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Presynaptic fibres of spiral neurons and reciprocal synapses in the organ of Corti in culture

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Summary

Isolated segments of the newborn mouse organ of Corti were explanted together with the spiral ganglion components. Within the innervation provided by the spiral neurons, we observed presynaptic vesiculated nerve endings that form reciprocal ribbon-afferent/efferent synapses with inner hair cells. These intracochlear presynaptic fibres are characteristically located between adjoining inner hair cells, on the modiolar side, low and close to the supporting cells. The presynaptic fibres display different modes of synaptic connectivity, forming repetitive reciprocal synapses on single inner hair cells or on adjoining hair cells, or connecting adjoining inner hair cells through simultaneous efferent synapses. Many presynaptic fibres exhibit a distinctive ultrastructure: defined clusters of synaptic vesicles, dense core vesicles, coated vesicles, and mitochondria. These organelles occur focally at the synaptic sites; beyond the efferent synaptic specializations, the endings appear quite nondescript and afferent-like.

We believe that the reciprocal synapses, although observed in cultures of the organ of Corti, represent real intracochlear synaptic arrangements providing a feedback mechanism between the primary sensory receptors and a special class of spiral ganglion cells that have yet to be recognized in the organ *in situ*.

Introduction

The term 'reciprocal synapse' refers to a synaptic arrangement in which two neuronal elements exhibit alternate pre- and postsynaptic relationships that provide a means for bidirectional synaptic activity. Together with dendro–dendritic synapses, reciprocal synapses are characteristic of many afferent sensory pathways and of the sensory processing areas in the CNS.

Reciprocal synapses were first described in the olfactory bulb, where they occur between the dendrites of granule and mitral, and possibly also tufted, cells (Hirata, 1964; Andres, 1965; Reese & Brightman, 1965; Rall *et al.*, 1966; Price, 1968; Hinds, 1970; Reese & Shepherd, 1972; Landis *et al.*, 1974; King *et al.*, 1975; Jackowski *et al.*, 1978; Rebière & Dainat, 1981; Wilson & Leon, 1988), and where they may amount to 90% of all dendro–dendritic connections.

Reciprocal synaptic arrangements were next discovered in the inner plexiform layer of the retina between the bipolar neuron terminals that form dyad ribbon synapses and the dendrites of amacrine cells (Dowling & Boycott, 1966; Raviola & Raviola, 1967; Dowling, 1968; Vaughn *et al.*, 1981; Tachibana & Kaneko, 1988; Strettoi *et al.*, 1990; Massey *et al.*, 1992).

Reciprocal synapses were also identified as a contingent of dendro–dendritic synapses in the lateral geniculate nucleus (Famiglietti, 1970; Lieberman & Webster, 1972; Lieberman, 1973), in the ventrolateral nucleus of the thalamus (Harding, 1971), in the nuclei of the dorsal column (Ellis & Rustioni, 1981), and in the substantia gelatinosa in the spinal cord (Gobel *et al.*, 1980). Lieberman (1973) commented that central neurons participating in reciprocal synapses are typically small-sized, anaxonal intrinsic cells that function as inhibitory interneurons, e.g. granule cells in the olfactory bulb or amacrine cells in the retina.

In the PNS, reciprocal synapses occur in the carotid body. They are formed between the afferent chemoreceptor–nerve endings and the dopaminergic glomus cells, and also between glomus cells themselves (King *et al.*, 1975; McDonald & Mitchell, 1975; McDonald, 1976). An intriguing finding revealed that, in serial sections, the large afferent calyces and the presynaptic vesiculated boutons both stem from the same afferent

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fibres (McDonald & Mitchell, 1975). Thus, some afferent endings may be pre- or postsynaptic to glomus cells or may form reciprocal synapses with them. This variety of immediate connections in the carotid body is truly astounding.

In the inner ear organs, reciprocal synapses are characterized by the presence of a presynaptic ribbon at the afferent site and a postsynaptic cistern at the efferent site. Reciprocal synapses between sensory cells and efferent fibres have been described in the basilar papilla of the chick (Tanaka & Smith, 1978) and in the organ of Corti of the guinea pig (Thorn *et al.*, 1972), although both authors stress the rarity of the finding. Occasionally, ribbon synapses of the sensory cells occur with vesiculated fibres in the macula utriculi and sacculi in the squirrel monkey (Engström *et al.*, 1972). In the paratympanic organ of the chick, reciprocal synapses between hair cells and efferent (vesiculated) fibres are frequent but may involve a limited number of sensory cells (Gianessi, 1989).

Reciprocal synapses between hair cells and afferent fibres were described in the crista ampullaris of the bullfrog by Dunn (1976, 1980), where they comprise 6–8% of the anterior ampullary nerve. Unique synaptic arrangements have been reported in the mammalian utricular and saccular maculae. There, the Type II hair cells form ribbon-afferent/efferent reciprocal synapses with the presynaptic vesiculated endings of intramacular origin, which constitute about 20% of the synaptic connections (Ross *et al.*, 1986, 1990).

In the cochlea, reciprocal synapses have been described by Nadol in the human (1981, 1983, 1984, 1990) and also in the chimpanzee (1988). These are considered to be specific for primates and restricted to the outer hair cells. In the human, reciprocal synapses constitute about 50% of all afferent endings on outer hair cells, and their frequency increases from the first to the third row (Nadol, 1984). Nadol believed that these reciprocal synapses could provide a feedback loop between the spiral neurons and the outer hair cells and could function as an immediate regulatory mechanism for several receptor cells at once. Recent electrophysiological studies on postsynaptic inhibition in avian auditory nerve fibres (in the pigeon, 51% of the neurons are influenced by postsynaptic inhibition) actually suggest the presence of reciprocal synapses at the dendritic terminals (Gummer, 1991).

In the present work, we demonstrate the occurrence of reciprocal synapses between the primary receptors (the inner hair cells) and presynaptic vesiculated fibres in cultures of the mouse organ of Corti, explanted together with the spiral ganglion. Presynaptic innervation in the isolated organ can only originate in the spiral ganglion. Our ultrastructural results suggest that similar synaptic arrangements could easily be overlooked *in situ*.

