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Unmyelinated Axons of the Auditory Nerve in Cats

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ABSTRACT

This paper describes some central terminations of type II spiral ganglion neurons as labeled by extracellular injections of horseradish peroxidase (HRP) into the auditory nerve of cats. After histological processing with diaminobenzidine, both thick (2–4 μm) and thin (0.5 μm) fibers of the auditory nerve were stained. Whenever traced, thick fibers always originated from type I spiral ganglion neurons and thin fibers always from type II ganglion neurons. Because the labeling of type II axons faded as fibers projected into the cochlear nucleus, this report is limited to regions of the ventral cochlear nucleus near the auditory nerve root. The central axons of type II neurons are unmyelinated, have simple yet variable branching patterns in the cochlear nucleus, and form both en passant and terminal swellings. Under the light microscope, most swellings are located in the neuropil but they are also found in the vicinity of cell bodies, nodes of Ranvier of type I axons, and blood vessels. Eighteen en passant swellings in the neuropil were located by light microscopy and resectioned for electron microscopy; two of these swellings exhibited ultrastructural features characteristic of chemical synapses. The data indicate that inputs from outer hair cells might be able to influence auditory processing in the cochlear nucleus through type II primary neurons.

Key words: cochlea, cochlear nucleus, hearing, horseradish peroxidase, primary afferents, spiral ganglion, synapse, type II neurons

Receptor cells (hair cells) of the inner ear stimulate primary neurons that deliver impulses to the brain. The cell bodies of the primary auditory neurons are located in the spiral ganglion within the bony cochlea. Morphological data demonstrate that there are at least two different types of spiral ganglion neurons based on cell body size, myelination, polarity, or cytoplasmic content (e.g., Kellerhals et al., '67; Spoendlin, '73; Ota and Kimura, '80; Kiang et al., '84; Berglund and Ryugo, '86) and peripheral innervation (Kiang et al., '82; Berglund and Ryugo, '87; Brown, '87). In cats, type I neurons, which constitute 90–95% of the ganglion cell population, tend to have large (20–30 μm in diameter), bipolar cell bodies and myelinated processes. Each type I neuron typically forms an unbranched peripheral process that contacts a single inner hair cell (IHC). Type II neurons are smaller (10–20 μm in diameter), pseudomonopolar in shape, and have unmyelinated processes. In number, type II neurons constitute only 5–10% of the ganglion cells although each peripheral process branches to form contacts with many outer hair cells (OHCs).

Demonstration that the two types of hair cells have separate afferent innervation has led to much speculation about their function (e.g., Stebbins et al., '69; Spoendlin, '71; Dallos et al., '72, '82; Ryan and Dallos, '75; Liberman and Kiang, '78, '84). Presumably, sensory information requiring rapid processing is carried by the type I neurons with their large myelinated axons. In contrast, the relatively small numbers of type II neurons and a failure to observe their central projections in cats raised the question of whether the OHC system contributed any significant input to the brain (Spoendlin, '79). Some functions of OHCs need not necessarily involve the afferent type II neurons directly since OHCs can apparently alter the responses of IHCs through mechanisms such as modulating the extracellular receptor current (Ryan and Dallos, '75; Dallos and Harris, '78) or mechanical coupling via the tectorial membrane (Neely and Kim, '83; Brown and Nuttall, '84; Kiang et al., '86). Although there may be functional interactions between the two types of hair cells, their

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afferent innervations are segregated. Whether OHC afferents have activities completely dissociated from those of IHC afferents cannot be determined until more is known about their respective central connections.

Basic issues regarding the morphology of the synapses of type II neurons, or whether type I and type II neurons can innervate the same neurons in the cochlear nucleus, have not yet been resolved. On the basis of tracer injections in the cochlear nucleus that retrogradely label spiral ganglion cells, type II neurons have been shown to project to the ipsilateral cochlear nucleus (Ruggero et al., '82; Leake-Jones and Snyder, '82; Jones et al., '84). It has also been suggested that type II neurons project preferentially to the dorsal cochlear nucleus because a pattern of fine-fiber degeneration correlates with OHC loss (Morest and Bohne, '83). Recently, the central axons from type II cells have been traced into the cochlear nucleus of gerbils and mice (Brown et al., '88; Brown and Ledwith, '90). The course and arborization patterns of type II axons have been revealed in these small rodents and the data are consistent with the notion that type II neurons can convey information to the brain about the status of OHCs. Ultrastructural details of these axons and endings in the central nervous system have been lacking up to now.

In the present paper, we describe the results of experiments on cats using anterograde and retrograde filling of type II neurons by horseradish peroxidase (HRP). This method permits the examination of individual axons with both the light and electron microscope. Thus, it was possible to determine whether the central axons of type II neurons were unmyelinated and whether the axonal swellings exhibited morphological features characteristic of chemical synapses.

MATERIALS AND METHODS

Surgery and HRP injections

Adult animals in good health, of either sex, and weighing from 1.2 to 3.5 kg were used in the present study. Our methods for anesthesia, surgery, and injection of HRP have been previously described (Kiang et al., '65; Ryugo and Fekete, '82). Briefly, animals were anesthetized with intraperitoneal injections (0.2 cc per kg body weight) of diallyl barbituric acid (100 mg/ml) in urethane solution (400 mg per ml). Supplemental doses were periodically administered in order to maintain areflexia to paw pinches. The skin and muscle layers of the head were removed so that the skull overlying the posterior fossa could be opened with rongeurs. The dura mater over the cerebellum was reflected, and the cerebellum retracted, revealing the auditory nerve between the internal auditory meatus and the cochlear nucleus. Extracellular injections of HRP (Sigma type VI, 10–35% w/v) were made through glass micropipettes (inner tip diameter = 5–80 μm) inserted into the auditory nerve. Typically, two to three injections separated by at least 1 mm were made into each nerve. The HRP was contained in a solution of 0.1 M Tris buffer (pH 7.6 or 8.6) and delivered to the tissue by pulsing 2–3 μA of positive current (50% duty cycle) for 2–4 minutes.

Histology

At the appropriate survival time, each animal was given a lethal dose of Nembutal and perfused through the heart with 50 cc of isotonic saline (37°C) with 0.1% $NaNO_2$, followed immediately by buffered fixative. For light microscopic analysis, the fixative contained 1.0% paraformaldehyde and 2.5% glutaraldehyde in 0.12 M phosphate buffer (pH 7.4). For electron microscopic analysis, the fixative was 500 ml of warm (37°C) fixative containing 0.5% paraformaldehyde, 1.0% glutaraldehyde (freshly purified), and 0.008% $CaCl_2$ in 0.12 M phosphate buffer (pH 7.4), followed by 1.5 liters of a second fixative (37°C) containing 1.25% paraformaldehyde, 2.5% glutaraldehyde (also freshly purified), and 0.008% $CaCl_2$ in the same buffer solution.

In studies that focused on retrograde staining of fibers and their cell bodies, eight animals were perfused 20–48 hours after the HRP injection. Immediately following the vascular perfusion, each cochlea was perfused with the second fixative through its round and oval windows. The cochlear capsules were thinned with stone burrs, and decalcified (4–7 days) in a 0.1 M EDTA solution. When decalcified, tissue blocks containing the cochlea and auditory nerve were prepared for sectioning on a freezing microtome. Tissue was cryoprotected by being placed in a solution of 30% sucrose, and then embedded in 20% gelatin and cut at a thickness of 60–80 μm.

In studies using anterograde staining of fibers, nine animals were perfused 2–24 hours after the HRP injections. Following vascular perfusion, the head was left in the second fixative at 5°C. The next day, the brain was removed from the skull, and the auditory nerve, cochlear nucleus, and adjacent brain stem were isolated in a single tissue block. This block was embedded in gelatin-albumin (Frank et al., '80) and cut at a thickness of 40–60 μm on a Vibratome. Sections were serially collected on "subbed" slides. In a few cases, cochlea, auditory nerve, and cochlear nucleus were processed together. All sections were reacted with diaminobenzidine (DAB) according to previously published procedures (e.g., Ryugo and Fekete, '82). In most cases, the tissue was counterstained with cresyl violet and coverslipped with Permount for light microscopic analysis. The counterstaining provided cytoarchitectonic and other spatial landmarks to aid in locating the HRP injection site and labeled elements. Measurements were collected from traced silhouettes of cell bodies and processes made using a light microscope and drawing tube (total magnification 2,600×). Drawings were digitized using an electronic planimeter.

In four other cats, DAB-reacted tissue was osmicated with 1% osmium tetroxide in 0.12 M phosphate buffer (30 minutes), stained en bloc with 2% uranyl acetate (1 hour) or 1% uranyl acetate (overnight), dehydrated, infiltrated with Epon, and embedded between two sheets of Aclar (Allied Chemical Co.). Once the Epon polymerized, each tissue section was numbered in sequence and then taped to a glass slide for analysis. Twelve HRP-labeled thin fibers were located within the auditory nerve with the aid of a light microscope; eight exhibited en passant swellings in the cochlear nucleus. Structures of interest were then excised from the tissue section using a razor blade and isolated within smaller Epon pieces. These smaller pieces were reem- bedded for thin sectioning with an ultramicrotome. Serial ultrathin sections spanning 3–20 μm of thin fiber length were collected, stained with lead citrate and uranyl acetate, and examined with a JEOL 100S or 100C electron microscope.

RESULTS

After HRP was injected into the auditory nerve and the tissue histologically processed, each injection site was iden-

Fig. 1. HRP-labeled thick and thin (arrow) fibers of the cat auditory nerve. Both fiber types are unbranched and maintain a relatively constant diameter throughout their trajectory in the nerve. Scale bar = 20 μm.

