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Morphological and physiological effects of long duration infusion of strychnine into the organ of Corti

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Summary

Acute strychnine administration has long been used as a method to eliminate the effects of efferent activity. It has been shown that long after termination of *chronic* strychnine infusion into the cochlea, the ear becomes more susceptible to acoustic trauma suggesting that chronic strychnine infusion results in long lasting or permanent disruption of efferent function. Much research has been directed towards the functional significance of the olivocochlear system. However, there is little information concerning the effect of long duration inactivation of the medial olivocochlear system in an awake behaving animal. This study was designed to determine the structural and functional consequences of inactivation of the efferents by chronic infusion of strychnine into the cochlear perilymph of guinea pigs for two weeks via an osmotic pump. Physiological evaluations showed that the strychnine infusion eliminated the efferent induced reduction of the cochlear whole-nerve action potential three weeks after cessation of strychnine infusion. Contralateral efferent function remained unaltered. Histological evaluation at the light and electron microscopic levels revealed disoriented efferent synapses under the outer hair cells.

Introduction

The organ of Corti receives dual feedback information via the olivocochlear bundle (OCB) which originates in and around the superior olivary complex. The OCB comprises two systems; the medial (MOCS) and the lateral (LOCS) olivocochlear systems. They are classified according to the location of their respective cell bodies, and their postsynaptic targets in the cochlea (Klinke & Galley, 1974; Warr *et al*., 1986). The LOCS axons innervate the afferent fibers under the inner hair cells (IHCs) while the MOCS gives rise to large, myelinated axons that innervate the outer hair cells (OHCs).

The effect of activation of the LOCS on cochlear function is not well understood. More is known about the function of the MOCS but it is not completely understood. Electrical stimulation of the crossed olivocochlear bundle (COCB) causes a decrease in the endocochlear potential, a small increase of the cochlear microphonics, and suppression of the cochlear whole-nerve action potential (CAP) (Desmedt, 1975; Wiederhold, 1986). It has been suggested that the MOCS has the following roles: i) exerting an antimasking effect (Nieder & Nieder, 1970; Kawase *et al*., 1993), thereby improving the detection of a signal in noise backgrounds (Dewson, 1968; Winslow & Sachs, 1984; Dolan & Nuttall, 1988) and ii) attenuating noise-induced trauma (Cody & Johnstone, 1982; Reiter & Liberman, 1995) by reducing the activation of the transduction channels at the apex of the OHCs (Patuzzi & Thompson, 1991). The MOCS might influence transduction processes by modulating the electromotility of the OHCs (Kujawa *et al*., 1992).

In acute experiments, application of strychnine eliminates the effects of MOCS activation (Bobbin & Konishi, 1974; Sridhar *et al*., 1995). In these experiments strychnine either acts directly on the efferent synapse or the receptor located on the OHC. We have shown that after *chronic* strychnine infusion into the cochlea, there is an increase in the permanent threshold shift following noise exposure (Yamasoba & Dolan, 1997, 1998). The implication of those results is that a two week infusion of strychnine into the cochlea causes chronic disruption of efferent function and is consistent with the interpretation that the efferents serve to attenuate noise induced hearing loss (Cody & Johnstone, 1982; Reiter & Liberman, 1995). These two studies (Yamasoba & Dolan, 1997, 1998) both assumed that the effects of the

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strychnine infusion acted at either the efferent synaptic terminal or the receptor on the OHC. Since the strychnine infusion resulted in no hearing loss, the more likely locus of the strychnine effect was the efferent terminal. The present study was designed to investigate the morphological effects of a two-week infusion of strychnine into the cochlea via an osmotic pump. The results indicate that the only discernible change in cochlear morphology is the efferent synapse. The histological evaluation at the electron microscopic level revealed disoriented and degenerated efferent synapses. Physiological evaluations, in the same animals, showed that the strychnine infusion eliminated the electrically activated efferent induced reduction of the CAP three weeks after cessation of strychnine infusion.

Materials and methods

EXPERIMENTAL ANIMAL

Six pigmented guinea pigs weighing between 250–400 g were used in this study. Two animals were surgically implanted with a strychnine pump and a round window electrode for later recordings of the CAP in the awake animal. One of these was eliminated due to middle ear infection. Four animals were surgically implanted with the strychnine infusion pump only. Two of these animals were eliminated due to middle ear infection. The physiological results presented in Figures 1–4 are from three different animals. All three animals were processed for histological examination. Similar histological results were obtained from each animal receiving the two-week strychnine infusion.

IMPLANTATION OF AN OSMOTIC PUMP

For cochlear infusion, a fine-tipped cannula was made as previously described (Yamasoba & Dolan, 1997) with a slight modification. A 7 mm piece of the infusion tip of the cannula was made by trimming a fine cannula (Polyimide tubing, Microlumen) and inserted in the end of 8 cm length of polyurethane tubing (Intravenous Medical Vinyl Tubing, Scientific Commodities, Inc.). The connection was secured with silicone rubber, leaving 3.75 mm extending as the fine infusion tip. A small drop of silicone rubber placed 0.5 mm from the fine tip was used so that the cannula tip could not be inserted too far to cause damage to the organ of Corti and prevent leakage of perilymph. The cannula was sterilized with ethylene oxide.

