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Synapses involving auditory nerve fibers in primate cochlea

(organ of Corti/hair cells/synaptic junctions/auditory periphery)

DAVID BODIAN

Departments of Otolaryngology and Anatomy, Johns Hopkins School of Medicine, Baltimore, Maryland 21205

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ABSTRACT **The anatomical mechanisms for processing auditory signals are extremely complex and incompletely understood, despite major advances already made with the use of electron microscopy. A major enigma, for example, is the presence in the mammalian cochlea of a double hair cell receptor system. A renewed attempt to discover evidence of synaptic coupling between the two systems in the primate cochlea, postulated from physiological studies, has failed. However, in the outer spiral bundle the narrow and rigid clefts seen between pairs of presumptive afferent fibers suggest the possibility of dendro-dendritic interaction confined to the outer hair cell system. The clustering of afferent processes within folds of supporting cells subjacent to outer hair cells is in contrast to the lack of such close associations in the inner hair cell region. The difference reinforces the suggestion of functional interaction of some sort between the outer hair cell afferent nerve processes.**

In discussing the future of science and technology recently, Frank Press, the Science and Technology Advisor to the President, lists some of the important research questions of national interest, as cited by members of the President's Cabinet. One of the two major biological questions mentioned is: "What are the mechanisms responsible for sensory signal processing, neural membrane phenomena, and distinct chemical operations of nerve junctions?" (1). An actively probed facet of this question concerns the synaptic structures involved in cell-to-cell transmission of neural signals. In this field, electron microscopy has already produced revolutionary changes in our concepts of how the nervous system and sensorimotor end-organs work (2, 3). Unsuspected structures and structural relationships have been especially evident in sensory regions of the brain and in sense organs such as the retina and organ of Corti.

Yet, despite intensive recent investigation, our knowledge of structural relations of neural elements in the auditory end-organ is insufficient to enable a reliable interpretation of physiological recordings. For example, one of the puzzles of both vestibular and auditory functions of birds and mammals is the presence of at least two distinct types of receptor cells in each receptor organ. In mammals the difference between the types is most dramatically expressed in the spiral organ of Corti in which, in addition to prominent cytological differences between the inner and outer hair cells (IHCs, OHCs), Spoendlin has found that the IHCs receive 95% of the afferent nerve fiber connections, whereas the OHCs, which are 3 times as numerous, are linked most importantly with synapses of the efferent olivocochlear system and with the afferent processes of a special type of ganglion cell (4).

A major facet of the puzzle of the double hair cell system in the organ of Corti is the unresolved question of whether, or how, the two subsystems may be coupled anatomically. Recently, evidence has been presented for functional interaction of the cochlear IHCs and OHCs (5). There remains, however, a gap in our understanding of the anatomical mechanism whereby a postulated inhibitory action of the efferent olivocochlear system, mainly mediated by the OHC system, may be transmitted to the IHC system or its auditory nerve fibers, which represent the bulk of the pathway from the organ of Corti to the auditory nuclei in the brain stem (4).

My purpose in this study was to explore the possibility that a previously unrecognized neural connection might exist between OHC and IHC. If such a connection could be discovered, or ruled out, one could focus attention on an appropriate theory.

In view of the fact that the spiral bundles at the base of the hair cells have been shown to be regions of synaptic interaction, namely the locus of efferent terminal synapses, it seemed important to establish whether other neuronal junctions, perhaps atypical cytologically, might also be present in the spiral bundles. These conceivably might mediate inhibitory influences from OHC to IHC as postulated by Zwislocki and Sokolich (5). According to these authors, "the recordings obtained from Mongolian gerbils, our preferred experimental animals, have lent themselves to only one explanation, namely, that the inner and outer hair cells do interact, and that the contribution of the outer hair cells involves an inhibitory synapse. This outcome has led to a model which accounts for the neural sharpening of the mechanical cochlear frequency analysis and for other hitherto unexplained response characteristics of the cochlear nerve. However, a clear anatomical substrate for the suspected interaction between the inner and outer hair cells remains to be found." Finally, Spoendlin, commenting on the possible significance of the elaborate efferent innervation at the receptor level, states: "In conclusion, it appears that the cochlear receptor is far more than a simple energy transducer. By means of the innervation pattern of the sensory cells, the interaction between afferent and efferent neurons and possibly the influences of the adrenergic innervation, an important integration and modification of the acoustic message is most probably accomplished within the cochlear receptor." (3).