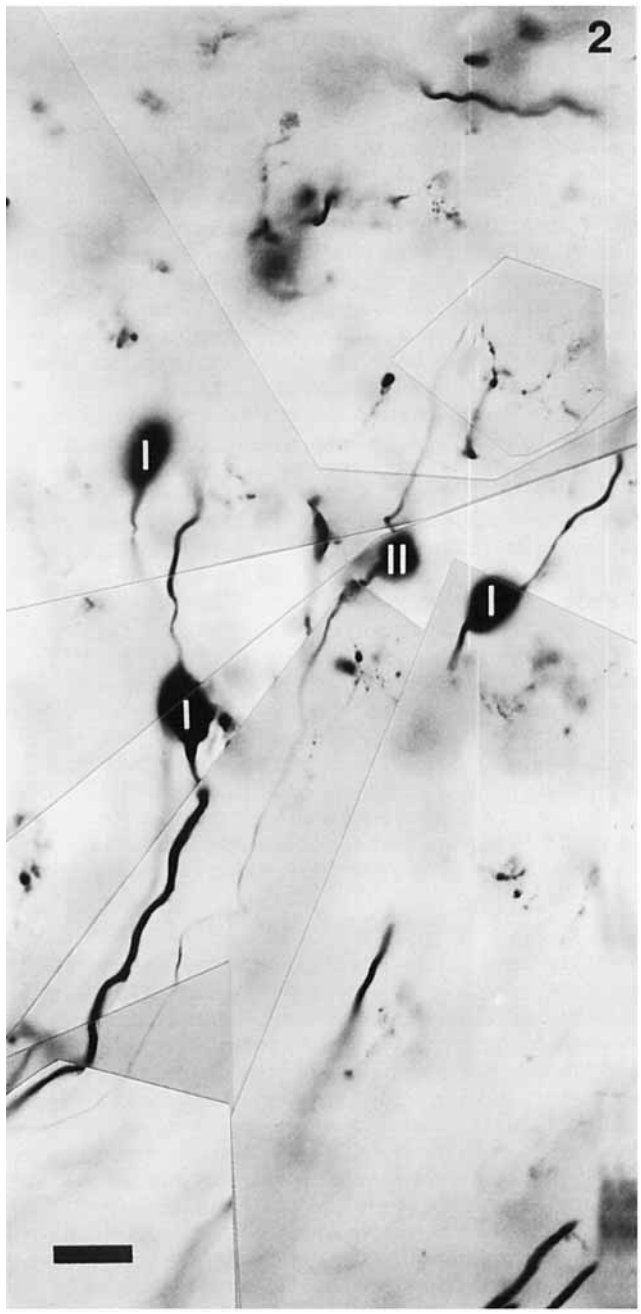

Fig. 2. HRP-labeled thick fibers were traced peripherally to the cell bodies of type I neurons (I), whereas thin fibers were traced back to the cell bodies of type II neurons (II). Note the thick central process and somewhat thinner peripheral process of the type I neurons, in contrast to the uniformly thin processes of the type II neurons. Scale bar = 20 μm.

tified as a circumscribed dark region from which stained fibers emanated. The opaque core of injection sites obscured the fibers as they passed through, making it difficult to trace individual fibers through injection sites. In every case, all stained fibers converged towards the injection site; no stained fibers bypassed the injection site. Stained axons appeared dark brown or black against a pale background. Some fibers could be traced peripherally from the injection site to their cell bodies in the spiral ganglion and occasionally even further to terminations in the cochlea. Other fibers could be traced centrally from the injection site into the cochlear nucleus. It was not possible to connect a particular fiber in both directions from the injection site.

Peripheral tracing

Most of the HRP-labeled fibers in our cats were "thick" in caliber with only an occasional "thin" fiber present (Fig. 1). The thick fibers were 2–4 μm in diameter and remained unbranched during their peripheral course in the nerve and cochlea. Individual thick fibers, followed through serial sections of the auditory nerve to the spiral ganglion, always arose from large cell bodies (Fig. 2). In the vicinity of each cell body, the peripheral process was characteristically thinner than the central process. In all cases in which the peripheral processes could be traced to their terminations in the cochlea (n = 67), it was determined that they innervated IHCs, as is typical of type I neurons in the cat (Kiang et al., '82; Liberman, '82; Liberman and Oliver, '84).

Thin fibers were less frequently labeled but when present, were clearly distinguishable from type I axons (Figs. 1, 2). Forty retrogradely labeled thin fibers were recovered from 11 nerves and cochleas of eight cats. All of these fibers were traced back to cell bodies in the spiral ganglion. Thin fibers

were 0.3–0.5 μm in diameter, unbranched, and fairly constant in caliber. Individual thin fibers traveled with neighboring thick fibers throughout the length of the nerve. For example, the labeled thick and thin fibers of Fig. 2 never strayed more than 100 μm from each other, spiraling through the nerve together in close proximity. Examples of thin fiber segments are shown in Fig. 3. Most of the labeled thin fibers appear similar to the two shown in the upper panel (A,B), where the axon is uniform in diameter. The lower panel (C,D) shows examples of a less common situation in which varicosities are present, occasionally in such close proximity to one another as to give the fiber a beaded appearance. Beaded and unbeaded thin fibers have been traced to small cell bodies typical of type II neurons (as described by Kiang et al., '82).

Thin unbranched fibers could be traced through serial sections from the injection site to their cell bodies, but not always as far as their peripheral terminations in the cochlea. In nine cases, thin fibers were traced to terminal swellings at the base of OHCs. In 31 other cases, reconstructed thin fibers arose from cell bodies having morphological features characteristic of type II neurons, using criteria established by Kiang et al. ('82). The cell bodies were relatively small in size, monopolar (Fig. 4C,D), pseudomonopolar (Fig. 4E–G), or bipolar (Fig. 4H,I) in shape, and their central and peripheral processes were comparable in diameter. Somatic silhouette areas and process diameters were determined, and the resulting data points were found to define a population separable from the population of larger ganglion cells innervating IHCs (Fig. 5). These results are consistent with the idea that within the auditory nerve, thick fibers are always axons of type I neurons and thin fibers are always axons of type II neurons.

Anterograde labeling

On the basis of these labeling data, it appears valid to assign neuronal type (e.g., type I or type II) to axons in the auditory nerve knowing only axon diameters. We applied the diameter criterion to labeled, centrally projecting fibers, and defined 48 thin fibers from 11 nerves and nuclei of nine additional cats. When HRP injection sites were located near the modiolus of the auditory nerve, long stretches of centrally stained fibers could be studied peripheral to the Schwann-glial border, unobscured by extraneous reaction product. Thin fibers were obviously different from thick fibers, and could be readily traced even when they weaved in and out among surrounding thick fibers. It was also helpful that relatively few thin fibers were stained in any single nerve. The distinction between the thick type I axons and the thin type II axons was easily made even when varicosities were present in the thin fibers (Fig. 6). When labeled thin fibers in each nerve were counted, their numbers were found to be close (within one or two) to the numbers of labeled type II cell bodies of the spiral ganglion in the corresponding cochlea. Thus, central to the injection site in the auditory nerve, the relationship between thin fibers and type II cell bodies was maintained.

Thin fibers entered the cochlear nucleus by passing the Schwann-glial border, and began branching after traversing a variable distance that depended on the place of innervation in the cochlea. This result is similar to the descriptions for rodent thin fibers (Brown, '87; Brown et al., '88; Berglund and Brown, '89). Because the reaction product labeling thin fibers faded before all the terminals were reached, we can only describe the arborizations of type II axons within the central region of the ventral cochlear nucleus (mostly in the vicinity of the nerve root). There was one fiber, however, whose ascending branch extended 1.9 mm from the bifurcation to the anterior part of the anteroventral cochlear nucleus (AVCN) and whose descending branch reached 2.5 mm beyond the bifurcation, well into the posterior part of the posteroventral cochlear nucleus before the labeling faded. This ascending branch exhibited 11 collaterals. Five collaterals were short (< 10 μm in length) and each ended in a terminal swelling roughly 1 μm in diameter. Short collaterals (termed pedunculated swellings) resembling dendritic spines are characteristic of thin fibers (Fig. 7A, arrow). One collateral extended 12 μm but had no terminal swelling and five were blunt, tongue-like extensions (< 3 μm in length) with no swellings. The descending branch gave rise to three labeled collaterals, two of which could not be followed to terminals before fading and one of which was short with a small terminal swelling. Although this aborization was the most extensively labeled in our sample, it nevertheless appeared representative of the population of thin fibers by virtue of the infrequent branching and the presence of short collaterals.

We concentrated on characterizing darkly stained collaterals that terminated in distinct swellings (approximately 1–2 μm in diameter) at their tips. When these swellings were located away from the tissue section surfaces, they provided light microscopic evidence that a particular stained collateral had terminated. The light microscopic appearance of these labeled terminal swellings resembled small versions of bouton endings as described for type I spiral ganglion neurons (e.g., Rouiller et al., '86). Most of the terminal swellings were found in neuropil away from neuronal cell bodies (Fig. 7A). In a few instances, a pedunculated swelling was observed to abut a node of Ranvier of a type I axon (Fig. 7B).

Other collaterals of thin fibers came into close apposition with the cell bodies of globular cells and glial cells (Fig. 8). In general, the collaterals are nearly always thinner than the parent fiber after a branch point, and branch points are often marked by a swelling. Most collaterals are unbranched, but some collaterals branch once or twice and a few exhibit elaborate branching patterns. Nearly a third of the fibers gave rise to collaterals that formed terminals near blood vessels (Fig. 9).

Under the light microscope, en passant swellings were present in fibers labeled by both anterograde and retrograde transport. These swellings usually became increasingly more numerous along the axon with greater distance from the cell body. The number of en passant swellings per 100 μm of axon length in the nerve ranged from 0 to 6 across all the traced fibers, and did not seem to be systematically related to methods of staining or any obvious aspect of thin fiber morphology.

Electron microscopic observations

Twelve thin fibers, eight of which exhibited swellings, were traced from deep within the auditory nerve into the cochlear nucleus. Segments of these fibers, ranging from 3 to 20 μm in length, were examined using serial section electron microscopy. The fibers were all unmyelinated throughout the examined lengths and their diameters were consistent with that determined by light microscopy (Fig. 10). Labeled structures studied using the light microscope were found with the electron microscope and identified on the basis of their distinctive shapes and spatial relation-