Animals were anesthetized with xylazine (10 mg/kg, i.m.) and ketamine (40 mg/kg, i.m.), supplemented with local infusion of 1% lidocaine. Chloramphenicol sodium succinate (50 mg/kg, i.m.) was injected to prevent postoperative infection. Under aseptic conditions, the left bulla was exposed from the occipito-lateral direction and a small hole was opened in the bony wall of the basal turn lateral to the round window with a sharp gimlet, allowing access to the scala tympani. The tip of the cannula pre-filled with Ringer's solution with strychnine (50 μM) was inserted into the hole until the silicone drop was seated against the otic capsule. The cannula was secured to the bulla with glue and dental acrylic cement, looped around a stainless steel screw placed in the skull, and secured to it with methyl methacrylate. Another stainless steel screw was placed at the vertex to be used as a reference electrode. A subcutaneous pocket was formed between the scapulae to accommodate the pump. The osmotic pump (model 2002, 0.5 μl/h; Alza Corp.), had been pre-filled with Ringer's solution with strychnine (50 μM) and placed in a 38°C, 0.9% saline bath for 4 h to allow the pump to be operable immediately upon implantation. The pump was then attached to the cannula and implanted. After suturing the wound, buprenorphin (0.5 ml/kg, i.m.) was injected to relieve postoperative pain.

PHYSIOLOGY

Two animals were implanted as described above with a strychnine filled osmotic pump. At the time of implantation a Teflon coated wire was placed on the round window membrane and secured at the bulla wall with dental cement. The wire was led to a connector-housing unit cemented to the skull. A second wire was placed in the muscle of the neck and led to a second port of the same connector. These wires served as the active and ground electrode for measurement of the CAP response while the animal was awake and restrained. The CAP measurements were obtained before, during and after the two week infusion of strychnine. One animal developed a hearing loss due to a middle ear infection and was eliminated from the study. The function of the medial efferent system was monitored for nine weeks after the termination of the strychnine infusion by acoustically activating the contralateral ear and measuring the effect on the ipsilateral CAP (Liberman, 1989; Smith *et al.*, 1994). At the end of the nine week period, this animal was surgically prepared for electrical stimulation of the COCB. Unfortunately, the connector-housing unit cemented to the skull disrupted the skull landmarks used for stereotaxic placement of the stimulating electrodes thereby preventing electrical stimulation of the COCB.

ELECTRIC STIMULATION OF COCB

Four additional animals were surgically implanted with strychnine pumps as described above. Two animals developed a hearing loss due to a middle ear infection and were eliminated from the study. Three weeks after termination of the two-week strychnine infusion, the animals were anesthetized (Nembutal, 13 mg/kg IP and Innovar-Vet, 0.4 ml/kg IM) and surgically prepared for stereotaxic electrical stimulation of the COCB at the floor of the 4th ventricle (Dolan & Nuttall, 1988; Dolan *et al.*, 1997). Maintenance of anesthesia was accomplished by supplemental doses of Innovar-Vet and Nembutal (at 1/2 initial amount) every 1 and 3 hours, respectively. Heart rate and body temperature were monitored and the animals were wrapped in a heating blanket to maintain the rectal temperature at 37.0 $\pm$ 0.5°C. A tracheotomy and tracheal cannulation was performed to maintain artificial respiration. In addition, a head-holder heater was used to maintain cochlear temperature near body temperature and preserve cochlear sensitivity (Brown *et al.*, 1983; Shore & Nuttall, 1985).

The right and left bullas were exposed by postauricular dissection. The CAP was recorded from each ear via a round window electrode cemented in place on each bulla wall. The CAP was used to assess auditory sensitivity to tone bursts (1.0 ms rise-fall time with 10 ms duration) ranging from 2.0 to 40.0 kHz delivered into a closed system (Dolan & Nuttall, 1988). After placement of the round window electrodes, the animal was rotated and prepared for stereotaxic placement of the bipolar stimulating electrode (insulated 32 gauge stainless steel wire). The animals were paralyzed with curare (0.3 mg, i.m.) after placement of the COCB stimulation electrode. The COCB was stimulated for 250 ms with a 75% duty cycle (bipolar pulses (0.4 ms duration at a rate of 250/s, 200–400 μA)) (Dolan & Nuttall, 1988).

OBSERVATION OF HAIR CELLS AND SYNAPTIC TERMINALS

After termination of the physiological experiments the distribution of efferent terminals in the organ of Corti were studied in the strychnine treated ear, the contralateral ear and a control ear receiving a two week infusion of artificial perilymph. To detect efferent terminals, synapsin-specific antibodies were used. Synapsin proteins are associated with the membrane of synaptic vesicles (Ueda *et al*., 1977; Greengard *et al*., 1993) and are therefore useful for identification of efferent nerve terminals (Frisancho *et al*., 1997; Ofsie & Cotanche, 1996; Zidanik & Fuchs, 1996).