With these considerations in mind, I began a survey of the organ of Corti of Old World monkeys to determine whether anatomical evidence for neuronal coupling of OHC and IHC systems could be found. The assumption was implicit that synaptic coupling might be atypical to a degree that it may have been missed in earlier studies. In the course of the survey, other findings of possible functional significance were encountered.

MATERIALS AND METHODS

Old World monkeys (*Erythrocebus patas* and *Macaca mulatta*) were used in this study. All were healthy adolescents or young adults and weighed from 2 to 4 kg. I used eight in preliminary studies to determine the best mode of access to the membranous

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Abbreviations: IHC, inner hair cell; OHC, outer hair cells.

cochlea and to test the adequacy of fixation. Eleven were finally used to supply tissue for electron microscopy.

Satisfactory fixation for electron microscopy was obtained by means of perfusion of the aorta, via the left ventricle, with fixative at room temperature, prior to opening the right atrium to permit flow-through. The fixative consisted of 2.5% glutaraldehyde and 1% paraformaldehyde in phosphate buffer at pH 7.2. Preliminary washing out of blood with saline was not found to be helpful. Approximately 2 liters of fixative, with the perfusion bottle at 1 meter in height, gave thorough perfusion to the extent that only occasional erythrocytes were seen in spiral vessels in stained sections. After initial washout of blood, the rate of perfusion was reduced to approximately 1 liter in 15 min.

The cochlea was approached by splitting the temporal bone above the internal auditory meatus with bone cutting forceps, after a cube of temporal bone surrounding the cochlea was removed with the aid of a Stryker bone saw. Exposure and removal of the membranous cochlea was accomplished with bone forceps rather than with a dental drill. This procedure was facilitated by dropping a few drops of osmic acid solution upon the exposed parts of the membranous cochlea and cochlear nerve. Pieces of the membranous cochlea were then carefully dissected and placed in 2% osmic acid in chilled Millonig's phosphate buffer for 2 hr. Millonig's phosphate buffer in 100-ml amounts is made by combining 83 ml of a stock solution of 2.26% monosodium phosphate with 17 ml of a stock solution of 2.52% sodium hydroxide. Embedding in epoxy resin (Epon) was carried out in the usual manner, with orientation of the tissue fragment prior to hardening.

In 2-μm sections stained with toluidine blue, it was usually seen that the organ of Corti structures, except for the vestibular membrane, were intact, along with attached portions of stria vascularis and osseous spiral lamina. Blocks were trimmed in accord with the appearance of stained sections, and thin sections in the radial plane of the cochlea were then obtained for electron microscopy. Sections were mounted on bare grids and stained with lead citrate and uranyl acetate in the usual manner.

OBSERVATIONS

Observations were largely directed to the recepto–neural junctional zone at the base of hair cells.

A prerequisite of any analysis of intercellular neural junctions is the identification of efferent axon processes, afferent ganglion cell processes, and supporting cell processes. The last are seen so frequently as extensions from the nucleated portions and are so characteristically poor in organelle content, that they are readily spotted in a cluster of profiles. Adjacent phalangeal cells and processes often exhibit tight junctions with each other in the basal hair cell regions. However, as they envelop neural profiles they are separated from the latter by a somewhat variable space of approximately 50–80 nm. In accord with the similar relationship of glial cells and neurons elsewhere in the nervous system, it seems likely that such spaces are to be regarded as unspecialized parts of the extracellular space. Even more critical for our purposes are the criteria for identification of synaptic structures.