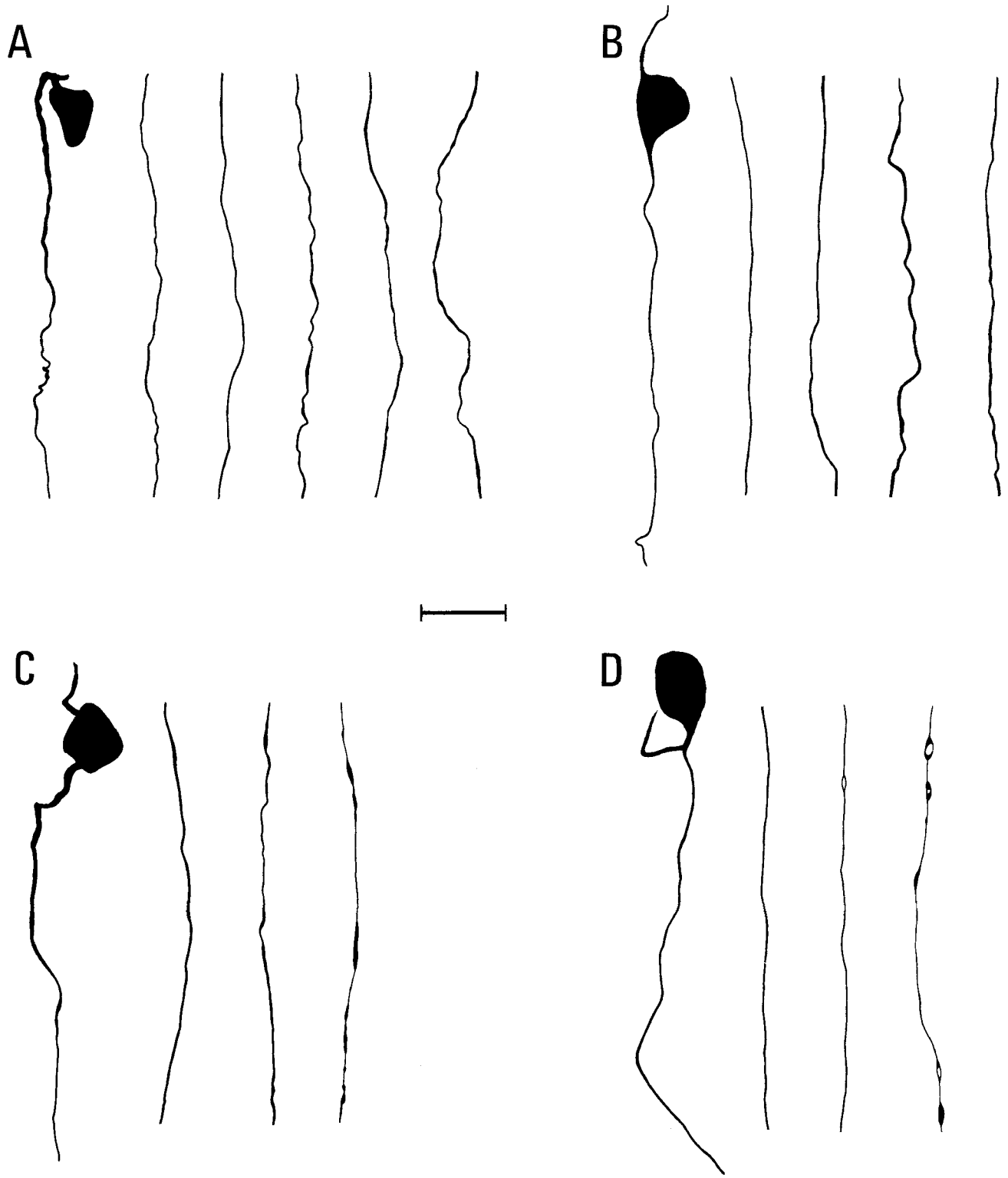

Fig. 3. Drawing tube tracings of cell bodies and central axons of HRP-labeled type II neurons. Each segment represents roughly 100 μm of length; individual segments were separated by 0.5 mm. The axons of type II neurons were typically free of varicosities along their lengths (**A,B**). On occasion, however, there appeared infrequent varicosities of various sizes and shapes (**C,D**). Note that when varicosities occurred, they formed away from the cell body. Scale bar = 20 μm.

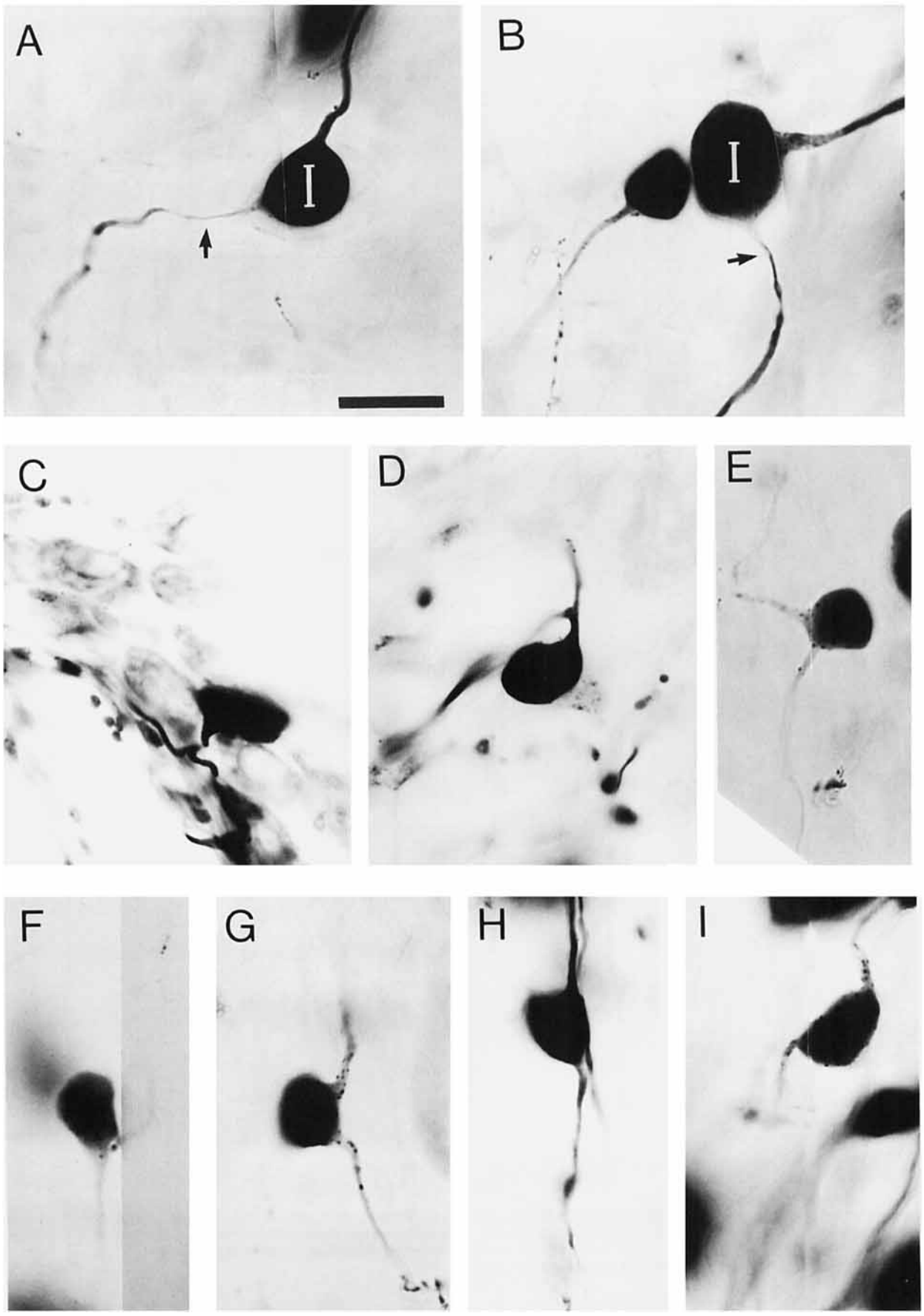

Figure 4

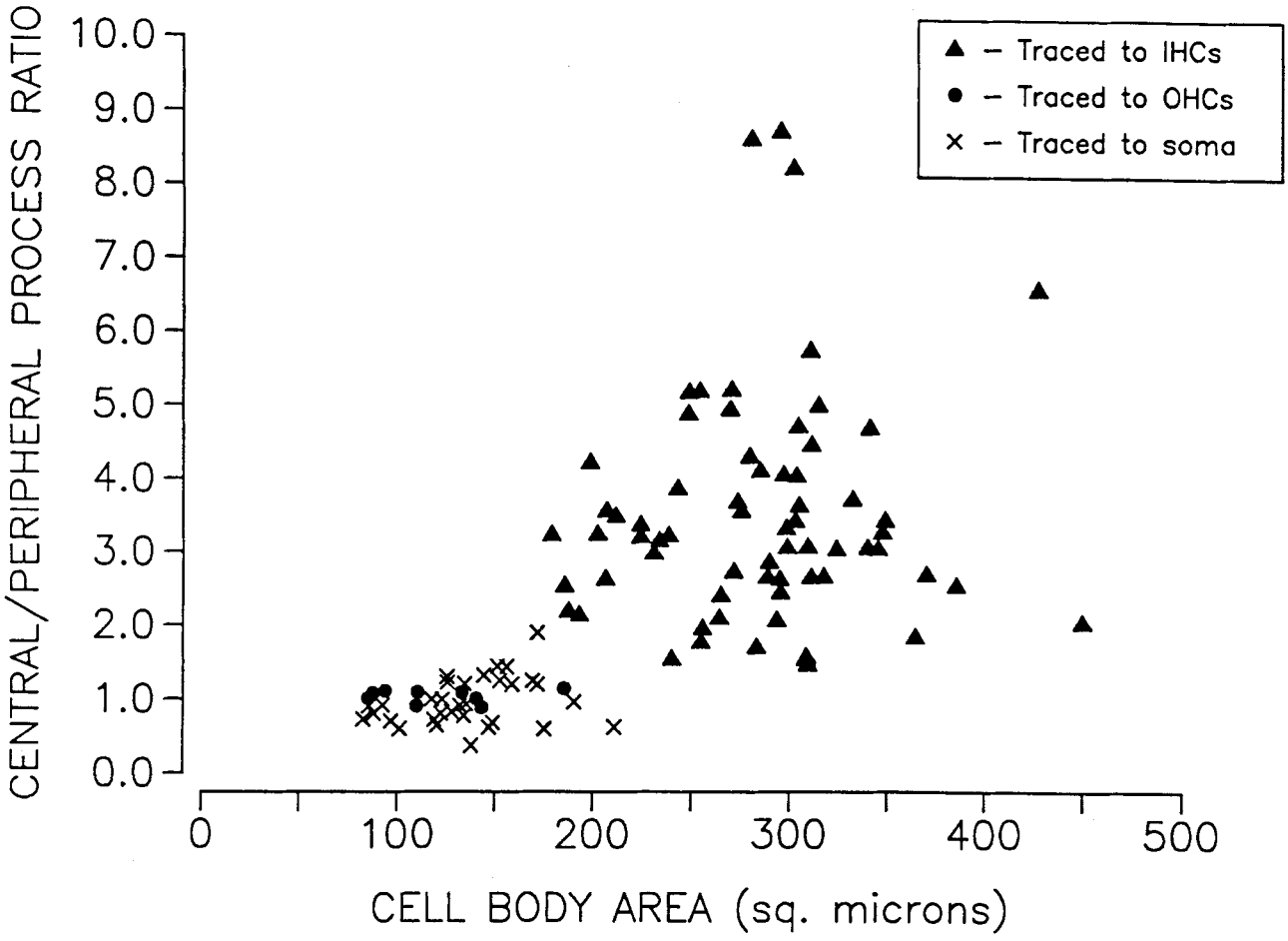

Fig. 5. Scatter plot of the somatic area of HRP-labeled ganglion cells versus the ratio of process diameters. All thick fibers (solid triangles) were traced to cell bodies typical of type I spiral ganglion neurons and whose peripheral processes innervated inner hair cells (IHCs). In contrast, all thin fibers were traced to cell bodies typical of type II neurons (crosses) whose peripheral processes (in nine cases) were traced to terminal swellings at the bases of outer hair cells (OHCs) (solid circles).

ships with other morphological features (such as blood vessels, fiber fascicles, and/or cell bodies). We paid particular attention to en passant swellings because similar structures have been associated with synapses (Peters et al., '76), and because they were plentiful and therefore more accessible for study using electron microscopy.