Following decapitation under general anesthesia with a mixture of xylazine (10 mg/kg, i.m.) and ketamine (50 mg/kg, IM), the bilateral temporal bones were immediately removed. The perilymphatic spaces were perfused with 2% paraformaldehyde in phosphate buffer and the specimens were immersed in the same solution for 1 hour. Then, the cochlea was isolated and the lateral bony wall of the cochlea was removed. After permeabilization with 0.3% Triton X-100 solution for 5 minutes, whole mounts were stained with anti-synapsin antibody (G-304 polyclonal, affinity purified rabbit antipeptide, a gift from Andrew Czernik, The Rockefeller University) for 80 minutes. Samples were then incubated with TRITC-conjugated goat anti-rabbit IgG for 40 minutes. The whole mounts were then stained for actin with FITC phalloidin for 30 minutes. The sensory epithelium including the organ of Corti was isolated from the modiolus, mounted on slides, and observed under a fluorescence microscope (Leica DMRB). Samples were photographed on A Kodak T-max 400 film.

TRANSMISSION ELECTRON MICROSCOPY OBSERVATIONS

The organ of Corti of the 3 animals was examined by TEM after the termination of the experiment and in one animal receiving artificial perilymph rather than strychnine. Following decapitation under deep anesthesia with a mixture of xylazine (10 mg/kg, IM) and ketamine (50 mg/kg, IM), the bilateral temporal bones were immediately removed and the perilymphatic spaces perfused with 2.5% glutaraldehyde in phosphate buffer for 2 hours. The specimens were then immersed in 3% EDTA with 0.3% glutaraldehyde. After decalcification, specimens were postfixed in 1% osmium tetroxide for 2 hours, dehydrated in a graded alcohol series, treated with propylene oxide, and embedded in epoxy resin. Ultrathin mid-modiolar sections of the cochlea were prepared from the basal turn, stained with uranyl acetate and lead citrate, and observed by LM and TEM.

The University of Michigan Committee on Use and Care of Animals approved all studies described in this report. Veterinary care and housing were provided by the Unit for Laboratory Animal Medicine of the University of Michigan in animal facilities approved by the AALAC.

Results

CAP data obtained from an awake and restrained animal are shown in Figs. 1 and 2. In each figure, the solid line shows the averaged CAP response to a tone burst presented to the ipsilateral ear. The dotted line shows the CAP response from the same ear, but with broadband noise presented to the contralateral ear. Prior to the infusion of strychnine into the ipsilateral cochlea, the effect of contralateral acoustic stimulation reduces the ipsilateral CAP (Fig. 1). The reduction in amplitude is caused by activation of the efferent system (Liberman, 1989; Smith *et al*., 1994). Figure 2 shows representative

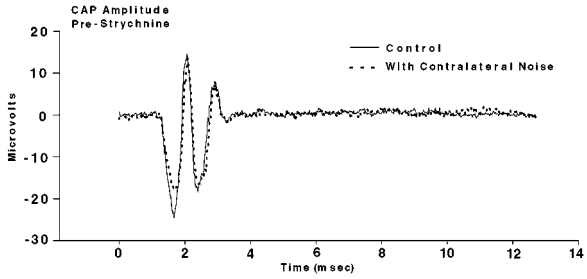

Fig. 1. The cochlear whole-nerve action potential (CAP) is recorded from an awake, restrained animal with a surgically implanted electrode on the round window. Prior to infusion of strychnine, the CAP amplitude to a 7.5 kHz toneburst (solid line) is reduced in the presence of noise presented to the contralateral ear (dotted line). The reduction in response amplitude is caused by contralateral acoustic activation of the efferent system.

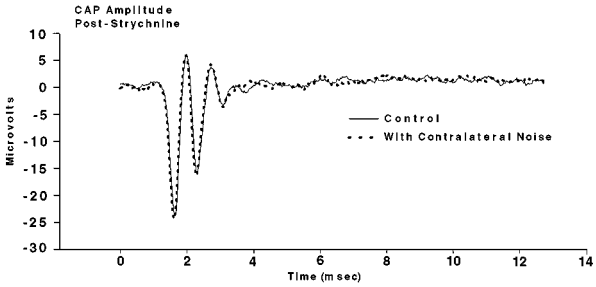

Fig. 2. The same stimulus conditions from Fig. 1 are shown here except that strychnine was perfused into the ipsilateral cochlea. During the strychnine infusion the reduction of the CAP response by acoustic stimulation of the contralateral ear is eliminated. The elimination of the efferent effect persists up to nine weeks (time of sacrifice) after termination of the infusion.

results from measurements obtained during the strychnine infusion and up to 9 weeks after infusion termination. During and after the strychnine infusion period, the efferent induced reduction in CAP response amplitude by contralateral acoustic stimulation is eliminated (Fig. 2).

