact between the membranes (e.g., tight junctions, gap junctions) was ever identified. One particularly interesting feature of the protrusion area of the contact was the electron density associated with the cell membranes (Fig. 3A,B). This density was distributed symmetrically on the presynaptic and postsynaptic membranes, making the protrusion area distinct from adjacent membranes.

Inside the protrusion, the hair cell cytoplasm was pale and appeared to be devoid of organelles but contained a fine granular material. The light cytoplasm, demarcated by electron-dense cell membranes, was often observed in cross section of the circular or oblique profiles, which were easily identifiable in sample sections. Circular profiles were completely surrounded by the afferent terminal, which was identified by the presence of neurofilaments, neurotubules, and small mitochondria (Fig. 3B). Hair cell profiles of this sort were found in every sample, indicating that these specialized contacts may occur relatively frequently.

Multiple hair cells sometimes made these specialized contacts with a single afferent terminal. Figure 2 shows the bases of four hair cells contacting a single afferent terminal. A fifth hair cell is also innervated by this afferent, out of the plane of section. In the absence of serial reconstructions, the precise ratio of hair cells to afferent fibers cannot be resolved.

Linear distance of the afferent contact

The electron-dense region demarcating the specialized contact area shown in Figure 2 can easily be identified in the micrographs (arrowheads). It should be noted that the electron density stopped at the mouth of the protrusion even though the terminal remained in contact with the hair cell beyond this point. The linear distance of the entire afferent contact was measured in single sections of 17 hair cells. In addition, we determined the proportion of that distance that contained electron-dense membranes.

We found that the total linear distance of the contacts in our samples ranged from 3 to 12.9 μm , and the electron-dense portion of the contact constituted 19–65% of the total. These values clearly do not represent the entire contact but are presented to provide a sense of the overall extent of the structure. Because the hair cell protrusion often appeared to be convoluted, much of the contact may have extended some distance beyond the plane of the

