DISAPPEARANCE OF AFFERENT AND EFFERENT NERVE TERMINALS IN THE INNER EAR OF THE CHICK EMBRYO AFTER CHRONIC TREATMENT WITH β-BUNGAROTOXIN

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ABSTRACT

β-Bungarotoxin(β-BT) was applied to chick embryos at 3-day intervals beginning on the 4th day of incubation to see the effect of chronically and massively applied β-BT, and to investigate the hair cell-nerve relationship in the developing inner ear by electron microscopy. On the 10th day of incubation, nerve terminals had achieved contact with differentiating hair cells, but the acoustico-vestibular ganglion cells of treated animals were decreased in number to one-third of those of the control. By the 14th day, most of the ganglion cells degenerated and disappeared, and only a few nerve terminals were seen in the neuroepithelium. At this time, most of the hair cells lacked synaptic contacts with nerve terminals, but their presynaptic specialization remained intact and they showed evidence of continuing differentiation. On the 17th day, the acoustico-vestibular ganglion cells were completely absent. All the hair cells were devoid of afferent and efferent innervation but were fully differentiated on the 21st day. β-BT was found to have a similar destructive effect on cultured spinal ganglion cells. The present study shows that β-BT kills acoustico-vestibular and spinal nerve cells when applied chronically and massively during development. Furthermore, the differentiation of hair cells proceeds normally, and their presynaptic specializations are maintained when nerve terminals are absent during later developmental stages.

β-Bungarotoxin(β-BT) has been known to inhibit the release of the neurotransmitter at the neuro-muscular junction (6, 7, 8) and also to affect the storage of several transmitters, norepinephrine, γ-aminobutyric acid, serotonin, and the acetylcholine ACh precursor, choline, in the rat synaptosome (44). It is supposed that β-BT has some presynaptic effect to inhibit the release of several neurotransmitters besides acetylcholine (ACh). The relationship between target cells and nerve has been extensively studied by many investigators. I tried to apply this neurotoxin as a tool for studying hair cell-nerve relationship during the development of the inner ear, and I also intended to investigate the action of β-BT when extensively and chronically applied in vivo and in vitro.

The role of the nerve in the differentiation and maintenance of the several target cells has been investigated extensively, since Von Vintshgau and Hönigschmied (42) first demonstrated the dependence of taste buds on the nerve supply. The majority of target cells studied have been receptor cells (taste buds, lateral line organs, cutaneous receptor cells, and muscle spindles) and skeletal muscle. Both receptor cells and skeletal muscle are somewhat dependent on the nerves. They fall

into degeneration or atrophy when their nerves are transected, and regeneration is induced by reinnervation (27, 47, 49). Thus, some neurotrophic factors are presumably transmitted by the nerves for the differentiation and maintenance of the target cells (19, 27). However, the degree of dependency of the target cells on the nerves differs among various species. In amphibians, the taste buds, lateral line organs and skeletal muscles are not necessarily dependent on the nerves for their differentiation and maintenance as in the fishes and mammals (13, 28, 29, 45). These results were obtained by surgical denervation and transplantation techniques which involved more or less local disturbances.

In the case of the inner ear, only a few studies have been undertaken, probably because of the technical difficulty of nerve transection between the sensory ganglion and the neuroepithelium. The cristae of the sensory epithelia degenerated when more than 75% of the ampullar nerve was severed in squirrel monkeys (26), and degeneration of the cristae was indicated 1 mo after destruction of the frog Scarpa ganglion (18). Nevertheless, a recent study in the frog showed that sensory cells in the labyrinth survived total denervation for 1-2 wk without typical signs of ultrastructural damage (17). The work of Orr (31) represents the only previous study of the effect of acoustico-vestibular innervation on hair cells. In her experiments, otocysts cultured with associated mesenchyme and acoustic ganglia developed completely differentiated sensory epithelial membranes, and it appeared that complete differentiation of hair cells was invariably accompanied by innervation. In the present study, β-BT has been applied to further investigate, in vivo, the hair cell-nerve relationship during the development of the chick inner ear.

MATERIALS AND METHODS

40 μg of pure β-BT(Miami Serpentarium Lab., Miami, Fla.) in 0.1 ml of sterile Ringer solution was injected into the yolk sacs of chick embryos on the 4th, 7th, 10th, 14th, and 17th days of incubation. Thus, the 21st-day embryo had received five successive injections. Only the Ringer solution was injected into the controls.

The embryos were sacrificed, and the inner ears were dissected on the 7th, 10th, 14th, 17th, and 21st days. After immersion in 2.5% glutaraldehyde, 2% formaldehyde in 0.1 M cacodylate buffer pH. 7.4 for 2-3 h, the tissue was postfixed in 1% OsO_4 in the same buffer for 1.5 h, stained en bloc in 2% uranyl acetate for 1 h, dehydrated in graded alcohols, embedded in Araldite,

and sectioned on an LKB microtome (LKB Instruments, Inc., Rockville, Md.). The ultrathin sections were stained with uranyl acetate and lead citrate and observed with an Hitachi HU-11DS electron microscope. Thick sections were stained with toluidine blue for light microscope observation. Most analyses concentrated on the basilar papilla (hearing organ) and saccular macula (gravity receptor organ).

In Vitro Study

The spinal ganglia of 10-day chick embryos were cultured in explant in the feeding medium, which consisted of 62.5% Eagle's minimal medium (Difco Laboratories, Detroit, Mich.), 25% human placental cord serum, and 12.5% chick embryo extract and containing 6 mg glucose/ml, by the Maximow double cover slip method at 37°C. On the 2nd day, after the nerve fibers had grown in culture, β-BT was added to the culture medium with a final concentration of 40 μg/ml. On the 4th day in culture, these spinal ganglia were fixed in 5% formalin, 70% ethanol, and 5% acetic acid FEA and stained by Bodian's silver impregnation method.

RESULTS

On the 21st day of incubation, β-BT-treated chick embryos show a marked reduction in body size and weight without gross malformation. The brain and spinal cord are atrophied. Furthermore, the spinal ganglia and sympathetic ganglia are completely absent. Striated muscles are degenerated and bone growth appears to be affected possibly as a consequence of muscle atrophy. The effects of β-BT on various tissues including the central nervous system (CNS) will be presented in detail in a future paper.

Structural Characteristics of the β-BT-Treated Inner Ear at the Final Embryonic Stage

NERVE TERMINALS: In control basilar papillae of the 21st-day chick embryo, the tall hair cell has several afferent synapses and small efferent synapses (Fig. 2). The afferent nerve terminal contains some mitochondria and a few vesicles and the postsynaptic density is present. Synaptic bodies with surrounding vesicles are situated on the presynaptic membrane of hair cells (Fig. 2). The short hair cell has a small afferent synapse and large chalicelike efferent synapses (25). The efferent synapse is filled with numerous clear, flat, and round vesicles and some mitochondria (Fig. 3). Subsurface cisternae are present adjacent to the efferent nerve terminals (Fig. 3).
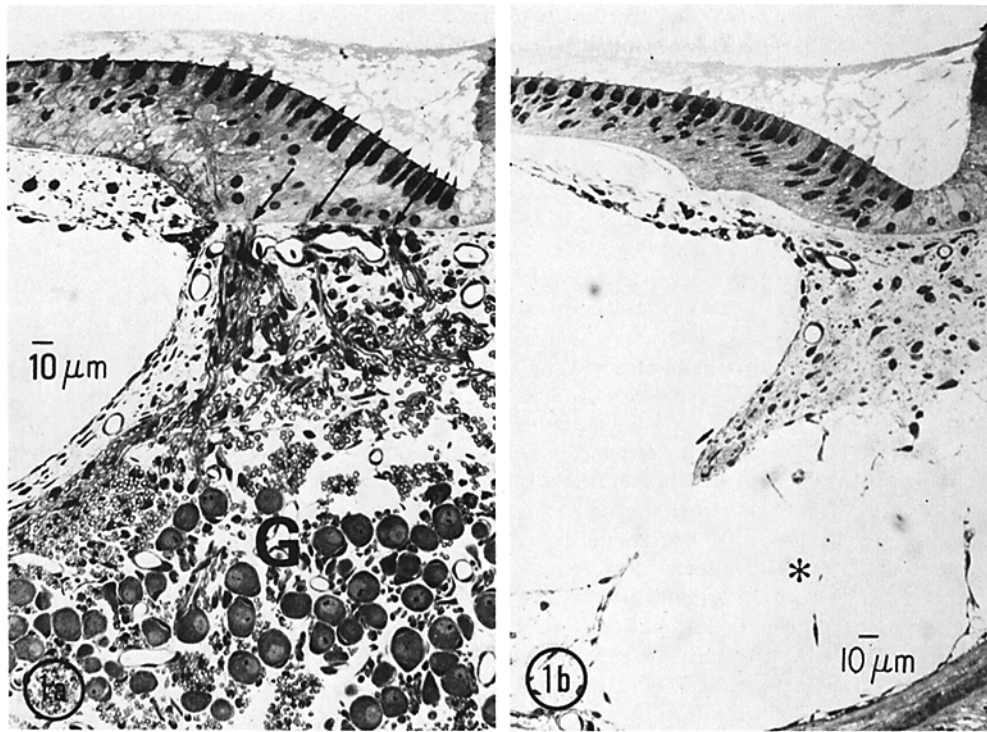
FIGURE 1 (a) Control basilar papilla. 21st day. The height of the hair cell decreases gradually from the tall to short hair cell. The spiral ganglion (G) is filled with numerous ganglion cells surrounded by satellite cells and myelinated nerve fibers. The nerve fibers penetrate into the neuroepithelium at the habenula perforata (arrows). × 245. Bar, 10 μm. (b) Basilar papilla. β-BT treatment. 21st day. The hair cells are distributed in the same manner as in the control. However, the spiral ganglion (*) is empty. No nerve fiber penetrates into the neuroepithelium. × 245. Bar, 10 μm.