Eighteen en passant swellings (16 in isolation and 2 at branch points) were studied. Under the electron microscope, four swellings contained mitochondria and exhibited round vesicular profiles; two of these were also associated with postsynaptic densities. In contrast, the other swellings contained only HRP reaction product and some empty spaces. It was not possible to predict the synaptic nature of swellings from light microscopic observations.

Fig. 4. Photomicrographs of type I (indicated by I in **A** and **B**) and type II (**C–I**) cell bodies retrogradely labeled with HRP. Note the thinner peripheral processes of type I neurons (arrows) in the vicinity of the cell body, and that not all type I cell bodies are strictly bipolar in shape. The processes of type II neurons in the vicinity of the cell body are approximately equal, and the shape of the cell bodies can appear monopolar (C,D), pseudomonopolar (E,F,G), or bipolar (H,I). Scale bar = 20 μm.

For our purposes, a synapse is defined as a region of the swelling having an obvious postsynaptic density and at least one vesicle within a distance of its diameter to the membrane specialization. Using these criteria, two swellings from two separate fibers gave rise to three synapses having clear round vesicles and an expanded intercellular cleft of regular width. There was no instance of synaptic ambiguity because no other membrane densities were found apposed to labeled thin fiber swellings. One swelling synapsed on a dendritic shaft and an immediately adjacent dendritic spine (Fig. 11); the other swelling synapsed on a dendritic shaft (Fig. 12A). The length of the membrane apposition (defined as where the pre- and postsynaptic membranes abut) and the length of the postsynaptic density were measured for all sections containing a postsynaptic density. Each synapse was characterized by a relatively long postsynaptic density: 42% of the apposition with the spine was synaptic, and 61% and 77% of the appositions with the shafts were synaptic. The swelling giving rise to two synapses contained vesicles having an average (±S.E.M.) diameter of 48.6 ± 0.3 nm (n = 194); vesicles in the other swelling had an average diameter of 46.3 ± 0.4 nm (n = 309).

The main observations for central synapses of type II neurons can be compared with those of type I neurons (Fig.

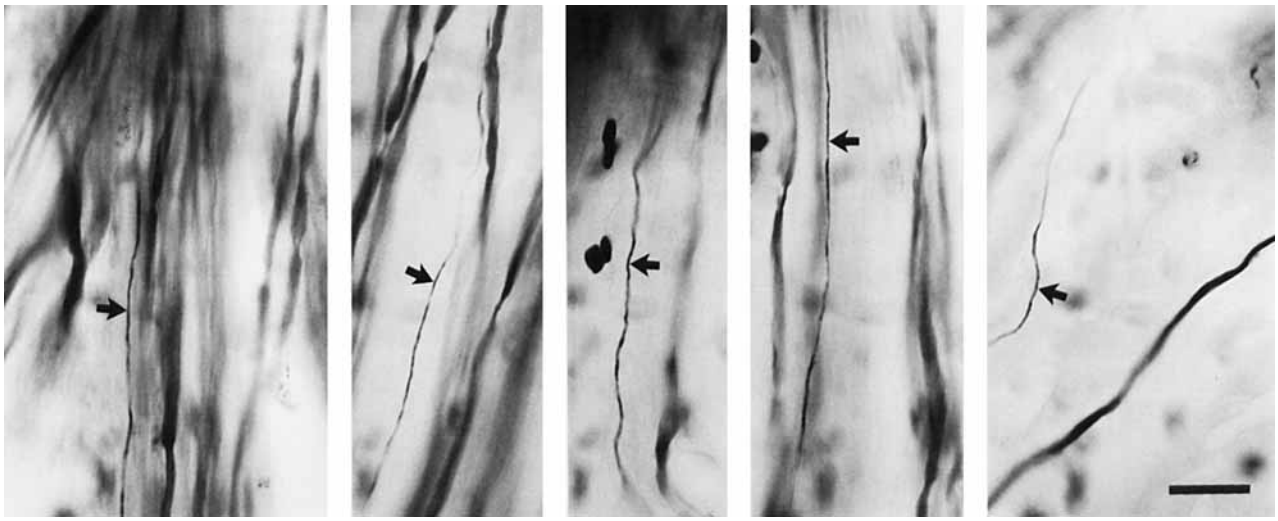

Fig. 6. HRP-labeled thick and thin fibers (arrows) as they appear central to the injection site in the auditory nerve. The respective caliber of these axons were virtually identical on both sides of the injection site. The size disparity between fiber types allows us to conclude that the thin fibers arise from type II ganglion cells and the thick fibers arise from the type I ganglion cells. Scale bar = 10 μm.

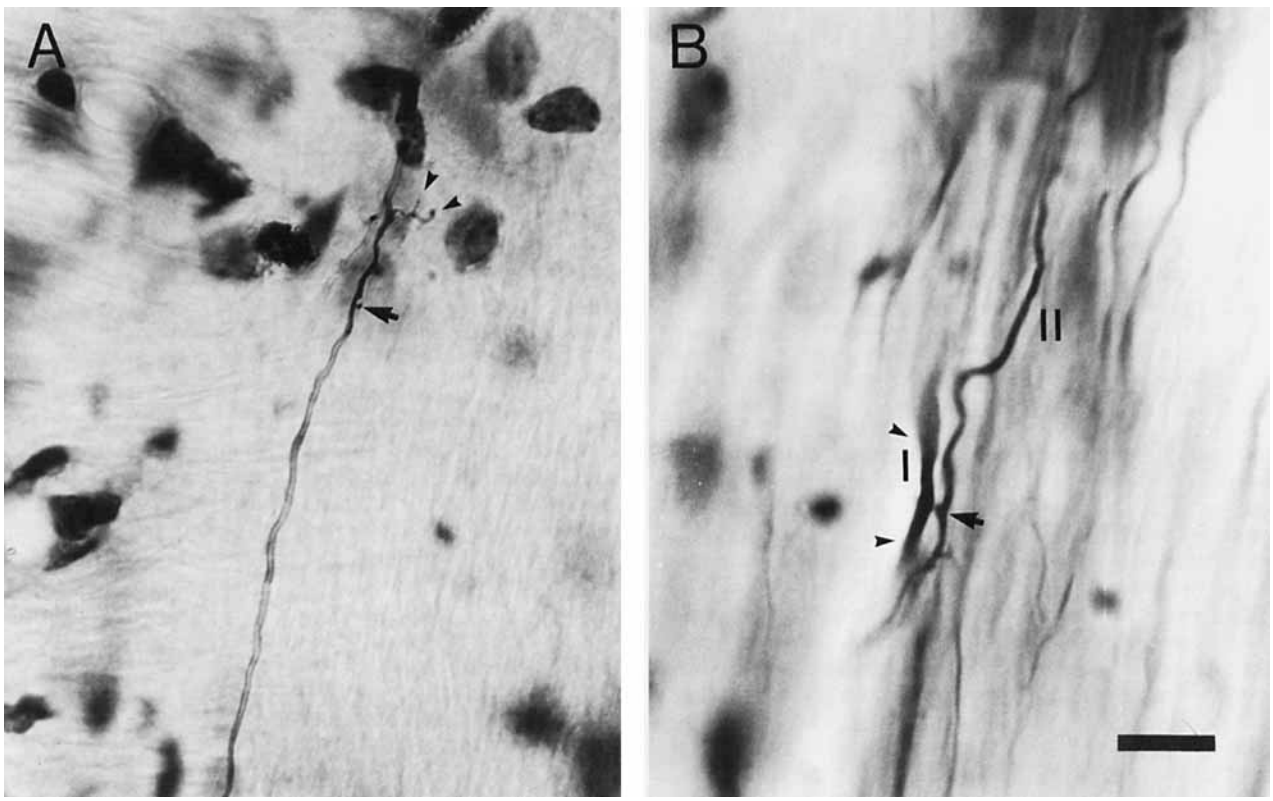

Fig. 7. Photomicrographs of thin fiber ramifications and swellings labeled by HRP in the cochlear nucleus. **A:** Pedunculated (arrow) and terminal (arrowheads) swellings are found in the neuropil. **B:** There were occasional indications that pedunculated swellings (arrow) from type II fibers (II) contacted type I fibers (I) at nodes of Ranvier (arrowheads). Scale bar = 10 μm.