The traditional method of efferent activation is by electrical stimulation of the at the floor of the fourth ventricle. The effect of such stimulation is to reduce the magnitude CAP response amplitude by an amount similar to reducing the stimulus intensity by 10–15 dB. Figures 3 and 4 show results from the two animals in which COCB electrical stimulation was performed

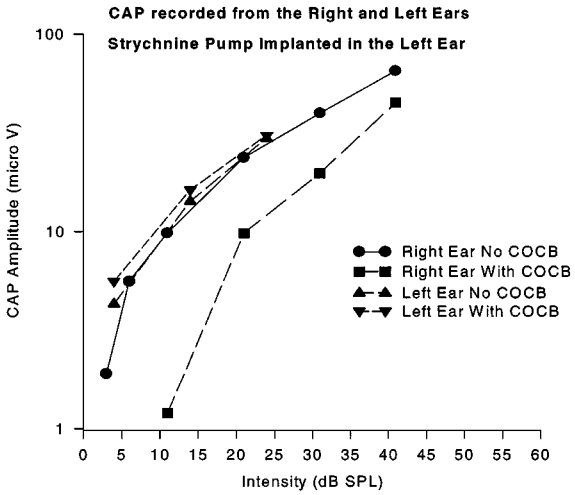

Fig. 3. Electrical activation of the crossed olivocochlear bundle (COCB) at the floor of the 4th ventricle reduces the CAP amplitude in the right ear but has no effect on the CAP in the left ear three weeks after termination of strychnine infusion.

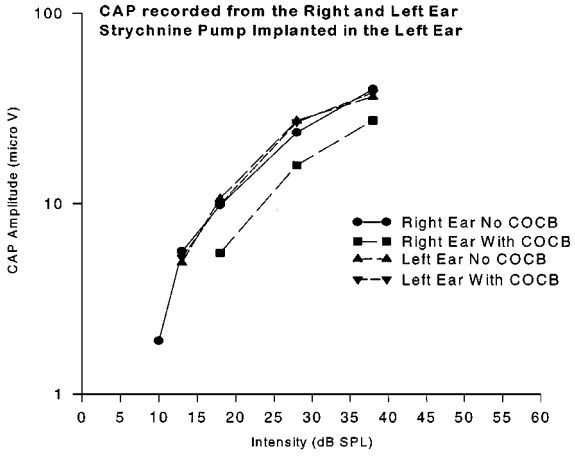

Fig. 4. Results from a different animal with the same experimental conditions as in Fig. 3 are shown in this figure. Electrical activation of the COCB at the floor of the 4th ventricle reduces the CAP amplitude in the right ear but has no effect on the CAP in the left ear three weeks after termination of strychnine infusion.

three weeks after termination of two-week strychnine infusion into the left ear. In each case, the effects of COCB stimulation on the CAP are compared for the right (no strychnine) and left (strychnine) ears. In each figure the solid line with closed circles shows the results from the right ear without COCB stimulation and dashed line with squares with COCB stimulation. The results from the left ear are shown with upward triangle (no COCB stimulation) and downward triangle (with COCB stimulation). In both animals, electrical stimulation of the COCB reduced the CAP response from the right ear, which did not receive the strychnine infusion. Electrical stimulation of the COCB had no effect for the same acoustic stimulus condition in the left ear, which did receive a strychnine infusion. In each case, the results obtained from the ear receiving the intracochlear infusion of strychnine are similar to results obtained in acute experiments where the strychnine is applied systemically (Bobbin & Konishi, 1974; Dolan & Nuttall, 1988; Sridhar *et al.*, 1995).

The I/O functions in Figs. 3 and 4 show comparable thresholds and response amplitudes between the two ears. The presence strychnine did not affect the sensitivity at these frequencies. Figure 5 shows more complete audiograms for the three animals represented in Figs. 1–4. These results indicate that the presence of strychnine in the cochlea did not alter the sensitivity at any frequency compared to the control ear.

Analysis of whole mounts of the organ of Corti show no obvious damage to hair cells in either strychnine-treated or contralateral ears. The nerve terminals beneath the inner and OHCs are well arranged in the

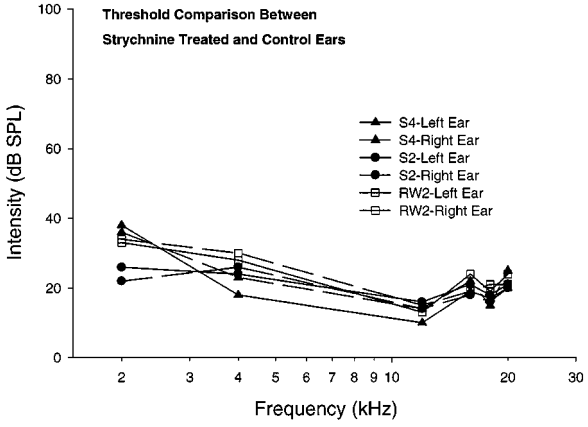

Fig. 5. The results shown in Figs. 1–4 are from three different animals. Threshold audiograms were obtained from each ear just prior to sacrifice (for each respective animal the strychnine ear is indicated by a solid line, the right, untreated ear, is shown with the dashed line). The figure shows that the two-week infusion of strychnine into the left ear had no significant effect on thresholds compared to the untreated ear. For animals S2 and S4, the measurement was made three weeks after infusion termination whereas the measurement was made nine weeks after termination for animal RW2.