In control saccular maculae, a large afferent nerve chalice containing some mitochondria, microtubules, neurofilaments, and a few vesicles surrounds several type I hair cells (Fig. 4). Bouton-shaped afferent and efferent nerve terminals synapse with the type II hair cell (Fig. 5). The efferent nerve terminal is filled with numerous flat and round vesicles just as in the basilar papillae.

It is a striking feature of β-BT-treated basilar papillae and saccular maculae that there are no afferent and efferent nerve terminals and nerve fibers in the neuroepithelium on the 21st day (Figs. 1 b and 6–10), and that vacant extracellular spaces are found between hair cells and supporting cells (Figs. 8 and 9). The presynaptic membrane specialization associated with the synaptic body is present in close contact with supporting cells or adjoining the empty spaces (Figs. 9 and 10). Subsurface cisternae are also observed at the basal part of short hair cells in the basilar papillae (Fig. 7).

Thus, the presynaptic specialization with the synaptic body and the postsynaptic specialization with subsurface cisternae of hair cells remain intact in the β-BT-treated neuroepithelium on the 21st day.

ACOUSTICO-VESTIBULAR GANGLIA: In the control, the acoustico-vestibular ganglion is filled with a large number of nerve cells surrounded by satellite cells and afferent and efferent nerve fibers (Fig. 1a). Numerous myelinated nerve fibers penetrate the basal lamina at the habenula perforata in the basilar papillae (Fig. 1a) and at many sites in the saccular maculae.

Another characteristic feature in the β-BT-treated inner ear is that nerve cells and nerve fibers are completely absent in the acoustico-vestibular ganglion (Fig. 1b). Neither Schwann cells nor satellite cells are observed in the ganglion at this stage.

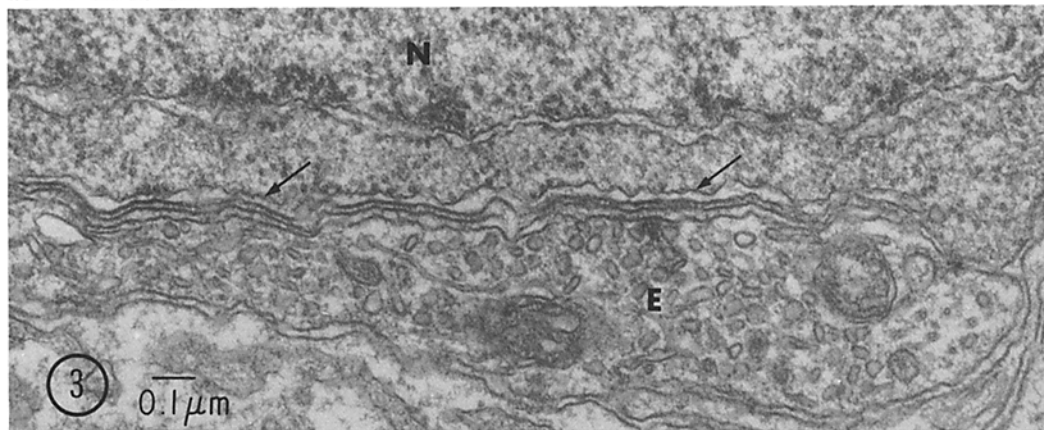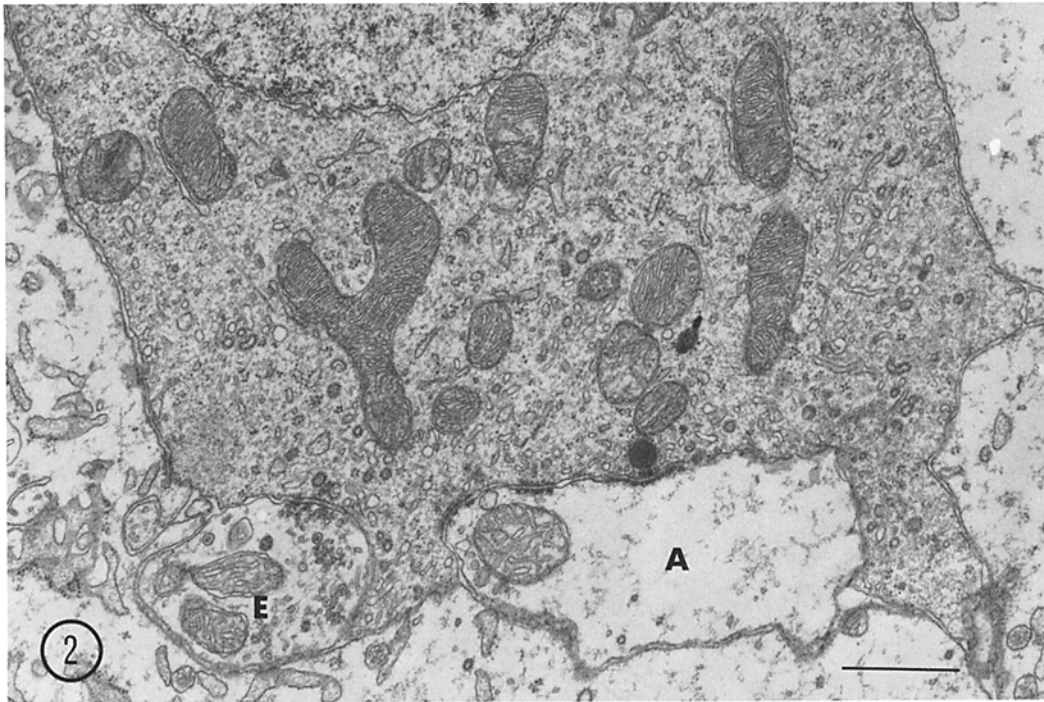HAIR CELLS: In spite of the complete absence of nervous elements, there are no morpho-

FIGURE 2   The infranuclear part of the tall hair cell in the control basilar papilla. 21st day. The afferent (A) and efferent (E) nerve terminals synapse with the hair cell. A synaptic body is seen at the presynaptic site. × 15,500. Bar, 1 μm.

FIGURE 3   The basal part of the short hair cell in the control basilar papilla. 21st day. A part of a large chalice-like efferent nerve terminal contains numerous flat and round vesicles. Note the subsurface cisternae (arrows) adjacent to the efferent nerve terminal (E). N: nucleus. × 58,000.

logical signs of degeneration in the hair cells of the β-BT-treated animals. The cells are fully developed, and their ultrastructure is similar to that of the controls.

Tall columnar and short thick hair cells are clearly distinguishable in the basilar papillae (Fig.

1b). In the tall hair cell, a kinocilium is present and a number of stereocilia arise from the cuticular plate (Fig. 6). The hair bundle is triangular in cross section, and the kinocilium is located at the apex of the triangle. The cuticular plate extends deep into the cytoplasm, which contains scattered
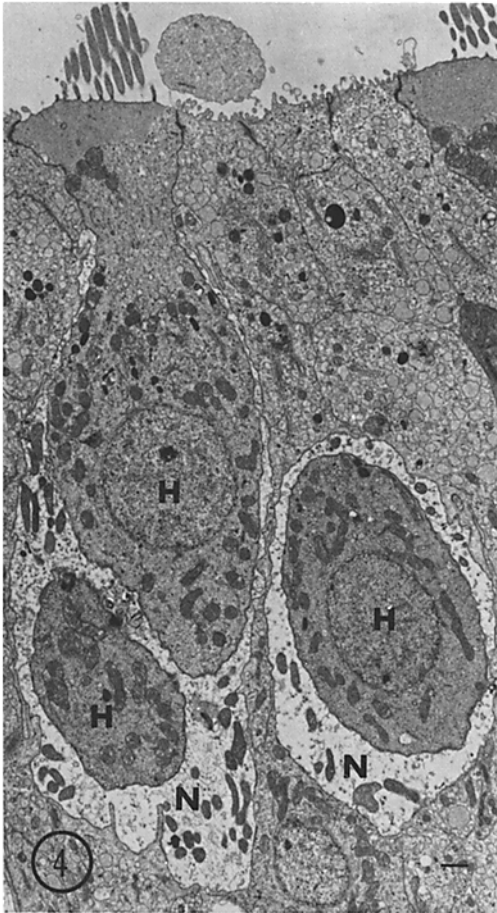
FIGURE 4 The type I hair cells (*H*) of the control saccular macula. 21st day. The type I hair cells are surrounded by large afferent nerve chalices (*N*). × 3,400. Bar, 1 μm.