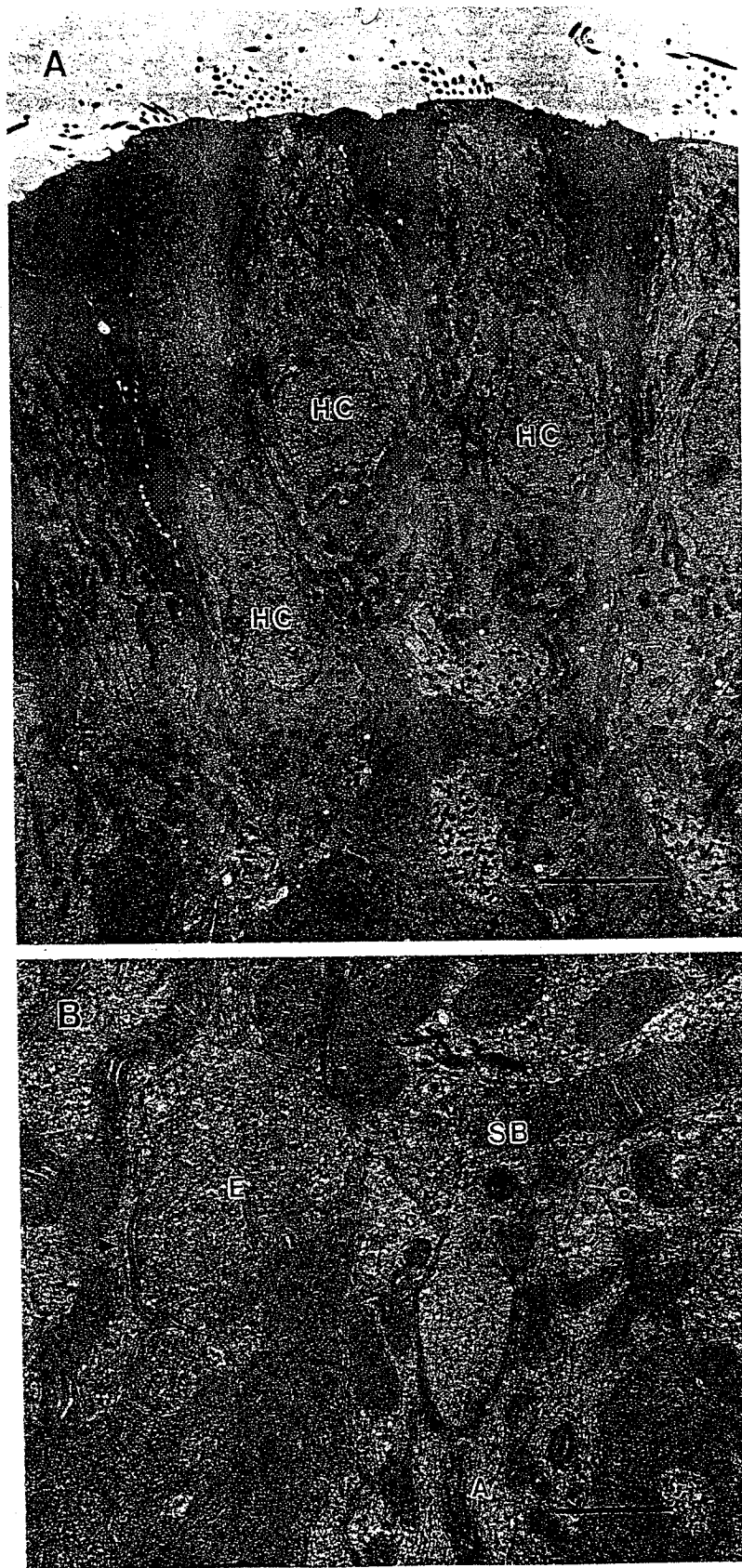


Fig. 1. Transmission electron micrographs of cells in the crista ampullaris of the goldfish *Carassius auratus*. A: The hair cell (HC) in the center of the figure is innervated by a specialized afferent terminal (boxed area). B: Boxed area in A enlarged and reoriented (adjacent section). In one area of the contact, the hair cell membrane is evaginated and is enveloped by the afferent terminal (A). The hair cell

and afferent membranes in this protruded region are regularly aligned and have a symmetrical electron density. A synaptic body (SB) is visible just beyond the mouth of the protrusion. An efferent terminal (E) synapses on this hair cell and on an adjacent hair cell (arrowheads). Scale bars = 5 μm in A, 1 μm in B.



Fig. 2. The specialized afferent terminals may contact more than one hair cell at a time. In this figure, four hair cells (HC) are contacted by a single large afferent terminal. A fifth hair cell contact was also present but is out of the plane of section. Contacts may be cup shaped or

may taper into smaller diameter fingerlike protrusions (arrow). Arrowheads mark the transition to the electron-dense region of the contact. Scale bar = 2 μ m.

section. Again, reconstruction of the tissue will be necessary to determine the total contact area.

Synaptic specializations

Both afferent and efferent synaptic junctions were present in the epithelia. Spherical synaptic bodies with associated vesicles were found within the hair cell at synapses between the hair cell and the afferent. These structures were not observed within the protrusion part of the contact but were very frequently observed at the mouth of the protrusion (Figs. 1B, 2). Efferent terminals often contacted the hair cell body directly at a position just above the limits of the afferent (Fig. 1B). The region of hair cell membrane opposed to efferent contacts contained subsynaptic cisternae similar to those described in other vertebrates (Wersäll, 1954, 1956; Hamilton, 1968).

There was also evidence of efferent-afferent contacts within the epithelium similar to those described in the goldfish and the toadfish *Opsanus tau* (Nakajima and Wang, 1974; Sans and Highstein, 1984). These terminals contained a large number of vesicles, including dense core vesicles, and the opposing afferent membrane did not display subsynaptic cisternae (Fig. 4, arrows). Interestingly, some efferents synapsed onto afferent fibers that contained the profiles of hair cell protrusions. Two afferent terminals shown in Figure 4 (I, II) each contain the profiles of three hair cell protrusions. An efferent terminal synapses onto one of these afferents (lower arrow) at a position

within the same plane of section as the protrusion profiles (arrowheads).

