Receptor synapses without synaptic ribbons in the cochlea of the cat

(neurocytology/hair cells/auditory system)

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Communicated by Don W. Fawcett, July 9, 1975

ABSTRACT This report describes recepto-neural junctions thought to be chemical synapses without synaptic ribbons or vesicle aggregates. Such synapses occur in the cat between the outer hair cells of the cochlea and auditory nerve fibers. These synapses are formed by flat or indented junctions having symmetric membrane complexes associated with smooth endoplasmic reticulum and coated vesicles in the outer hair cell cytoplasm. Inner hair cells have recepto-neural junctions with asymmetric membrane complexes; synaptic ribbons and vesicles gather at one type of junction, while the other type associates with endoplasmic reticulum. Gap junctions are not seen at cochlear synapses but do link supporting cells. The results suggest that inner and outer hair cell synapses have different properties and raise questions about the role of synaptic vesicles and endoplasmic reticulum in the storage and release of transmitters.

Chemical agents for synaptic transmission may reside in vesicles until released at a junction [vesicle hypothesis (1)]. Transmitter may pass from vesicles to the intercellular space by exocytosis (2). This hypothesis has been most thoroughly studied at neuromuscular junctions, where it might explain acetylcholine release in discrete quantities by individual vesicles [quantal transmission (1)]. The neuromuscular junction has been used as a model for chemical transmission in other parts of the nervous system. The present study demonstrates synapses, apparently dependent on chemical transmission, which may not fit this model. These synapses occur between hair cell receptors and auditory nerve fibers in the cat's cochlea. Synaptic ribbons with vesicles are reported in inner hair cells (IHC's) and outer hair cells (OHC's) opposite auditory nerve endings in guinea pig (3, 4), monkey (5), man (Kimura, personal communication), and mouse (6) but not in the OHC's of chinchilla (7) or cat (8, 9). In the cat, Rodríguez Echandía (8) noted few "synaptic vesicles" in the OHC, which he thought might not need to store a transmitter agent in vesicles, although observations on vesicles are hard to evaluate because of the fixation artifacts often seen in the cochlea. In the present study uniformly well-preserved cochleas and correlations with Golgi preparations (10, 11) were used to distinguish the kinds of auditory nerve endings. Thus clearly identified recepto-neural junctions (RNJ's) were reconstructed with electron microscopy by tracing fibers to their endings at the hair cell bases in interrupted serial sections.

MATERIALS AND METHODS

The most critical observations are from two cats (*Felis catus*, Boston), 32 and 8 months old, bred, born, and raised with no

history of illness or physical defects. The findings were confirmed in seven other cats deemed less than optimal for clinical or technical reasons. Sodium pentobarbital (35 mg/kg) was given intraperitoneally, 1-2 hr before preparation for electron microscopy as described elsewhere (12), but sodium acetazolamide was omitted and the fixative was 1% formaldehyde and 2.5% glutaraldehyde in 0.12 M cacodylate buffer (pH 7.3) and 0.008% calcium chloride. Without a preceding saline wash-out, the fixative was perfused simultaneously through the aorta and the round window of the cochlea, after the middle ear had been entered and the oval window opened. Artificial respiration with 95% O₂ and 5% CO₂ up to the start of the perfusion was considered essential because of the susceptibility of the inner ear to hypoxia. The cochlea was perfused at a rate of 1-3 ml/min, the aorta, at 50-70 ml/min. The cochleas were kept in fixative at 4° overnight. After rinsing in three changes of 0.18 M sucrose, 0.12 M sodium cacodylate, and 0.008% calcium chloride for 15 min and post-fixation with 2% osmium tetroxide in the rinsing solution, the bony labyrinth was partially removed and the cochlea was stained at 4° with 1% uranyl acetate. Dissection was completed after embedding in Epon 812. Thin sections stained by 1.5% alcoholic uranyl acetate and 0.2% lead citrate were examined with an RCA-3G or Jeolco 100B electron microscope.