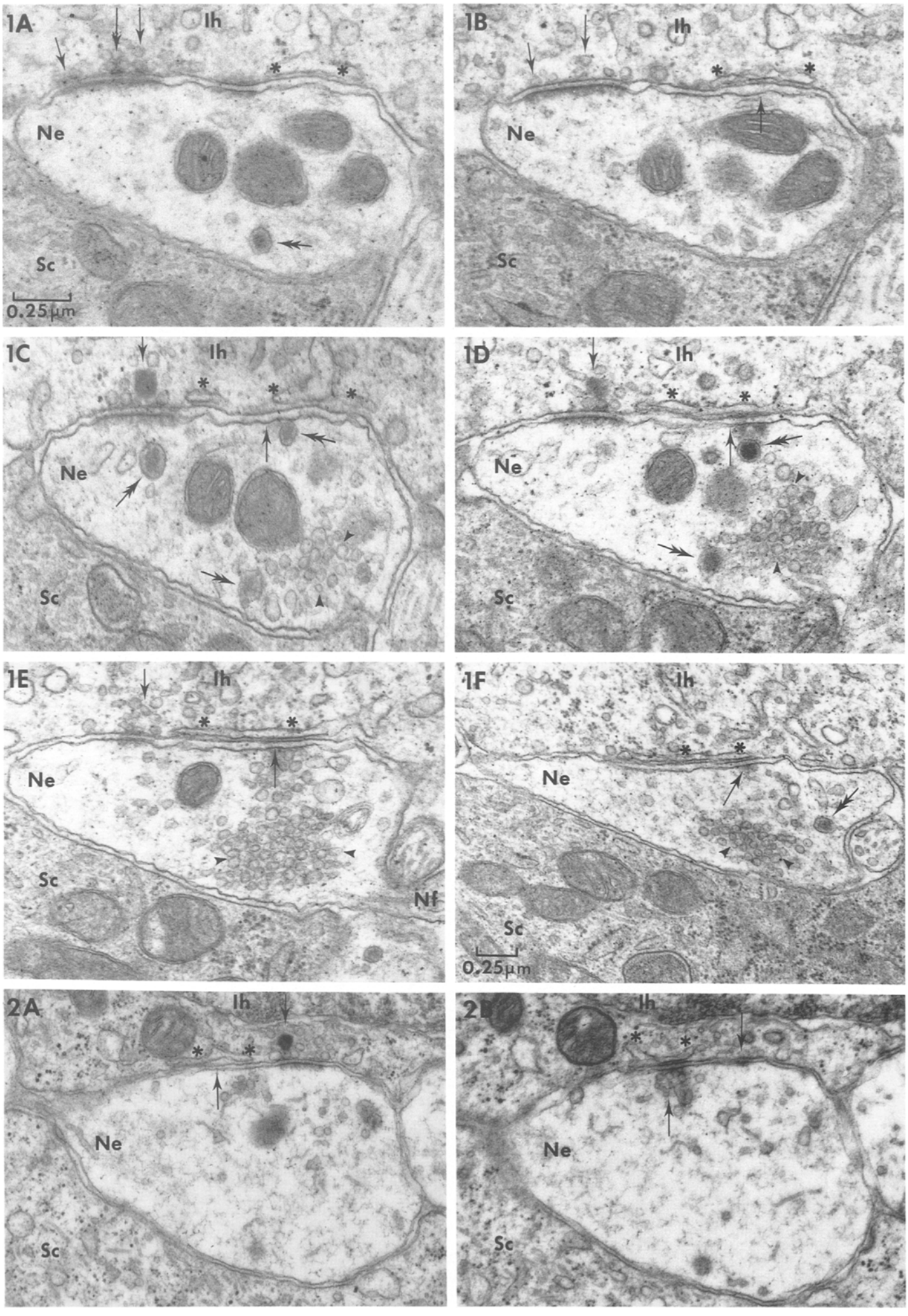
Materials and methods

The technique of preparation and maintenance of cultures of the newborn mouse organ of Corti together with the corresponding segment of spiral ganglion is described by Sobkowicz and colleagues (1975, 1993). The methods for preparing cultures for electron microscopical study are described by Guillery and colleagues (1970). Presynaptic vesiculated endings were looked for in eight cultures of neonatal organ of Corti, 3–14 days *in vitro* (DIV). Two of these cultures were experimental: in one, part of the sensory region was injured mechanically at 3 DIV. The culture was fixed after four days of recovery (total 7 DIV), and only the control, noninjured area was used for the present study. The

Abbreviations: Ih, inner hair cell; Is, inner spiral sulcus; Ne, nerve ending; Nf, nerve fibre; Oh, outer hair cell; Sc, supporting cell.

Fig. 1. Six of 12 serial sections through a vesiculated ending in culture, forming repetitive reciprocal synapses with an Ih. Arrows point in the assumed direction of synaptic transmission: the upper arrow points to the ribbon-afferent presynaptic specializations; the lower arrow points to the efferent-type synapse, characterized by discrete presynaptic densities and a postsynaptic cistern (asterisks). Arrowheads delimit clusters of clear synaptic vesicles. Dense core vesicles are marked by double arrows. (A) (section no. 4) Remnants of a multiribbon afferent synapse are at left. A postsynaptic cistern at right indicates the site of an incipient efferent synapse, but synaptic vesicles in the nerve ending are absent. Note the large dense core vesicle. Scale in A also applies to B–E. (B) (section no. 5) The ribbons at left are gone, some synaptic vesicles mark their place, and the postsynaptic density persists. A subsurface cistern is aligned at right in the postsynaptic position, but synaptic vesicles are still not present. (C) (section no. 8) A second ribbon synapse is formed. The ending now shows dense core vesicles (note the one at the presynaptic membrane) and a cluster of clear synaptic vesicles. The postsynaptic cistern is present at right. (D) (section no. 9) A ribbon afferent synapse is now side by side with a fully developed efferent synapse. Some of the synaptic vesicles are close to the densities of the presynaptic membrane. The postsynaptic cistern is in position. (E) (section no. 10) The ribbon is no longer present, and the site of the afferent synapse is marked by only a few vesicles; the efferent synapse is fully developed. (F) (section no. 12) The afferent synapse is no longer present. The postsynaptic cistern is still present, but the cluster of synaptic vesicles is now away from the presynaptic membrane.

Fig. 2. A reciprocal synapse involving an Ih in two consecutive sections. (A) A ribbon afferent synapse (upper arrow) formed on a nondescript 'afferent' type of nerve ending. (B) An efferent type of synapse (lower arrow) forms next to the afferent site: presynaptic vesicles are aligned at the presynaptic membrane of the nerve ending; a postsynaptic cistern (asterisks) is formed by the Ih along the apposition. Scale in 1F also applies to 2A & B.


