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Fine structure of the afferent synapse of the hair cells in the saccular macula of the goldfish, with special reference to the anastomosing tubules

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Summary

The fine structure of the afferent synapse has been studied in the hair cells of the goldfish saccular macula.

A spherical dense body which is surrounded by synaptic vesicles is observed in association with the presynaptic membrane. An alternating, parallel arrangement of dense bars and of rows of synaptic vesicles is observed on the presynaptic membrane beneath the dense body. Each row consists of five to six immediately available synaptic vesicles, and five to six such rows of vesicles are observed per synapse.

Sometimes anastomosing tubules are found around the dense body. The tubules are formed by direct infolding of the plasma membrane. Many coated vesicles are found at the periphery of the anastomosing tubules.

A possible role of the anastomosing tubules in the turnover of the synaptic vesicle membrane is discussed.

Introduction

The hair cells of the saccular macula of the goldfish are the receptor cells sensitive to vibratory stimuli. The apical surface of the sensory cell is studded with sensory hairs consisting of a kinocilium, and 40–70 regularly arranged stereocilia. Two types of nerve terminal, afferent and efferent, contact the basal surface of the cells as is commonly seen in the acoustico-lateralis organs (Engström, 1958, 1960; Spoendlin, 1959, 1960; Iurato, 1961, 1962; Smith and Sjöstrand, 1961; Kimura *et al.*, 1964; Loewenstein *et al.*, 1964; Flock, 1965; Hama, 1965; Jande, 1966; Takasaka and Smith, 1971; Düring *et al.*, 1974). Cell excitation caused by shearing motion of

the sensory hairs is synaptically transmitted to the afferent nerve fibres. The afferent synapse is characterized by the adjacent, presynaptic spherical dense body which is surrounded by synaptic vesicles. The anastomosing tubules and related vesicles are located in the basal cytoplasm near the afferent synapse (Hama, 1969). The present paper deals with the fine structure of the afferent synapse with special reference to the anastomosing tubules. The origin of synaptic vesicles, the mode of transmitter release and the fate of the synaptic vesicle membrane are not known in the hair cells of the acoustico-lateralis system. A possible function of the anastomosing tubules in the turnover of the synaptic vesicle membrane is discussed.

Some of our results were reported previously (Hama and Saito, 1974).

Material and methods

Animals used were common Japanese goldfish, 10–15 cm in crown–caudal length.

The saccule was dissected out from the animals, and the otolith was removed before immersion into the fixative. Care was taken not to damage the sensory epithelium because the otolith is closely attached to the surface of the epithelium.

Preparation of saccules for electron microscopy

a) The specimen was fixed for 3 h with a fixative consisting of 2.5% glutaraldehyde, 2% paraformaldehyde and 0.1M sodium cacodylate buffer (pH 7.2) at room temperature, rinsed for 30 min in buffer containing 8% sucrose, and then postfixed for 2 h in cold 2% osmium tetroxide in the same buffer.

b) Lanthanum was used as a marker for the extracellular space.

The specimen was soaked for 5–10 min in saline solution containing 0.5% lanthanum hydrosol at room temperature, and then fixed for 2 h with 1% osmium tetroxide in 0.1M sodium cacodylate buffer (pH 7.8) containing 0.5% lanthanum hydrosol and 8% sucrose.

c) For the peroxidase experiment, the specimens were soaked in fish physiological saline containing 0.25% horseradish peroxidase HRP (Sigma Chemical Co., St. Louis, Missouri: Type II) for 2 h at room temperature. After a brief rinse, the specimens were immersed in a fixative consisting of 2.5% glutaraldehyde, 2% paraformaldehyde, 0.1M phosphate buffer (pH 7.2) and 6% sucrose for 3 h at room temperature. They were then rinsed overnight in cold 0.1M cacodylate (pH 7.2) with 6% sucrose. Fixed specimens were soaked in the diaminobenzidine medium of Graham and Karnovsky (1966) for 1–2 h at room temperature. Tissue was postfixed in cold 1.3% OsO_4 in 0.1M cacodylate buffer (pH 7.2) for 1.5 h, stained *en bloc* with uranyl acetate, and processed for thin sectioning as usual.

d) For the demonstration of coated vesicles the specimen was fixed for 2 h with unbuffered 4% osmium tetroxide, and then postfixed for 2 h with unbuffered 8% glutaraldehyde at room temperature (Kanaseki and Kadota, 1969).

After fixation, all specimens were dehydrated through an ascending series of ethyl alcohol followed by propylene oxide and embedded in Epon 812 (Luft, 1961).

Thin sections were stained with Millonig's lead (Millonig, 1961) alone, or with 2% uranyl acetate in 70% alcohol and lead hydroxide, and observed with the Hitachi HU–12 electron microscope.

Results

Two types of nerve terminal are found on the basal surface of the receptor cell. One contains no vesicles, and the other contains a cluster of vesicles 60–70 nm in

diameter. They have been identified as afferent and efferent synapses, respectively (Hama, 1969).