DISCUSSION

TEM of the goldfish crista ampullaris has shown that some of the hair cells in these endorgans made structurally specialized contacts with postsynaptic afferent terminals. These terminals enveloped an evaginated portion of the hair cell basolateral membrane. Such contacts have not been previously described in the goldfish ear, and ultrastructural details of this sort have not been included in studies of other anamniotic vertebrates.

We discuss below two possible interpretations of these findings. First, we examine the structural similarities between this unusual afferent terminal and the structure of calyx afferents in amniotes. Second, we discuss the relationship between the structure of these afferents and the development and innervation of new hair cells in the goldfish crista.

Similarities to the amniote calyx

Figure 5 compares the ultrastructural features of cells in the goldfish crista such as we have described here and type I cells from an amniote. A number of important structural similarities exist between the two. First, afferent terminals in the goldfish crista enveloped a portion of the basolateral hair cell membrane. As in amniotes, this region of the

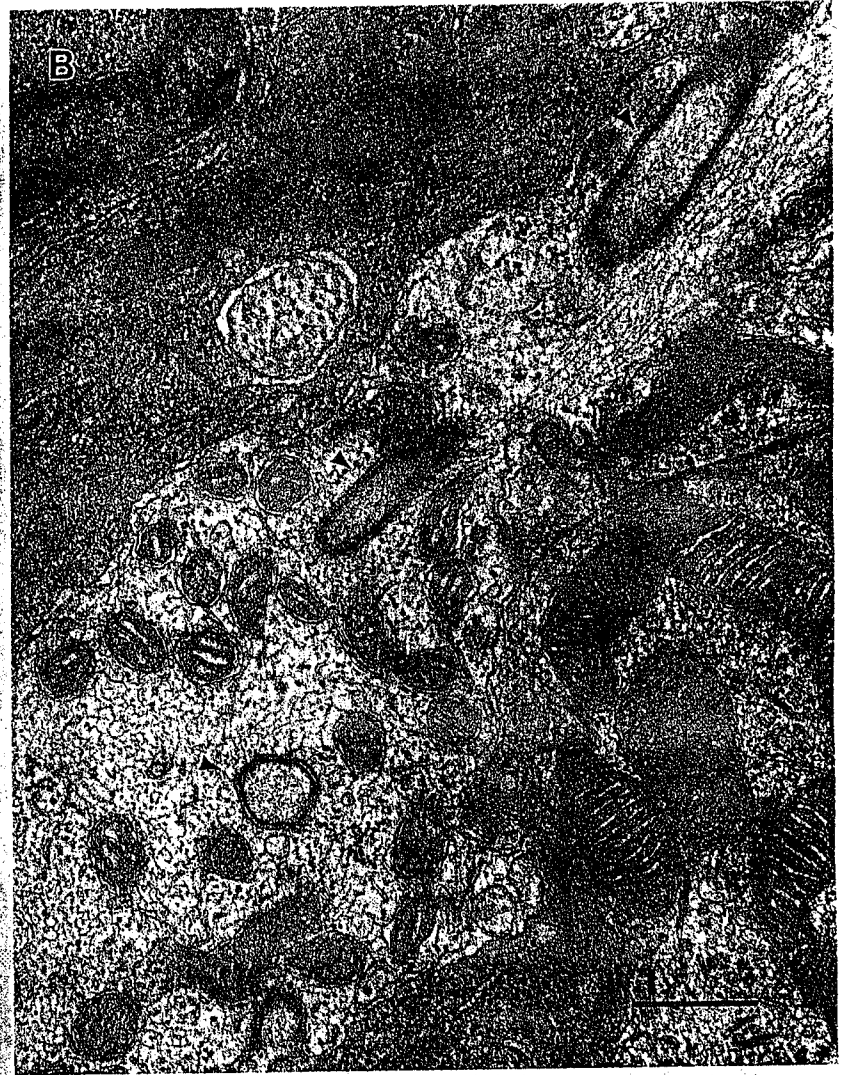
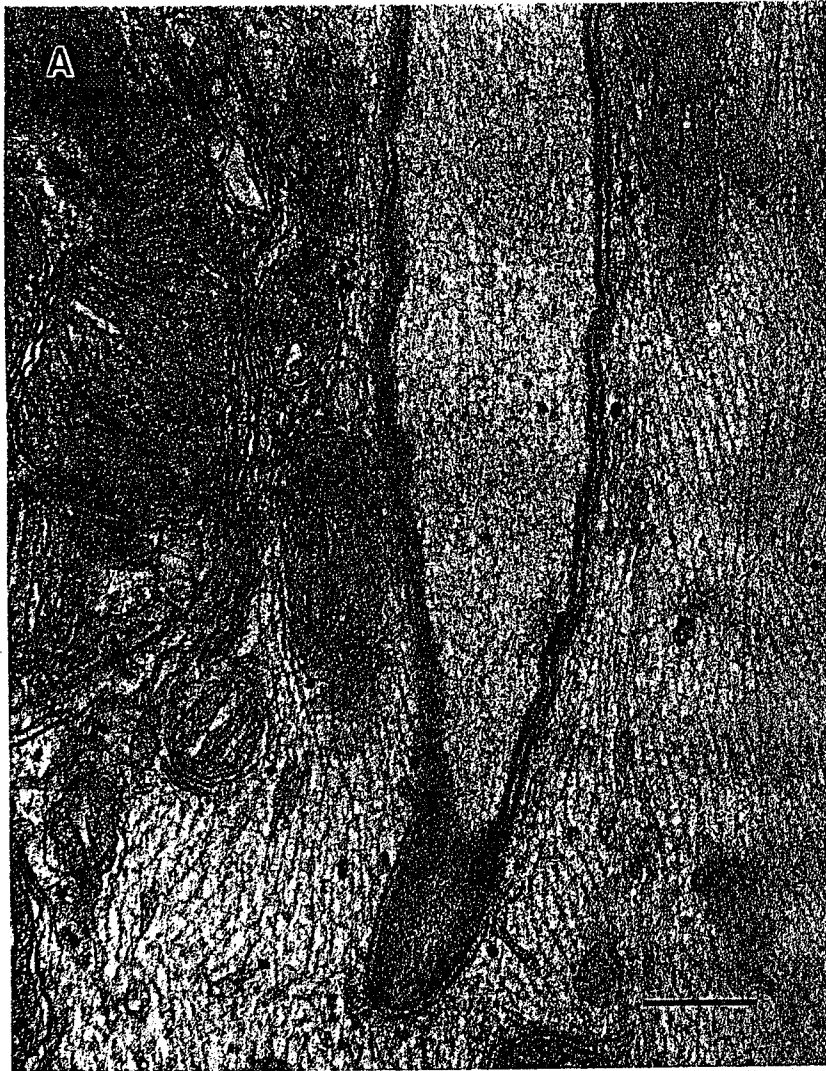


Fig. 3. High magnifications of hair cell protrusions enveloped by afferent terminals. **A:** Micrograph shows the electron-dense region of the afferent contact. The surrounding afferent terminal is identified by the presence of neurofilaments, neurotubules, and small mitochondria. The hair cell and afferent membranes are regularly aligned, at 8–12 nm outerleaflet distance. The density of the hair cell cytoplasm in the extreme tip of the protrusion is probably due to the plane

of section and is most likely associated with the inner surface of the hair cell membrane. **B:** Circular or oblique profiles of hair cell protrusions within afferent terminals (arrowheads) were observed frequently throughout the tissue. Circular profiles were always completely surrounded by the afferent. Scale bar = 0.25 μm in A, 0.5 μm in B.