1A
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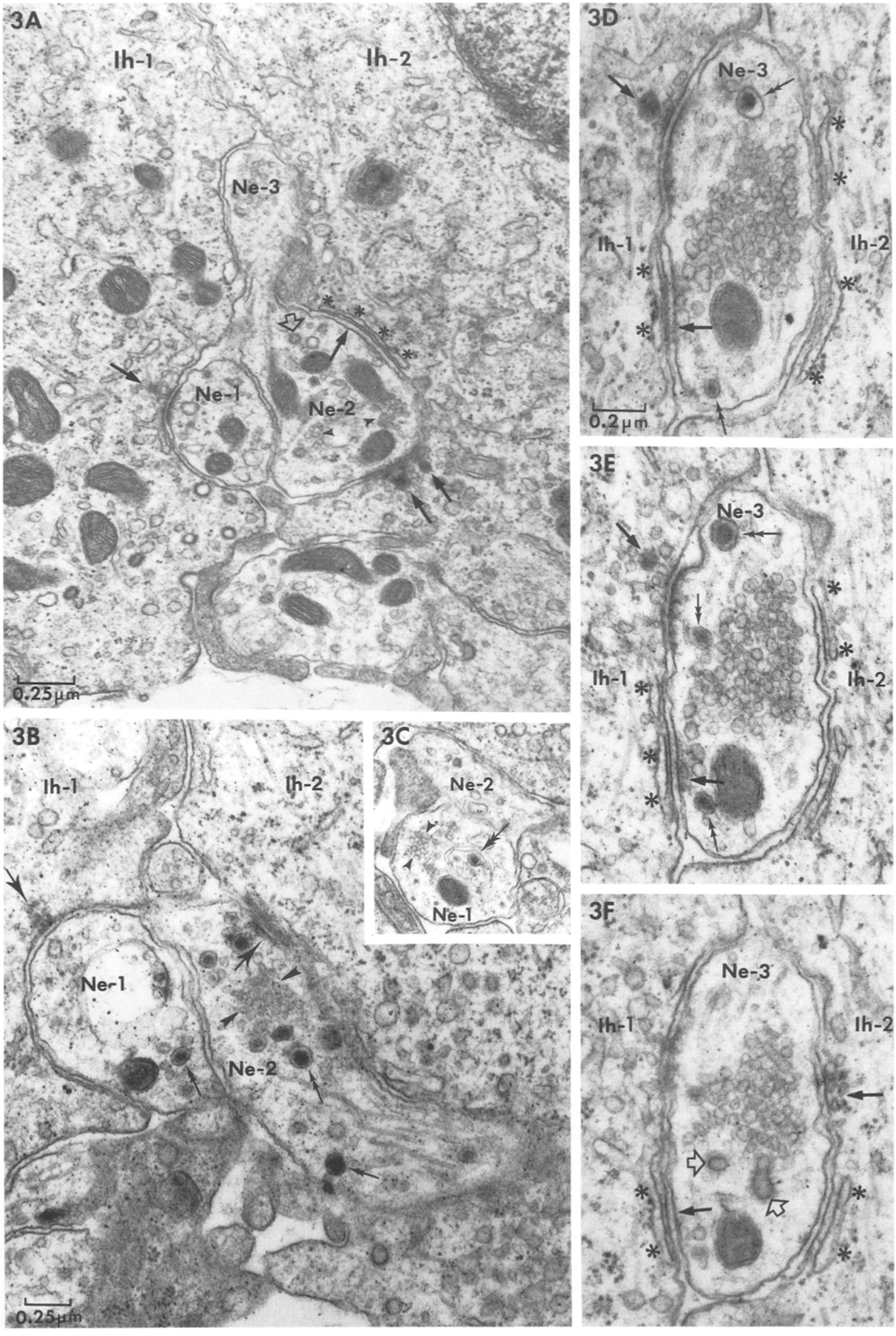
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Ih-1
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Ne-1
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0.25µm
3B
Ih-1
Ih-2
Ne-1
Ne-2
0.25µm
3C
Ne-2
Ne-1
3D
Ne-3
Ih-1
Ih-2
0.2µm
3E
Ne-3
Ih-1
Ih-2
3F
Ne-3
Ih-1
Ih-2

other culture, 9 DIV, was uninjured but fed for six days with feeding solution from recovering postinjury cultures (to demonstrate possible diffusible mitogenic factors produced in postinjury cultures (Sobkowicz *et al.*, 1992)).

Reciprocal synapses are difficult to find. In screening cultures for presynaptic fibres, we section the explants tangentially, alternating between 1 μm sections for light microscopy and 2.0–2.5 μm sections for re-embedding. In this way, the entire organ of Corti is sectioned within one or two days, yet we can go back to any given area or structure if needed. Sections that display a continuous row of inner hair cells, cut low through their receptor pole but containing nuclei and clear inner spiral fibres, are most favourable in searching for reciprocal synapses.

Results

The reciprocal synapses in our material occur between the primary receptors (the inner hair cells) and presynaptic vesiculated fibres originating from the spiral ganglion (Figs 1 & 2).

PRESYNAPTIC VESICULATED ENDINGS

The occurrence in culture of presynaptic vesiculated endings, reminiscent of the lateral olivocochlear fibres innervating the organ *in situ*, have been reported by us previously (Sobkowicz *et al.*, 1984; Sobkowicz, 1992). These endings, however, appeared to be rare. They have since been noted in eight cultures of the neonatal cochlea at 3–14 DIV, in all three turns of the organ of Corti. At first, the occurrence of an occasional vesiculated presynaptic ending did not suggest any pattern, except for their evident restriction to the innervation of inner hair cells. Two methodological adjustments suddenly brought more of these endings into view. First was the change in the plane of sectioning, from the conventional cross-sections, showing one inner and three outer hair cells, to the tangential plane that includes a continuous row of inner hair cells. Second was the serial mapping of suggestive endings.

Location

Vesiculated endings in culture are characteristically located on the modiolar side of the inner hair cells, where they seem to nest between two adjacent inner hair cells, preferably close to the supporting cells nearby (Fig. 3A).

Occurrence

Screening of consecutive or close sections (accounting

Fig. 4. (A) Vesiculated Ne-1 connects with both Ih's through efferent synapses (arrows). Ne-2, which is possibly continuous with Ne-1 (arrowheads) also forms an efferent synapse with Ih-2 (arrow). Note dense core vesicles (double arrows). (B) Unexpectedly, some distance away, ending Ne-2 is free of synaptic vesicles and receives a ribbon synapse (arrow). The empty arrow marks a coated vesicle. Scale in Fig. 6 also applies to Figs 4B, 5A and 5B.

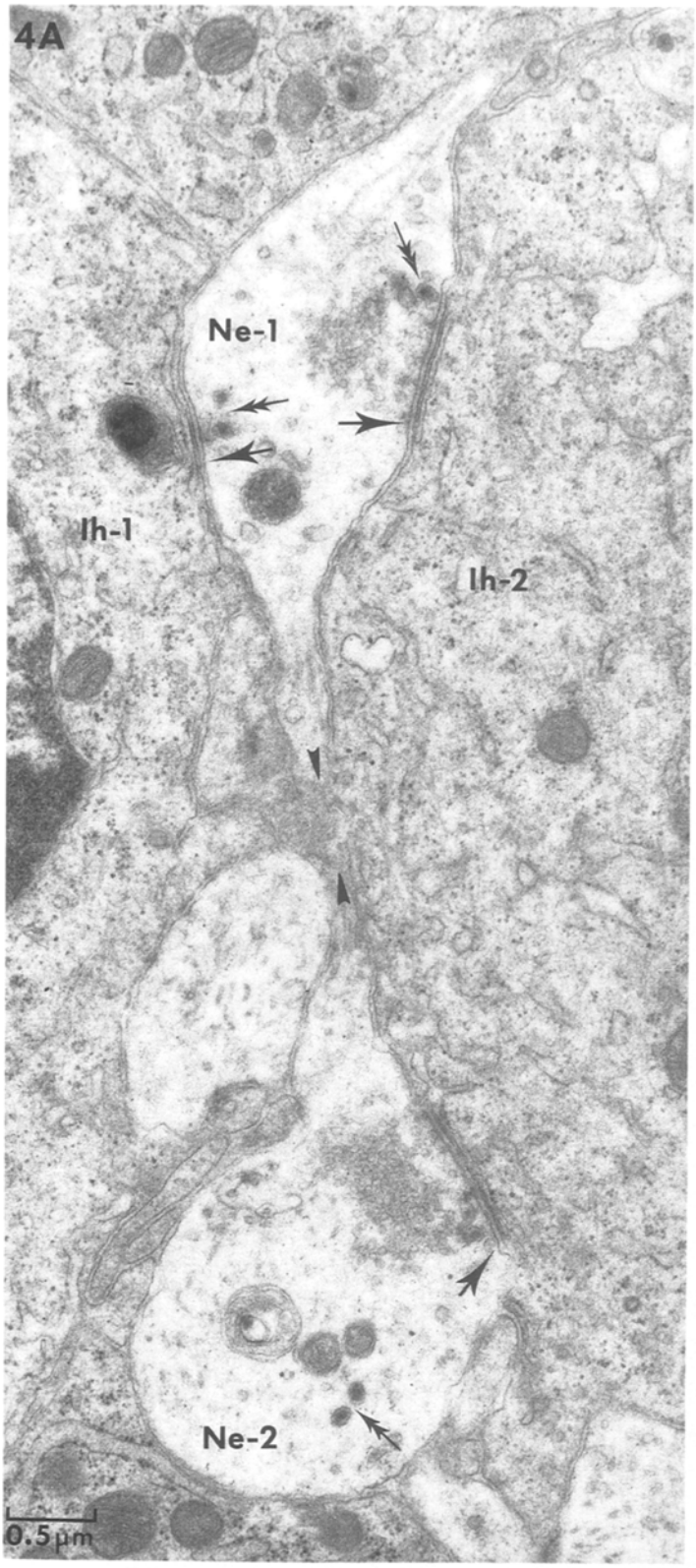
Fig. 5. Another example of a presynaptic vesiculated ending connecting two Ih's through efferent synapses (small arrows) followed in 13 consecutive sections. (A) Note the distinct efferent specializations with Ih-2: The synaptic vesicles (arrowheads) extend to the presynaptic membrane which is electron dense with presynaptic densities; the postsynaptic cistern (asterisks here and in (B) is also electron dense, its upper membrane decorated with ribosomes; fuzzy material fills the cleft. Arrow in the upper right points to a free cytoplasmic ribbon. The empty arrow points to a coated vesicle. (B) A second efferent synapse (lower arrow) forms with Ih-1 three sections away.