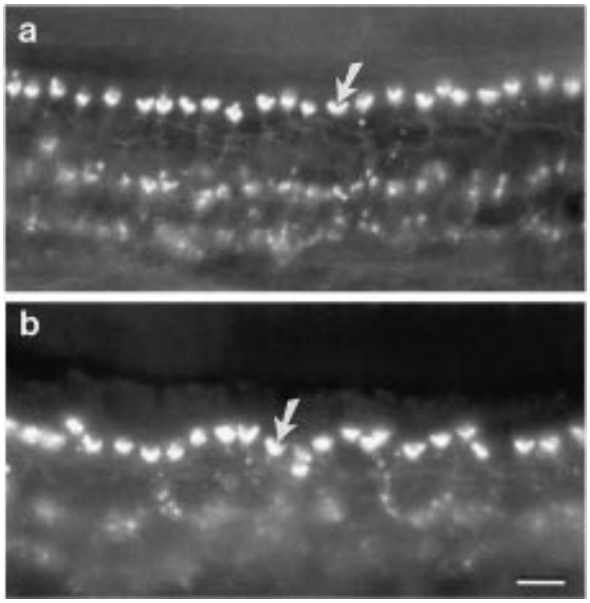

Fig. 6. Whole mount of the third cochlear turn stained with anti-synapsin antibody in the contralateral control (a) and strychnine-treated ear (b). Arrows in each micrograph depict a set of efferent terminals under one outer hair cell (first row). In the non-treated ear (a) there is a nearly straight line of terminals at this focal place. In a similar focal plane, terminals beneath the first row of outer hair cells in the strychnine-treated ear are displaced resulting in a disorganized zigzag shaped line.

contralateral controls. In strychnine-treated ears, the terminals beneath the OHCs show some disarrangement especially in the third turn (Fig. 6). Instead of a relatively straight line of terminals under each row of OHCs (Fig. 6a), treated ears display zigzag-shaped organization of terminals (Fig. 6b). The terminals beneath the IHCs, as well as most terminals beneath the OHCs in the basal and second turns, are normally arranged (results not shown).

The morphology of the organ of Corti in the contralateral control ears is normal at the electron microscopic level (Fig. 7a). The strychnine-treated ears, however, show pathological changes in the medial efferent terminals beneath the OHCs. In most of the medial efferent terminals vacuoles are observed. These vacuoles vary in size and number between terminals (Fig. 7b–e). Nevertheless, the synaptic connections between the medial efferent terminals and OHCs are relatively well preserved (Fig. 7d and e). The postsynaptic membrane is clearly observed in the region where the medial efferent terminals show severe degeneration. We do not observe conspicuous degenerative signs in the sensory hair cells or supporting cells.

In contrast to the conspicuous damage to the medial efferent system, we observe no lesion of the lateral efferent system or afferent nerves in any area of the cochlea (Fig. 8). The inner, tunnel, and outer spiral bundles are well preserved. No apparent lesion is found in the synaptic connection of the lateral efferent terminals with the afferent nerves beneath the IHCs.

Additional control ears which were treated with artificial perilymph determine if the surgical intervention used in these experiments causes any ultrastructurally-detectable lesion. TEM analysis reveals normal morphology of hair cells and nerve terminals in both inner and outer hair cell areas (Fig. 9).

Discussion

The results from this study show that chronic infusion of strychnine into the cochlea has no effect on cochlear sensitivity (Figs. 3–5) but does result in long term inactivation of the MOCS (Figs. 2–4). The results are similar to the effects of acute application of strychnine (Bobbin & Konishi, 1974; Sridhar *et al*., 1995). Our previous studies (Yamasoba & Dolan, 1997, 1998) showed that chronic infusion of strychnine into the cochlea of awake behaving animals rendered the treated ears susceptible to acoustic trauma. We assumed in those studies (Yamasoba & Dolan, 1997, 1998) that the protection from acoustic trauma provided by the efferent system was eliminated by the strychnine inactivation of the MOCS. The results from this study confirm that a two-week cochlear infusion of strychnine eliminates the electrically evoked COCB effect three weeks after infusion termination. Our previous studies (Yamasoba & Dolan, 1997, 1998) are also consistent with other results (Cody & Johnstone, 1982; Rajan & Johnstone, 1983, 1988, 1989; Puel *et al*., 1988) suggesting that the efferents play a role in protecting the ear from acoustic trauma.

The histological evaluation of strychnine treated ears showed that the efferent terminals, while present, display morphological changes. More experiments are necessary to determine if the lesion is reversible, or reflecting a step in the degeneration of terminals in the strychnine treated ears. The abnormality in the MOCS is restricted to the terminal since the TSB is normal (Fig. 8a), as is the LOCS terminating on the afferent synapse under the IHC (Fig. 8b–d). The morphology of the OHCs is not affected by the strychnine, which is consistent with the physiological results.