FIGURE 5 The infranuclear portion of the type II hair cell in control saccular macula. 21st day. Afferent (*A*) and efferent (*E*) nerve terminals synapse with the hair cell. The synaptic bodies (arrows), fine bridgelike structures in the cleft, and postsynaptic membrane thickening are present. × 15,200. Bar, 1 μm. *Inset:* two synaptic bodies adjacent to an afferent nerve terminal. × 41,600. Bar, 0.1 μm.
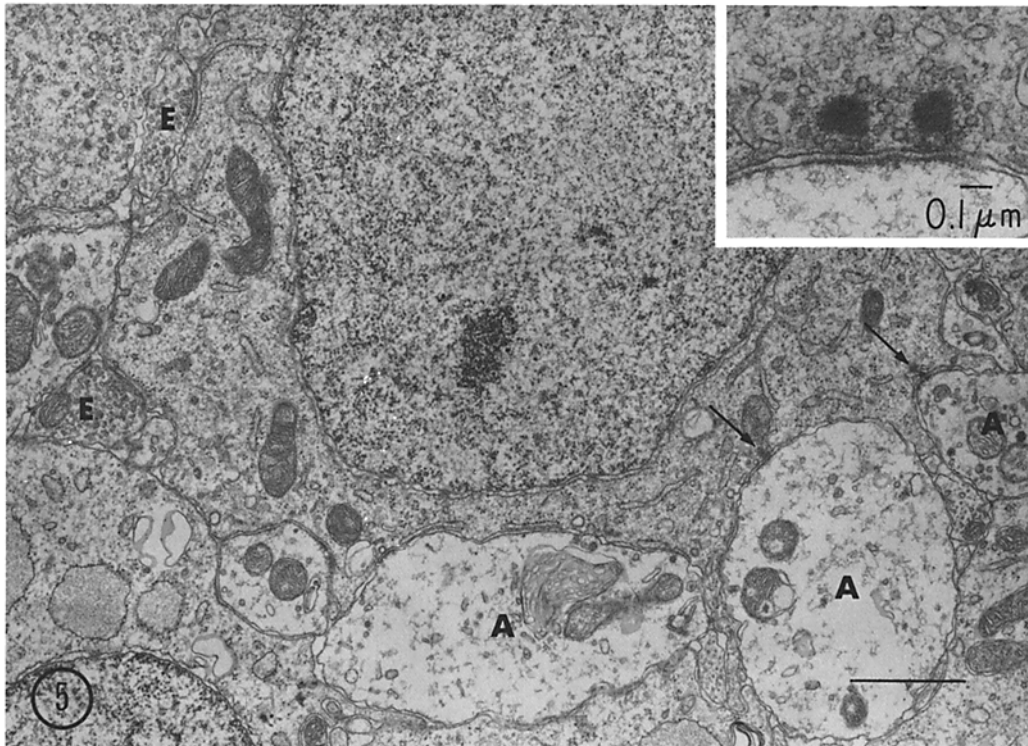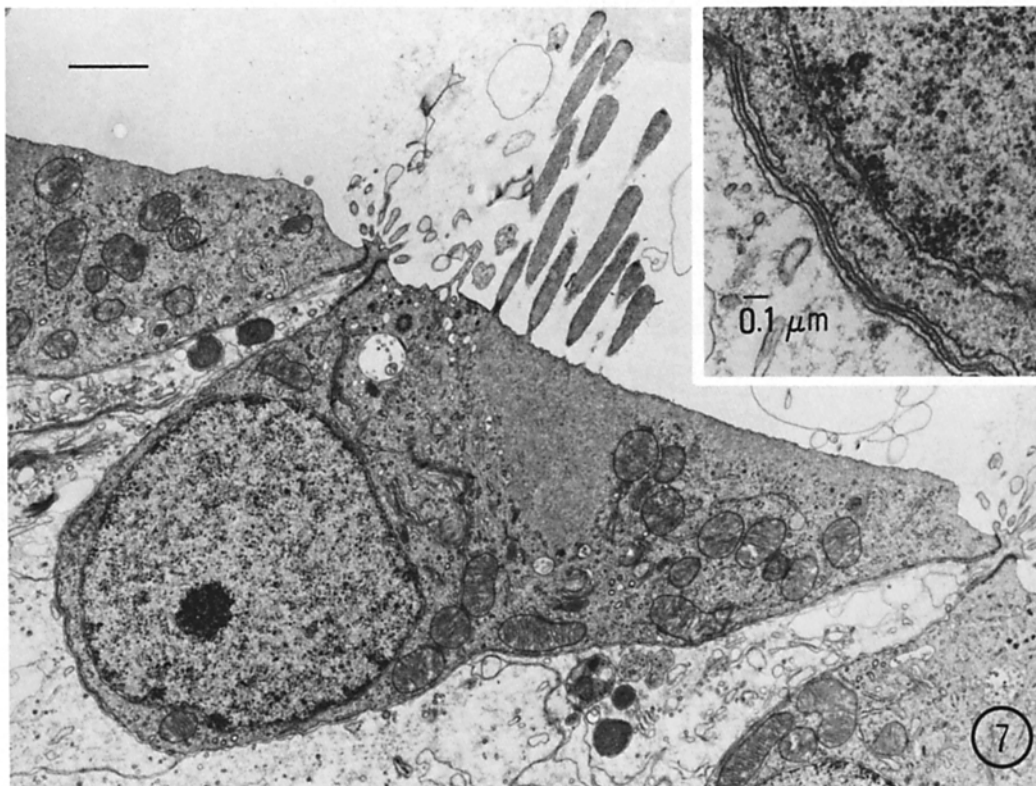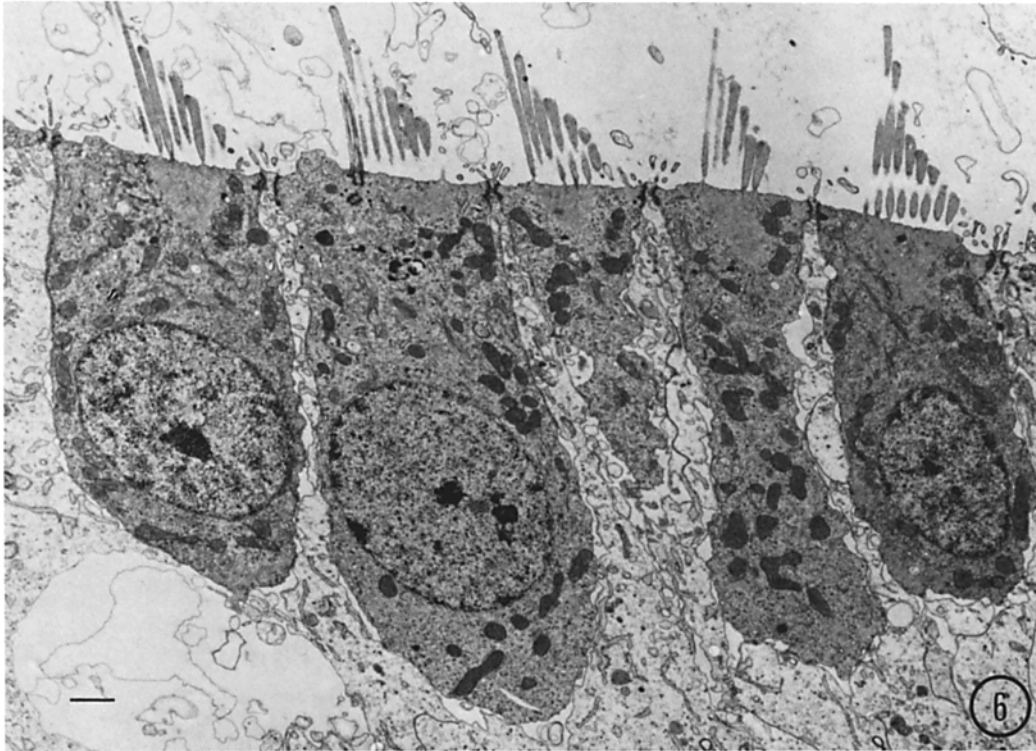
FIGURE 6 The tall hair cells of the β-BT-treated basilar papilla. 21st day. A kinocilium and regularly arranged stereocilia protrude from the top surface. Some extracellular empty spaces are present at the basal parts of the hair cells. Note the absence of the afferent and efferent nerve terminals. × 5800. Bar, 1 μm.

FIGURE 7 The short hair cell of β-BT-treated basilar papilla. 21st day. There is no nerve terminal in contact with the hair cell. × 10,400. Inset: the basal part of the short hair cell. Note the subsurface cisternae adjoining the supporting cell. × 31,500. Bar, 0.1 μm.
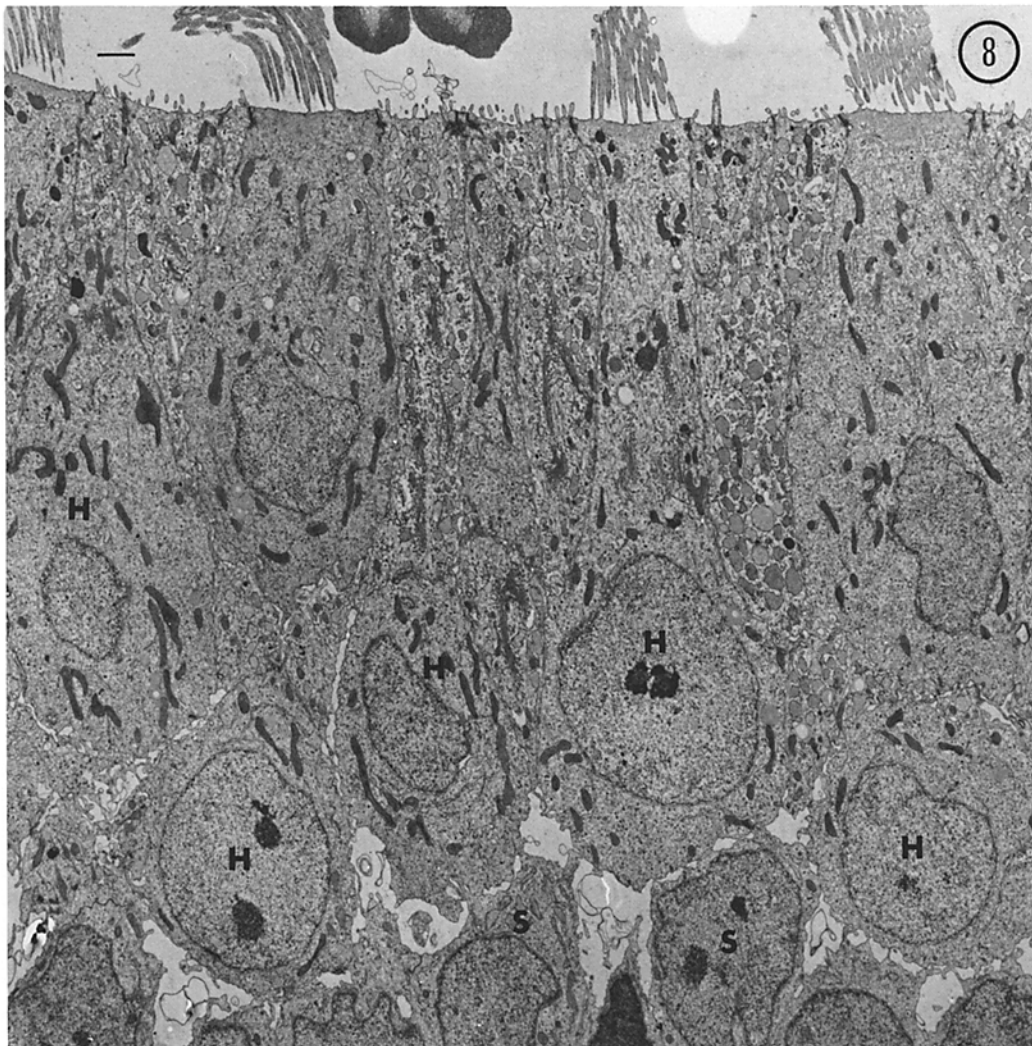
FIGURE 8   The saccular macula. β-BT treatment. 21st day. The nuclei of the supporting cells (S) are present at the lower part of the neuroepithelium. The well developed hair cells are present and surrounded by the processes of the supporting cells. Between the basal parts of the hair cells and supporting cells some extracellular vacant spaces are found. No neural element is present in the neuroepithelium. × 5000. Bar, 1 μm.

mitochondria, cisternae of rough and smooth endoplasmic reticulum, numerous ribosomal clusters and coated vesicles (Fig. 6). However, specialized presynaptic membrane and synaptic bodies adjoin supporting cells or empty extracellular spaces instead of afferent nerve terminals (Fig. 9).