Fig. 6. (Upper right of plate) A vesiculated ending characterized by a clump of synaptic vesicles (arrowheads) and engaged synaptically with the Ih through a ribbon afferent synapse.

Fig. 7. (A) A climbing afferent-like fibre alongside the Ih forms a reciprocal synapse (arrow/asterisks) *en passant*. (B) The two synaptic contacts at higher magnification. (C) Several sections away, another cluster of synaptic vesicles appears; curved arrows in (A) and (C) approximate corresponding locations. Note, both in (B) and (C), a close relationship between the vesicles and the mitochondria, which is characteristic in nerve fibre shafts. Scale in Fig. 7B also applies to Figs 7C and 8.

Fig. 8. Three neuronal profiles caught *en passant* displaying well-defined clusters of synaptic vesicles (arrowheads). The empty arrow indicates a coated vesicle.

Fig. 3. A cluster of three nerve endings nesting between two adjacent Ihs was followed through 16 sections. (A) (section no. 7) All three endings are shown in their characteristic location adjoining both Ihs, rather low, on the modiolar side. Ne-2 and -3 are connected, while Ne-1 appears only to share their location. Ne-1 forms a ribbon afferent synapse with Ih-1 (arrow); the vesiculated Ne-2 connects with Ih-2 through a double ribbon afferent and possibly an efferent synapse (arrows). An arrow in Ne-2 points toward the apposing subsurface cistern along the hair cell membrane. An empty arrow points to a coated vesicle. (B) (section no. 1) Ne-1 shows a remnant of another ribbon synapse with Ih-1 (left arrow); Ne-2 forms an efferent synapse with Ih-2 (right arrow). Note several dense core vesicles (double arrows) and a well-defined group of clear synaptic vesicles (arrowheads). (C) (section no. 12) Ne-1 unexpectedly reveals a well-defined group of synaptic vesicles (arrowheads); Ne-2 is inconspicuous and is no longer present in section 13. (D) (section no. 13) A side-by-side afferent/efferent reciprocal synapse with Ih-1 (arrows). Postsynaptic cisterns (asterisks) also align along the side of Ih-2. Note dense core vesicles (double arrows). Scale in 3D also applies to E and F. (E) (section no. 14) The reciprocal synapse between Ne-3 and Ih-1 continues. (F) (section no. 15) A ribbon synapse is now forming on Ih-2 (arrow). Postsynaptic cisterns (asterisks) align along the postsynaptic membrane of both Ih-1 and Ih-2. Note coated vesicles (empty arrows).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Ih-1
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Ne-2
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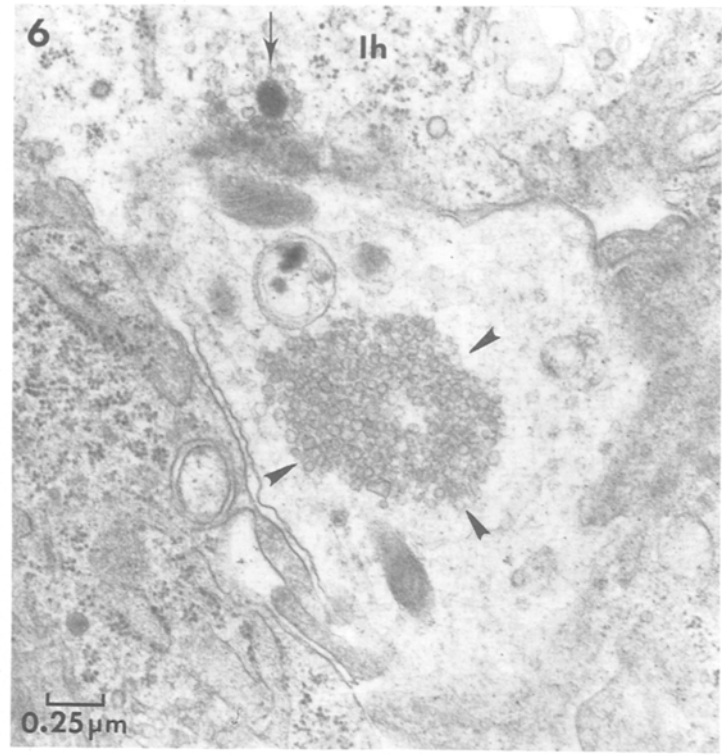
6
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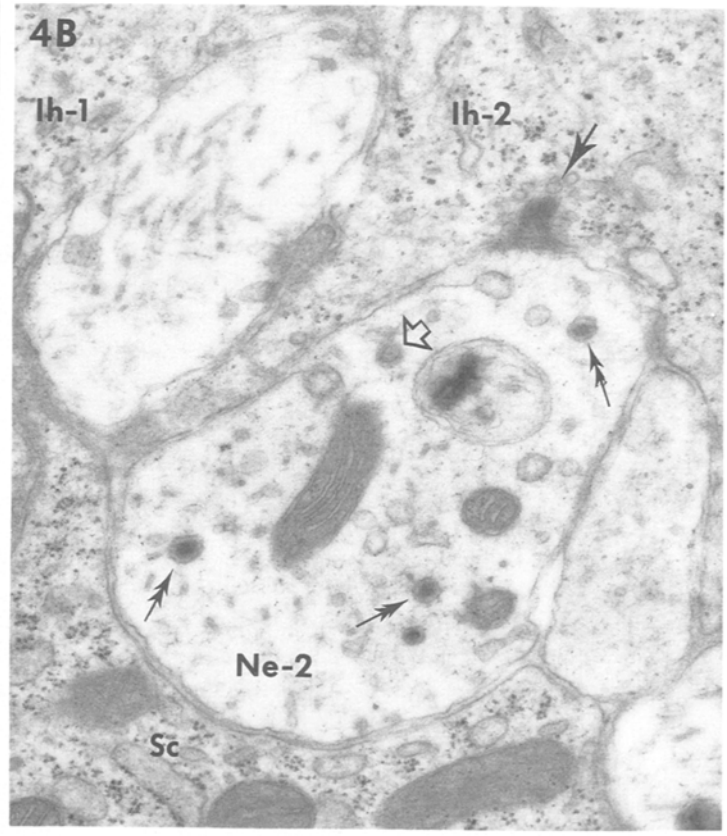
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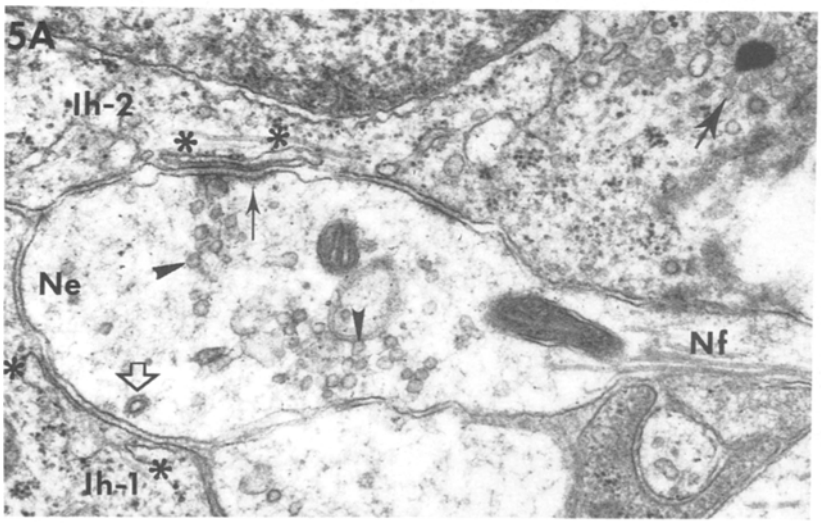
Ih-1
Ih-2
Ne-2
Sc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Ih-2
Ne
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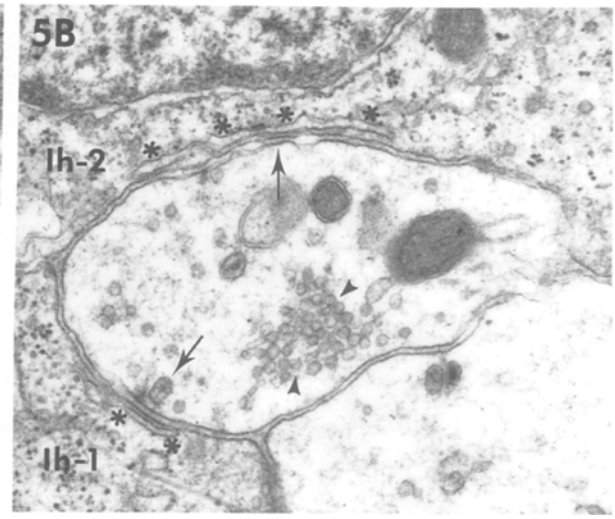
Ih-1