Morphological Characteristics Associated with Synaptic Properties. Known chemical synapses are associated with certain distinctive junctional structures. These are a synaptic cleft 20–30 nm wide usually outlined by increased density of the junctional membranes, cleft material such as filaments, clusters of microvesicles at the presynaptic membrane, subsynaptic cisterns, and various pre- and postsynaptic electron-dense structures, such as the so-called presynaptic "ribbons" and subsynaptic "web". Some, or even most, of these structures may be absent. For example, certain mammalian OHC synapses on cochlear afferent postsynaptic terminals are apparently devoid of synaptic microvesicles, a *sine qua non* of chemical transmission theory (6). One is therefore forced to conclude at this time that synaptic junctions possessing the minimal characteristics of a cleft outlined by dense membranes, 20–30 nm wide, and containing dense material such as cleft filaments, may be associated with some type of synaptic activity.

Identification of Afferent and Efferent Neuronal Processes. The identification of unmyelinated nerve cell processes as afferent or efferent in character also poses a formidable problem. Although there is a tendency for afferent processes to contain microtubules and efferent processes to contain microfilaments, as noted by Spoendlin (7), this distinction does not appear to hold for the finest processes, which exhibit microtubules irrespective of afferent or efferent character (8). In the following description, each type of neural structure will be assessed in terms of its possible role as a presynaptic or postsynaptic unit.

Afferent Processes and Their Hair Cell Junctions. Afferent processes are distinctly different cytologically, depending upon whether they terminate upon OHCs or IHCs. Those that appear to be postsynaptic to OHCs form terminal expansions which are usually well preserved, and which contain either a dense filamentous material with scattered microvesicles (Fig. 1*d*) or a more "open" cytoplasm with numerous microtubules (Fig. 2*a*). The microtubules are usually oriented at right angles to the long axis of the hair cell. The synaptic cleft is about 20 nm in diameter, contains cleft filaments, and is bordered pre- and postsynaptically by narrow densities. In the filamentous terminals the synaptic cleft has a wavy appearance indicating an interdigitation of pre- and postsynaptic knob-like processes. The presynaptic region of the OHC usually contains many coated vesicles, but aggregates of synaptic vesicles are conspicuously absent. Finally, it must be noted that the synaptic pole of the OHC, well basal to the nucleus, is densely crowded with mitochrondria, endoplasmic reticulum, ribosomes, and coated vesicles. The high density of cytoplasmic organelles indicates an unusual degree of metabolic activity. The two types of afferent terminals forming synaptic junctions with OHCs tend to be distributed so that "filamentous" terminals are seen mainly in the basal coil and "microtubular" terminals are seen mainly in apical coils.

Afferent processes that are postsynaptic to an IHC pass directly to the IHC from the habenula perforata and usually make side-to-side contact by means of slightly swollen terminals (Fig. 1 *a* and *b*). The latter are subject to extreme swelling and dissolution, unless rapidly fixed after cessation of the blood circulation. Afferent processes that are postsynaptic to an IHC contain microtubules, but these are usually replaced in the synaptic swelling by scattered reticulum and scattered vesicles ranging from 40 nm to 100 nm in diameter. Junctions with the base or sides of the IHC are of two kinds. Symmetrical densities of variable length, not associated with any other synaptic specialization, are common. Perhaps of greater significance in terms of synaptic function are asymmetrical junctions with quite heavy synaptic web on the postsynaptic side of a cleft of about 35 nm (Fig. 1 *a* and *b*). These junctions also exhibit a remarkable presynaptic dense body, in the shape of a ring, surrounded by a "halo" of synaptic vesicles. The synaptic "rings" are sufficiently numerous so that one might infer their presence in relation to each afferent process, but occasionally two "ring" synapses are seen in relation to a single asymmetrical junction (Fig. 1*b*). In one instance the "ring" synapse complex was found at a distance of several micrometers from the hair