12). Terminals of both fiber types contain mitochondria and clear, round synaptic vesicles. The vesicles of terminals from type I fibers, however, have larger average diameters (54.6 ± 0.4 nm, n = 505) than do those of type II fibers. Furthermore, the relatively long postsynaptic densities of type II fibers contrast with the punctate densities of four completely reconstructed synapses of type I terminals. Postsynaptic densities for the type I fibers represented 16% of the total apposition (range 8–26%). Although the sample is still small, if these observations prove to be reliable, it will

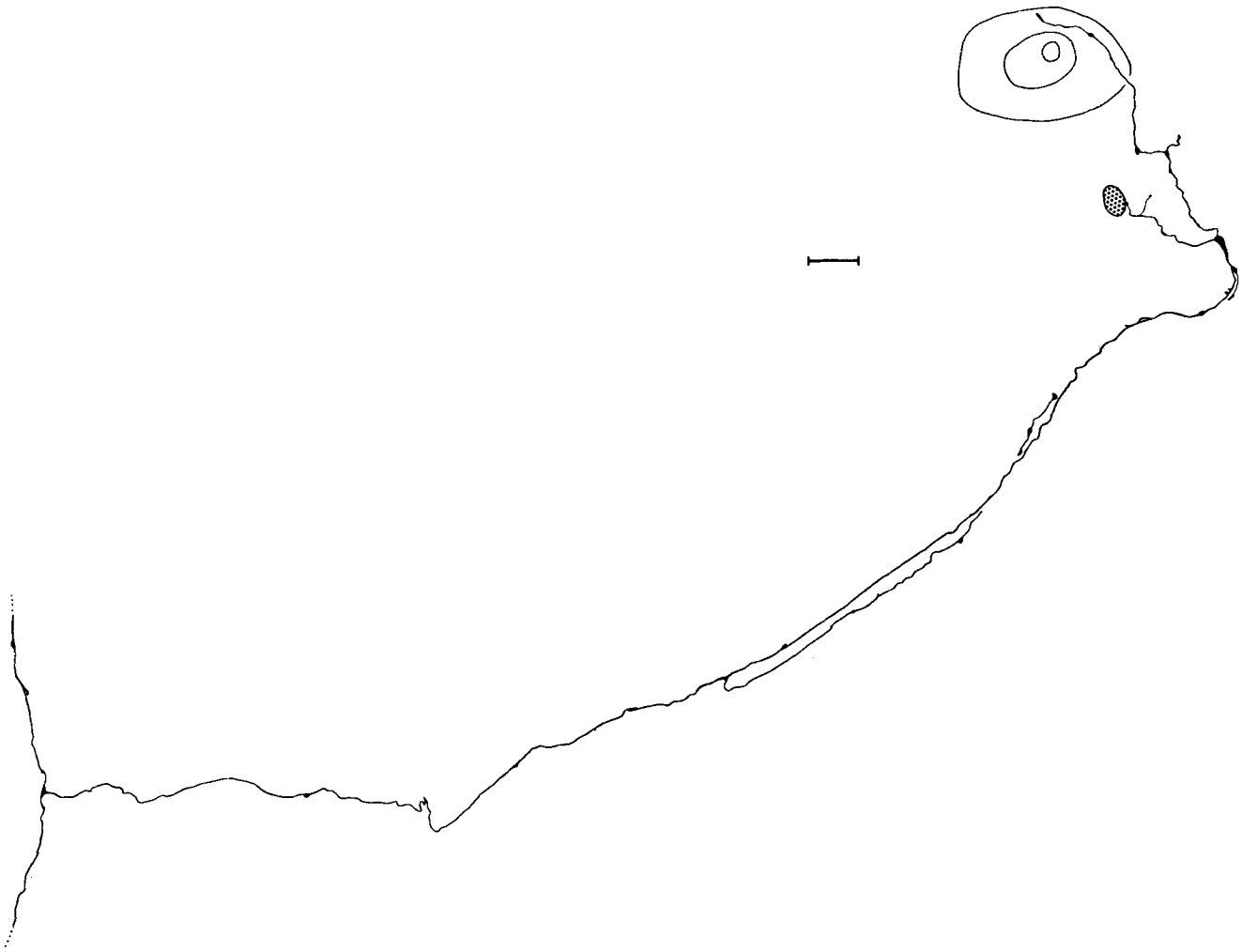

Fig. 8. Drawing tube reconstruction of thin fiber coming into close apposition with the somata of a globular cell (drawn with nucleus and nucleolus) and a glial cell (stippled). Other en passant and terminal swellings were located in neuropil. Scale bar = 10 μm.

then be possible to use these descriptive criteria to help identify terminal types of primary fibers in unlabeled material where morphological features are less obscured by reaction product.

DISCUSSION

Recent studies on small rodents have shown that the general course of both type I and type II primary auditory fibers are similar and entirely confined to the cochlear nucleus (Brown, '87; Brown et al., '88; Brown and Ledwidth, '90). In this paper, we describe the morphological characteristics of thin fibers in the auditory nerve of cats. Although it was not possible in our material to follow individual thin fibers to all of their terminations in the cochlear nucleus, sufficient pieces were stained for us to identify them as parts of type II spiral ganglion cells. This identification is based on observations from extracellular injections of HRP into the auditory nerve, in which labeled thick and thin fibers maintained their relative thickness throughout their trajectory in the nerve. Whenever traced to their origin, the thick fibers (2–4 μm in diameter) were shown to arise from type I spiral ganglion cells. The thin fibers (0.3–0.5 μm in diameter) were unmyelinated and whenever traced were shown to arise from type II ganglion cells. There were never any indications that within the nerve, segments of thick fibers could be thin or that segments of thin fibers could be thick. Furthermore, there was no evidence that either fiber type branched within the nerve (between the Schwann-glial border and the spiral ganglion). In the cochlear nucleus, however, there are thin fibers that could be fine collaterals of type I neurons, so it is essential to trace thin fibers in the nucleus back into the nerve in order to assign whether they are type I or type II neurons using light microscopy. Our observations on auditory nerve fibers are wholly consistent with data from other studies of primary fibers in cats (e.g., Ryugo and Fekete, '82; Fekete et al., '84; Liberman and Oliver, '84) and rodents (Brown, '87; Brown et al., '88).

We can clearly put to rest the notion (Spoendlin, '79) that type II spiral ganglion neurons in cats fail to project to the cochlear nucleus. Our results are completely consistent with the data from rodents and suggest that the fading of labeling is because of the longer lengths of fibers in cats. Not only have we traced the unmyelinated thin axons from

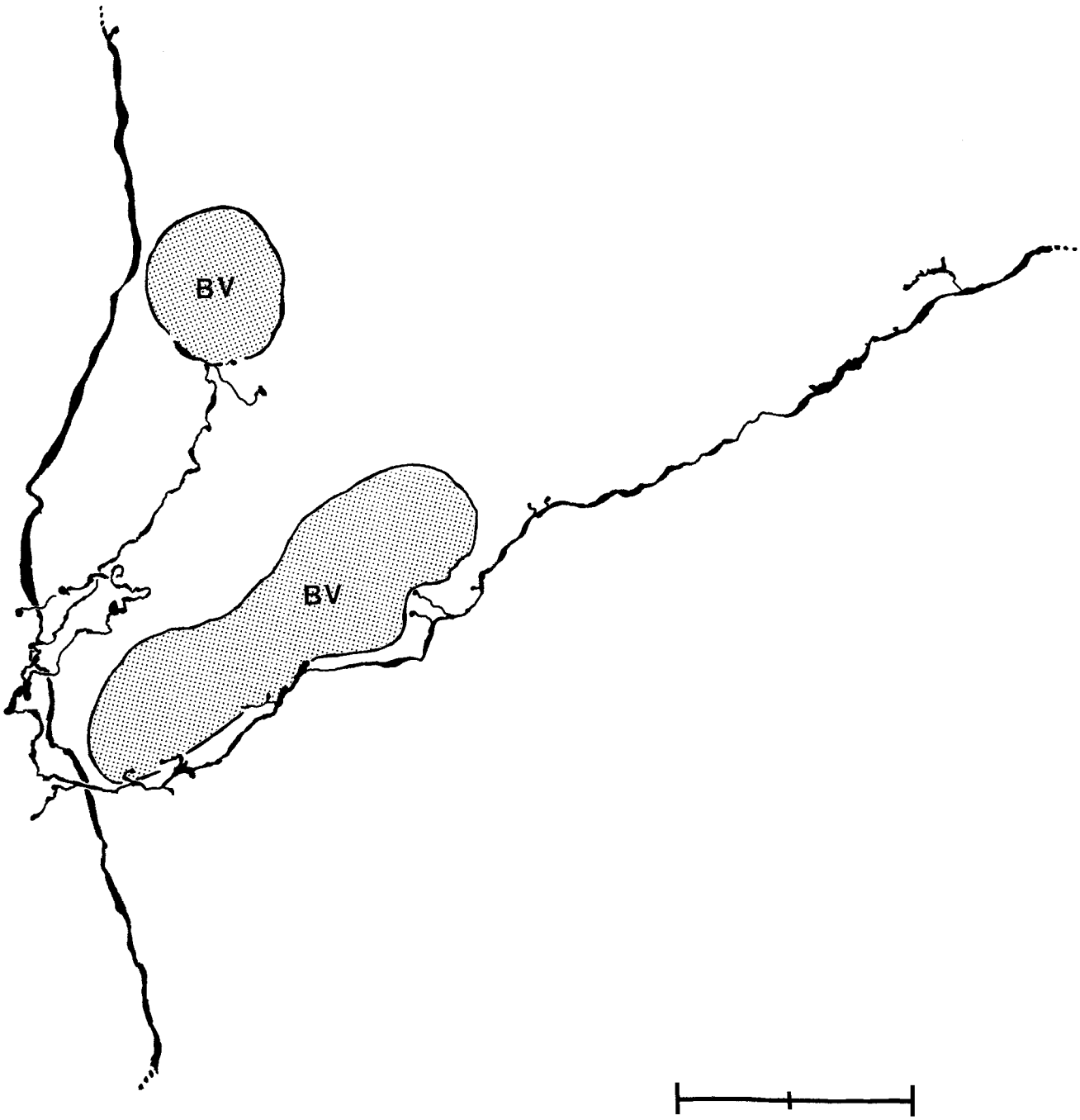

Fig. 9. Drawing tube reconstruction of an HRP-labeled thin fiber that emits multiple short collaterals and one longer one that terminate in the vicinity of blood vessels (BV). Short collaterals with terminal swellings are characteristic of the thin fiber population. Many thin fibers exhibited an obvious association with the vasculature, yet it was equally clear that en passant and terminal swellings were also associated with other structures. Scale bar = 20 μm.

the nerve into the nucleus, but we have also demonstrated that a small proportion of en passant swellings have morphological characteristics suggestive of chemical synapses. These terminals contain round vesicles, exhibit an expanded intercellular cleft with some intervening dense material, and appose a postsynaptic density. The synapses of type II neurons are identifiable because their membrane apposition to target structures is marked by a relatively large postsynaptic density in contrast to the small postsynaptic density typical of the synapses of type I neurons. The functional significance of these size differences in postsynaptic densities remains to be determined.