5B
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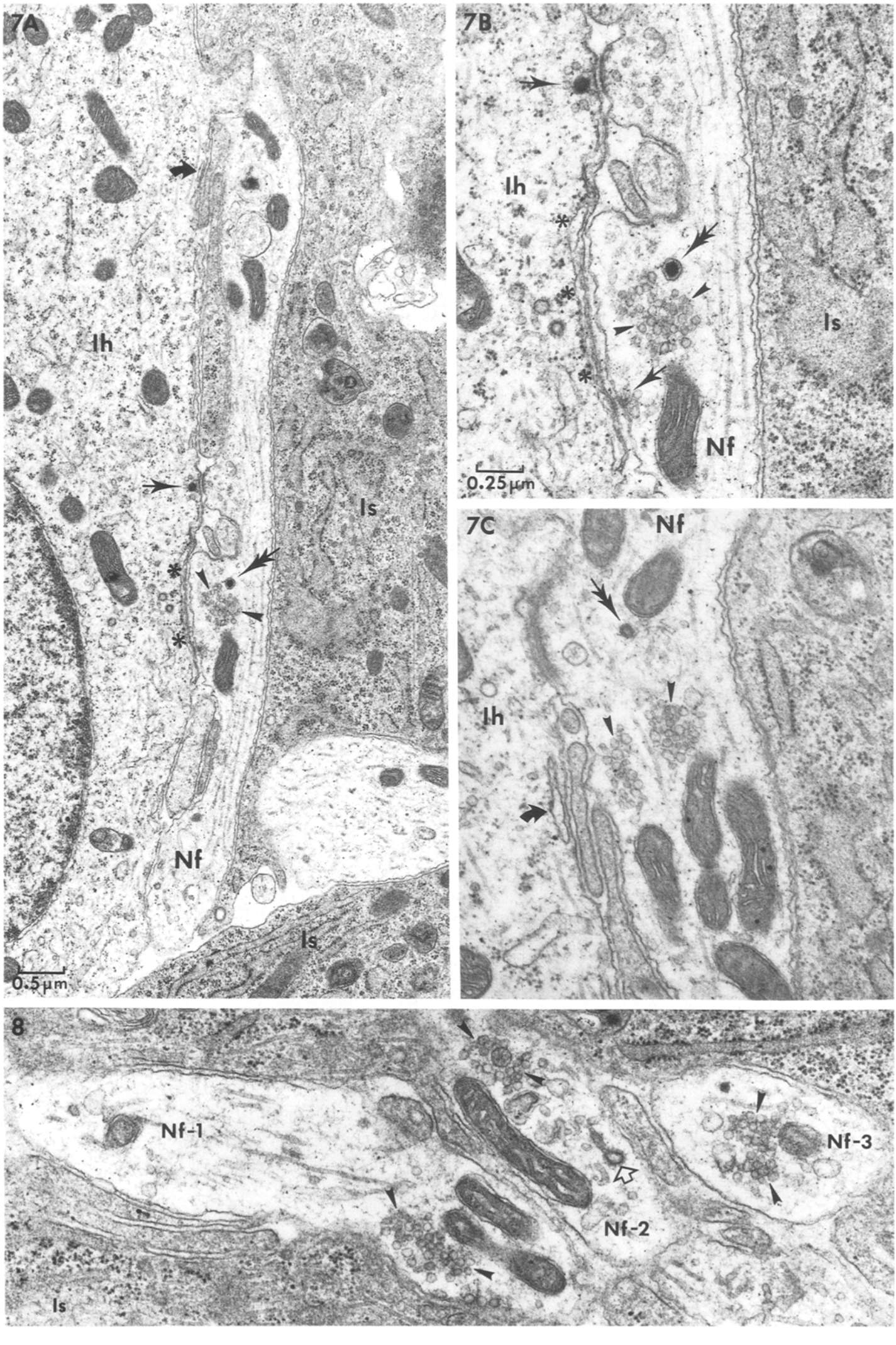
7A
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7C
Nf
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8
Nf-1
Nf-3
Nf-2
ls

for some missed under grid bars) through 22 adjacent inner hair cells, spanning a depth of 1.5 μm, revealed that the distribution of the presynaptic endings is spread rather than focal. This suggests that all or most inner hair cells may be interconnected through these endings, but does not exclude the existence of single presynaptic endings occurring on individual inner hair cells.