RESULTS

The main findings are from the middle turn of the cochlea supplemented by the apical and basal turns and include serial sections of the synaptic zones of dozens of hair cells and unselected sections from dozens more. The Golgi method defines two classes of spiral ganglion cells (10): those with radial fibers that innervate one or two IHC's and those with outer spiral fibers that innervate many OHC's. Both kinds of spiral ganglion cells send axons into the auditory nerve, which projects to the brain. Efferent axons contact radial fibers beneath the IHC's or cross the inner tunnel to the OHC bases. In electron micrographs the efferent axons and endings differ from the afferents (11) (Figs. 1-3).

Radial fibers have microtubules and neurofilaments; the synaptic endings contain mitochondria, smooth endoplasmic reticulum (SER), and coated vesicles in a granulo-filamentous matrix (Fig. 1). Synaptic vesicles, as distinct from coated vesicles or circular profiles of SER, are not seen in this ending. At the synapse, in the IHC, synaptic vesicles surround a ribbon (Fig. 1, top). The ribbon ends in ridges, where vesicles approach the presynaptic membrane, which is separated by a cleft of 12 nm from the thicker postsynaptic density. Flanking the ribbon formation are protuberances of the radial fiber, where the membrane asymmetry is less

Abbreviations: IHC, inner hair cell; OHC, outer hair cell; RNJ, recepto-neural junction; SER, smooth endoplasmic reticulum.

FIG. 1. *Bottom*: Inner hair cell recepto-neural synapse. G, gap junction (compare Fig. 3 inset); IHC, inner hair cell; N, neurofilaments in radial fiber; R, synaptic ribbon; RE, radial fiber ending; SC, supporting cell processes; T, microtubules. Radial section; $\times 27,000$. *Top*: Detail of recepto-neural junctions. SER, smooth endoplasmic reticulum; C, coated vesicles; I, indented junction. $\times 45,000$. Note: In the figures the bases of hair cells are facing down.

FIG. 2. Outer hair cell synapses. E, efferent ending with spherical to ovoid vesicles; MV, multivesicular body; SE, spiral fiber ending traced from a spiral fiber in serial sections; SF, outer spiral fiber; *, digitiform invaginating appendage; other abbreviations as in Fig. 1. Radial section; $\times 27,000$.

pronounced, the gap slightly wider, and granular material, coated vesicles, and SER gather in the IHC (Fig. 1, I).

Beneath the OHC's, outer spiral afferents contain many microtubules but no neurofilaments in the middle and basal turns (Figs. 2 and 3). Traced to OHC bases, the 1–2 μm wide endings contain mitochondria, SER, and coated vesicles in a granulo-filamentous matrix like that in dendritic appendages of central auditory neurons (13). Vesicle aggregates do not appear. *The afferent synapse* comprises flat and indented junctions and coated invaginations. A single fiber usually makes several invaginations and junctions of each type alternately with an OHC (Fig. 3). *The flat RNJ* has symmetrical membrane thickenings facing a narrow, irregular space, 12–22 nm wide (Figs. 3 and 4). Granular material encrusts the pre- and postsynaptic membranes. Similar junctions occur between supporting cells (Fig. 3, FS). *Indented junctions* form alternately at protuberances and indentations of the hair cell and the endings (Figs. 2 and 3). These junctions have intercellular separations of 10–20 nm and symmetrical membrane thickenings invested with fine granular material that extends back into the pre- and postsynaptic cytoplasm. *In the OHC branching canaliculi* of SER, microtubules, and small mitochondria stream from the subnuclear zone to the RNJ's (Figs. 3 and 4). Tubules of SER gather at the presynaptic membrane and seem to fuse with it in places. Many coated vesicles of various sizes collect on the edges of the

FIG. 3. Outer hair cell synapses. D, dense-cored vesicles in efferent endings; F, flat recepto-neural junctions; F1, terminal appendage of spiral fiber; FS, flat junctions between supporting cell processes; PC, postsynaptic cistern; SN, space of Nuel; other abbreviations as in Fig. 2. $\times 27,000$. *Inset*: Part of a gap junction between supporting cell processes from another preparation. $\times 370,000$; Radial sections.

junctions and are often fused with the plasmalemma or open to the intercellular space (Figs. 2 and 3).

