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Mossy Fiber Projections From the Cuneate Nucleus to the Cochlear Nucleus in the Rat

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ABSTRACT

A reciprocal connection is known to exist between the cuneate nucleus, which is a first-order somatosensory nucleus, and the cochlear nucleus, which is a first-order auditory nucleus. We continued this line of study by investigating the fiber endings of this projection in the cochlear nucleus of rats using the neuronal tracer *Phaseolus vulgaris* leucoagglutinin in combination with ultrastructural and immunocytochemical analyses. In the cochlear nucleus, mossy fiber terminals had been described and named for their morphologic similarity to those in the cerebellum, but their origins had not been discovered. In the present study, we determined that the axonal projections from the cuneate region gave rise to mossy fiber terminals in the granule cell regions of the ipsilateral cochlear nucleus. The cuneate mossy fibers appear to be excitatory in nature, because they are filled with round synaptic vesicles, they make asymmetric synapses with postsynaptic targets, and they are labeled with an antibody to glutamate. The postsynaptic targets of the mossy fibers include dendrites of granule cells. This projection onto the granule cell interneuron circuit of the cochlear nucleus indicates that somatosensory cues are intimately involved with information processing at this early stage of the auditory system.

Indexing terms: auditory system, glutamate, somatosensory system, synapses, ultrastructure

The cochlear nucleus is the first central auditory nucleus, and it receives primary input from the receptor cells of the cochlea via the auditory nerve. The cuneate and gracile nuclei, which, together, form the dorsal column nuclei, occupy an analogous position in the somatosensory system, in that they receive direct primary input from dorsal root ganglion cells that innervate touch, vibratory, and proprioceptive receptors on the body surface.

A pathway between the cochlear nucleus and the cuneate nucleus region was previously demonstrated anatomically in rat and cat with wheat germ agglutinin-horseradish peroxidase (WGA-HRP) tracing studies (Itoh et al., 1987; Weinberg and Rustioni, 1987). These studies indicated that cells from the dorsolateral edge of the cuneate nucleus, which is thought to receive sensory input from the back of the head and the pinna (Miller and Basbaum, 1975; Maslany et al., 1991), send a projection to the granule cell regions of the cochlear nucleus (Weinberg and Rustioni, 1987). More recently, electrophysiological studies in the cochlear nucleus of the cat have demonstrated that electrical stimulation in the vicinity of the dorsal column nuclei and the spinal trigeminal nucleus, or direct tactile stimulation of the pinna in particular, evokes activity in cochlear nucleus neurons (Saadé et al., 1989; Young et al., 1995). The presence of pinna-based spectral cues for sound localization has been reported in cats (see, e.g., Rice et al., 1992). These studies indicate that somatosensory information related to pinna position is an important adjunct to auditory processing in the cochlear nucleus. In the present study, our goal was to characterize the synaptic interactions of cuneate fibers with known neuronal populations in the cochlear nucleus.

In the dorsal cochlear nucleus (DCN), the auditory nerve forms synapses with giant cells in the deep layers (Gonzalez et al., 1993) and with pyramidal cells more superficially (Osen, 1970; Ryugo and May, 1993), and these cells, in turn, project to the inferior colliculus (Osen, 1969; Roth et al., 1978; Ryugo et al., 1981). In addition, the cochlear nucleus has a complex interneuronal circuitry, including a granule cell system that is in a position to influence the output of the DCN (Hirsch and Oertel, 1988; Manis, 1989). The granule cell axons run parallel to the pial surface of the DCN and form synapses with the apical dendrites of pyramidal cells (Mugnaini et al., 1980) and with interneurons such as cartwheel cells (Wouterlood and Mugnaini, 1984) that have extensive input onto the pyramidal cells (Berrebi and Mugnaini, 1991). The granule cell regions receive a variety of auditory and nonauditory inputs in

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addition to those from the cuneate, including projections from the raphe nuclei (Keppler and Herbert, 1991), the locus coeruleus (Kromer and Moore, 1980), and the auditory cortex (Feliciano et al., 1993; Weedman et al., 1995) as well as from the unmyelinated type II auditory nerve fibers (Brown et al., 1988a) and collaterals of medial olivocochlear efferents (Brown et al., 1988b).