The top surface of the short hair cell is wider than that of the tall hair cell. However, the infranuclear portion of the cytoplasm of the short hair cell is smaller than that of the tall cell. Although long subsurface cisternae are often seen adjacent

to the basal plasma membrane, other cell organelles are similar to those seen in the tall hair cells (Fig. 7).

In the β-BT-treated saccular macula, it is difficult to discriminate type I from type II cells because of the complete lack of nerve terminals in the neuroepithelium (Fig. 8). The hair cell has a kinocilium and a bundle of regularly arranged stereocilia. The cytoplasm contains scattered mitochondria, SER, RER, well developed Golgi bodies, and multivesicular bodies (Fig. 8). Synaptic
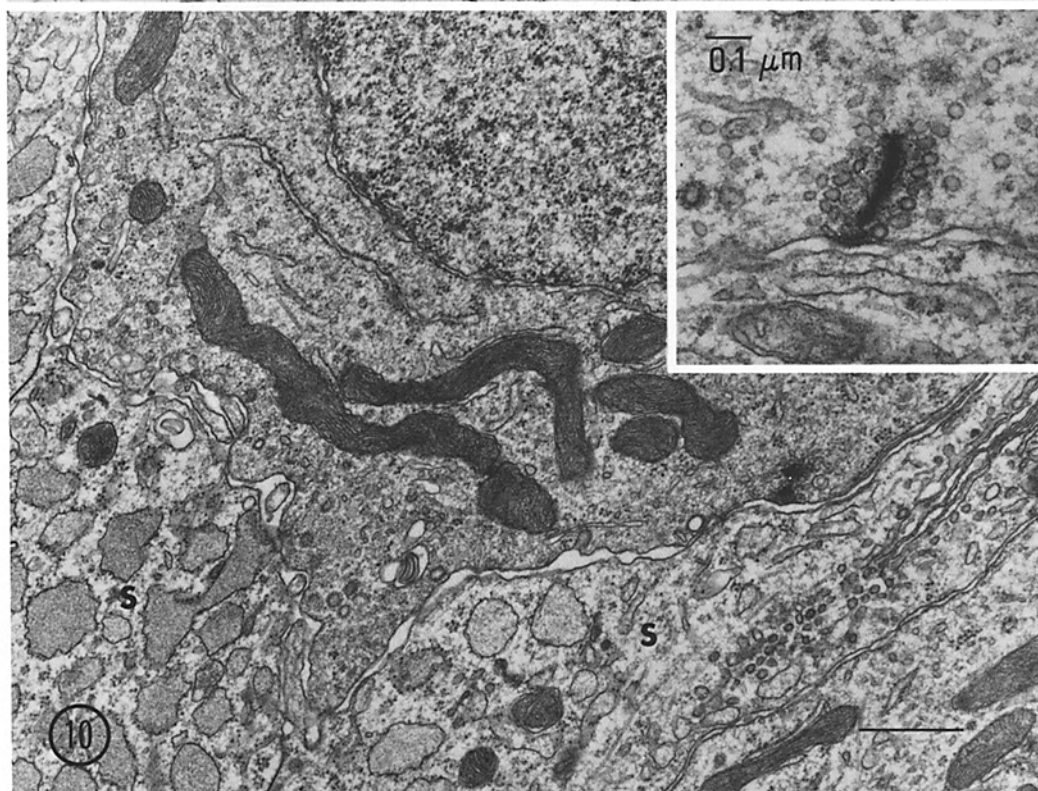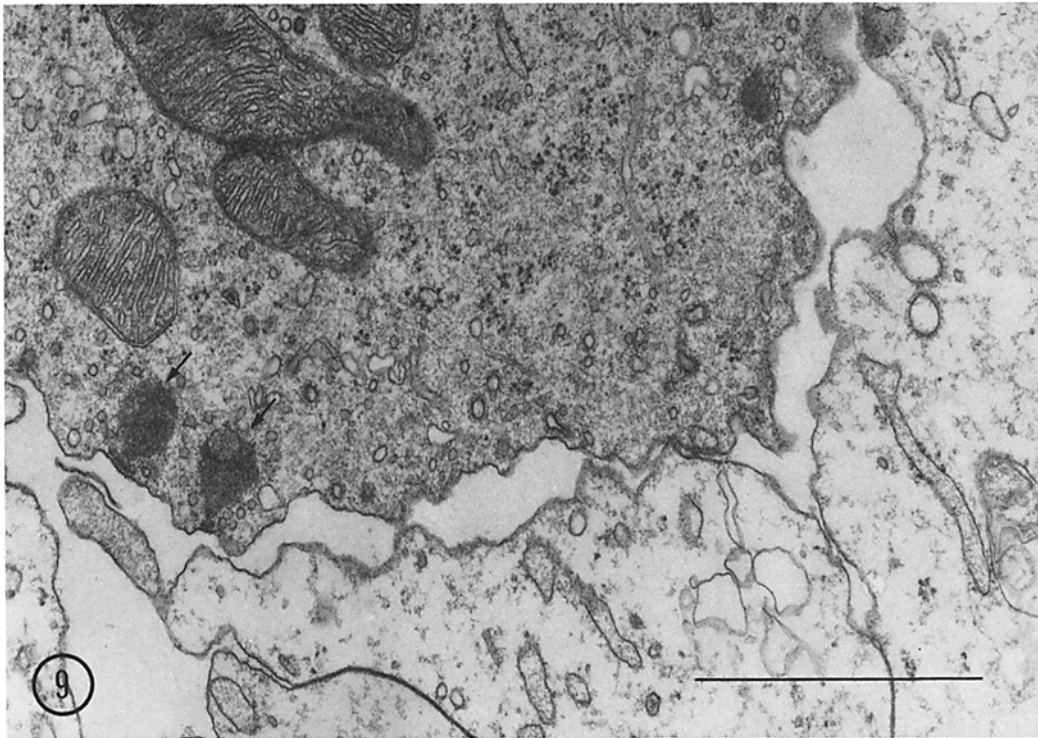
FIGURE 9   The basal part of a tall hair cell in the β-BT-treated basilar papilla. 21st day. The presynaptic specializations associated with synaptic bodies (arrows) are seen devoid of contact with postsynaptic elements. Between hair cell and supporting cell some extracellular space remains. Numerous coated vesicles are present in the hair cell. × 38,300. Bar, 1 μm.

FIGURE 10   The infranuclear portion of the hair cell in the β-BT-treated saccular macula. 21st day. The hair cell adjoins directly the supporting cells (S). The nerve terminals are not present. × 13,900. Bar, 1 μm. *Inset:* the synaptic body adjacent to the supporting cell. × 50,900. Bar, 0.1 μm.

bodies, which face supporting cells or vacant extracellular spaces, and numerous coated vesicles are present in the basal region of these hair cells (Fig. 10).

The Development of the β-BT-Treated Basilar Papilla and Saccular Macula

NERVE TERMINALS: In control basilar papillae, contact between nerve terminals and some of the undifferentiated neuroepithelial cells already exists on the 7th day of incubation. Hair cells with kino- and stereocilia and the supporting cells have differentiated by the 9th day. At the same time, nerve terminals are interposed between hair cells and supporting cells. However, membrane specialization as a morphological sign of synaptogenesis is not yet found on the nerve terminals which are attached to supporting cells in the same manner as to hair cells. Numerous nerve terminals are in close apposition with the base of hair cells in basilar papillae and saccular maculae by the 10th day. The occasional presence of slight membrane thickening at this stage is the first sign of synaptogenesis. The nerve terminals contain a few vesicles and flocculent material. Synaptic bodies are found in the basal cytoplasm of hair cells adjacent to nerve terminals or supporting cells. By the 14th day, several afferent and occasional efferent nerve terminals synapse with hair cells (Fig. 13). The afferent nerve terminals contain several mitochondria, microtubules, fine filaments, and vesicles. Efferent nerve terminals have a large number of flat or round vesicles and several mitochondria.

In basilar papillae and saccular maculae of β-BT-treated embryos, a smaller number of nerve terminals are attached to hair cells than in the controls by the 10th day of incubation (Fig. 12). Slight membrane thickenings are occasionally seen on these nerve terminals at this stage. On the 14th day, numerous large empty spaces are observed under hair cells and between supporting cells (Figs. 11 d, 14, and 15). Nerve terminals that have synaptic contact with hair cells are very few (Figs. 14 and 15). Free nerve terminals are occasionally found near hair cells (Fig. 14), and they appear to have detached from the hair cell. Fine filaments, a few vesicles, and flocculent material are present in those nerve terminals. Degenerative nerve terminals containing markedly swollen mitochondria and vacuoles as seen in surgically denervated neuroepithelium (17, 36) have not been found. It is speculated that the nerve terminals have retracted

rather than having left the debris. By the 17th day, no afferent and efferent nerve fibers and terminals are seen in the β-BT-treated basilar papilla and saccular macula (Fig. 17a and b).

ACOUSTICO-VESTIBULAR GANGLIA: On the 10th day of incubation, dendrites of ganglion cells have penetrated the basal lamina and entered the neuroepithelium at the habenula perforata in basilar papillae and at several sites in saccular maculae of the control animals (Fig. 11a). A large number of nerve cell bodies are present in the ganglion (Fig. 11a and c). These neurons are surrounded by satellite cells, and their cytoplasm contains some mitochondria, prominent Golgi bodies and numerous microtubules and neurofilaments. Well-developed RER and many ribosomal clusters are also present within this cytoplasm (Fig. 16c). Nerve fibers within the ganglion are wrapped by Schwann cells.