In our material, both types of primary afferent endings contain round synaptic vesicles. Some workers have suggested that endings with round vesicles exert excitatory action on the postsynaptic targets (Uchizono, '65; Larra-

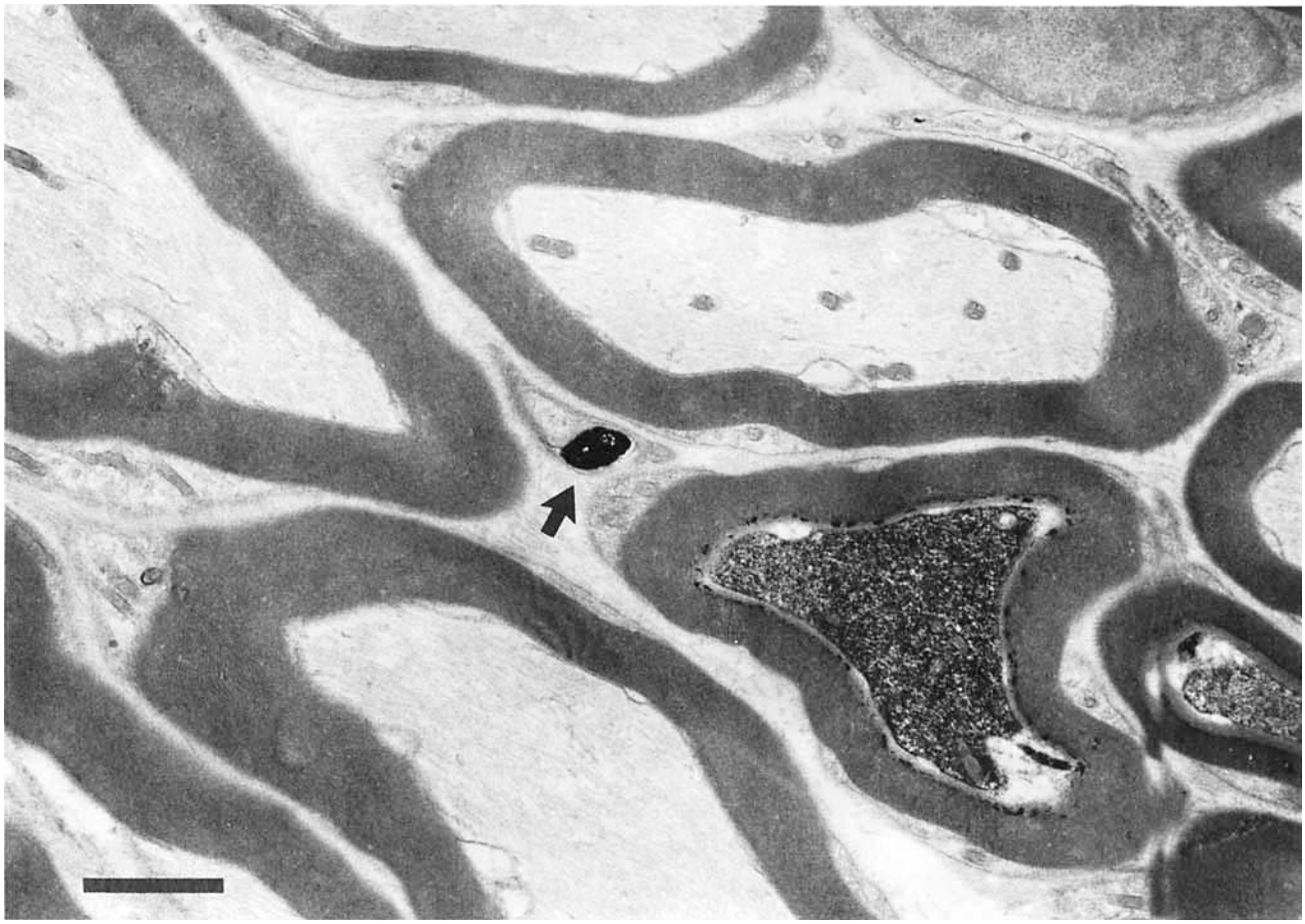

Fig. 10. Electron micrograph of HRP-labeled thick and thin fibers in the auditory nerve. Note that the thin fiber is unmyelinated (arrow) and the thick fibers are myelinated. Scale bar = 0.5 μm.

mendi et al., '67; Atwood et al., '72), whereas endings having pleiomorphic or flattened vesicles would have inhibitory actions (Ribak et al., '77; Jahr and Nicoll, '80; Nowycky et al., '81; King and Bishop, '82). Ultimately, however, synaptic action will depend not only on the chemicals released but also on the nature of the postsynaptic receptor(s) and ion channel(s). In any case, there is evidence that large round synaptic vesicles of type I afferents (e.g., Cant and Morest, '79) are associated with excitatory postsynaptic effects on at least some cochlear nucleus cells (Kiang, '75). Although the terminals of both type I and type II primary afferents have round vesicles, the vesicles differ in size for the two types of afferents. Such a finding is reminiscent of spherical vesicles for terminals of different primary afferents in the dorsal horn (Ralston and Ralston, '79): Small round vesicle profiles in the dorsal horn have been demonstrated to be immunoreactive for substance P, cholecystokinin, and somatostatin, whereas large vesicle profiles are not immunoreactive and are presumably glutamatergic (de Lanerolle and LaMotte, '83; LaMotte and de Lanerolle, '86; LaMotte, '86). Thus, vesicles of similar shape but of different size may contain different transmitter substances. The round vesicles in type I and type II spiral ganglion neurons may therefore contain different chemical transmitters.

The electrical behavior of both IHCs and OHCs is consistent with the notion that they are acoustic transducers, although perhaps with different functional characteristics (Dallos, '85). The afferent neurons of both types of hair cells have the structural machinery for chemical synaptic action in the cochlear nucleus. Nevertheless, the central effects must be rather different. The IHC innervation is divergent in that a single hair cell transmits information to many type I neurons (Spoendlin, '73; Liberman, '82), each of which is highly frequency selective (Kiang et al., '65). By contrast, the OHC afferents are convergent in that many OHCs along the organ of Corti transmit their messages through a single type II neuron (Spoendlin, '73; Smith, '75; Berglund and Ryugo, '87; Brown, '87). The axonal arborizations of both types of neurons appear to be divergent. Although it has not yet been possible to demonstrate the activity patterns of type II neurons (e.g., Robertson, '84), it is safe to assume that neural signals would be transmitted more slowly because of slow conduction times along unmyelinated axons (Gasser, '55).

There seems to be no question that type I neurons transmit information from IHCs to neurons of the cochlear nucleus (Kiang, '75). The data in this paper show for the first time that type II neurons have a morphology consistent with the capability to convey "sensory" information from OHCs directly to neurons in the cochlear nucleus.

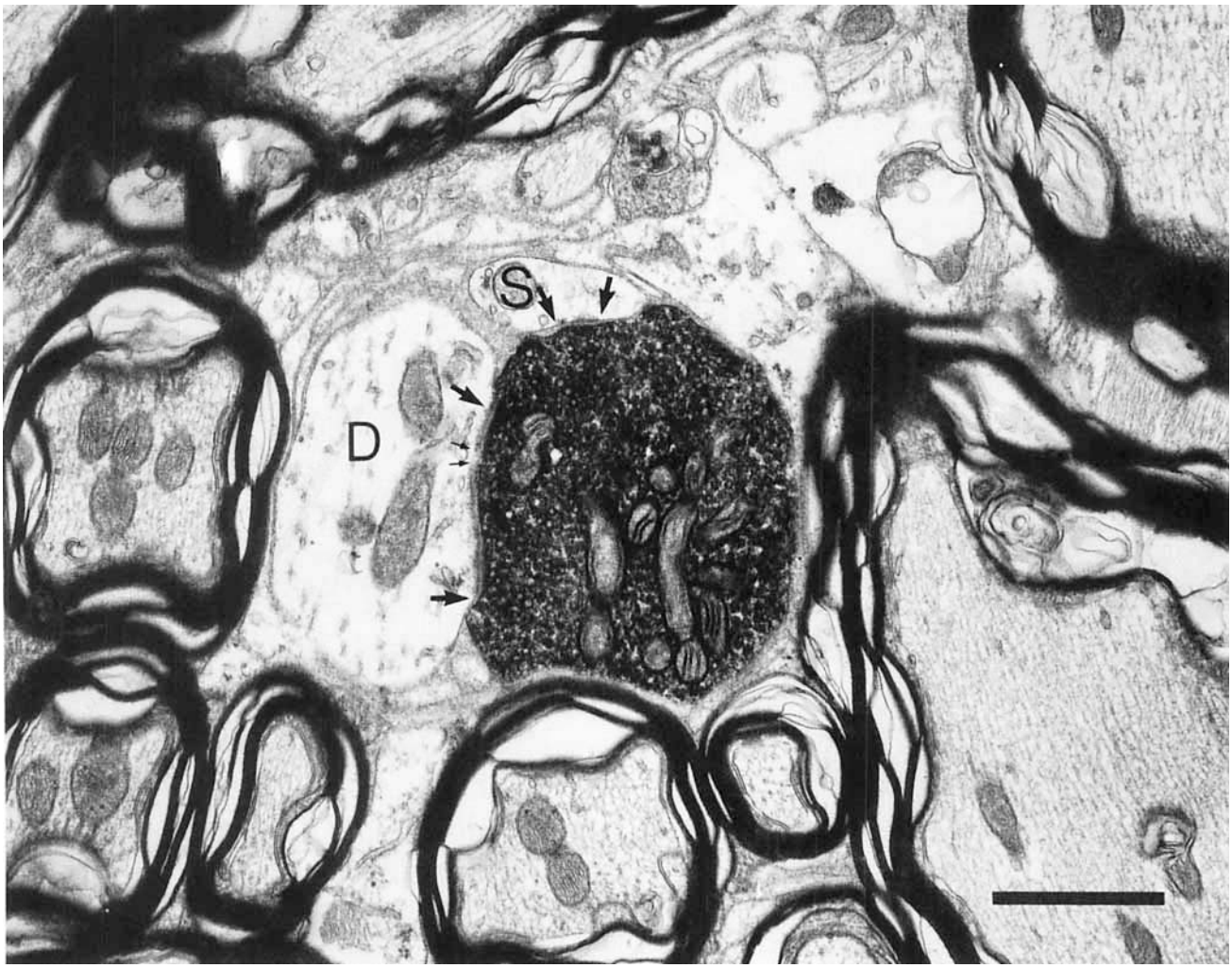

Fig. 11. Electromicrograph of an HRP-labeled swelling of a thin fiber within the auditory nerve root region of the AVCN. The swelling forms synapses (bounded by the larger arrows) with a dendritic shaft (D) and a dendritic spine (S). A pore in the synaptic plaque on the shaft is indicated by small arrows. The spine connects to the shaft 160 nm deeper. Scale bar = 1 μm.

Such information may not necessarily be directly related to the immediate acoustic stimulus but might instead pertain to the functional status of the OHC, perhaps analogous to muscle spindle afferents or nociceptors. Such a conclusion, however, does not preclude OHCs from participating in mechanisms that determine the response of IHCs and thus, influencing the information carried by type I neurons (e.g., Brown and Nuttall, '84; Liberman and Kiang, '84; Dallos, '85; Kiang et al., '86). Nor does it preclude the possibility that both sets of afferents will converge on more central targets. What is required to clarify further the role of OHCs in the central processing of acoustic information is to record the activity of type II neurons and to identify the postsynaptic targets of these neurons.