Fine structure of the afferent synapse

The dense body surrounded by synaptic vesicles is seen in the receptor cell cytoplasm beneath the presynaptic membrane juxtaposed to the afferent nerve terminal (Figs. 1a–c). Sometimes membrane-bounded, tubular structures are seen in direct continuity with the synaptic vesicles around the dense body (Fig. 1a). The juxtaposed synaptic membranes are separated by a cleft 15–20 nm wide. An electron-opaque intercalated layer is seen midway between the pre- and postsynaptic membranes (Fig. 1a).

Beneath the dense body some synaptic membrane specialization is observed. Pre- and postsynaptic membranes show increased electron opacity and are accompanied by opaque material on the cytoplasmic side (Fig. 1a). The pre- and postsynaptic membranes show a wave-like profile (Fig. 1a). Many caveolae with bristles are found on the basal plasma membrane of the receptor cell (Figs. 1a, 3 and 4).

The dense body is separated from the presynaptic membrane by a space of about 60 nm (Figs. 1a–c). Although the dense body is usually seen as a round profile about 0.3 μm in diameter, it varies in size and shape from specimen to specimen (Figs. 3, 5, and 7). In the cytoplasm between the dense body and the presynaptic membrane, dense projections of about 40 nm basal diameter and synaptic vesicles 40–50 nm in diameter are alternately arranged on the presynaptic membrane (Fig. 1a). The dense projections are seen on the protrusions of the membrane (Fig. 1a). The centre-to-centre distance between adjacent dense projections is approximately 60 nm. The tips of the dense projections are connected to the basal surface of the dense body (Fig. 1a).

The appearance of the presynaptic area of the afferent synapse depends upon the plane of section. In some sections, the dense projections and the synaptic vesicles are arranged alternately on the presynaptic membrane, as mentioned above. In others, only synaptic vesicles (Fig. 1b) or only an electron-dense rodlet about 0.2 μm long (Fig. 1c) is seen on the presynaptic membrane. In oblique section, some of the synaptic vesicles are arranged in a row between the rodlets, which are seen between the dense body and the postsynaptic density (Fig. 1d). In tangential section to the presynaptic membrane through the dense projections, alternately arranged rodlets about 0.2 μm long and rows of synaptic vesicles lie parallel to one another (Fig. 2). The centre-to-centre distance between adjacent dense rodlets is approximately 60 nm. Thus the dense projections seen in Fig. 1a represent cross sections of rodlets.

From the observations mentioned above, a scheme of the afferent synapse can be drawn as in Fig. 10. At the presynaptic side of the active zone of the afferent synapse, tunnels are formed between the dense body and the presynaptic membrane. The lateral walls of the tunnels are formed by the adjacent rodlets, and a row of synaptic vesicles is seen within each tunnel.

Each row of synaptic vesicles contains 5–6 vesicles. In a plane of section in which alternating rows of rodlets and synaptic vesicles are observed, there is a total of 6–7 rows. This configuration is reminiscent of that described in ampullary electric receptors in Amiurus (Mullinger, 1964). Since 5–7 active zones involving juxtaposition of the hair cell and afferent synapses are usually observed per hair cell in the saccular macula, one can estimate that about 150–300 immediately available synaptic vesicles are arranged on the presynaptic membrane in each hair cell.

Anastomosing tubule

In the basal cytoplasm, an elaborate membrane system composed of anastomosing tubules about 55 nm in diameter is seen near the afferent synapse (Fig. 3). The extent of the network varies from specimen to specimen (Figs. 2, 4, 5 and 8). Sometimes, the tubular network completely surrounds the dense body (Fig. 5). Buddings of the membrane with a bristle-like coat are observed at the periphery of the tubular network (Figs. 3, and 4). The tubular networks are frequently observed in continuity with the plasma membrane of the cell (Fig. 4). In lanthanum and peroxidase treated specimens, electron-opaque deposits are observed within the intercellular space, lumen of the tubular network and cavity of some coated vesicles (Figs. 6, 7).