Ultrastructural characterization

There are several features that make presynaptic endings of spiral ganglion fibres distinctive. Perhaps most characteristic is the clustering of clear presynaptic vesicles that occupy only a limited part of the ending (Figs 4–6). Between the clusters of synaptic vesicles, these endings display the typical nondescript appearance of afferents (Figs 2A, 4B & 7). This fleeting afferent/efferent-like appearance can only be appreciated in serial sections and is demonstrated repeatedly in most of our material. These endings also display dense core vesicles (some adjacent to the presynaptic membrane (Figs 1, 3, 4 & 7)), coated vesicles (Figs 3, 5 & 8), and mitochondria. Presynaptic endings do not appear to differ in size from typical afferent endings. Some fibres *de passage* in the regions of the presynaptic endings also display discrete islands of clear synaptic vesicles, usually adjacent to mitochondria (Figs 7 & 8).

RECIPROCAL SYNAPSES

Reciprocal synapses in our material have so far been observed between the presynaptic vesiculated fibres of spiral neurons and the inner hair cells. Reciprocal synapses consist, on the inner hair cell side, of the presynaptic ribbon complex and the postsynaptic cistern, both side by side (Figs 1, 2, & 3D–F) or within a distance of a few sections (Figs 4A & B). The nerve ending also often shows a lateralization into pre- and postsynaptic sites; synaptic vesicles cluster at the densities of the presynaptic membrane which is fairly straight and electron-dense; the postsynaptic density underlines the dome of the ribbon complex (Figs 3D & E). Electron-dense material is present within the clefts at each synaptic site. Thus, despite the adjacent location, both the afferent and efferent synapses retain their usual ultrastructural features. Figures 1A–F show a series of sections through a reciprocal synapse in a 7–day culture. The series emphasizes how easily reciprocal synapses could be missed *in situ* and how essential it is to follow the nerve endings serially. Of the six sections, the reciprocity is fortuitously expressed in only one section, Fig. 1D, in which the ribbon afferent synapse at left coincides with the efferent synapse at right. Even to an experienced eye, the nerve profiles in Figs 1A and B would appear as afferent endings engaged in a ribbon synapse with an inner hair cell. The adjacent subsurface cistern could be easily dismissed in Figs 1A–C as incidental, especially when seen in the developing organ. Figures 1E and F, although somewhat puzzling in culture, would be readily identified *in situ* as efferent olivocochlear synapses. In Figs 1C–F, the lateralization within the presynaptic ending is nicely expressed, with the synaptic vesicles remaining at the right. The question remains: is the persistence of the subsurface cistern in the position of the incipient efferent synapse incidental, or is it indicative of the presynaptic nature of the ending? The presence of dense core vesicles should be noted in this series and in other figures, since they may reflect a biochemically distinctive character of at least some of the intrinsic presynaptic endings.

Another example of a reciprocal synapse is shown in Fig. 2, where, in A, an inner hair cell forms a ribbon afferent synapse with a nondescript 'afferent'-like ending, which, in B, unexpectedly becomes vesiculated and forms an efferent synapse. The ending in Fig. 2A would look like a typical afferent, were it not for the adjacent subsurface cistern. In Fig. 2B, the same ending looks very similar to young efferent fibres in the developing organ *in situ* (compare with Fig. 22 in Emmerling *et al.*, 1990).

COMPOUND RECIPROCAL SYNAPSES

Our material suggests that the presynaptic endings in culture can be divided into those that form reciprocal synapses with single inner hair cells (Figs 1 & 2) and those that connect adjacent inner hair cells through a chain of repetitive reciprocal synapses which we call 'compound reciprocal synapses' (Figs 3A–F). Figure 3A illustrates the prevalent location of the presynaptic endings between two adjacent inner hair cells. Of the three endings, ending 1 (Fig. 3A) is engaged in a ribbon synapse with inner hair cell 1, despite being vesiculated (Fig. 3C). The exact fate of ending 2 is unknown: it engages in a ribbon afferent and possibly efferent synapse with inner hair cell 2 (Figs 3A & B) and gives rise to ending 3 (Fig. 3A); afterwards it becomes nondescript and fades away (Fig. 3C). In further serial sections (Figs 3D–F), ending three synapses with both inner hair cells. The ending forms at least one ribbon synapse with both inner hair cells and an efferent synapse with inner hair cell 1. The persistence of the subsurface cistern along the membrane of hair cell 2 suggests an incipient efferent contact as well. Thus, ending 3 provides a pre- and postsynaptic link between both inner hair cells. The ultrastructure of ending 3 is very similar to that in Fig. 1; both contain clustered clear vesicles, distinct dense core vesicles and coated vesicles.

CONNECTING PRESYNAPTIC FIBRES

Another group of presynaptic endings appears to link adjacent hair cells through efferent-like synapses only. It is not yet entirely clear if these endings

represent a class of their own or are part of reciprocal arrangements. Figure 4A shows two vesiculated endings, possibly connected to each other. Ending 1 simultaneously synapses with both inner hair cells, while ending 2 synapses with inner hair cell 2 only. Note the 'classical' efferent appearance of these endings. They are virtually indistinguishable from the olivocochlear endings of the lateral tract in the organ of Corti *in situ* (compare with Fig. 13B of Emmerling *et al.*, 1990). In a distant section (4B), nerve ending 2 unexpectedly changes its character and receives a ribbon synapse from inner hair cell 2. Ending 1 becomes silent and disappears within the nine following sections. Another ending, similar to no. 1 in Fig. 4A, is shown in Fig. 5. In 13 consecutive sections, the ending demonstrates only two efferent synapses in close proximity, one with each of two adjoining inner hair cells. All of the remaining profiles were nondescript and afferent-like, but no afferent ribbon synapses were found. On occasion, a vesiculated ending in synaptic contact with an inner hair cell and an afferent ending was observed in a 9 DIV culture of an apical turn.

RIBBON SYNAPSES ON VESICULATED FIBRES

Another synaptic variant may be formed when presynaptic fibres containing conspicuous synaptic vesicles receive afferent ribbon synapses (Fig. 6). Here, the ending contains a defined island of synaptic vesicles, but we saw no evidence of further efferent synaptic specialization.

VESICULATED FIBRES DE PASSAGE

Shafts of afferent-like fibres that contain clusters of synaptic vesicles are seen next to or in the neighbourhood of inner hair cells, or within the inner spiral bundle (Figs. 7, 8). The clusters of vesicles are commonly associated with mitochondria and microtubules. In Figs 7A and B, the inner hair cell forms an efferent synapse with the vesiculated fibre or, at the very least, apposes a subsurface cistern, possibly in recognition of the presence of the synaptic vesicles.

We do not know if the various synaptic configurations we have seen reflect different synaptic modes or are fragments of a special neuronal system forming pre- and postsynaptic chains that interconnect inner hair cells.

Discussion

We demonstrated in cultures of the organ of Corti the presence of presynaptic vesiculated endings and of reciprocal synapses with inner hair cells. Both findings are entirely new. Since these presynaptic endings were discovered in culture, they evidently stem from a special class of spiral ganglion neurons. In the interpretation of our results, there are two options: first, the presence of these new synaptic formations is related to the tissue culture conditions; or second, these new synaptic arrangements are specific for the local circuitry of the peripheral auditory organ but have not been recognized previously.

Are reciprocal synapses in the organ of Corti induced in culture?