Based on light microscopic observations, we propose some possible target structures in the interstitial region of the cochlear nucleus: Terminal swellings are found in close apposition to the somata of globular and glial cells, nodes of Ranvier of type I axons, and blood vessels. Each of these candidate structures opens many possibilities for speculation about function. Most swellings are found in neuropil, so our electron microscopic observations up to now have been limited to these, revealing synapses against profiles indicative of dendritic shafts and spines. The origin of these profiles with respect to cell types, however, has not yet been determined. It now seems imperative to identify these thin fiber targets by electron microscopic analyses, especially since the feasibility of such studies has been established. How type II neurons interact with specific neuron populations, or with the vasculature for that matter, will undoubtedly have powerful consequences for theoretical mechanisms in both normal and pathologic hearing.

An important issue is whether terminals of type I and type II ganglion cells can converge on the same neurons in the cochlear nucleus, or whether the two types of ganglion cells synapse on entirely different targets. Such a situation is unresolved at present because both fiber types have regionally overlapping projection fields in the nucleus (Brown et al., '88), and only data with higher resolution can be useful. Our HRP-labeled material shows that the question of synaptic convergence may be directly addressable because the synapses of type I and type II axons appear to

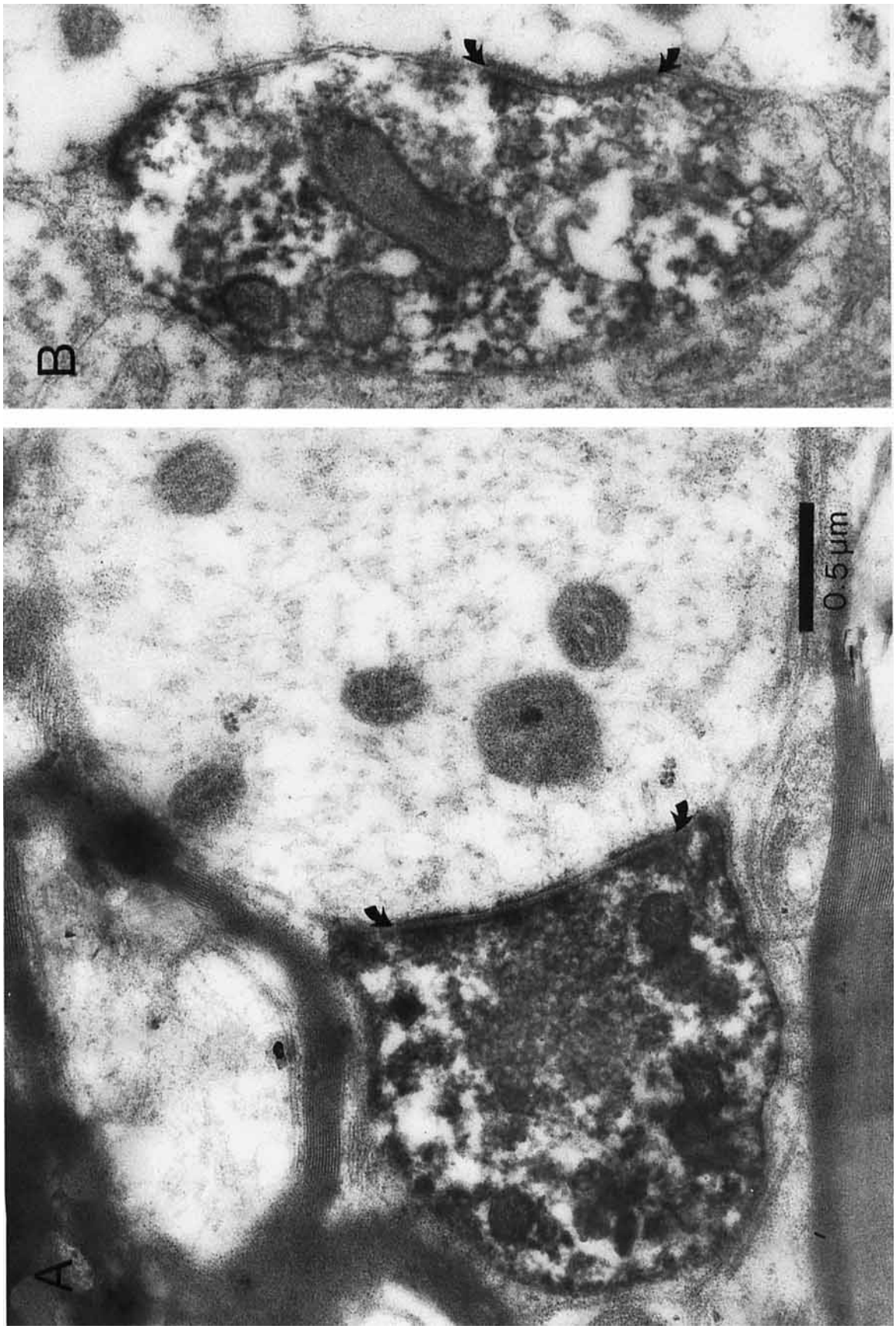

Fig. 12. Electron micrographs comparing synapses from terminals of type II (**A**) and type I (**B**) ganglion cells. The terminal of the type II neuron contains small round synaptic vesicles, small mitochondria, and a membrane apposition that is largely synaptic (flanked by arrows). Pores in the postsynaptic density are also visible. In contrast, the terminal of the type I neuron contains larger round synaptic vesicles, larger mitochondria, and a punctate postsynaptic density (arrows).

be distinguishable by their ultrastructural features. Although both types of terminals exhibit round synaptic vesicles and form asymmetric contacts, they differ in important ways. The terminals of type II neurons arise from unmyelinated axons, contain smaller synaptic vesicles, and have a large proportion of the membrane apposition that is apparently synaptic. In contrast, the terminals of type I neurons arise from myelinated axons, contain larger synaptic vesicles, and exhibit a punctate synaptic apposition.

One consideration of the possible function of inputs from OHCs is that like some other thin fiber systems (e.g., the unmyelinated somatic c fibers), they signal cell damage giving rise to the sensation of pain. Such a function, for instance in the presence of traumatic noise, has survival value and would be consistent with a slowly conducting system with less localized representation than the IHC afferents have. If this idea has merit, then the central projections of type II neurons might well be substantially different from those of type I neurons.

It is of some interest to consider the significance of swellings that did not exhibit features of "junctional" relationships, that is, those lacking postsynaptic densities. Under the light microscope, such nonjunctional swellings could not be distinguished from junctional swellings. In the electron microscope, however, they contained distinctly fewer vesicles and gave no evidence of membrane thickenings or regular widenings of the intercellular space. As such, these nonjunctional swellings resembled cortical monoamine terminals (Itakura et al., '81; Beaudet and Descarries, '84). The association of swellings with blood vessels in the cochlear nucleus coupled with the report of diffuse release (and site of action) of transmitter in the peripheral autonomic nervous system (Merrillees et al., '63) raises the possibility that sensory stimulation might evoke adjustments in circulation. More information at both the light and electron microscopic level is still needed, but the observations are related to other reports indicating that vascular function may be modulated by fibers of various origins, including autonomic (Edvinsson et al., '72, '73), sensory (Feindel et al., '60), and/or central (Falck et al., '65; McDonald and Rasmussen, '77). Our results, in fact, may be directly relevant to the classic textbook report of the "axon reflex," whereby collaterals of sensory fibers are thought to have dilatory effects on local blood vessels (e.g., Mountcastle, '74). The cochleotopic projection of the type II axons into the cochlear nucleus (Berglund and Brown, '89) plus the reports on activity-dependent changes in regional cerebral blood flow (e.g., Raichle et al., '76) elicit images of tonotopic influences on cochlear nucleus vasculature. Intense auditory stimulation could exert local vasomotor effects such that neural activity generated by type I fibers would be accompanied by vascular changes evoked by the type II fibers. Along with direct stimulation of cochlear nucleus neurons, type II neurons may have a more generalized regional effect by increasing circulation as neuronal activity is increased. Such a mechanism may help account for changes in blood supply to auditory regions of the brain as a result of acoustic stimulation, or for that matter, to other active brain areas (Heiss et al., '85).

It is possible that nonsynaptic en passant swellings represent transient boli of organelles being transported along the axon. Then again, nonsynaptic en passant swellings may represent pathological artifacts resembling the beading of a degenerating axon (e.g., Guillery, '70). All of these speculations generated by the structural descriptions need further evaluation. More information is required regarding the distribution and targets of endings of both type I and type II spiral ganglion neurons, and such studies promise to provide a fertile direction for future ultrastructural analyses. It is especially important to obtain information regarding the synaptic nature of terminal swellings of type II fibers. These kinds of data represent minimal considerations for formulating a comprehensive functional picture of the cochlear nucleus, a key structure in the auditory nervous system.

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LITERATURE CITED

Atwood, H.L., F. Lang, and W.A. Morin (1972) Synaptic vesicles: Selective depletion in crayfish excitatory and inhibitory axons. Science *176:*1353–1355.

Beaudet, A., and L. Descarries (1984) Fine structure of monoamine axon terminals in cerebral cortex. In L. Descarries, T.R. Reader, and H.H. Jasper (eds): Monoamine Innervation of Cerebral Cortex. New York: Alan R. Liss, Inc., pp. 77–93.

Berglund, A.M., and D.K. Ryugo (1986) A monoclonal antibody labels type II neurons of the spiral ganglion. Brain Res. *383:*327–332.

Berglund, A.M., and D.K. Ryugo (1987) Hair cell innervation by spiral ganglion neurons in the mouse. J. Comp. Neurol. *255:*560–570.

Berglund, A.M., and M.C. Brown (1989) Axonal trajectories of type-II spiral ganglion cells from various cochlear regions in mice. Soc. Neurosci. Abstr. *15:*742.

Brown, M.C. (1987) Morphology of labeled afferent fibers in the guinea pig cochlea. J. Comp. Neurol. *260:*591–604.

Brown, M.C., and A.L. Nuttall (1984) Efferent control of cochlear inner hair cell responses in the guinea-pig. J. Physiol. (Lond.) *354:*625–646.

Brown, M.C., and J.V. Ledwith III (1990) Projections of thin (type-II) and thick (type-I) auditory nerve fibers into the cochlear nucleus of the mouse. Hearing Res. *49:*105–118.

Brown, M.C., A.M. Berglund, N.Y.S. Kiang, and D.K. Ryugo (1988) Central trajectories of type II spiral ganglion cells. J. Comp. Neurol. *278:*581–590.

Cant, N.B., and D.K. Morest (1979) The bushy cells in the anteroventral cochlear nucleus of the cat. A study with the electron microscope. Neuroscience *4:*1925–1945.