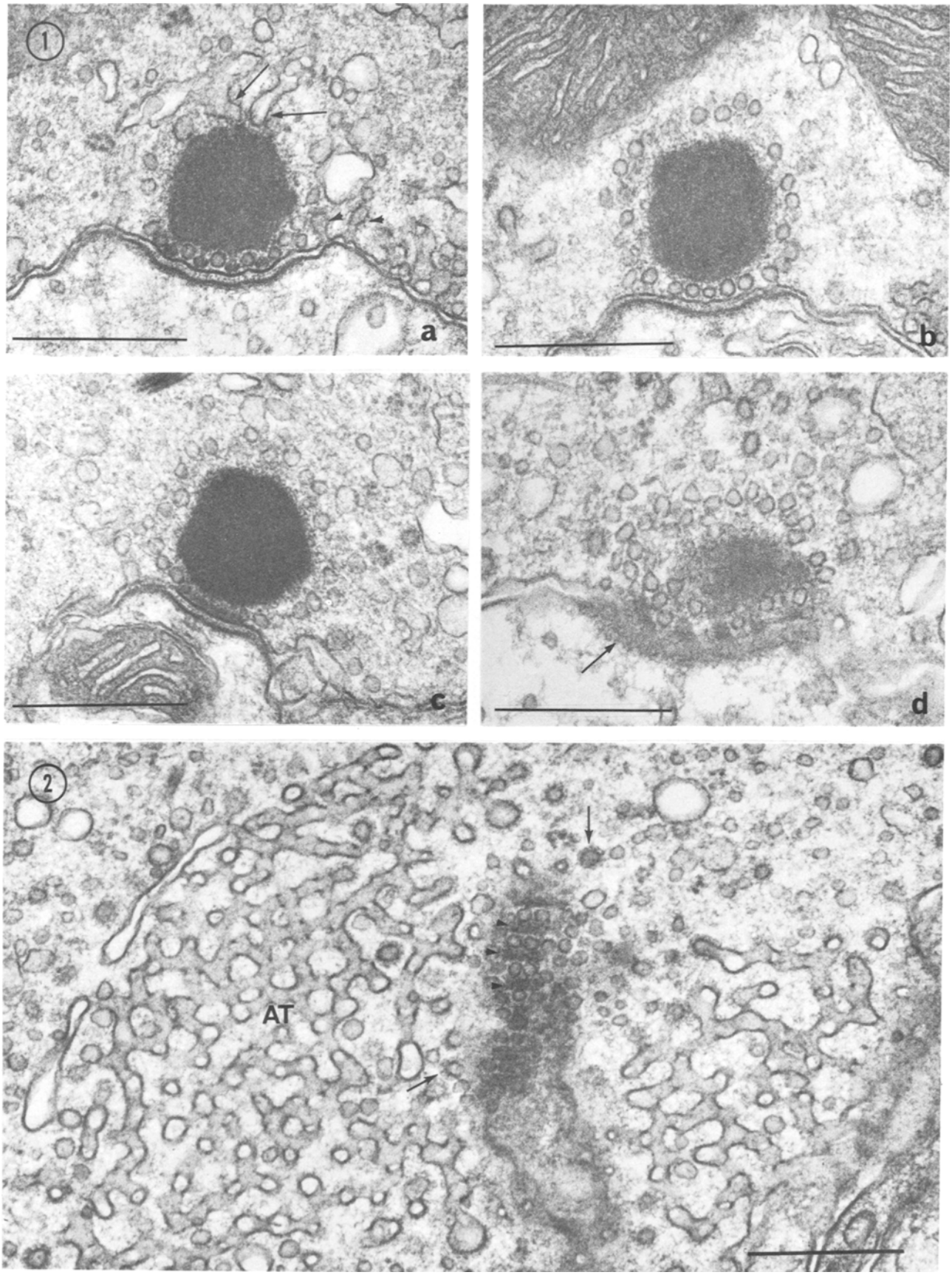
Flattened cistern of the endoplasmic reticulum, coated and smooth vesicles

A flattened cistern with attached ribosomes, and coated and smooth vesicles are observed around the tubular network and the dense body (Figs. 3 and 9). Sometimes, there is close apposition of the flattened cistern and the anastomosing tubules (Fig. 3).

Many coated vesicles are seen at the periphery of the tubular network (Figs. 3–5) and beneath the plasma membrane near the afferent synapse (Fig. 3). They are also seen between the tubular network and the dense body (Figs. 2 and 5). Those seen around the dense body have the size of the synaptic vesicles, ~50 nm in diameter.

Fig. 1. Electron micrographs showing afferent synapses. (The bars represent 0.5 μm) a) Synaptic vesicles and dense projections are alternately arranged between the dense body and the presynaptic membrane. Profiles suggesting a direct continuity between the endoplasmic reticulum and the synaptic vesicles are observed at many places (arrows). Caveolae with bristles are seen on the presynaptic membrane near the dense body (arrow heads). x 64 000. b) In this plane of section only synaptic vesicles are observed between the dense body and the presynaptic membrane. x 64 000. c) A dense rodlet occurs between the dense body and the presynaptic membrane. No synaptic vesicles are observed on the presynaptic membrane. x 64 000. d) Dense rodlets are observed as short bars situated beneath the dense body. Synaptic vesicles are located between the dense rodlets. The arrow indicates the postsynaptic membrane. x 64 000.

Fig. 2. A tangential section at the level between the dense body and the presynaptic membrane. Rows of synaptic vesicles occur between the dense rodlets (arrowheads). Smooth and coated vesicles (arrows) are seen between the anastomosing tubules (AT) and rows of synaptic vesicles. x 54 000.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In tissues fixed with unbuffered osmium tetroxide and unbuffered glutaraldehyde, coated vesicles and the shells without vesicles described by Kanaseki and Kadota (1969) are seen in the cytoplasm around the tubular network (Fig. 8).

Smooth vesicles, 45–50 nm in diameter, are observed between the tubular network and the dense body, intermingled with coated vesicles (Figs. 2 and 5). The diameter of the smooth vesicle is similar to that of the synaptic vesicle. The smooth vesicles are also seen between the endoplasmic reticulum and the dense body and between the endoplasmic reticulum and the tubular network (Fig. 9). Some of the smooth vesicles are observed attached to the endoplasmic reticulum and the tubular network (Figs. 5 and 9).