Could the specific anatomical conditions introduced by isolation of the organ from the higher centres in culture be responsible for the new circuitry? Synaptogenesis and the preservation of organotypic neurosensory relationships in the developing organ of Corti in culture are striking (Sobkowicz *et al.*, 1975, 1982; Sobkowicz & Rose, 1983; Sobkowicz, 1992). A hair cell *in situ* is presynaptic to the afferent endings of the peripheral fibres of the spiral neurons and postsynaptic to the efferent olivocochlear endings. Each synaptic specialization is conspicuously marked by the synaptic ribbon at an afferent synapse or by the postsynaptic cistern at an efferent synapse. Afferent synaptogenesis proceeds *in vitro* despite the absence of the efferent system. This is especially noticeable in outer hair cells, which *in situ* (Fig. 9A) become innervated mainly by large vesiculated efferent endings (Smith & Sjöstrand, 1961; Saito, 1990). During the first week of afferent synaptogenesis, the total population of synaptic ribbons decreases to about 20% in the outer hair cells, both *in situ* and *in vitro* (Sobkowicz *et al.*, 1982). *In situ*, the arriving efferent endings occupy the predominant space of the sensory receptor poles. In culture (Fig. 9B), the postsynaptic cisterns delineate a substantial part of the sensory receptor pole despite the failure of the efferent endings to appear. Regardless of the denuded receptor pole, a small group of afferent endings cluster at the opposite side (Sobkowicz *et al.*, 1984). Thus, the characteristic pattern of innervation of outer hair cells in culture demonstrates basically a deficit in the presence of efferent innervation.

Inner hair cells *in situ* (Fig. 9C) are presynaptic to the afferent endings of the spiral neurons. Vesiculated endings of the lateral olivocochlear tracts are supposed to synapse solely with the afferent endings of the spiral neurons (Pujol *et al.*, 1986). Thus, the possible culture-induced changes in the synaptic specializations in inner hair cells deprived of efferent innervation are expected to be negligible. The distribution of synaptic ribbons (synaptic/misplaced/free ribbons) and afferent synaptogenesis in the developing inner hair cells *in situ* and *in vitro* differ very little (Sobkowicz *et al.*, 1986). In culture, the presynaptic ribbon complexes and postsynaptic cisterns may coexist along the same afferent fibre (Sobkowicz *et al.*, 1984), but the same configurations are observed in the intact, maturing (12 PN) mouse (unpublished observation). The occurrence of efferent or reciprocal

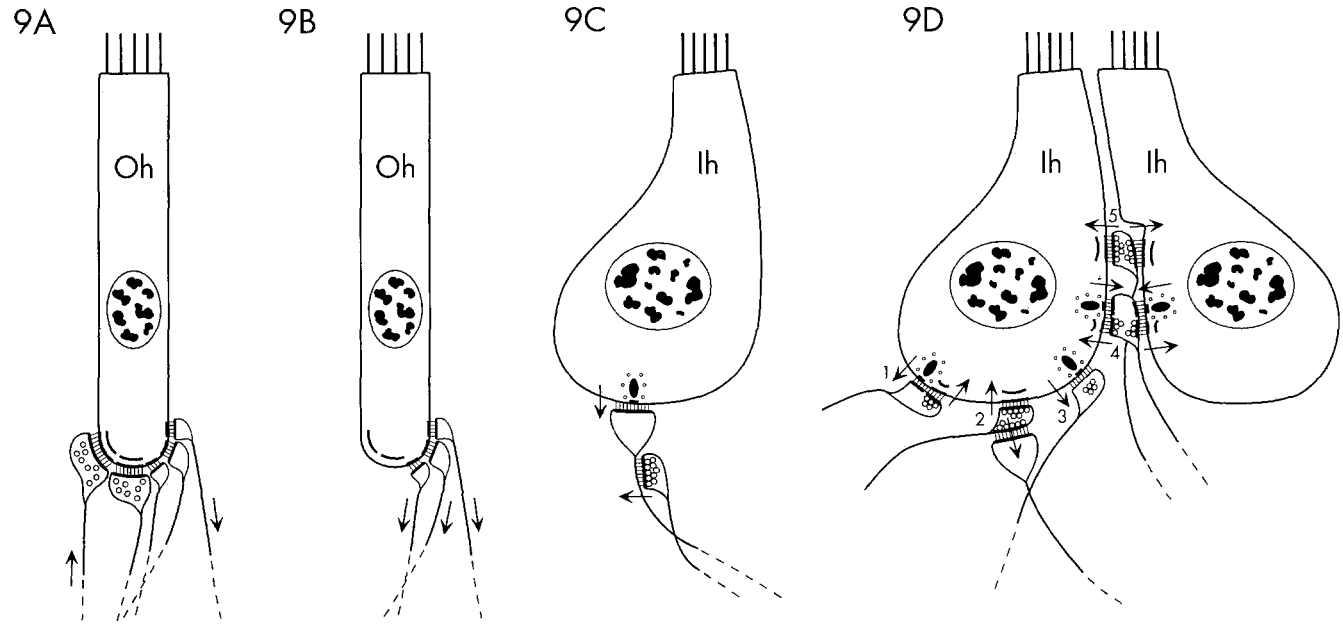

Fig. 9. Diagrams A–D summarize differences in synaptic connectivity between auditory hair cells *in situ* and in culture. (A) A prevalent mode of innervation of the outer hair cell *in situ*. Large vesiculated efferent endings occupy the predominant part of the receptor pole (Smith & Sjöstrand, 1961; Saito, 1990). (B) An outer hair cell in culture (compare with Fig. 4.39 in Sobkowicz *et al.*, 1984). Three afferent endings cluster at one side of the receptor pole while the remaining denuded area is lined with subsurface cisterns as if in anticipation of the arrival of the efferent endings. (C) The commonly accepted mode of innervation of the inner hair cell *in situ* (compare with Fig. 7 in Pujol *et al.*, 1986). Endings of the peripheral fibres of the spiral neurons solely adjoin the receptor pole of the hair cell which forms ribbon afferent synapses with them. The vesiculated efferent endings of the lateral olivocochlear system connect with the afferent endings alone, without direct contact with the receptor cell itself. (D) The diversity of synaptic connections between the endings of spiral neurons and the inner hair cells in culture: (1) – a single reciprocal synapse (Figs 1 & 2); (2) – a presynaptic vesiculated ending in synaptic contact with a hair cell and an afferent ending; (3) – a ribbon synapse on a vesiculated ending (Fig. 6); (4) – a compound reciprocal synapse in which a presynaptic ending forms a reciprocal synapse with adjoining inner hair cells (Figs 3D–E); (5) – a presynaptic fibre connecting adjoining inner hair cells through the efferent synapses (Figs 4A & 5).

afferent-efferent synapses of inner hair cells, shown in this paper (Fig. 9D), is so defined that it can hardly be attributed to culture-induced synaptic remodeling.

Could the presynaptic vesiculated endings, evidently derived from some spiral ganglion cells, result from the severance of the central axons or the deprivation from the postsynaptic cells in the cochlear nucleus? The spiral neuron is a bipolar cell whose peripheral fibre innervates either inner or outer hair cells and whose central fibre connects with the cochlear nucleus. Spiral neurons in culture demonstrate many different modes of survival and growth (Sobkowicz *et al.*, 1975, 1980). Most frequently seen are spiral neurons which become unipolar. The peripheral fibre remains synaptically engaged with a receptor cell; the central fibre is discarded. Strangely enough, the peripheral fibres of the surviving neurons (explanted from the foetal or newborn mouse) maintain and continue to acquire synaptic contacts.