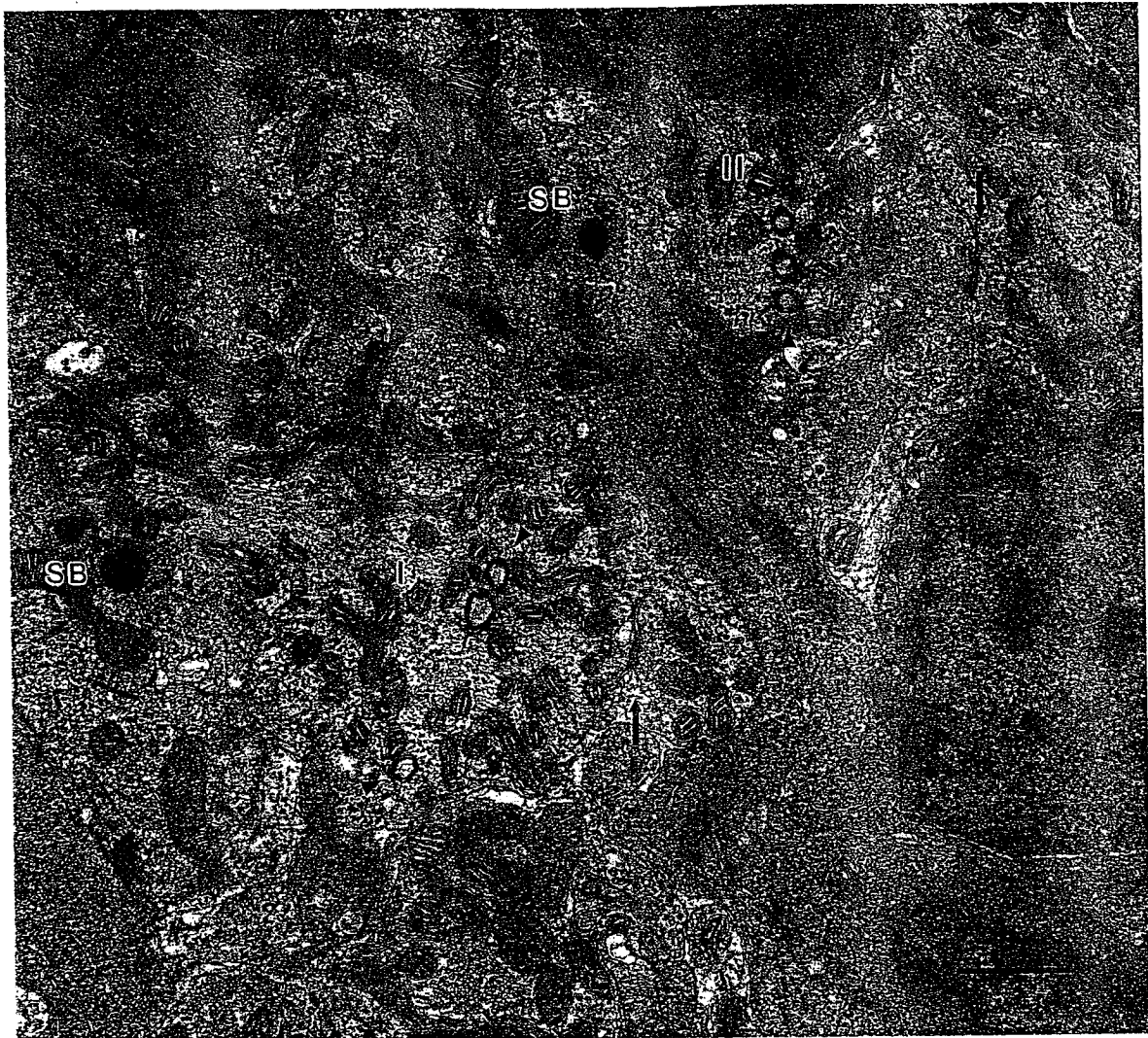


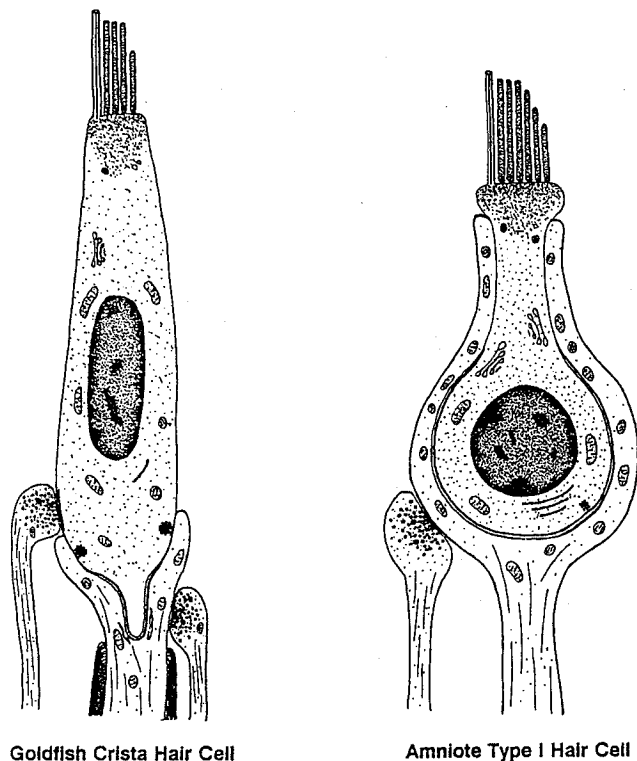
Fig. 4. Efferent-to-afferent contacts are present in the epithelium (arrows). Two afferents (I, II) each contain the profiles of hair cell protrusions (arrowheads). One of these afferents (I) is contacted by an efferent synapse. Note also the presence of an afferent synapse (left) and synaptic bodies (SB). Scale bar = 1 μ m.

goldfish hair cell was nearly surrounded by the afferent terminal. The area of contact is not as extensive in the goldfish crista as it is in the amniote type I hair cell. However, some examples of "incomplete calyces" that do not completely surround the hair cell have been described in the amniote literature. Engström (1960) mentioned the presence of "half-chalice" afferent terminals in the vestibular endorgans of the guinea pig. Baird and Lowman (1978) demonstrated incomplete calyces in the papilla neglecta of the lizard *Anolis carolinensis*. In the lizard, efferent synapses not only make contact with the calyx afferent but also frequently terminate on the hair cell body at a position just above the apical lip of the calyx. This configuration of afferent and efferent contacts is comparable to the arrangement found in the goldfish crista.

Similarities between the goldfish crista afferents and the amniote calyx are also found in the fine structure of the contact. The electron-dense area of the afferent contact in goldfish is strikingly similar to the membrane apposition