Discussion

The hypothesis of a local recycling of the synaptic vesicle membrane was postulated for synapses in the C.N.S. (Gray and Willis, 1970), retinal receptor (Gray and Pease, 1971; Ripps *et al.*, 1976; Schacher *et al.*, 1976) and at the frog neuromuscular junction (Ceccarelli *et al.*, 1973; Heuser and Reese, 1973; Heuser *et al.*, 1974). Taking the life span of the vesicle membrane into account, Teichberg *et al.* (1975), mentioned the role of the lysosome system in the turnover of the synaptic vesicle membrane in addition to local recycling.

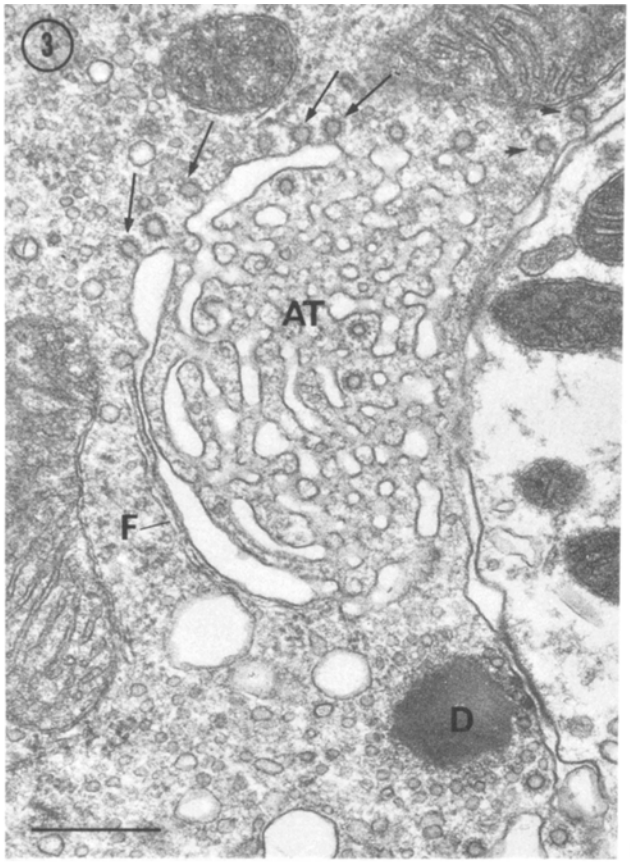
A relationship between the endoplasmic reticulum and synaptic vesicles has already been reported in the retina of the rat (Pellegrino de Iraldi and Suburo, 1971), dog, monkey, and human (Lovas, 1971), in motor end-plates (Düring, 1967) and rat spinal and chick ciliary ganglia (Droz *et al.*, 1975). In the goldfish tissue we examined, smooth membrane-bounded tubular structures are found around the dense body. These structures are frequently observed in direct continuity with some of the vesicles around the dense body, suggesting an origin from the endoplasmic reticulum of the synaptic vesicles.

The anastomosing tubules found around the dense body are continuous with the plasma membrane at many places. The limiting membrane of the anastomosing tubules has the same thickness as the plasma membrane and is thicker than that of the endoplasmic reticulum. Moreover, HRP and lanthanum used as extracellular

Fig. 3. Profiles of the afferent synapse and the anastomosing tubules (AT). Buddings with a bristle coat are seen on the periphery of the anastomosing tubules (arrows). Caveolae with bristles (arrowheads) are observed on the plasma membrane. A flattened cistern (F) is closely associated with the distended anastomosing tubules. D: dense body. x 34 000. (The bars represent 0.5 μm.)

Fig. 4. Anastomosing tubules in a regular network. The arrows indicate openings to the extracellular space. Buddings with a bristle-like coat are seen at the periphery of the tubular network..x 40 000.