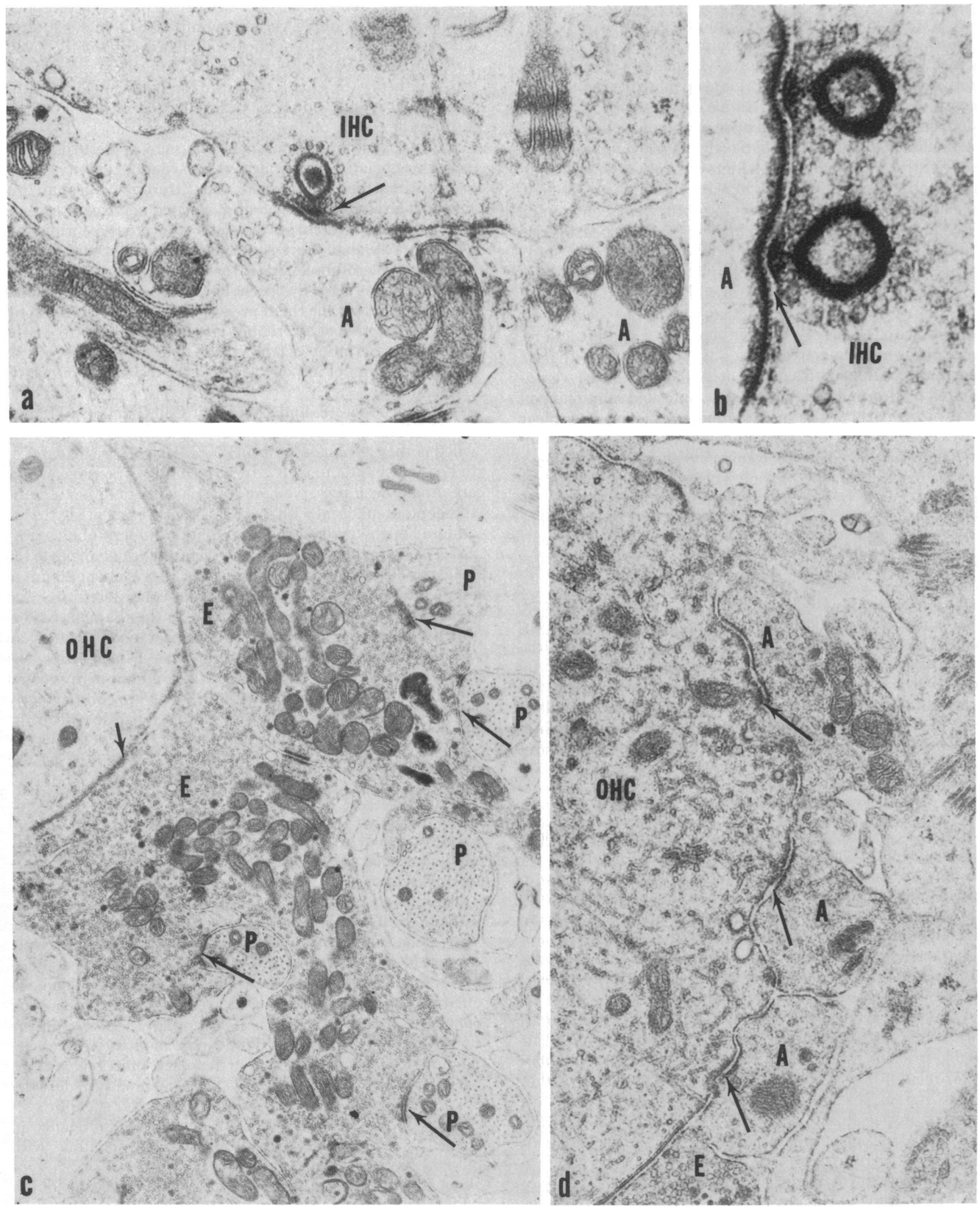

FIG. 1. Synapses of postsynaptic afferent nerve processes with hair cells (A) and with large efferent terminals or their collateral processes (P). Arrows indicate synaptic junctions of (*a* and *b*) IHCs and (*c* and *d*) OHCs of basal coil. E, presynaptic terminals of the efferent olivocochlear system and their synaptic junctions (short arrow). (*a*, ×20,000; *b*, ×41,000; *c*, ×7600; *d*, ×15,000.)