The granule cell system of the cochlear nucleus has many anatomical, biochemical, and electrophysiological similarities with the granule cell circuit in the cerebellar cortex (see, e.g., Mugnaini and Morgan, 1987; Berrebi and Mugnaini, 1991; Manis et al., 1994), including mossy fiber inputs that participate in synaptic glomeruli (Mugnaini et al., 1980; Osen et al., 1984). The mossy fibers of the cochlear nucleus are large terminals that resemble those of the cerebellum (Mugnaini, 1972; Palay and Chan Palay, 1974), in that they contain numerous round synaptic vesicles and mitochondria and form a central terminal that makes synapses with surrounding dendritic and axonal profiles (Mugnaini et al., 1980). The postsynaptic targets of the cochlear nucleus mossy fibers are thought to include Golgi and granule cells (Mugnaini et al., 1980) and unipolar brush cells (Wright and Ryugo, 1995). The sources of these mossy fibers have been speculated to arise from auditory nerve fibers (Kane, 1974) or from olivocochlear efferents (McDonald and Rasmussen, 1971; Osen et al., 1984), but neither idea has been substantiated.

In the present study, we demonstrate that projections from the cuneate region form mossy fiber terminals in the granule cell domains of the cochlear nucleus and make direct contact with the granule cells. The terminals are large and numerous, and they are enriched in glutamate. Therefore, the cuneate mossy fibers are likely to provide a significant excitatory input to the granule cell circuit of the DCN.

MATERIALS AND METHODS

Neuronal tracing

Data from seven male Sprague Dawley rats (200–250 g) are presented in this study. Rats were first deeply anesthetized with Nembutal (45 mg/kg), and then an incision was made in the skin and muscle along the posterior cranium to the rostral cervical spinal cord. The dura was opened to expose the dorsal brainstem surface at the level of the obex. Iontophoretic injections were made into the cuneate nucleus just lateral and rostral to the obex, using 15–30 μm (ID) pipettes filled with 2.5% *Phaseolus vulgaris* leucoagglutinin (PHA-L; Vector Laboratories) in 0.01 M phosphate buffer, pH 8.0. The tracer was delivered using a 5 μA DC-positive current (7 seconds on/7 seconds off) for up to 20 minutes. The incision was sutured closed, and, after a 6–12 day survival period, the rats were again anesthetized with Nembutal (50 mg/kg) and were transcardially perfused with 4% paraformaldehyde. After postfixing in 4% paraformaldehyde for at least 1 hour at 4°C, the medulla and pons were cut coronally on a Vibratome into 50-μm-thick serial sections.

Cochlear nucleus tissue from four of the seven rats was processed using the following procedures. Unless otherwise stated, all immunocytochemical processing was performed at room temperature in Tris-buffered saline (0.05 M, pH 7.6). Sections for light microscopy were treated with 0.1% Triton X-100 (TX) in all processing steps in order to permeabilize tissue for antibody labeling. The ultrastructure was better preserved in electron microscopic material by treating sections with a 0.1% solution of the mild detergent Photo-Flo during the primary and secondary antibody incubations (Wouterlood et al., 1988). Following permeabilization, tissue sections were blocked in 5% nonfat dry milk for 2 hours and were then incubated overnight with an anti-PHA-L antibody (Dako) at a concentration of 1:2,000. After washes with buffer, sections were incubated in a goat anti-rabbit immunoglobulin (IgG) secondary antibody (1:50) for 1 hour. Sections were washed in buffer and then treated with rabbit peroxidase antiperoxidase (Sternberger Monoclonals) at 1:400 for 2 hours. Washes with buffer were followed by a 5–10 minute incubation in 0.05% 3,3′-diaminobenzidine tetrahydrochloride (DAB) and 0.015% H_2O_2 in Tris buffer (0.05 M, pH 7.6). Sections for light microscopy were washed and mounted on gelatin-coated slides, air dried, and then cleared and coverslipped. Electron microscopic material was postfixed in 1% OsO_4 for 20 minutes, stained overnight en bloc with 1% uranyl acetate, and then dehydrated, infiltrated, and embedded in Epon between two sheets of Aclar. Individual mossy fiber terminals were dissected out and reembedded in BEEM capsules. Ultrathin (70 nm) sections were cut on a microtome, collected on Formvar-coated grids, and counterstained with uranyl acetate and lead citrate.