In β-BT-treated otocysts, on the 10th day of incubation the neuroepithelium of basilar papillae and saccular maculae is penetrated by nerve fibers, but the number of cell bodies in the ganglia are apparently decreased to one-third of that seen in the controls (Fig. 11b). The number is estimated by counting the cells in every 20th section taken from a series of 1-μm thick serial sections of the ganglion. By the 14th day only a few ganglion cells remain, and occasional nerve fibers penetrate the basal lamina of the neuroepithelium (Fig. 11d). Macrophages are often found in the ganglion and they are sometimes resting inside the capsule of satellite cells (Fig. 16b). Degenerated cells, which contain swollen mitochondria with dense deposits, vacuoles, and lysosomes, are also seen to be surrounded by satellite cells (Fig. 16a). They are probably degenerating ganglion cells. The remaining ganglion cells are somewhat smaller in size than cells in untreated ganglia, and the RER and Golgi apparatus in these cells are less differentiated than in controls. By the 17th day, the ganglion cells are completely absent and no nerve fibers penetrate the neuroepithelium.

HAIR CELLS: In the β-BT-treated embryos, hair cells go through the same developmental processes as those of the controls. By the 10th day of incubation, hair cells, which have already differentiated during the previous stages, achieve long cylindrical shapes in basilar papillae and saccular maculae (Fig. 11b). A kinocilium and numerous stereocilia protrude from the top surface. At the basal part of the hair cell some mitochondria, RER and numerous polysomes are found (Fig. 12). However, coated vesicles are fewer than in
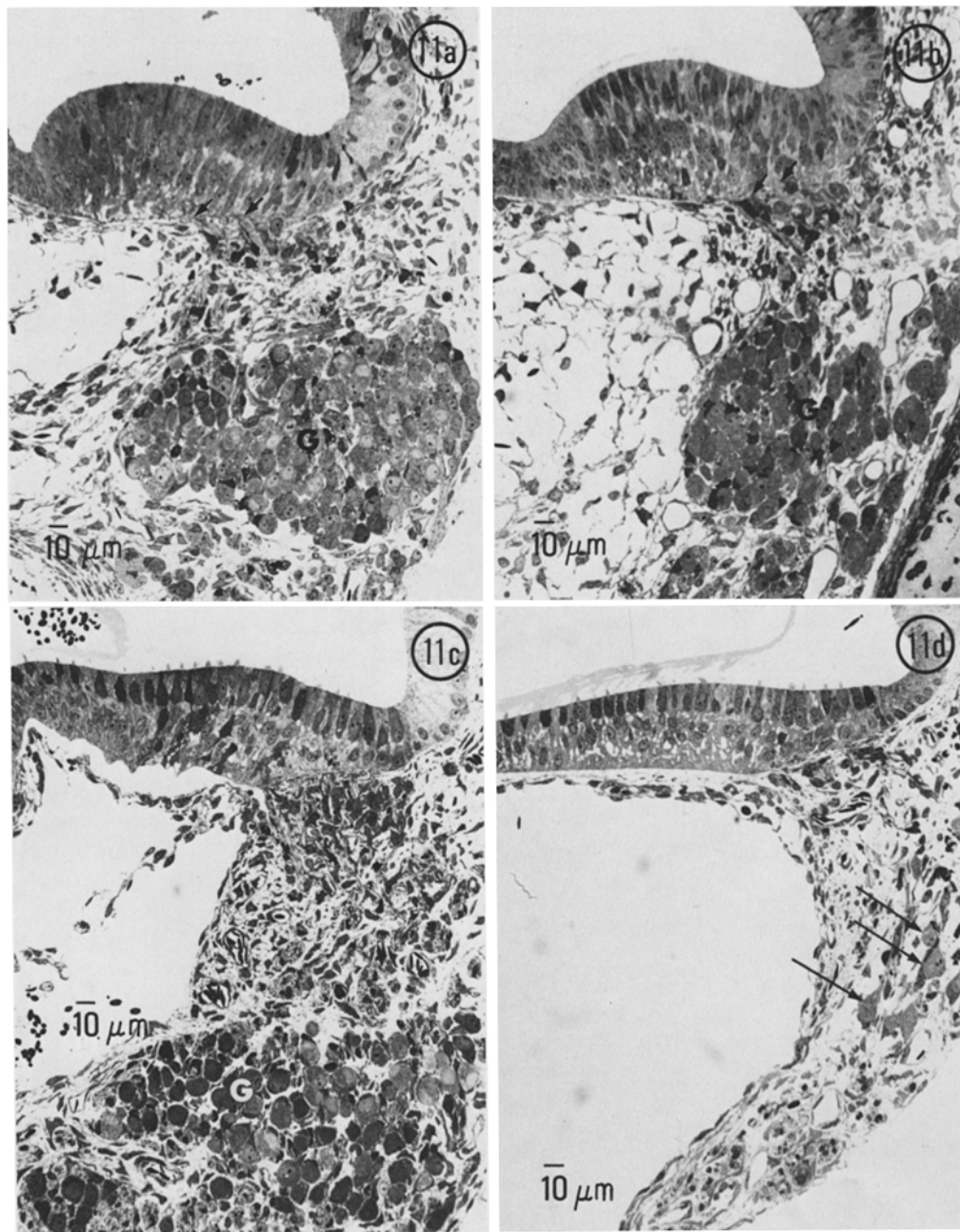
FIGURE 11 (a) The control basilar papilla. 10th day. The differentiating hair cells are distributed in the upper part of the neuroepithelium. The unmyelinated nerve fibers enter into the neuroepithelium at the habenula perforata (arrows). G: Spiral ganglion. × 260. Bar, 10 μm. (b) The basilar papilla. β-BT-treatment. 10th day. The number of the spiral ganglion cells (G) is fewer than in the control, but the nerve fibers still penetrate into the neuroepithelium (arrows). × 260. Bar, 10 μm. (c) The control basilar papilla. 14th day. × 260. Bar, 1 μm. (d) The basilar papilla. β-BT treatment. 14th day. Only a few ganglion cells (arrows) remain in the spiral ganglion. Note the numerous vacant spaces under the hair cells and among the supporting cells. × 260. Bar, 10 μm.
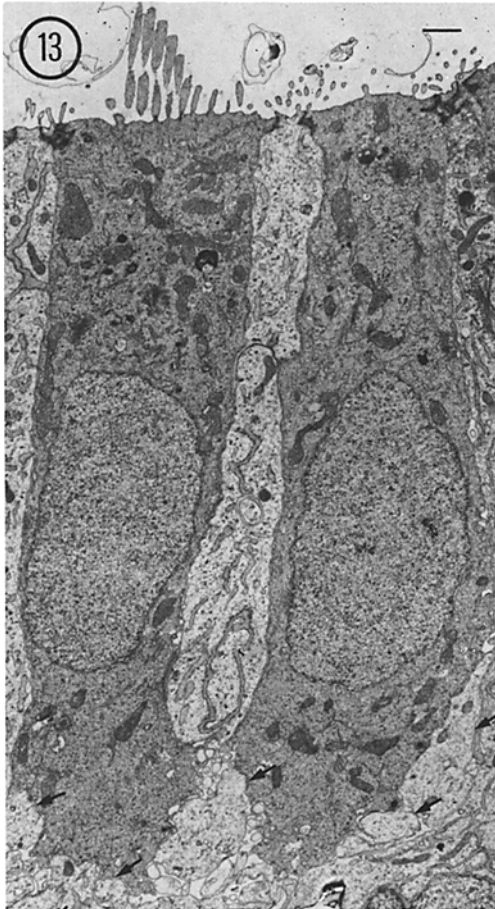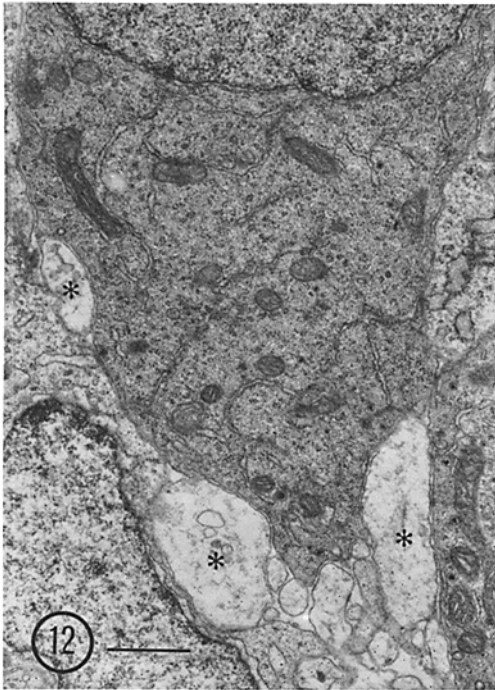
the later stages, and synaptic bodies are rarely seen. The ultrastructure of hair cells does not differ from that of the controls. On the 14th day, the hair cells can be classified into two groups in basilar papillae: the tall hair cells and the short hair cells. The top surface of the short hair cell is not so wide as it is in the later stages. The synaptic bodies associated with presynaptic membrane specializations are mostly situated near large vacant extracellular spaces or in contact with supporting cells (Fig. 15). By the 17th day, differences between tall and short hair cells become more conspicuous than in the early stages.

By the 14th day in the controls, synaptic bodies are present either adjacent to afferent nerve terminals or close to supporting cells (Fig. 13). When the synaptic bodies are located close to nerve terminals, fine bridgelike structures can be seen in the synaptic cleft. Subsurface cisternae are visible inside the hair cells adjacent to efferent nerve terminals. On the 14th day of incubation, most of the nerve terminals have completely disappeared from β-BT-treated inner ears. Nevertheless, the presynaptic specializations and associated synaptic bodies remain within the hair cells. Therefore, the presynaptic specializations with synaptic bodies found in the β-BT-treated inner ear after this stage either have never been in contact with nerve terminals or have lost their once established contact with them.