Fig. 5. Dense body (D) surrounded by anastomosing tubules. Smooth vesicles (short arrows) are associated with the dilated ends of the anastomosing tubules. Coated and smooth vesicles are seen between the anastomosing tubules and the dense body. The long arrow indicates an opening of the anastomosing tubules to the extracellular space. x 60 000.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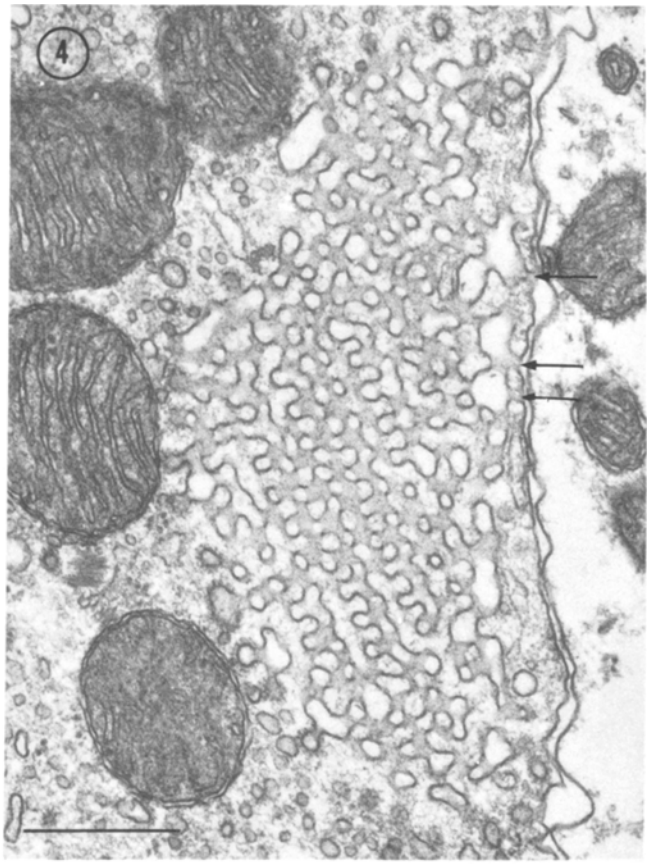
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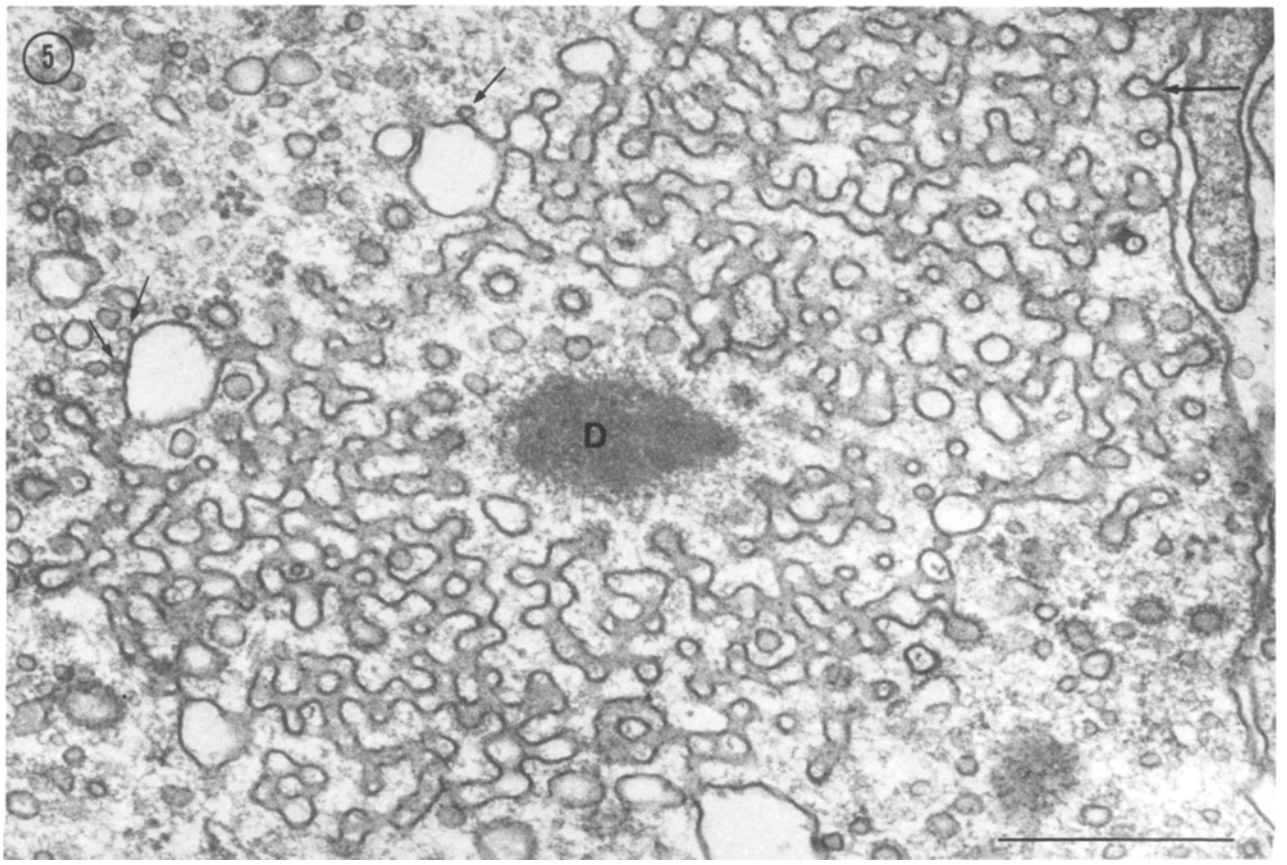
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space tracers penetrate into the tubular network. Thus, it is apparent that the anastomosing tubules near the afferent synapse are formed by the tubular infolding of the plasma membrane, unlike the irregular membrane-walled cistern in the frog neuromuscular junction which has been reported to be an intracytoplasmic membrane system (Heuser and Reese, 1973) or the smooth endoplasmic reticulum which continues from the perikaryon to the axon terminal (Droz *et al.*, 1975).