ACh is the likely neurotransmitter of the efferent fibers innervating the OHC (Guth *et al*., 1976; Klinke, 1981; Bobbin *et al*., 1984; Eybalin, 1993) based on the number of anticholinergic agents that block the effects of efferent stimulation. Various pharmacological experiments suggest that the receptor mediating the MOCS is unresponsive to nicotine or muscarine but paradoxically is blocked by both nicotinic and muscarinic antagonists such as curare, α-bungarotoxin and atropine. The most potent blocker of efferent activation is strychnine (Bobbin & Konishi, 1974; Sridhar *et al*., 1995), a nontraditional antagonist of ACh receptors. A likely candidate for the ACh receptor on OHCs

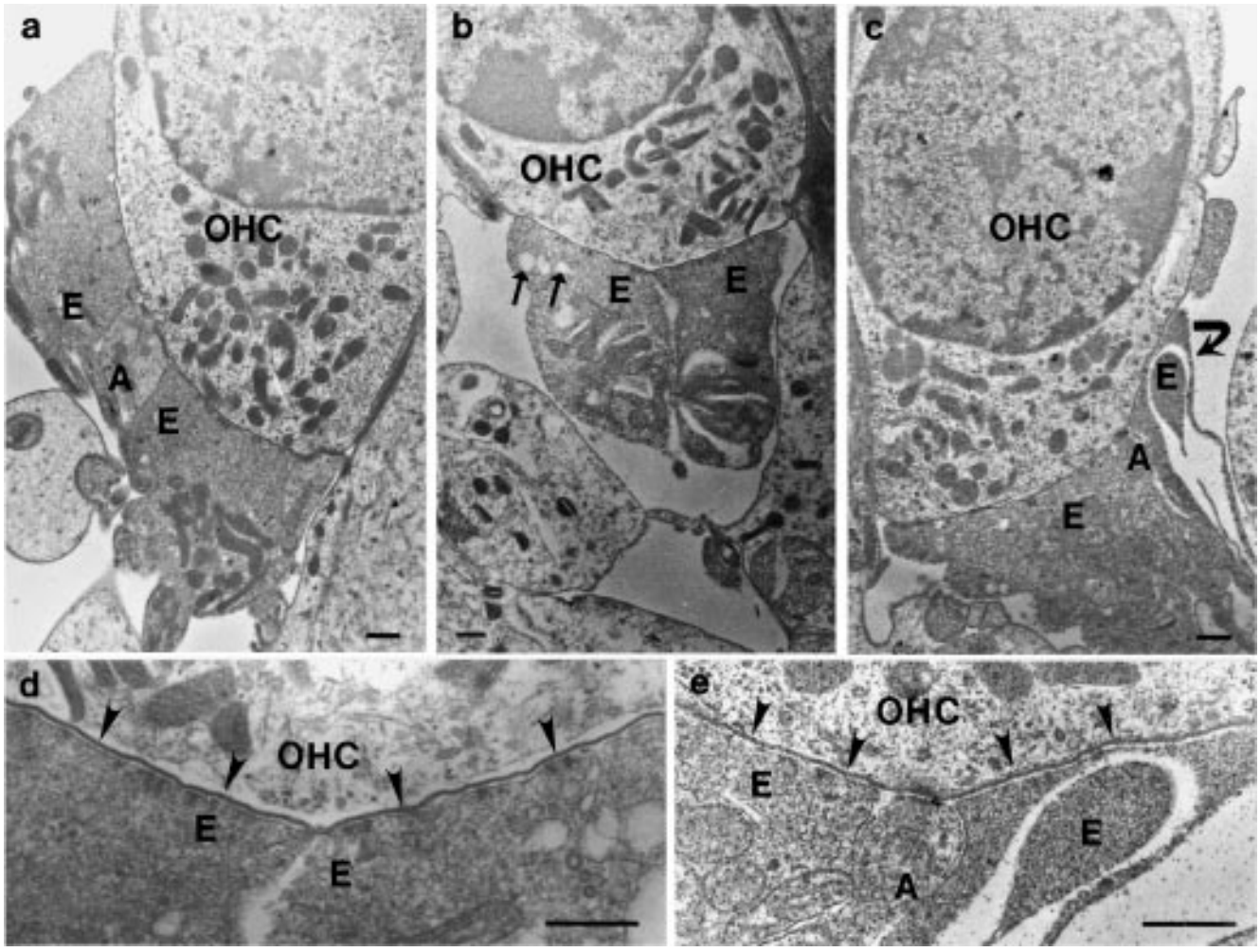

Fig. 7. TEM micrographs (a–c) showing the basal domain of outer hair cells (OHC). (a) A normal control OHC with contacts to medial efferent (E) and afferent (A) nerve terminals. (b) A strychnine-treated ear, showing vacuole formation (arrows). (c) A strychnine-treated ear, showing degenerative signs in the efferent terminal (arrows). (d, e). High magnification of Figs. b and c. Postsynaptic membranes are well preserved (arrowheads). Bar, 0.2 μm.

is a α9 subunit (Elgoyhen *et al*., 1994). The pharmacology of this isolated receptor is similar to the cholinergic receptor in the cochlea (Housley & Ashmore, 1991; Kakehata *et al*., 1993; Elgoyhen *et al*., 1994; Erostegui *et al*., 1994; Kujawa *et al*., 1994). The site of action of *acutely* applied strychnine (either systemically or intracochlear) is presumably at the ACh receptor on the OHC. This blockage is thought to prevent the ACh induced hyperpolarization of the OHC by blocking the inward Ca^{2+} current that leads to the Ca^{2+}-dependent outward K^+ current (Housley & Ashmore, 1991; Doi & Ohmori, 1993; Kakehata *et al*., 1993; Erostegui *et al*., 1994; Evans, 1996). The rise in Ca^{2+} may result by Ca^{2+} crossing the OHC membrane through a cation channel gated by a nicotinic-like receptor (Housley & Ashmore, 1991; Evans, 1996;) or a release of Ca^{2+} from inside the OHC through the action of a muscarinic receptor linked to a G protein (Kakehata *et al*., 1993).