Dallos, P. (1985) Response characteristics of mammalian cochlear hair cells. J. Neurosci. *5:*1591–1608.

Dallos, P., and D. Harris (1978) Properties of auditory nerve response in absence of outer hair cells. J. Neurophysiol. *41:*365–383.

Dallos, P., M.C. Billone, J.D. Durrant, C.-Y. Wang, and S. Raynor (1972) Cochlear inner and outer hair cells: Functional differences. Science *177:*356–358.

Dallos, P., J. Santos-Sacchi, and A. Flock (1982) Intracellular recordings from cochlear outer hair cells. Science *218:*582–584.

de Lanerolle, N.C., and C.C. LaMotte (1983) Ultrastructure of chemically defined neuron systems in the dorsal horn of the monkey. I. Substance P immunoreactivity. Brain Res. *274:*31–49.

Edvinsson, L., K.C. Nielsen, C. Owman, and B. Sporrong (1972) Cholinergic mechanisms in pial vessels: Histochemistry, electron microscopy and pharmacology. Z. Zellforsch. *134:*311–325.

Edvinsson, L., M. Lindvall, K.C. Nielsen, and C. Owman (1973) Are brain vessels innervated also by central (non-sympathetic) adrenergic neurones? Brain Res. *63:*494–499.

Falck, B., G.I. Mchedlishvili, and C. Owman (1965) Histochemical demonstration of adrenergic nerves in cortex-pia of rabbit. Acta Pharmacol. Toxicol. *23:*133–142.

Feindel, W., W. Penfield, and R. McNaughton (1960) The tentorial nerves and localization of intracranial pain in man. Neurology *10:*555–563.

Fekete, D.M., E.M. Rouiller, M.C. Liberman, and D.K. Ryugo (1984) The central projections of intracellularly labeled auditory nerve fibers in cats. J. Comp. Neurol. *229:*432–450.

Frank, E., W.A. Harris, and M.B. Kennedy (1980) Lysophophatidly choline facilitates labeling of CNS projections with horseradish peroxidase. J. Neurosci. Methods *2:*183–189.

Gasser, H.S. (1955) Properties of dorsal root unmedullated fibers on the two sides of the ganglion. J. Gen. Physiol. *38:*709–728.

Guillery, R.W. (1970) Light- and electron microscopical studies of normal and degenerating axons. In W.J.H. Nauta and S.O.E. Ebbesson (eds): Contemporary Research Methods in Neuroanatomy. New York: Springer-Verlag, pp. 77–105.

Heiss, W.-D., C. Beil, K. Herholz, G. Pawlik, R. Wagner, and W. Wienhard (1985) Atlas of Positron Emission Tomography of the Brain. Berlin: Springer-Verlag.

Itakura, T., T. Kasamatsu, and J.D. Pettigrew (1981) Norepinephrine-containing terminals in kitten visual cortex: Laminar distribution and ultrastructure. Neuroscience *6:*159–175.

Jahr, C.E., and R.A. Nicoll (1980) Dendrodendritic inhibition: Demonstration with intracellular recording. Science *207:*1473–1475.

Jones, D.R., D.K. Morest, D.L. Oliver, and S.J. Potashner (1984) Transganglionic transport of D-aspartate from cochlear nucleus to cochlea—a quantitative autoradiographic study. Hearing Res. *15:*197–213.

Kellerhals, B., M. Engstrom, and H.W. Ades (1967) Die Morphologie des Ganglion Spirale cochlea. II. Das normale Spiralganglion. Acta Otolaryngol. [Suppl.] (Stockh) *226:*6–33.

Kiang, N.Y.S., T. Watanabe, E.C. Thomas, and L.F. Clark (1965) Discharge Patterns of Single Fibers in the Cat's Auditory Nerve. Cambridge, MA: MIT Press.

Kiang, N.Y.S. (1975) Stimulus representation in the discharge patterns of auditory neurons. In D.B. Towers (ed): The Nervous System, Vol. 3, Human Communication and its Disorders. New York: Raven Press, pp. 81–96.

Kiang, N.Y.S., J.M. Rho, C.C. Northrop, M.C. Liberman, and D.K. Ryugo (1982) Hair-cell innervation by spiral ganglion cells in adult cats. Science *217:*175–177.

Kiang, N.Y.S., M.C. Liberman, J.S. Gage, C.C. Northrop, L.W. Dodds, and M.E. Oliver (1984) Afferent innervation of the mammalian cochlea. In L. Bolis, R.D. Keynes, and S.H.P. Maddrell (eds): Comparative Physiology of Sensory Systems. Cambridge: Cambridge University Press, pp. 143–161.

Kiang, N.Y.S., M.C. Liberman, W.F. Sewell, and J.J. Guinan (1986) Single unit clues to cochlear mechanisms. Hearing Res. *22:*171–182.

King, J.S., and G.A. Bishop (1982) The synaptic features of horseradish peroxidase-labelled recurrent collaterals in the ganglionic plexus of the cat cerebellar cortex. J. Neurocytol. *11:*867–880.

LaMotte, C.C. (1986) Organization of dorsal horn neurotransmitter systems. In T.L. Yaksh (ed): Spinal Afferent Processing. New York: Plenum Pub. Corp., pp. 97–116.

LaMotte, C.C., and N.C. de Lanerolle (1986) VIP terminals, axons, and neurons: Distribution throughout the length of monkey and cat spinal cord. J. Comp. Neurol. *249:*133–145.

Larramendi, L.H., H.L. Fickenscher, and N. Lemkey-Johnson (1967) Synaptic vesicles of inhibitory and excitatory terminals in the cerebellum. Science *156:*967–969.

Leake-Jones, P.A., and R.L. Snyder (1982) Uptake and transport of HRP by cochlear spiral ganglion neurons. Hearing Res. *8:*199–224.

Liberman, M.C. (1982) Single-neuron labeling in the cat auditory nerve. Science *216:*1239–1241.

Liberman, M.C., and N.Y.S. Kiang (1978) Acoustic trauma in cats: Cochlear pathology and auditory-nerve activity. Acta Otolaryngol. [Suppl.] (Stockh.) *358:*1–63.

Liberman, M.C., and N.Y.S. Kiang (1984) Single-neuron labeling and chronic cochlear pathology. IV. Stereocilia damage and alterations in rate- and phase-level functions. Hearing Res. *16:*75–90.

Liberman, M.C., and M.E. Oliver (1984) Morphometry of intracellularly labeled neurons of the auditory-nerve: Correlations with functional properties. J. Comp. Neurol. *223:*163–176.

McDonald, D.M., and G.L. Rasmussen (1977) An ultrastructural analysis of neurites in the basal lamina of capillaries in the chinchilla cochlear nucleus. J. Comp. Neurol. *173:*475–496.

Merrillees, N., G. Burnstock, and M. Holman (1963) Correlation of fine structure and physiology of the innervation of smooth muscle in the guinea-pig vas deferens. J. Cell. Biol. *19:*529–550.

Morest, D.K., and B.A. Bohne (1983) Noise-induced degeneration in the brain and representation of inner and outer hair cells. Hearing Res. *9:*145–151.

Mountcastle, V.B. (1974) Pain and temperature sensibilities. In V.B. Mountcastle (ed): Medical Physiology. St. Louis: C.V. Mosby Co., p. 357.

Neely, S.T., and D.O. Kim (1983) An active cochlear model showing sharp tuning and high sensitivity. Hearing Res. *9:*123–130.

Nowycky, M.C., K. Mori, and G.M. Sherpherd (1981) GABAergic mechanisms of dendrodendritic synapses in isolated turtle olfactory bulb. J. Neurophysiol. *46:*639–648.

Ota, C.Y., and R.S. Kimura (1980) Ultrastructural study of the human spiral ganglion. Acta Otolaryngol. (Stockh.) *89:*53–62.

Peters, A., S.L. Palay, and H. deF. Webster (1976) The Fine Structure of the Nervous System. Philadelphia: W.B. Saunders Co.

Raichle, M.E., R.L. Grubb, M.H. Gado, J.O. Eichling, and M.T. Ter-Pogossian (1976) Correlation between regional cerebral blood flow and oxidative metabolism. Arch. Neurol. *8:*523–526.

Ralston, H.J., and D.D. Ralston (1979) The distribution of dorsal root axons in laminae I, II, and III of the macaque spinal cord: A quantitative electron microscope study. J. Comp. Neurol. *184:*643–684.

Ribak, C.E., J.E. Vaughn, K. Saito, R. Barber, and E. Roberts (1977) Glutamate decarboxylase localization in neurons of the olfactory bulb. Brain Res. *126:*1–18.

Robertson, D. (1984) Horseradish peroxidase injection of physiologically characterized afferent and efferent neurones in the guinea pig spiral ganglion. Hearing. Res. *15:*113–121.

Rouiller, E.M., R. Cronin-Schreiber, D.M. Fekete, and D.K. Ryugo (1986) The central projections of intracellularly labeled auditory nerve fibers: An analysis of terminal morphology. J. Comp. Neurol. *249:*261–278.

Ruggero, M.A., P.A. Santi, and N.C. Rich (1982) Type II cochlear ganglion cells in the chinchilla. Hearing Res. *8:*339–356.

Ryan, A.F., and Dallos, P. (1975) Effect of absence of cochlear outer hair cells on behavioral auditory threshold. Nature *253:*44–46.

Ryugo, D.K., and D.M. Fekete (1982) Morphology of primary axosomatic endings in the anteroventral cochlear nucleus of the cat: A study of the endbulbs of Held. J. Comp. Neurol. *210:*239–257.

Smith, C.A. (1975) Innervation of the cochlea of the guinea pig by use of the Golgi stain. Ann. Otolaryngol. *84:*443–458.

Spoendlin, H. (1971) Degeneration behaviour of the cochlear nerve. Arch. Klin. Exp. Ohr.-Nas.-u. Kehlk. Heilk. *200:*275–291.

Spoendlin, H. (1973) The innervation of the cochlear receptor. In A.R. Moller (ed): Basic Mechanisms in Hearing. New York: Academic Press, pp. 185–234.

Spoendlin, H. (1979) Neural connections of the outer hair cell system. Acta Otolaryngol. *87:*381–387.

Stebbins, W.C., J.M. Miller, L.-G. Johnsson, and J.E. Hawkins (1969) Ototoxic hearing loss and cochlear pathology in the monkey. Ann. Otol. Rhinol. Laryngol. *78:*1007–1025.

Uchizono, K. (1965) Characteristics of excitatory and inhibitory synapses in the central nervous system of the cat. Nature *207:*642–643.