Many coated vesicles are formed at the periphery of the anastomosing tubules. The dense deposits of lanthanum and peroxidase reaction product are seen inside the small coated vesicles and the smooth vesicles around the presynaptic dense body. Because the epithelium is soaked in the saline solution containing lanthanum or peroxidase before fixation, it can be considered that the coated vesicles containing the dense deposits are derived from the tubular network, although the possibility that these profiles may represent cross sections of the tubular structures cannot be ruled out. Technical difficulty experienced in the tracer experiments in this study resulted from the fact that the epithelium of the saccular macula of the goldfish has a well-developed tight junctional network at the luminal surface and extensive gap junctions which connect the supporting cells near the basal surface. These features prevent the rapid spread of tracers into the epithelium (Hama and Saito, 1977). Those coated vesicles formed at the periphery facing the dense body, about 50 nm diameter, are intermingled with smooth vesicles of about the same size. Many empty shells (Kanaseki and Kadota, 1969) are found among these small coated vesicles. These observations suggest that the small coated vesicles become the synaptic vesicles after losing their bristle-like coat as reported by Gray (Gray and Willis, 1970; Gray and Pease, 1971), although little evidence for coated vesicle participation in recycling was seen by Ceccarelli *et al.* (1973) in their study of the frog neuromuscular junction. Similar tubular networks were also described in the Schwann cell surrounding squid (Hama, 1962) and lobster (Holzman *et al.*, 1970) giant axons. They are probably involved in the rapid passage of ions and water across the Schwann cell sheath to control the composition of the medium surrounding the axon surface (Holzman *et al.*, 1970).

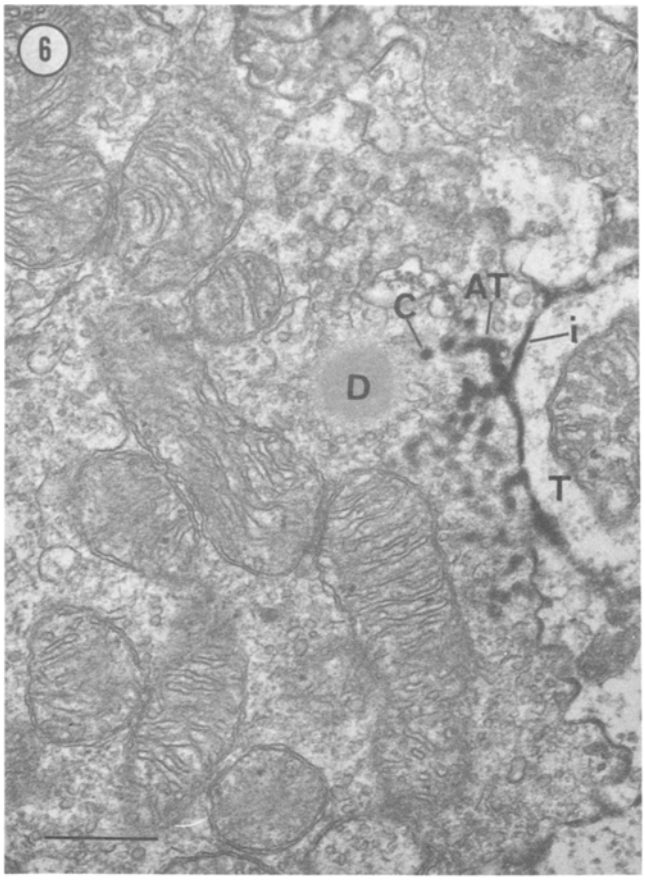
Continuity between the cytoplasmic tubular membrane and the plasma membrane was also observed in the growth cone of isolated sympathetic neurons in culture

Fig. 6. Colloidal lanthanum-treated material. Electron-opaque deposits are seen within the intercellular space (i), the lumen of the anastomosing tubules (AT), and the cavity of a coated vesicle (CV). D: dense body, T: afferent nerve terminal. x 30 000. (The bars represent 0.5 μm.)

Fig. 7. Peroxidase treated material. Opaque deposits are seen in the intercellular space (i), the lumen of the tubular structure (AT) and in the vesicular profile (V) near the dense body (D). T: afferent nerve terminal. x 38 000.

Fig. 8. In this specimen fixed with unbuffered osmium tetroxide and glutaraldehyde, many coated vesicles and shells without vesicles (arrows) are seen. T: nerve terminal, D: dense body. x 46 000.