In two of the seven rats, PHA-L was visualized using immunofluorescence. For these experiments (data used to construct Figs. 1 and 2), the procedure was the same as above for light microscopy, except that sections were incubated in primary antibody (Dako) overnight at a concentration of 1:500 followed by an FITC-conjugated goat anti-rabbit secondary antibody (Jackson, 1:25) for 2 hours at room temperature. After rinsing, sections were mounted on microscope slides with Vectashield mounting medium (Vector Laboratories), and the slides were viewed with a fluorescence microscope.

Postembedding immunogold

One of the seven rats was used for postembedding immunogold double-labeling experiments, where cuneate terminals were first labeled by PHA-L and were then processed with antibodies directed against neurotransmitter markers. We used the procedure outlined by Smith and Paré (1994). Briefly, the PHA-L neuronal tracing method was similar to that described above, except that the rat was first perfused with 50 cc of a cold (4°C) oxygenated solution containing 126 mM NaCl, 26 mM $NaHCO_3$, 3 mM KCl, 1.2 mM KH_2PO_4, 1.3 mM $MgSO_4$, 2.4 mM $CaCl_2$, 5 mM HEPES, and 15 mM dextrose followed by 500 cc of a cold (4°C) 2% paraformaldehyde/1% glutaraldehyde fixative solution. After dissection of the cochlear nuclei, tissue was immediately sectioned at 50 μm in the coronal plane.

Prior to immunoprocessing, tissue sections were treated with a 1% solution of sodium borohydride in phosphate-buffered saline (PBS) for 20 minutes, washed, then permeabilized by cryoprotecting (25% sucrose, 10% glycerol in PBS 0.05 M, pH 7.4, for 20 minutes), and then freezing the tissue for 20 minutes at −80°C. After blocking with a solution containing 1% normal goat serum (NGS) and 1% bovine serum albumin (BSA) for 1 hour, sections were incubated with a biotinylated goat anti-PHA-L antibody (Vector Laboratories; 1:200) solution containing 1% BSA and 1% NGS overnight at room temperature. The antibody was visualized using ABC (standard; Vector Laboratories) at 1:100 in PBS with 1% BSA for 90 minutes followed by

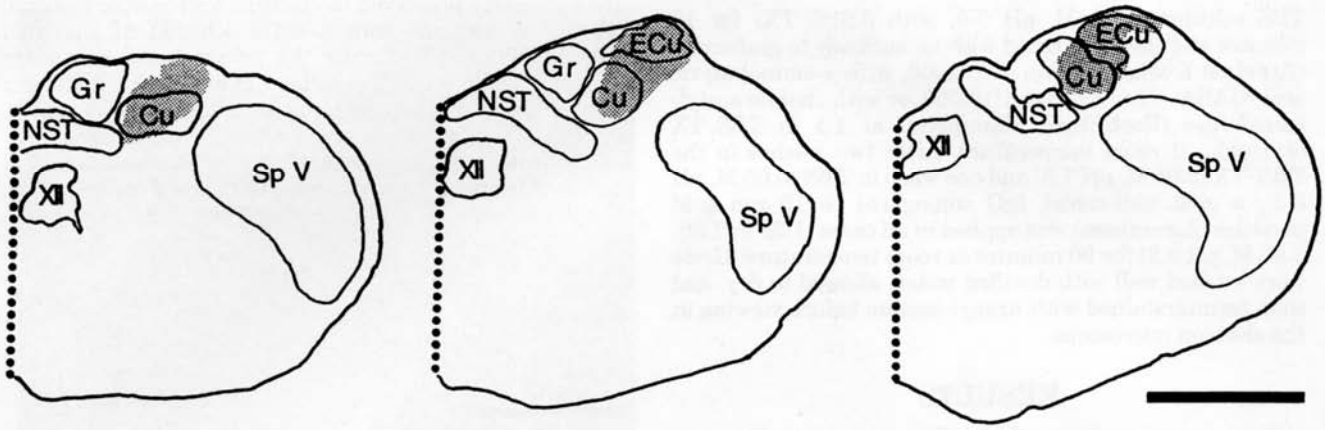

Fig. 1. Schematic drawing of injection site spanning 0.85 mm from caudal (**right**) to rostral (**left**). Injections of *Phaseolus vulgaris* leucoagglutinin (PHA-L) were made lateral and slightly rostral to the obex. Coronal sections of the brainstem contained tracer in the cuneate nucleus. Sometimes, tracer extended into the region of the external cuneate nucleus. The injection site (shaded area) illustrated in this figure is from the experiment with the most extensive labeling of projections into the cochlear nucleus. Anatomic boundaries were created by superimposing drawings of experimental sections onto coronal sections from a rat brain atlas (Swanson, 1992) to approximate the brainstem nuclei at the different levels. ECu, external cuneate; Cu, cuneate nucleus; Gr, gracile nucleus; NST, nucleus of the solitary tract; Sp V, spinal nucleus of the trigeminal nerve; XII, hypoglossal nucleus. Scale bar = 1 mm.