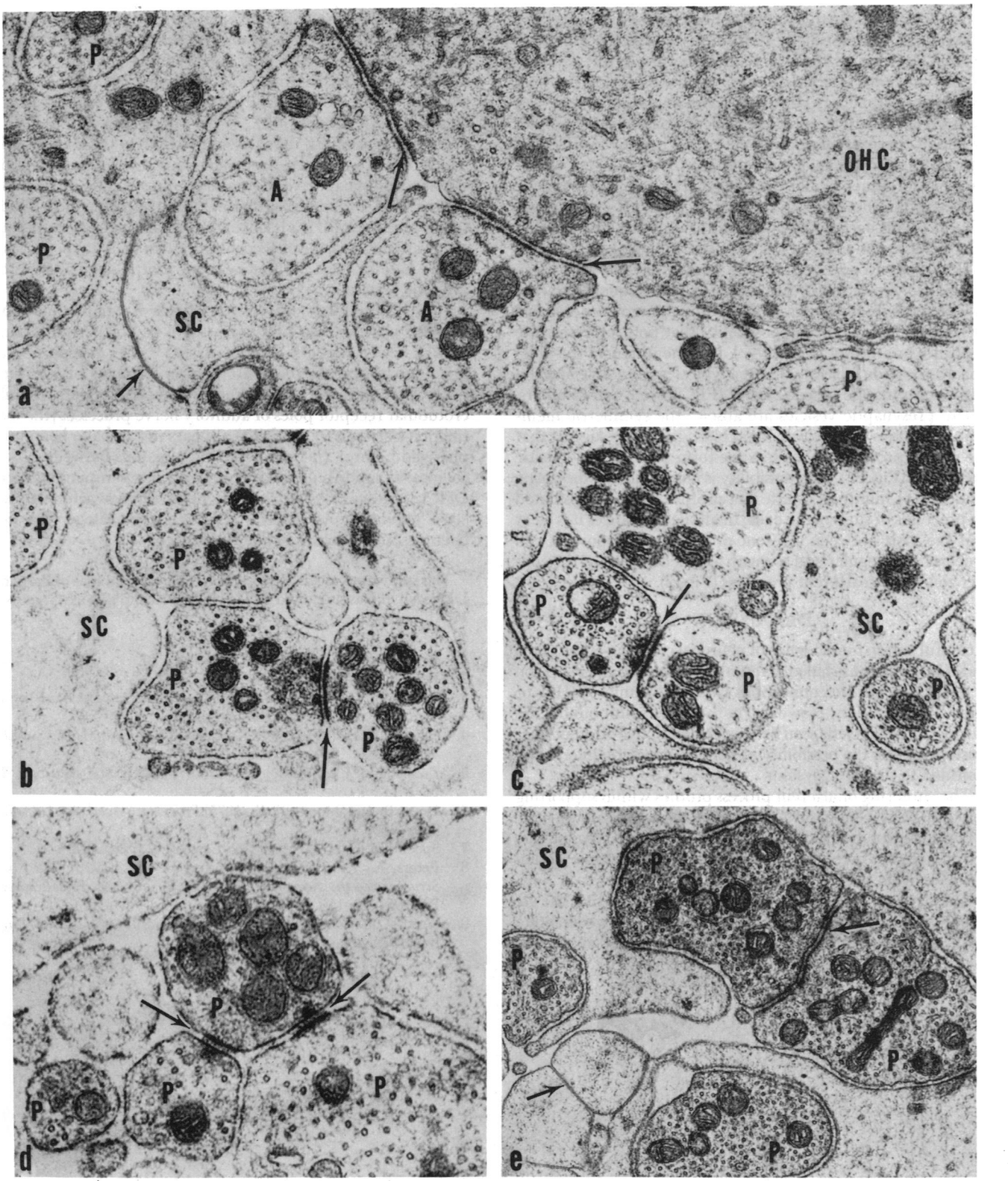

FIG. 2. Synapses of postsynaptic afferent nerve processes (A) with (*a*) OHC of apical coil, and (*b*, *c*, *d*, and *e*) various junctions between presumptive afferent processes (P) in the outer spiral bundle (long arrows). SC, supporting cells. Short arrows, gap junctions between processes of supporting cells (*a* and *e*). (*a*, *b*, and *e*, ×14,550; *c* and *d*, ×22,310.)

cell surface. The ring density appears to be composed of several layers, and the hollow of the ring may contain a very dense central body or a small amount of dense material.