Fig. 9. Profiles of the anastomosing tubules (AT) and endoplasmic reticulum (ER) are observed in close proximity. Ribosomes stud the surface of the endoplasmic reticulum in places. An attachment between a smooth vesicle and the endoplasmic reticulum is indicated (arrow). Many coated vesicles (arrowheads) are seen between the anastomosing tubules (AT) and dense bodies. x 40 000.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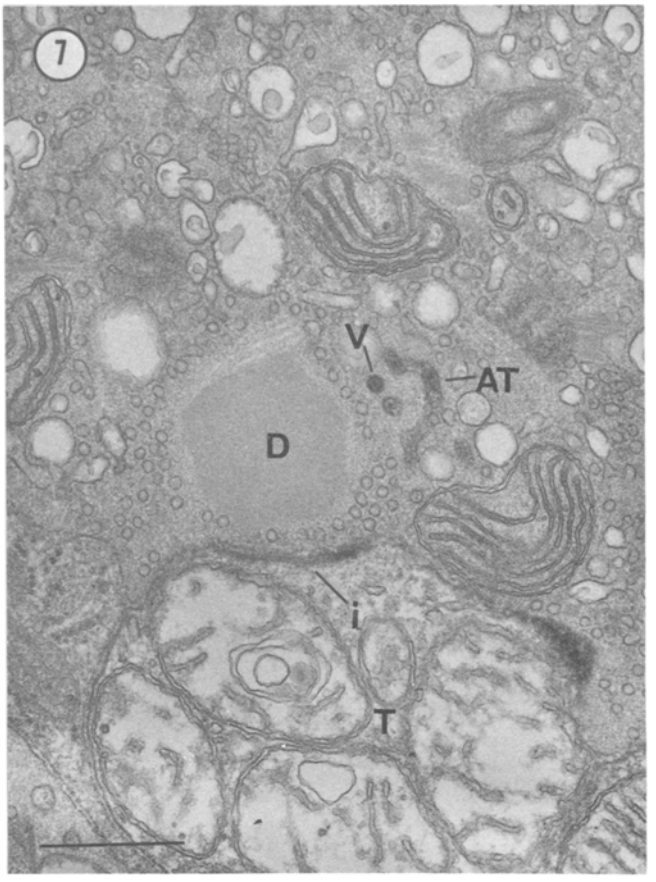
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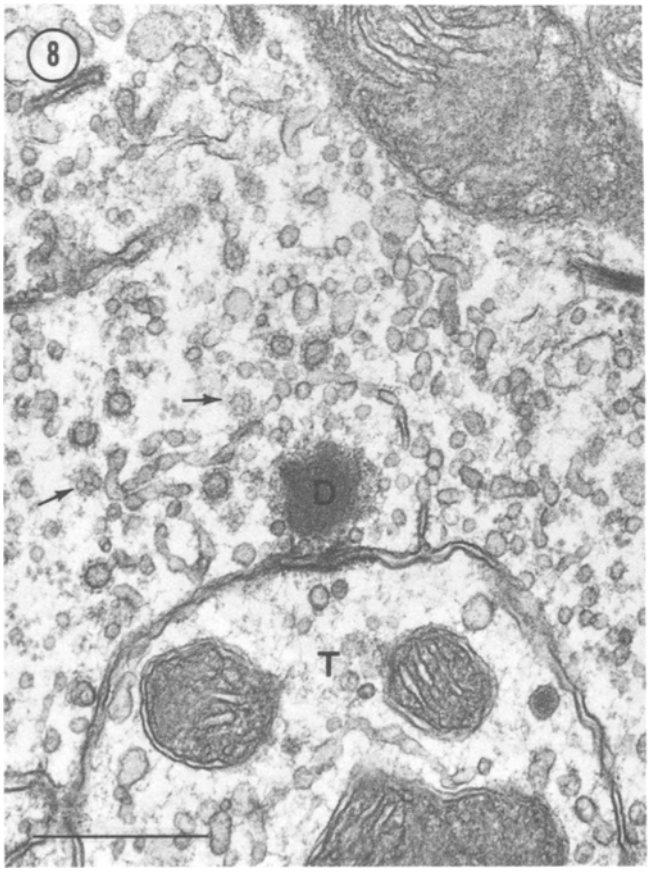
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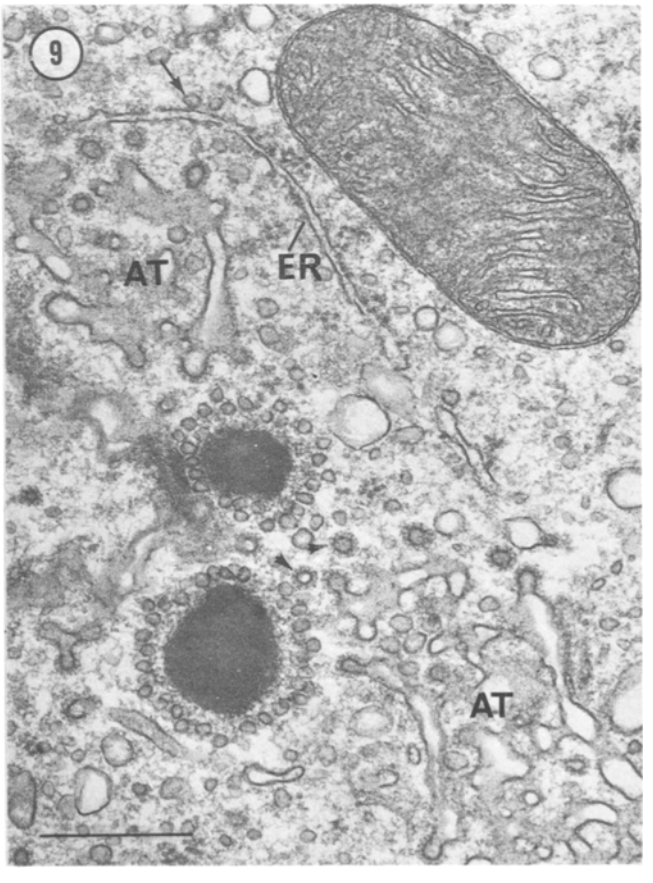
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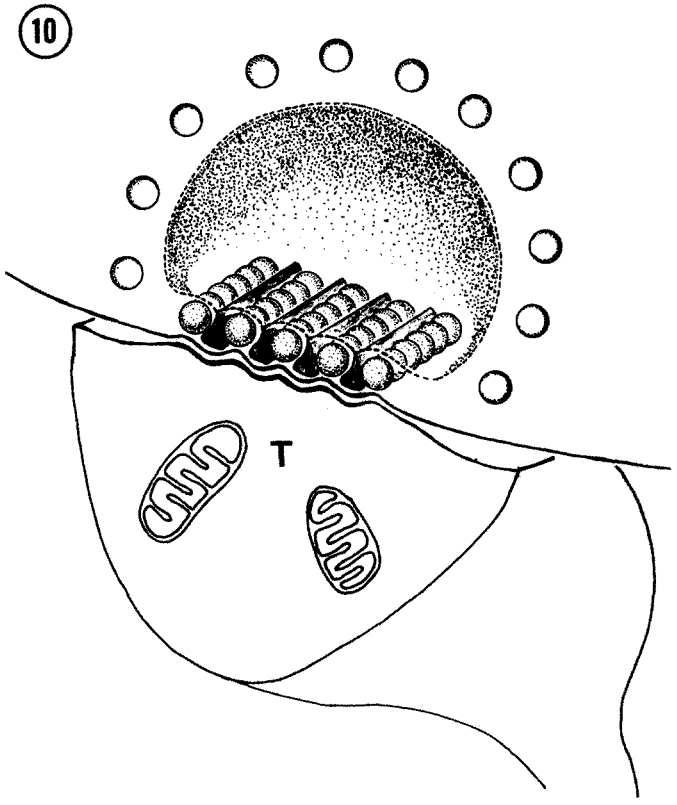