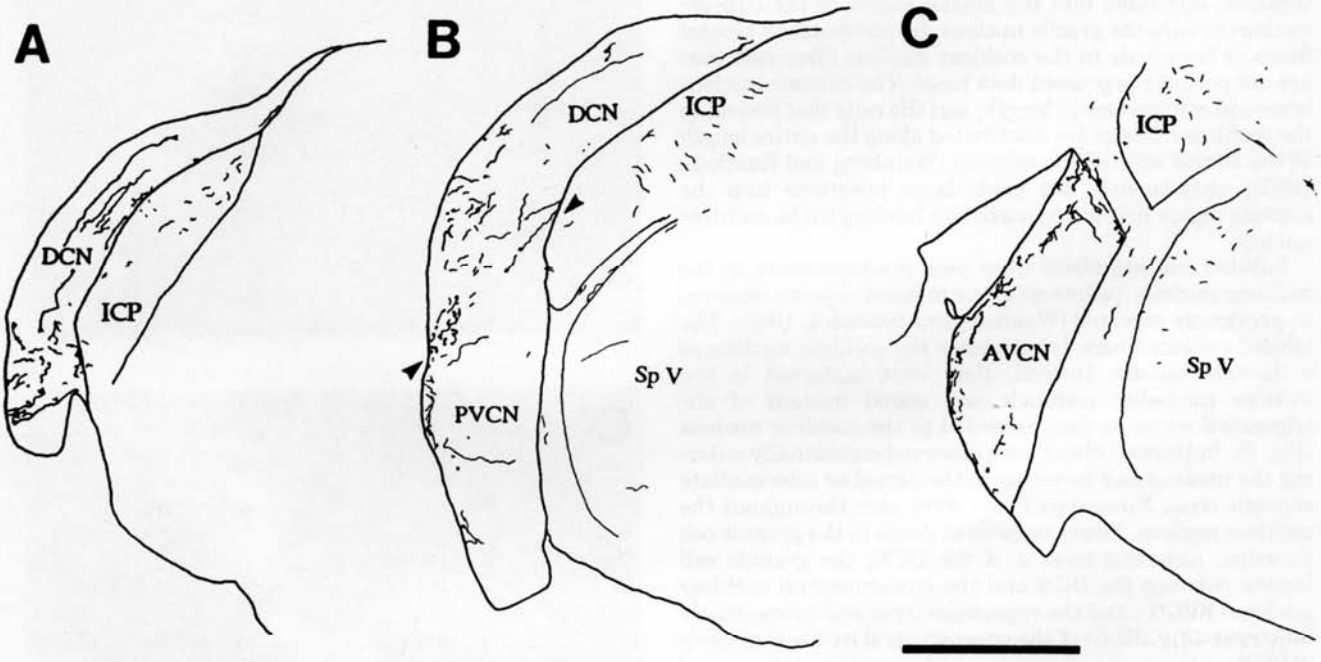

Fig. 2. Fibers and their terminals in the cochlear nucleus following an injection of PHA-L into the ipsilateral cuneate nucleus. The distribution of this fiber projection is shown in drawings of three 50 μm coronal sections, which represent the caudal (**A**), middle (**B**), and rostral (**C**) aspects of the cochlear nucleus. The labeled cuneate fibers course through and terminate in the caudal dorsal cochlear nucleus (DCN; A), which contains mostly layer 2. Fibers and terminals in the main body of the nucleus congregate in the granule cell lamina indicated between arrowheads in B), which separates the DCN from the posteroventral cochlear nucleus (PVCN), layer 2 of the DCN, the lateral edge of the PVCN (B), and the lateral and dorsal borders of the anteroventral cochlear nucleus (AVCN; C). ICP, inferior cerebellar peduncle; Sp V, spinal nucleus of the trigeminal nerve. Scale bar = 1 mm.