The results of this study show that a 2-week infusion of strychnine leads to in a disruption of the efferent terminal 3 weeks after the cessation of the infusion. The classic view has been that the ACh receptor resides in the membrane of the OHC. The observed disruption of the efferent terminal may result from a blockage of this receptor site resulting in an accumulation of transmitter substance or Ca^{2+} that is unhealthy for the terminal. Two other possibilities exist. The strychnine may block transmitter release and cause an accumulation of the transmitter that results in the terminal reaction. The third possibility is the existence of presynaptic and/or preterminal ACh receptors (for review see Wonnacott, 1997). Presynaptic or preterminal receptors are thought to modulate transmitter release. Their existence has not been previously described in the efferent fibers in the ear. If they exist in the efferent fibers, the presence of strychnine may block the receptors, resulting

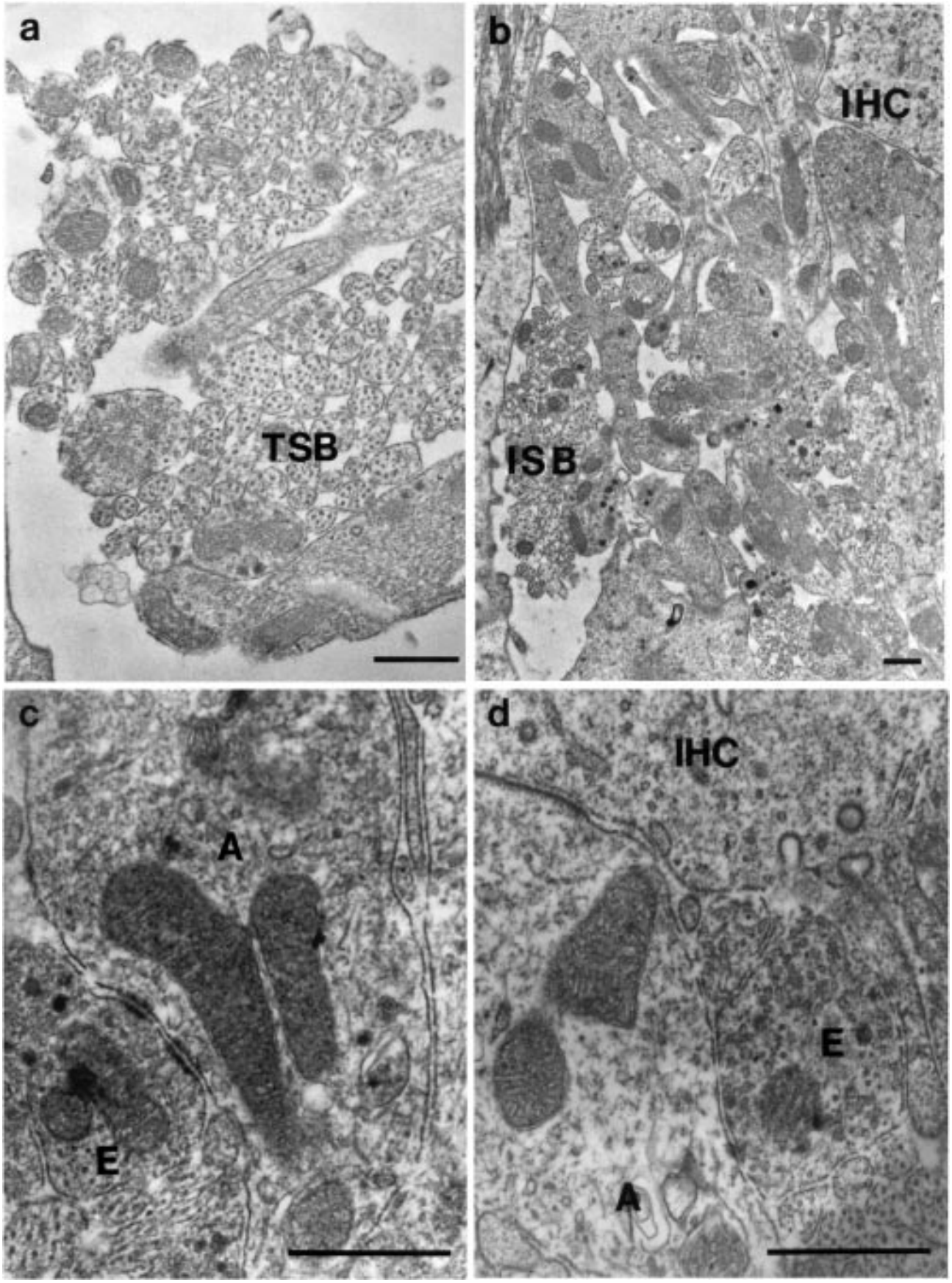

Fig. 8. TEM micrographs of the lateral efferent fibers. (a) The tunnel spiral bundle (TSB) in the strychnine-treated ear appear normal. (b–d) The region beneath the inner hair cell (IHC) in the strychnine-treated ear showing a normal appearing inner spiral bundle (ISB), afferent nerves (A) and lateral efferent fibers (E). Bars, 0.2 μm.