Fig. 10. A diagram showing the afferent synapse. T: afferent nerve terminal. For explanation see text.

(Bunge, 1973). The contribution of cytoplasmic tubular membranes to a rapid increase of the surface membrane as suggested by Bunge (1973) is unlikely in the sensory hair cells. In our material, the tubular network is always located near the afferent synapse and presumably is related to synaptic function. The tubular structures continuous with the extracellular space have been described in the hair cells of the lateral-line canal organ (Flock and Wersäll, 1962). However, those authors did not describe a relationship between the tubular structures and synaptic vesicles.

Based on the present observations, the following hypothesis on the turnover of synaptic vesicles in the hair cells of the saccular macula of the goldfish could be proposed (Fig. 11). The primary source of the synaptic vesicle is probably the endoplasmic reticulum. The synaptic vesicles are formed by pinching off from the endoplasmic reticulum and accumulate around the dense body. The transmitter is presumably released from the presynaptic cell by the process of exocytosis. The

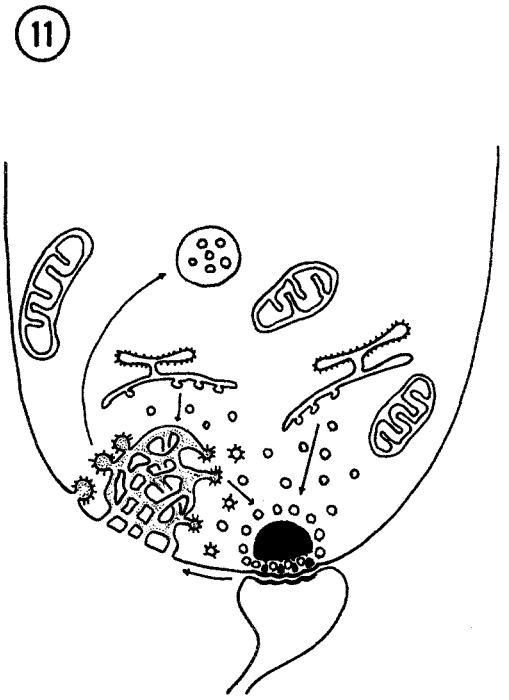

Fig. 11. A diagram showing the possible pathway of the synaptic vesicle membrane in the hair cell. For explanation see text.

synaptic vesicle membrane is added to the presynaptic membrane by this process, and then the excess membrane is probably taken into the cell by coated vesicle formation at the periphery of the active zone and is decomposed by the lysosome system as mentioned by Teichberg *et al.* (1975). Sometimes, probably at hyperfunction of the cell, excess membrane is taken into the cell by tubular infolding of the plasma membrane near the active zone. The complicated anastomosing tubules are formed by the fusion of these tubular infoldings. The coated vesicles are pinched off from the distal surface of the anastomosing tubules and are probably destroyed by the lysosome system together with the coated vesicles formed at the plasma membrane. The coated vesicles are also formed at the periphery of the anastomosing tubules facing the dense body. There is a possibility that coated vesicles are re-utilized as synaptic vesicles after losing their shell as described by Heuser and Reese (1973) and Gray and Pease (1971). The cistern of endoplasmic reticulum around the anastomosing tubules probably contributes material to the anastomosing tubules.

Thus anastomosing tubules can be considered as a membrane reservoir regulating the turnover of the synaptic vesicle membrane although other functions of the tubular network cannot be ruled out, e.g. exchanging material between the receptor cell and extracellular space.

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