In the control saccular maculae, the type I hair cell and type II hair cell have already differentiated by the 17th day. The former is surrounded by a large afferent nerve chalice, but not so completely as the hair cell in the later stages (see Fig. 4). Consequently, most of the cell body of the type I hair cell is separated from adjacent cells by the nerve chalice. In contrast, the type II cell has several bouton-shaped afferent and efferent synapses at its basal end.

In the β-BT-treated saccular maculae, discrimination between type I and type II hair cells is often possible by the 17th day. The type I hair cell is surrounded almost entirely by several empty

FIGURE 12 The basal part of the hair cell in the β-BT-treated basilar papilla. 10th day. Several nerve terminals (*) touch the hair cell. × 10,800. Bar, 1 μm.

FIGURE 13 Control basilar papilla. 14th day. Several nerve terminals synapse with the hair cell (arrows). The synaptic bodies are situated opposite the afferent nerve terminals. × 51,000. Bar, 1 μm.
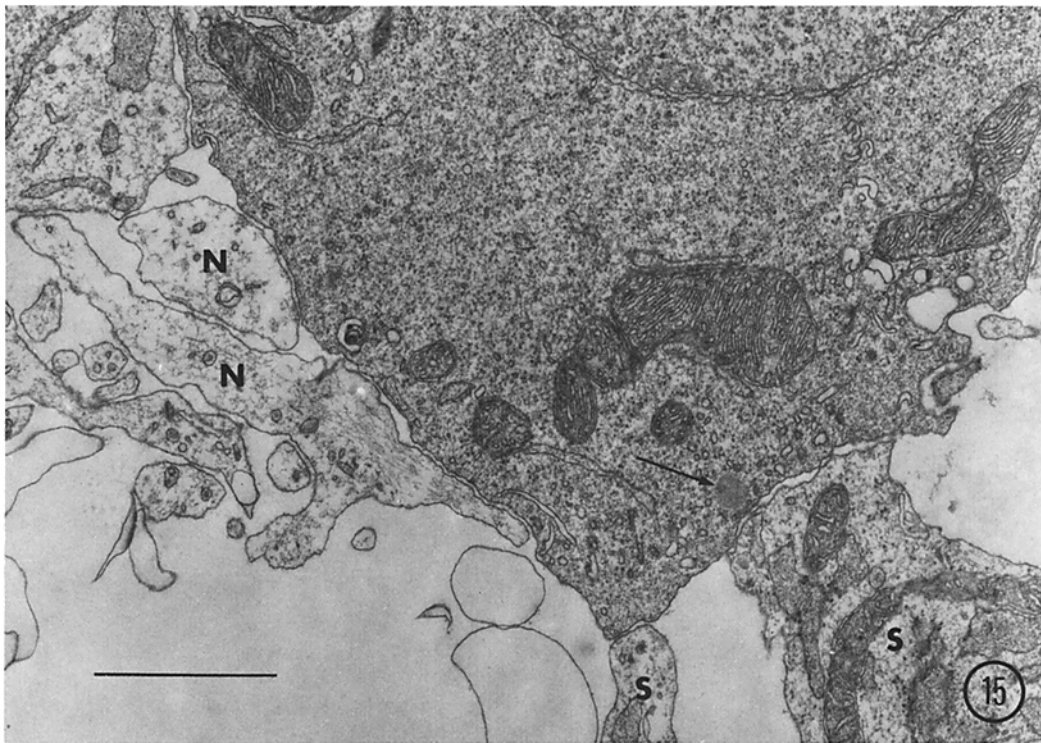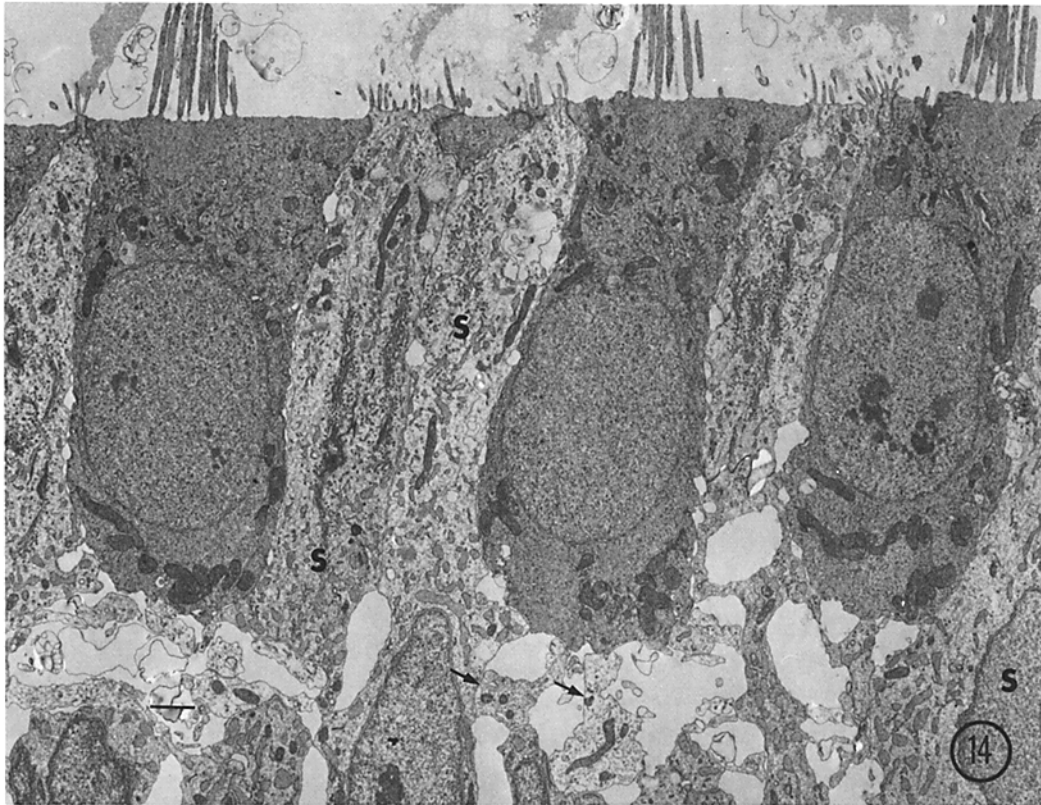
spaces (Fig. 17a), but the type II cells adjoin the few vacant spaces mainly at its base. At some points, hair cells are in close apposition with each other because of the absence of nerve fibers separating them (Fig. 17a and c). The synaptic bodies and adjacent presynaptic membrane specializations are as intact as those in the basilar papillae (Fig. 17b). Occasionally these structures are located adjacent to the neighboring hair cells, and dense material is found in the cleft between them (Fig. 17c). At the later stages, the supporting cells tend to occupy the empty spaces, and one cannot easily recognize the difference between type I and type II hair cells in the β-BT-treated saccular maculae (Fig. 8). However, the ultrastructure of the hair cell cytoplasm, including the regular arrangement of the kinocilium and stereocilia, remains similar to that of the controls (Figs. 4 and 8).

In Vitro Study

Numerous nerve growth cones grow radially from the spinal ganglion after 24 h of culture. On the 4th day, groups of spinal ganglion cells are recognized in the center of the control explants. The cells are round in shape and contain spherical nuclei with conspicuous nucleoli. Numerous nerve fibers extend from the explants (Fig. 18a).

In the β-BT-treated explants, to which β-BT is applied after 24 h of culture, a small number of darkly stained, degenerating ganglion cells without nuclei and nucleoli are found by the 4th day of culture (Fig. 18b). Intact ganglion cells are almost lacking. A few degenerating nerve fibers are also visible (Fig. 18b). No conspicuous changes are noted in Schwann cells and fibroblasts.

DISCUSSION

Destruction of Nerve Cells by β-BT

The acoustico-vestibular ganglion cells degenerate in the inner ear of chick embryos after chronic and massive application of β-BT. The efferent nerve fibers are also affected, but the efferent nerve cell bodies in the central nervous system have not been examined in the present study. The nerve cells in the cultured spinal ganglion also degenerate after treatment with β-BT.

It is well known that the botulinus toxin has a blocking effect on the transmitter release at the neuromuscular junction that is somewhat similar to that of β-BT (7). Previous workers have demonstrated atrophy in chick embryo skeletal muscle after application of the botulinus toxin. This may be due to the blockage of ACh release from the nerve terminal, or it may result from the disruption of some trophic function of the nerve (11, 16). The skeletal muscles of β-BT-treated chick embryos also undergo neurogenic degeneration as they do after botulinus toxin application (unpublished data). Botulinus toxin may affect cholinergic systems, while β-BT is supposed to affect not only cholinergic synapses, but also noncholinergic synapses (44). In the present in vivo study, the question arises whether nerve cells degenerate because of the possible blocking by β-BT of some trophic factor released from the hair cells in the same manner that the botulinus toxin causes blocking at the neuromuscular junction or whether they are destroyed directly by β-BT, but this question has been settled by the in vitro study which has been done in order to answer it. The results do support the possibility that β-BT directly destroys the nerve cells and fibers. Wernick et al. (44) reported that synaptosomes prepared from brain tissue treated with β-BT have a reduced ability to accumulate radioactive norepinephrine, γ-aminobutyric acid, serotonin, and ACh precursor, choline; and they suggested that the toxin may affect the cholinergic and noncholinergic synapses. The present study evidently suggests that the toxin does affect both the cholinergic and noncholinergic nervous systems.

β-BT has been thought to inhibit only the mech-

FIGURE 14 The basilar papilla. β-BT treatment. 14th day. Numerous large empty spaces are present adjacent to the hair cells or between the supporting cells. Several profiles of probable nerve terminals (arrows), which contain numerous microtubules and neurofilaments, remain in the neuroepithelium. S: supporting cell. × 5700. Bar, 1 μm.