Aggregates of synaptic vesicles cannot be identified in the OHC opposite the RNJ's. In this location few examples of groups of circular profiles can be found out of the hundreds of RNJ's examined. These circular profiles, when studied in serial sections or in nearly complete series, are best interpreted as cross sections of tubular SER (Fig. 2: SER). There may be isolated vesicles in the SER, but they do not show any

tendency to gather at the RNJ's. No structures like synaptic ribbons are seen; slight presynaptic projections of the junctional density do occur. Gap junctions are not found at the synapse, although they (also refs. 7 and 14; Mulroy and Nadol, personal communication) often link supporting cells (Figs. 1, 3, and 4). Minute conical tips appear on all cell types and do not form junctions in the present material (Figs. 3 and 4). Occasionally small desmosomes or attachment plaques between fiber and hair cell flank the RNJ's.

FIG. 4. Outer hair cell synapses. I', protuberances of hair cell that make indented recepto-neural junctions in adjacent sections; P, small conical tips on supporting cell processes; arrow, coated vesicle fused with endoplasmic reticulum. Radial section; other abbreviations as in preceding figures; $\times 45,000$.

DISCUSSION

At the IHC the *asymmetrical* synaptic membrane complexes of both types of RNJ resemble excitatory synapses in

the auditory system (15). As in other receptors (5, 16) synaptic ribbons with vesicles occur but only at one type of RNJ. At the OHC, the RNJ's have *symmetrical* membrane com-

plexes, which are not as a rule associated with synaptic ribbons or vesicle aggregates. These observations may reflect functional differences between IHC's and OHC's, as implied by their separate innervations (10). Perhaps the inner and outer hair cell synapses involve different principles of transmission or different transmitter agents. The apparent lack of synaptic ribbons and vesicle aggregates at the OHC's could be an artifact of preparation or interpretation. However, in the present aldehyde-fixed material, the synaptic zone is filled by organelles and nearly free of fixation artifacts. The observations are consistent with those of Rodríguez Echandía (8), using osmium fixation. Other artifacts, which cannot be ruled out, might result from chemical fixation, general anesthesia, or uncontrolled acoustic stimulation. However, it would be hard to explain the lack of such artifacts at the other cochlear synapses, including the synaptic ribbon formations of IHC's; in any case, the inner and outer hair cell synapses seem to have different structural properties. Although a few "synaptic" vesicles have been reported in OHC's (8), one need not interpret a few scattered profiles of SER as a population of synaptic vesicles, especially when they do not gather at the RNJ's. However, canaliculi and cisterns of SER closely approach and possibly fuse with the presynaptic membrane and so could provide a direct path from the cell's metabolic center to the synapses. Perhaps these forms of SER in the OHC correspond to the vesicle aggregates of other synapses (8). Coated vesicles could take part in synaptic transmission but may be involved in membrane recycling and endocytosis (17).

Given the present findings, one may ponder the nature of the sensory synapses. Although chemical transmission and quantal release may occur in the goldfish sacculus (18), which is known to have synaptic ribbons (14), there are no comparable data for the mammalian cochlea. Neither is there a morphological basis for electrical transmission at these synapses, since gap junctions are not seen at the RNJ's. If chemical transmission occurs at the RNJ, one may question if synaptic vesicles normally store transmitters in the OHC. If synaptic vesicles release transmitter at the presynaptic membrane, they must be too few and too ephemeral to be recognized in our experiments. The same considerations apply to the nonvesiculated RNJ of the IHC and to similar junctions in photoreceptors (16). Perhaps quantal release does not require synaptic vesicles (19, 20). Static histological observations cannot resolve these issues, when the synaptic activity at the time of fixation is unknown.

We thank Debbye Yurgelun for technical aid and members of the Massachusetts Eye and Ear Infirmary for personal communications and criticism. This work was supported by the Deafness Research Foundation and by U.S. Public Health Service grants 5 T01 GM00406, 5 R01 NS06115, and 1 K04 NS42,538.

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