Synaptic Association of Presumptive Afferent Processes in the Outer Spiral Bundle. I am not aware of references to various junctional associations of nerve fiber profiles in the outer spiral bundle that may be of functional importance. One may assume that junctions are olivocochlear axodendritic synapses in which the presynaptic member of a pair of profiles has clustered microvesicles at an asymmetric junction, and abundant microfilaments, and the postsynaptic member possesses abundant microtubules, so is likely to be an afferent process (Fig. 1*c*). This perhaps has led to the assumption that no other synaptic associations are present in the outer spiral bundles.

However, if one examines the bundle beyond the region immediately subjacent to the OHC, one finds frequent instances in which the microtubular content of both members of an associated pair suggests the possibility that both processes are dendritic or afferent (Fig. 2 *b–e*). In some instances the junctional association is clearly that of a typical asymmetric junction, with a presynaptic vesicle cluster (Fig. 2 *b–d*), and in others, where presynaptic vesicles are absent, the symmetric membrane densities and narrow, rigid cleft diameters suggest that the pair of profiles may be in some form of dendro–dendritic association (Fig. 2*e*). This possibility is heightened by the strong tendency of multiples of afferent profiles to be encapsulated by processes of supporting cells. In all such instances it is clear that the cleft between afferent process and supporting cell process is less regular and as much as twice as wide as the contact zone of two afferent processes (Fig. 2).

DISCUSSION

A careful search for evidence of neuronal coupling of the OHC and IHC systems has failed to reveal evidence in the IHC region of dendro–dendritic connections from OHC units to IHCs or their afferent fibers. However, in the outer spiral bundle the narrow and rigid clefts seen between presumptive afferent fiber clusters suggest the possibility of dendro–dendritic interaction confined to the OHC system.

The clustering of afferent process profiles within supporting cell folds, subjacent to OHCs, is to be contrasted with the lack of close association of such clusters found in the IHC region. The difference reinforces the suggestion of functional interaction of some sort between OHC afferents.

The strong impression is obtained in this study that the afferent processes of spiral ganglion cells may be involved in quite atypical junctions in which "synaptic" vesicles play a minor role, and in which quantal chemical transmission is therefore problematic. This applies not only to the two types of afferent processes postsynaptic to OHCs (6), but also to a type of "dendrodendritic" synaptic junction between pairs of presumptive afferent processes in the outer spiral bundle. To these unusual relationships may be added the well-established synapses of efferent olivocochlear terminals upon afferent processes associated with OHCs (Fig. 1*c*).

In striking contrast, the afferent processes to IHCs are clearly associated with typical synaptic junctions involving asymmetric densities, presynaptic vesicles, and the presynaptic dense body or ring (Fig. 1 *a* and *b*). Other evidence of connections to afferent processes of IHCs is lacking, although there are numerous axon varicosities of the olivocochlear system (4, 9) in the vicinity of the base of the IHC, but without morphological signs of synaptic junctions.

It is proposed that the following types of synaptic junction impinge upon the receptor processes of auditory nerve fibers: (*i*) IHC receptoneural junctions, (*ii*) OHC receptoneural junctions to granulo-filamentous receptor poles of auditory nerve processes, (*iii*) OHC receptoneural junctions to microtubular receptor poles of auditory nerve processes [the distinction between (*ii*) and (*iii*) appears not to have been emphasized before], (*iv*) axo-dendritic junctions between olivocochlear synaptic bulbs and afferent processes impinging upon OHCs, (*v*) dendro–dendritic junctions between afferent processes impinging upon OHC (this presumptive category has not been described before to our knowledge).

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