between type I hair cells and their calyx contacts. In the cat, rat, and guinea pig, the calyx and hair cell membranes are regularly aligned and have a symmetrical electron density very similar to that found in the goldfish. This electron density is present only on the lower half of the afferent contact and the interleaflet distance is 15–35 nm (Hamilton, 1968; Favre and Sans, 1979; Gulley and Bagger-Sjöbäck, 1979). In the goldfish crista, the symmetrical electron density is present only at the base of the contact, and interleaflet distance is similar, although slightly smaller at 8–12 nm.

Certainly, not all features of the goldfish crista cells resemble those of amniote type I cells. In addition to differences in the overall extent of the afferent contact, the goldfish hair cells also differ from amniote type I hair cells in general shape; none of the goldfish cells have the typical flask-shaped appearance of amniote type I hair cells. It should be noted, however, that the shape of hair cells may not necessarily be a good indicator of "type." For example,



Goldfish Crista Hair Cell

Amniote Type I Hair Cell

Fig. 5. Schematic drawing shows the goldfish crista hair cell and a "typical" type I hair cell of an amniote. Drawings illustrate the configuration of the hair cell and afferent contact and positions of efferent terminals. In goldfish, efferents may contact the hair cell body, the afferent terminal, or possibly both. Serial sectioning will be required to determine the exact distribution of efferent contacts on the goldfish hair cell.

some type I hair cells in the papilla neglecta of the lizard also do not have the typical flask-shaped morphology and are similar in general appearance to the hair cells in the goldfish crista (Baird and Lowman, 1978). In fact, studies in the pigeon (Kevetter et al., 1995) and the guinea pig (Scarfone et al., 1991) have shown that amniote type I hair cells may have a variety of shapes.