FIGURE 15 The infranuclear part of a hair cell in the β-BT-treated basilar papilla. 14th day. Occasional profiles of probable nerve terminals (N), in which the cytoplasm is less dense than that of the supporting cell, are observed in contact with the hair cell. Arrow: synaptic body. Note the large empty space under the hair cell. × 24,000. Bar, 1 μm.
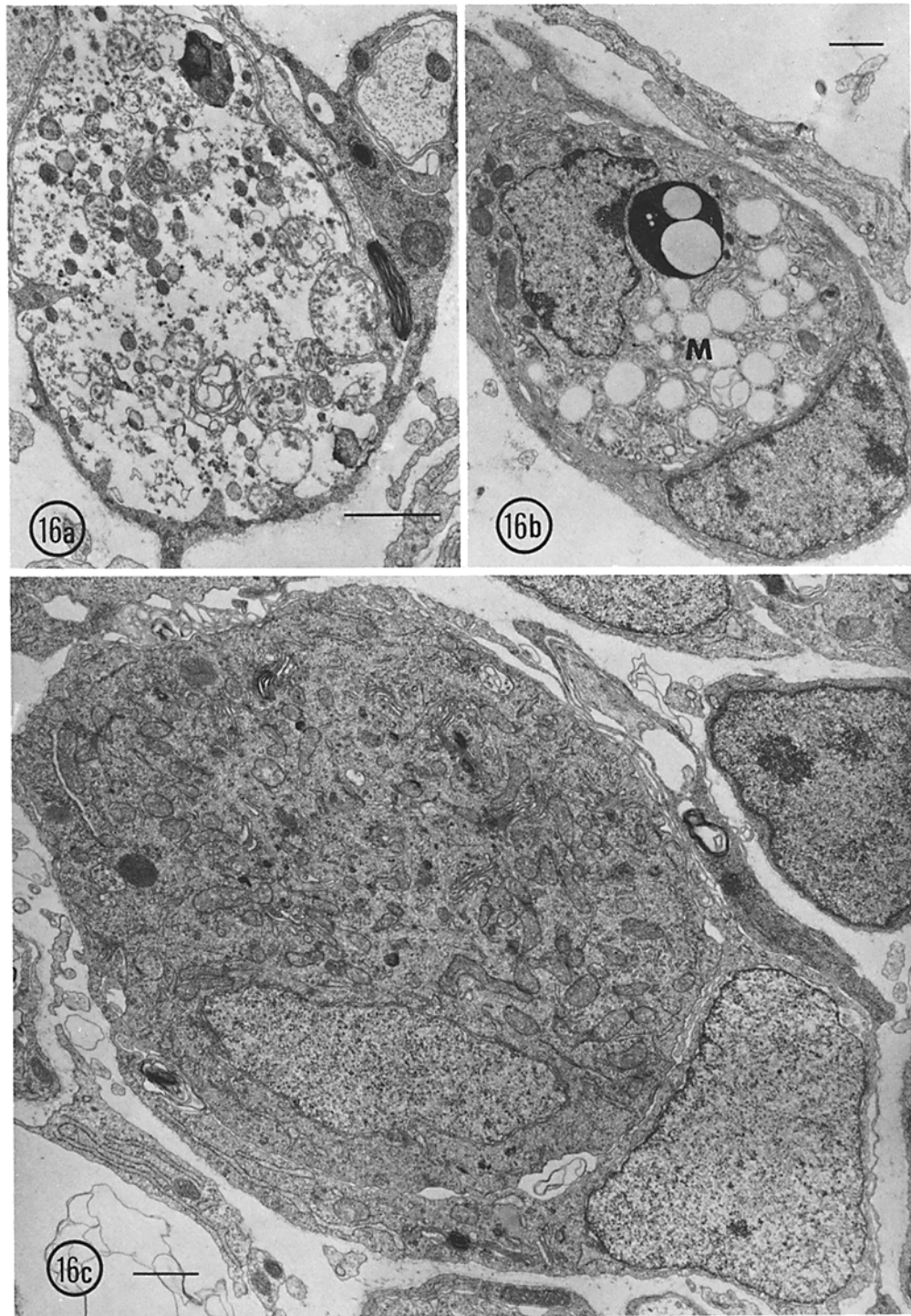
FIGURE 16 *a* and *b*   The spiral ganglion. β-BT treatment. 14th day. (*a*) The degenerating nerve cell containing swollen mitochondria and numerous lysosomes is surrounded by the satellite cell. × 13,900. Bar, 1 μm. (*b*) A macrophage (*M*) is wrapped by the satellite cell. × 7900. Bar, 1 μm. (*c*) The control spiral ganglion cell. 14th day. × 9,600. Bar, 1 μm.
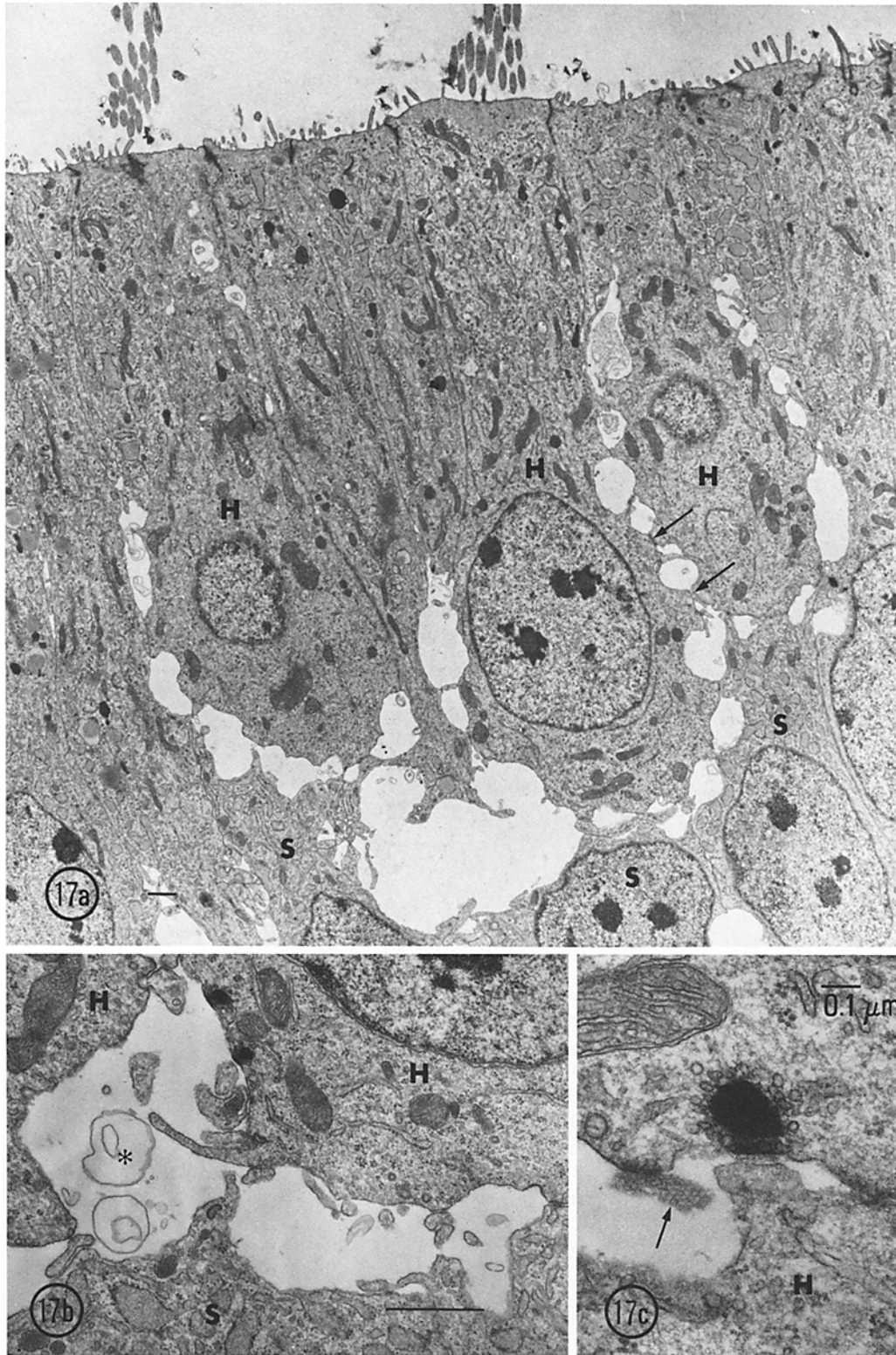
FIGURE 17 The saccular macula. β-BT treatment. 17th day. (a) Three probable type I hair cells (H) are surrounded by many vacant spaces. At several points the hair cells make contact with each other (arrows) and supporting cells (S). × 4400. Bar, 1 μm. (b) Two synaptic bodies are situated apposing the vacant space (*). × 15,500. Bar, 1 μm. (c) A synaptic body adjacent to the neighboring hair cell. In the cleft, dense material can be found. The dense material shows the regular lattice-like structure (arrow). × 59,900. Bar, 0.1 μm.