DAB (0.025% with 0.01 M imidazole and 0.006% H_2O_2 in Tris buffer). Tissue was then osmicated (1% in 0.1 M PB, pH 7.4) and dehydrated in a series of graded alcohols (35, 50, 70, 95, and 100%). The tissue was incubated in the 70% alcohol solution with 1% uranyl acetate for 30 minutes in order to enhance contrast in the electron microscope. Following the 100% alcohol step, tissue was treated with propylene oxide, embedded in Durcupan ACM, and allowed to harden at 60°C for 48 hours. PHA-L-labeled axon terminals in the cochlear nucleus were cut out and reembedded in BEEM capsules for ultrathin sectioning.

Postembedding immunogold techniques were carried out on 70-nm-thin (silver-gray) sections that had been collected on Formvar-coated nickel grids. Grids were washed with a

TBS solution (0.05 M, pH 7.6, with 0.01% TX) for 10 minutes and then incubated with an antibody to glutamate (Arnel) at a concentration of 1:5,000, with γ-aminobutyric acid (GABA; Chemicon) at 1:10,000, or with choline acetyltransferase (Boehringer Mannheim) at 1:1 in TBS/TX overnight at room temperature. After two washes in the TBS/TX (0.05 M, pH 7.6) and one wash in TBS (0.05 M, pH 8.2), a goat anti-rabbit IgG conjugated to 10 nm gold particles (Amersham) was applied in all cases (1:25 in TBS, 0.05 M, pH 8.2) for 90 minutes at room temperature. Grids were washed well with distilled water, allowed to dry, and then counterstained with uranyl acetate before viewing at the electron microscope.

RESULTS

The present results are drawn from seven rats. Two rats were studied using fluorescent tracing methods to map the cuneate projections, and five rats were prepared for light and electron microscopy of DAB-HRP reaction product following injections of the anterograde tracer PHA-L into the cuneate and external cuneate regions of the rat brainstem (Fig. 1). Injection of tracer into the lateral edge of the cuneate nucleus labeled fibers in the cochlear nucleus. In contrast, injections into the medial aspect of the cuneate nucleus or into the gracile nucleus did not result in labeled fibers or terminals in the cochlear nucleus (four rats that are not part of the present data base). The cuneate nucleus is several millimeters in length, and the cells that project to the cochlear nucleus are distributed along the entire length of the lateral edge of the nucleus (Weinberg and Rustioni, 1987); consequently, we made large injections into the cuneate region in order to maximize labeling in the cochlear nucleus.

Labeled cuneate fibers were seen predominantly in the cochlear nucleus ipsilateral to the injected cuneate nucleus, as previously reported (Weinberg and Rustioni, 1987). The labeled cuneate fibers did not enter the cochlear nucleus as a discrete bundle; instead, they were scattered in the inferior cerebellar peduncle and spinal nucleus of the trigeminal nerve as they ascended to the cochlear nucleus (Fig. 2). Individual fibers were observed occasionally entering the nucleus and traveling in the dorsal or intermediate acoustic stria. Numerous fibers were seen throughout the cochlear nucleus. Fibers were most dense in the granule cell domains, including layer 2 of the DCN, the granule cell lamina between the DCN and the posteroventral cochlear nucleus (PVCN), and the superficial layer and subpeduncular corner (Fig. 2B,C) of the anteroventral cochlear nucleus (AVCN).

Labeled cuneate fibers in the cochlear nucleus are thin (0.3–2.0 μm) and give rise to en passant and terminal swellings that range in size from 2 to 20 μm in diameter (Figs. 3, 4B, 5B, 6B; inset in Fig. 8). Most swellings occur in regions with large numbers of granule cells. The fibers can appear beaded, giving rise to multiple en passant swellings (Fig. 3A), or they can be uniform in diameter with large terminal swellings that give off thin collaterals (Fig. 3B). Fibers also emit small terminal boutons (Fig. 3C). This variation in size and shape of the swellings does not appear to correlate with their location in the cochlear nucleus.