Calyx terminals are found in all of the vestibular epithelia in amniotes, unlike the goldfish, where these unusual terminals have been identified in only the semicircular canal cristae. Although enlarged terminals are present in the saccule of the goldfish (Sento and Furukawa, 1987), our electron microscopic studies of that endorgan have shown that those terminals do not contain the specific ultrastructural features (hair cell evagination and membrane density) described here (Lanford and Popper, 1993). In fact, there is no evidence to date that the specific ultrastructural features described in the goldfish crista afferents are present in the any other anamniotic ear. In teleost fish, large cup-shaped afferent contacts are present in the crista of *Opsanus tau* and the lagena of *Colisa labiosa* (Wegner, 1981; Boyle et al., 1991) and in the crista of the lamprey *Lampetra fluviatilis* (Lowenstein, et al., 1968). However, electron micrographs of these contacts are available for only one of these species (*Colisa labiosa*), and in that study it is unclear whether or not these contacts contain the same ultrastructural characteristics as those described in the goldfish crista. Clearly, more detailed information at the

electron microscopic level is necessary to determine the presence or absence of these structures in anamniotes other than the goldfish.

Relationship to developing hair cells

Results of recent studies using the mitotic S-phase marker bromodeoxyuridine (BrdU) indicate that postembryonic cell proliferation occurs in the goldfish ear (Lanford et al., 1995) and in the oscar (Popper and Hoxter, 1984, 1990). It appears from these studies that cell proliferation in the goldfish ear is present in all endorgans, including the semicircular canal cristae. Therefore, it is possible that the unusual configuration of the afferent terminal is a transient structure associated with the ingrowth of terminals and the innervation of newly generated hair cells. A similar kind of glove-and-finger configuration has been noted in human embryos and cultured embryonic mouse cochleas (Sans and Dechesne, 1985; Sobkowicz and Slapnick, 1992).

We believe, however, that the specialized structure of these afferents is probably not part of the developmental process. Our previous TEM examinations of the goldfish saccule (Lanford and Popper, 1993; Saidel et al., 1995) did not reveal the presence of such afferent terminals as described here. Because our BrdU studies indicate that proliferation occurs in the saccule, one would expect these specialized contacts to be present there if, in fact, this afferent morphology is related to the development of new hair cells. In addition, the ultrastructural characteristics of the afferent structure indicate to us that it is part of the mature configuration of the cell. The electron density and close apposition of the membranes are quite robust and are not disrupted even under poor fixation conditions, suggesting that they are a permanent structure. Nevertheless, ultrastructural studies of hair cell development in the goldfish crista are necessary to verify this conclusion.

CONCLUSIONS

Our findings in the goldfish ear strengthen the argument (Chang et al., 1992; Popper et al., 1993) that structural and functional diversity in the inner ear is not an exclusive characteristic of amniotes. The goldfish crista contains not only the simple boutonlike afferents described in anamniotes but also contains at least a second type that envelopes the base of the hair cell. The presence of enlarged afferent terminals in the ears of such widely divergent species as the toadfish and lamprey indicates that afferent diversity may have evolved much earlier than previously supposed. It remains unclear, however, whether or not the enlarged afferent terminals found in those species contain the ultrastructural specializations found in the goldfish crista. A comprehensive examination of the crista epithelia in a variety of amniotes will be required to determine this point and to resolve the evolutionary questions raised by these findings.

Many of the structural features present in this second type of afferent terminal are similar to those of the amniote calyx. However, because the precise function of the calyx remains uncertain, it is impossible to determine whether or not the specialized afferent structures found in the goldfish crista are functionally analogous to the calyx. Physiological investigations of the goldfish crista may provide some insights into the function of these specialized terminals and may shed light on the function of calyx terminals in the amniote ear.

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