Can other conditions in culture, i.e. in this case the contact with the feeding solution exposed to the regenerating cultures or the sensory organ regenerating itself (see Materials and methods), induce reciprocal synapse formation? A reciprocal synapse was first seen in an uninjured culture at 14 DIV (Sobkowicz, 1992). In our experience, an additional injury in culture results in new sprouting of afferent endings and synapse formation (Sobkowicz & Slapnick, 1992) but not in a change of fibre morphology.

Experimental neuroanatomy provides examples of neurons that, in a changed cellular milieu, display different modes of synaptic activity, developing presynaptic dendritic sites or connecting with different targets. In the mutant or X-irradiated cerebella, some cell populations may be either eliminated or misplaced (Sotelo, 1977, 1982). The surviving neurons, notably Golgi II cells, may change their polarity and develop presynaptic sites, not only in their dendrites but also within the neuronal soma, and make direct synaptic contacts with Purkinje cells. In the dorsal lateral geniculate nucleus of the cat following chronic disconnection from the visual cortex, the relay neurons develop presynaptic dendrites which enables them to establish new synaptic connections, replacing those lost in ablation (Hámori & Silakov, 1980). In the monkey, however, these neurons normally exhibit

presynaptic dendritic sites and reciprocal synapses with other dendrites; cortical excision only increases these connections (Pasik *et al.*, 1986).

Organotypic cultures of neuronal systems display a high degree of developmental autonomy and retain morphological characteristics intrinsic to a given region (Seil, 1993): for spinal cord, see Guillery and colleagues (1968), Sobkowicz and colleagues (1968, 1973) and Calvet and colleagues (1992); for hypothalamus, see Hild (1954), Sobkowicz and colleagues (1974a,b) and Toran-Allerand (1978); for hippocampus, see Gähwiler (1984) and Zimmer & Gähwiler (1984); for cerebellum, see Wolf (1970), Wolf & Dubois-Dalcq (1970), Allerand (1971), Seil (1979), Herndon and colleagues (1981) and Blank & Seil (1982). Kim (1974) described a sporadic occurrence of presynaptic specialization within the soma of a single granule cell in culture, but similar synaptic arrangements occur in the granule cells of the molecular layer that are regularly distributed in the cerebellar cortex of the rabbit and hare (Špaček *et al.*, 1973). Thus, the cause of their synaptic specializations remains unknown. Seil and colleagues (1992) induced several modifications in the cellular milieu and the connectivity within the cerebellar cultures but never described formation of reciprocal synapses. Reciprocal synapses have not been described previously in any neuronal cultures (not even in the retina (La Vail & Hild, 1971)).

Do reciprocal synapses in cultures of the organ of Corti reflect aspects of normal circuitry?

We interpret the presence of reciprocal synapses in cultures of the organ of Corti as possibly reflecting an aspect of normal synaptic connectivity. Several arguments speak for the specificity of these neurosensory arrangements: (1) the reciprocal synapses appear to be restricted to inner hair cells, where they interconnect adjoining cells through repetitive reciprocal or efferent synapses; (2) their location is consistent, i.e. mostly in the areas between adjoining hair cells, low on the modiolar side of the receptor pole; (3) the presynaptic endings display a characteristic ultrastructure, with clustered, clear, round synaptic vesicles interspersed with large dense core and coated vesicles. The ultrastructural appearance of the endings is fleeting, changing from nondescript, afferent-like to vesiculated, efferent-like within a few sections.

The presence of dense core vesicles characterizes catecholaminergic endings (Hager & Tafuri, 1959; De Robertis & Pellegrino de Iraldi, 1961; Richardson, 1962, 1966; Borg *et al.*, 1974). Nerve endings of a dopaminergic nature have been identified in the organ of Corti *in situ* (Jones *et al.*, 1987; Usami *et al.*, 1988; Gil-Loyzaga & Parés-Herbute, 1989). They are confined to the inner spiral bundle and inner tunnel fibres and – like our presynaptic fibres – to the puncta of nerve endings beneath inner hair cells (Usami *et al.*, 1988). So far, they are considered to be a counterpart of the lateral olivocochlear bundle (Fex & Altschuler, 1986). Our results suggest that at least some of the endings containing prominent dense core vesicles are of intracochlear origin.

As presently recognized, the innervation of the organ of Corti *in situ* is provided by two systems: an entirely afferent system from the spiral ganglion neurons, and an entirely efferent system from the olivocochlear bundle. Thus, the peripheral auditory organ serves to transfer acoustic stimuli (Figs 9A–C), and any modulating or synchronizing role is played exclusively by the CNS, especially by the medial and lateral superior olivary complex. This arrangement is singularly different from the synaptic modes used by other sensory organs that usually possess inner relays and utilize autonomic control. Perhaps the best example is the complex synaptic circuitry of Type II hair cells in both the utricular and saccular maculae, where intramacular presynaptic fibres originating from the afferent calyces provide reciprocal relays within the organ itself (Ross & Donovan, 1984; Ross, 1985; Ross *et al.*, 1986, 1990).

Of the two innervation pathways provided by the olivocochlear bundle, the medial tract supplying the outer hair cells is much better understood than the lateral tract supplying the inner hair cells. Nevertheless, it is generally accepted that the lateral olivocochlear fibres terminate exclusively on radial afferents and do not make direct connections with the inner hair cells themselves. We have observed, however, a variety of different modes of connectivity between inner hair cells, radial afferent fibres and lateral olivocochlear efferent endings, at least up to 12 postnatal days in a hearing animal. A fairly frequent mode is the direct and simultaneous synaptic connection of an efferent ending with an inner hair cell and with its afferent (see Fig. 15 in Emmerling *et al.*, 1990). Direct synaptic connections between efferent endings and inner hair cells (see also Fig. 16 in Emmerling *et al.*, 1990) occur in our material up to 22 postnatal days (the oldest specimen studied (unpublished)) and thus do not appear to be transitory developmental events, as suggested by Pujol and colleagues (1980). Recently, we also found vesiculated efferent endings connecting two adjoining inner hair cells in a 12-day postnatal organ of Corti, exactly as shown in Fig. 5 and in Fig. 9D, nerve ending no. 5. Our data imply that to view an inner hair cell as a receptor that connects exclusively with afferent fibres may be too limiting. It must be mentioned, however, that in serial reconstruction of two sets of two adjacent inner hair cells in the adult cat cochlea, Liberman (1980) did not find any novel synaptic patterns.

As we have stressed, intracochlear vesiculated endings cannot be readily differentiated *in situ* from lateral

olivocochlear endings. Since, in culture, only a fraction of spiral neurons survive (Sobkowicz *et al.*, 1975), spotting the special class of 'presynaptic dendrite cells' must be treated as very fortuitous. Among the best candidates for the presynaptic dendrites would be fibres derived from a small class of GABA/GAD-positive (GAD = glutamic acid decarboxylase) spiral ganglion cells (Whitlon & Sobkowicz, 1989).

Conclusions

The isolated cochlea in culture presents a singular model of the organ of Corti innervated exclusively by locally present spiral neurons. The presence of intracochlear presynaptic endings innervating inner hair cells in culture suggests the existence of effector- or interneurons within the spiral ganglion that may provide internal integration circuits within the organ itself. In culture, these vesiculated presynaptic endings appear to connect the inner hair cells, possibly serving to synchronize them.

Reciprocal synapses provide a morphological basis for an immediate feedback loop between the presynaptic endings of spiral ganglion neurons and the inner hair cells. We believe that these intracochlear synaptic arrangements are specific to the local circuitry, but we have yet to identify reciprocal synaptic arrangements in the organ of Corti *in situ*.

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