With electron microscopy, a number of labeled cuneate terminals ($n > 25$) were observed to form the classic appearance of mossy fibers in the cochlear nucleus. That is, each labeled terminal formed a central core surrounded by dendritic and axonal processes. The sizes and shapes of

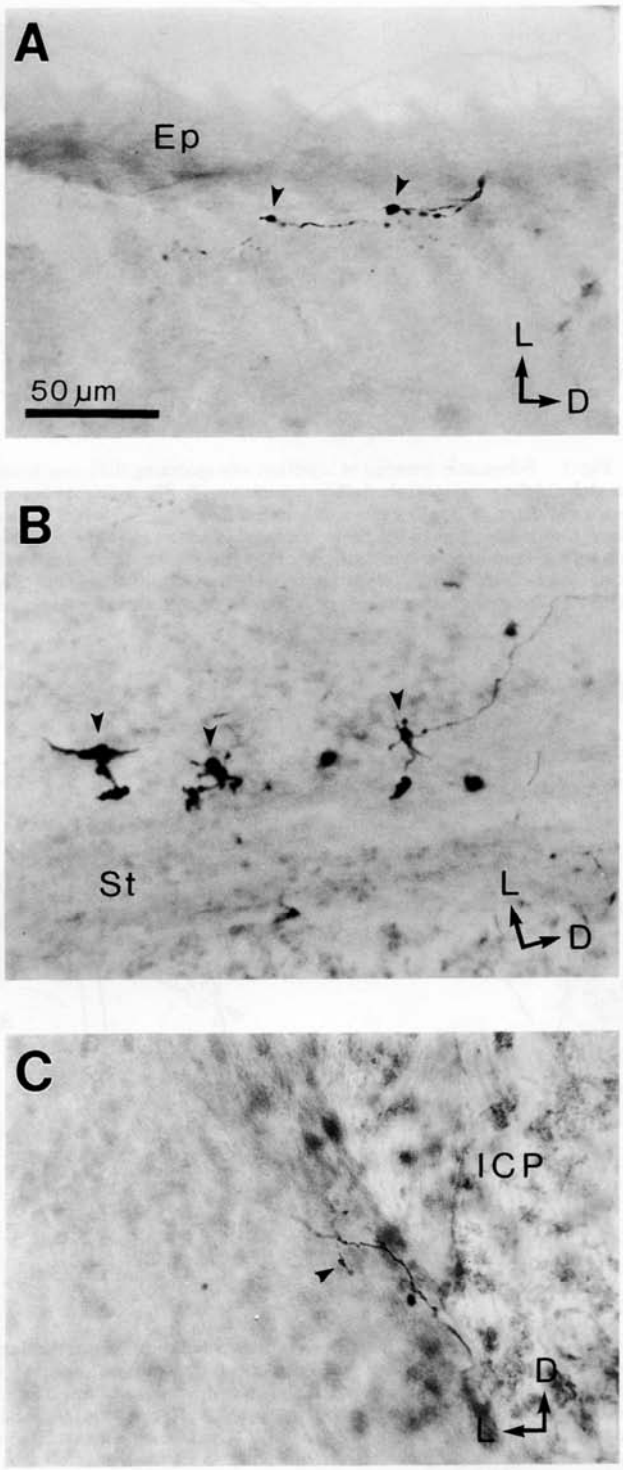

Fig. 3. Photomicrographs of en passant and terminal swellings of cuneate fibers. **A:** Two fibers adjacent to the ependymal (Ep) surface of the DCN form round en passant swellings (arrowheads). **B:** Fibers can also form large irregular terminals (arrowheads) with thin appendages that have terminal swellings. These particular terminals are located in the lamina at the strial border (St) of the PVCN. **C:** A labeled fiber traveling along the inferior cerebellar peduncle emits several small terminal boutons (one is indicated by an arrowhead) in the subpeduncular corner of the AVCN. These examples illustrate that the terminals can vary greatly in size and shape. L, lateral; D, dorsal. Scale bar applies to A–C.