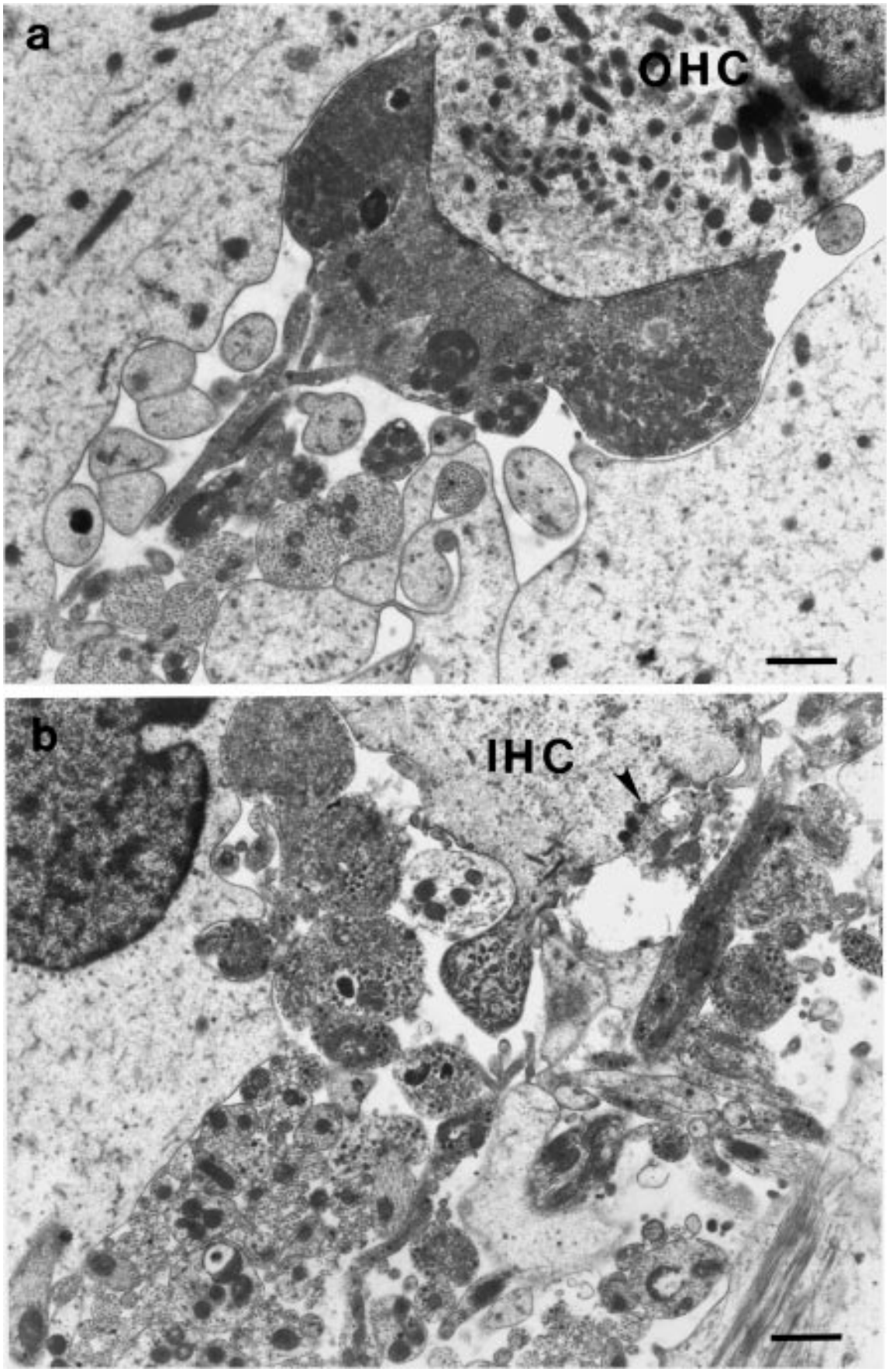

Fig. 9. Outer hair cell (a) and inner hair cell (b) regions in a control ear treated with artificial perilymph. Both types of hair cells and their innervation appear normal.

in metabolite depletion and oxygen deprivation due to continuous neurotransmitter synthesis without feedback inhibition and thereby neuronal damage. In both latter possibilities, a disturbance in the homeostatic mechanisms to control the presence of Ca^{2+} may be involved with the disruption of the efferent terminal. Further studies are necessary to determine the exact cause of strychnine-induced disruption of the MOCS terminal. It will also be necessary to determine the fate of the OHCs in the longer term following strychnine-induced lesions in the efferent terminals.

Acknowledgments

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