FIGURE 18 (a) Cultured spinal ganglion cells. Control. The well developed nerve cells containing round nuclei with conspicuous nucleoli send out numerous nerve fibers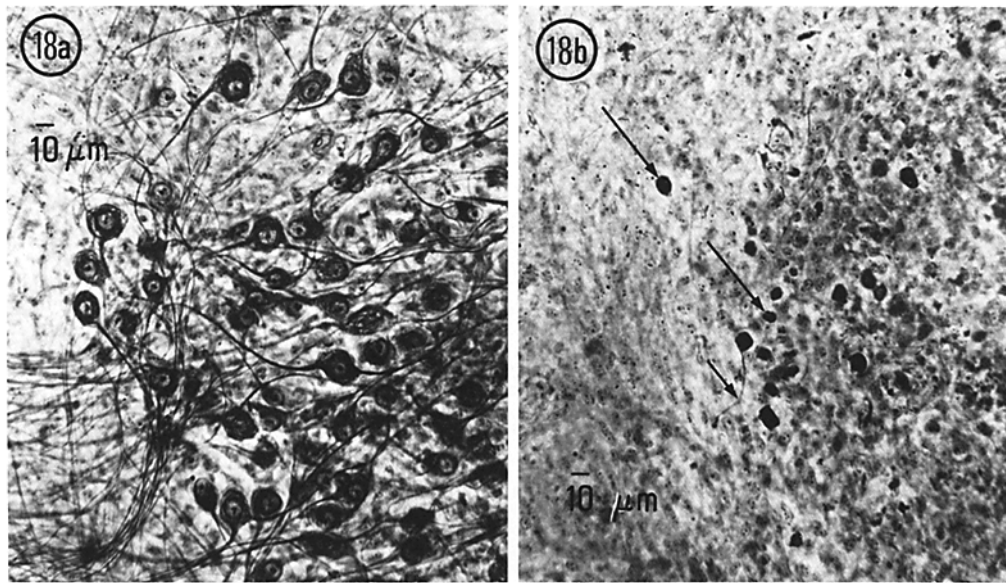. × 255. Bar, 10 μm. (b) Cultured spinal ganglion cells. β-BT treatment. Several darkly stained degenerating nerve cells are present (long arrows). Occasionally the remaining nerve fibers are visible (short arrow). × 255. Bar, 10 μm.

anism of neurotransmitter release without having any postsynaptic action on striated muscle and any effect on conduction in the nerve fiber at the neuromuscular junction (6, 8). Strong et al. (39) reported that β-BT has phospholipase A2 activity. They suggested that the phospholipase acts preferentially on the presynaptic neuronal membrane to modify the release of transmitter by altering the properties of the nerve plasma membrane. Considering the extensive effects of β-BT in the present study, we assume that the binding sites of β-BT are spread broadly on the nerve plasma membrane, unlike the binding sites of botulinus toxin. The binding sites of the botulinus toxin are localized at restricted presynaptic areas of the nerve fibers (24, 46).

In the β-BT-treated inner ear the hair cells and supporting cells are quite intact, whereas the Schwann and satellite cells disappear in the acoustico-vestibular ganglion. It is difficult to trace the degenerating Schwann and satellite cells in vivo. In vitro, Schwann cells of 10-day chick spinal ganglia do not appear to be affected by β-BT treatment. The question whether the Schwann and satellite cells migrate out, degenerate, or transform into macrophages (43) in vivo is left open. The age difference at which β-BT treatment was started in vivo (4th day) vs. in vitro (11th day)

might effect Schwann cell survival.

It is, however, clear that nerve cell destruction is extensive after β-BT treatment. The toxin can, therefore, be used as an effective means of denervation in studies of peripheral nerve-target cell relationships. In this regard, β-BT application is superior to surgical denervation, because it does not disrupt local circulation and it can be used where surgical denervation is difficult.

Hair Cell-Nerve Relationship

Various receptor cells are dependent on nerve cells for their differentiation and maintenance. The degree of dependency varies among receptor cells and among species of experimental animals.

Taste buds in fish disappear 11-19 days after denervation (30) and in frog 40 wk after denervation (34). In mammals, early signs of degeneration are detected at 8-12 h (3, 15) and taste buds degenerate totally by 10 days after denervation (15). The taste buds can be induced by nongustatory nerves, and they are able to develop without innervation in amphibians (33, 45). Recent study shows that also in mammals the taste buds are induced by nongustatory nerves in experimental situations (48).

Lateral line organs seem to disappear within a short time after denervation, and innervation is

necessary for their development in fish (4, 10, 32). In addition, ultrastructural changes have been reported for synaptic bodies in the ampullar organ of catfish several hours after denervation and of *Torpedo* 3 days after denervation (41, 9). However, the lateral line organ persists without conspicuous changes for long periods after repeated nerve section and differentiates without innervation from early developmental stages in amphibians (2, 13, 22, 29, 37, 38).

In general, target cells are dependent on nerves to some extent for their differentiation and maintenance. Although the degree of this dependency differs among various species, the amphibians appear to be an exceptional group in that their receptor cells are largely independent of the nerve.

Little information is available about the sensory cell-nerve relationship in the inner ear. Igarashi and Miyata (26) observed degenerative vestibular sensory cells after cutting ampullar nerves in squirrel monkeys. However, their observations are opposed by the work of Silverstein and Makimoto (35) who saw by light microscopy no signs of vestibular cell degeneration in cats when the vessels were intact at nerve sectioning. Sensory cells in the frog crista ampullaris survived total denervation for 1-2 wk without typical signs of ultrastructural damage (17). Gribenski (18) showed advanced degeneration of ampullar cristae in frog 4 wk after nerve division. The results of these studies are somewhat confusing, partly due to technical difficulties in transection of nerve fibers between sensory ganglion and neuroepithelium. A number of in vitro studies have shown that hair cells differentiated in cultures of otocysts that contained acoustico-vestibular ganglion cells (12, 14, 31). Furthermore, the work of Orr (31) on cultured chick otocysts suggests that the complete cytodifferentiation of hair cells is critically dependent upon innervation.

The present study shows that the sensory cells in the vestibulo-cochlear system of the chick embryo undergo differentiation quite independent of the nerve during the late developmental stages. However, there is a definite possibility that nerve terminals at some time made contact with hair cells in the present experimental material. Consequently, the possibility of neural influence at the early stages cannot be neglected. My developmental study[1] of the chick inner ear shows that hair cell

[1] Hirokawa, N. 1977. Synaptogenesis in the chicken basilar papilla. Manuscript in preparation.

differentiation begins on the 8th-9th day. At this time, some nerve terminals are interposed between differentiating hair cells and supporting cells. At this stage, the manner of contact of nerve terminals with hair cells is similar to that with supporting cells. No specific membrane differentiation of the nerve terminals can be observed. These observations suggest that not the nerve but the hair cell itself takes the initiative for the differentiation at the starting point. Nerve terminals probably exert the same influence on the hair cells as on the supporting cells. Orr observed that hair cells can differentiate in noninnervated sensory membrane in vitro but that this differentiation is not so pronounced as it is in the innervated membrane (31). Therefore, if the nerve has some effect on the differentiation of the sensory cells as Orr has suggested (31) in the inner ear of chick embryo, it may play some role only in their early stage of differentiation. The nerve appears to play no role in the subsequent development and maintenance of the hair cells.

It has generally been assumed that both pre- and postsynaptic elements are necessary for complete differentiation of synaptic specializations (1, 5, 20). In the inner ear of chicks, however, the exact sites of the presynaptic specialization on the hair cell membrane seem to be determined and the structure of it is maintained without participation of the postsynaptic element.

On the 10th day the slight membrane thickening, i.e., the first sign of the synaptogenesis, appears on the afferent nerve terminal. At this stage the synaptic body can already be seen in the hair cell. It is situated on the plasma membrane of the hair cell adjacent to either a nerve terminal or a supporting cell. On the 14th day the afferent synapses with the synaptic body, bridgelike structures in the synaptic cleft and the postsynaptic membrane thickening are present. However, observations on serial sections revealed that the synaptic bodies associated with presynaptic membrane specializations are often found on the plasma membrane where the nerve terminal has not yet made synaptic contact in the control.[1] In the β-BT-treated neuroepithelium, quite a few nerve terminals already achieved contact with hair cells. No nerve terminals are found in the neuroepithelium at the later stages. However, synaptic bodies and presynaptic specializations are maintained until the final developmental stage. The presynaptic specializations with synaptic bodies found in the β-BT-treated inner ear at the final stage either have
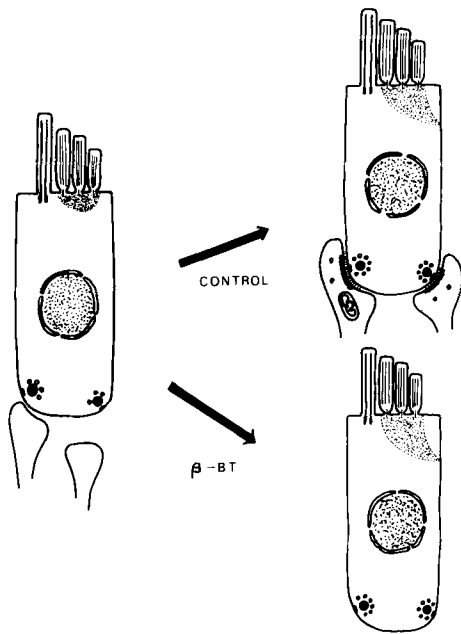
FIGURE 19 Possible course of the synaptogenesis in the control and β-BT-treated inner ear.

never been in contact with nerve terminals or have lost once established contact with them (Fig. 19). So the presynaptic specialization may be able to remain intact at least for 7-11 days (from 10-14th to 21st day) if it has never made synaptic contact with the nerve terminal.

In the control, although the presynaptic specializations with synaptic bodies are often found devoid of contact with postsynaptic elements by the 14th day of incubation as mentioned above, they adjoin the afferent nerve terminals at the final stages. Therefore, during normal development the afferent nerve may seek out the site of the preexisting presynaptic specialization on the hair cell to make a final synaptic contact of the adult type (Fig. 19).

The differentiation of the postsynaptic membrane thickening occurs independent of the presynaptic terminals on the Purkinje dendrite in the cerebellum of the weaver mouse which congenitally lacks granular cell (21, 23). Furthermore, Sytkowski et al. (40) have suggested that the ACh receptors may be the determinants of synapse recognition and that receptor cluster formation may be a normal step in synapse formation. These studies suggest that during synaptogenesis the differentiation of the synaptic membrane of the target cell (defined only as a cell toward which the

nerve process grows) occurs first and that nerve fibers actively seek out and recognize these preexisting synaptic sites on the target cells. The preexisting synaptic sites are the presynaptic specialization with the synaptic body in the hair cells, the postsynaptic specialization in the Purkinje dendrites and possibly, but not necessarily, the postsynaptic receptor sites in the muscles.

The present study on the chick embryo inner ear leads to the view that precise sites of the presynaptic specialization on the hair cell membrane may be determined and its structure maintained without participation of the postsynaptic element. This investigation also indicates that differentiation of the hair cells at the later developmental stages proceeds independently of the nerve cells. The exact mechanism of nerve destruction by β-BT, the binding sites of the toxin, and the influence of it on the other nervous tissues are being studied.

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