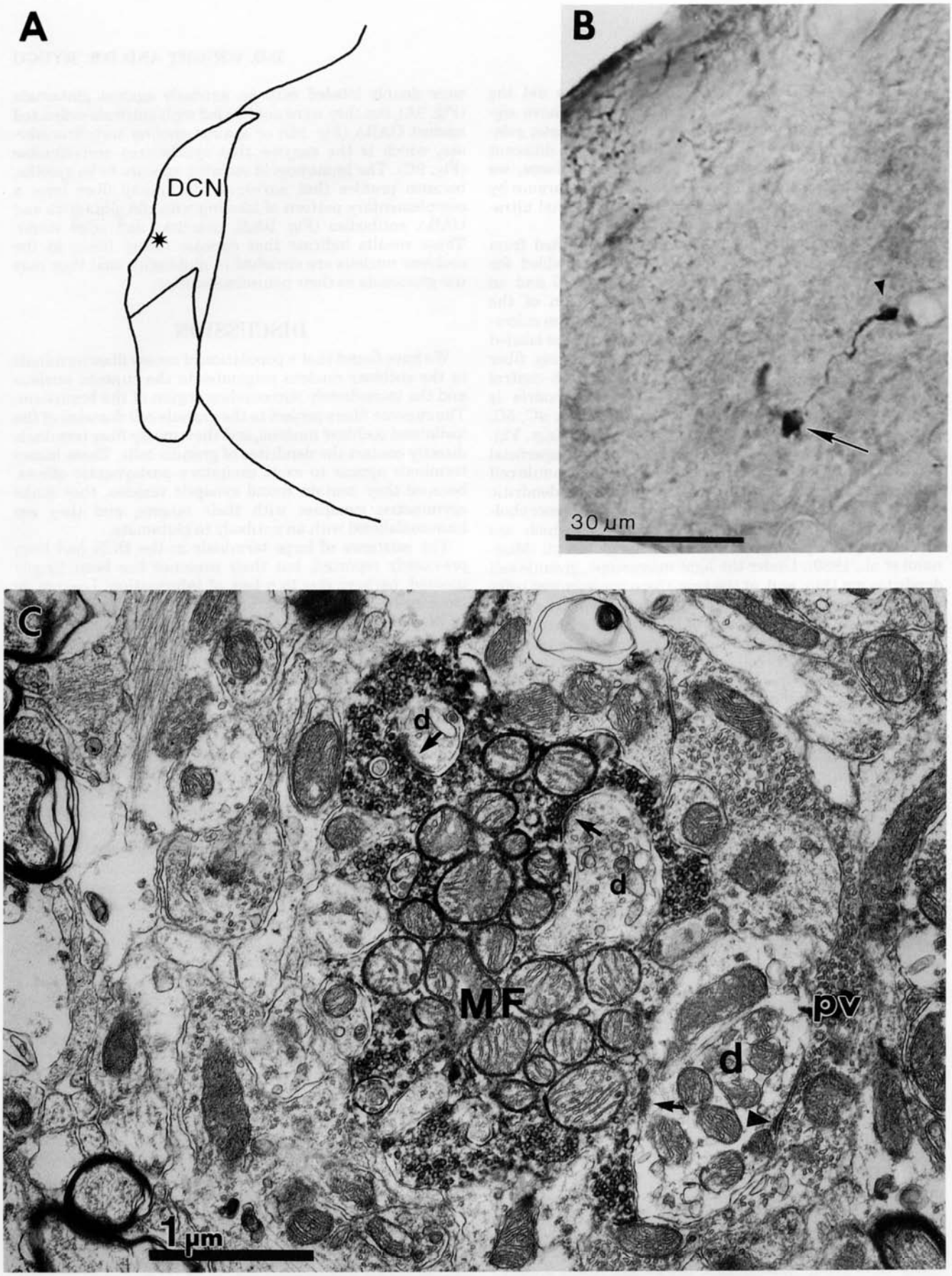

Fig. 4. Cuneate fibers form mossy fiber terminals in the DCN. **A:** Location of a labeled fiber that terminates (star) in layer 2 of the DCN **B:** A light micrograph of the fiber shows that it forms two terminals (arrow and arrowhead). **C:** An electron micrograph of one of the terminals (arrow in B) demonstrates mossy fiber-like characteristics, including numerous mitochondria and round synaptic vesicles. The mossy fiber (MF) forms asymmetric synapses (arrows) upon small dendrites (d). An unlabeled axon terminal filled with pleomorphic vesicles (pv) makes a synapse (arrowhead) with a dendrite contacted by the mossy fiber.

these central terminals varied considerably, as did the surrounding processes. These variations could have signaled fundamentally different pre- and postsynaptic relationships, or they could have simply reflected different views of a complex structure. To address this issue, we selected terminals that differed distinctly in appearance by light microscopy and then prepared them for serial ultrathin electron microscopic examination.

Eight swellings of cuneate fibers were identified from light microscopic study and were then reembedded for analysis with the electron microscope. Terminal and en passant swellings, each from a different region of the cochlear nucleus, were studied in light and electron micrographs (Figs. 4–6). At the ultrastructural level, the labeled swellings exhibit the classic appearance of mossy fiber terminals of cochlear nucleus glomeruli, where a central terminal full of synaptic vesicles and mitochondria is surrounded by numerous dendritic profiles (Figs. 4C, 5C, 6C). All swellings are found among granule cells (e.g., Fig. 5C). Mossy fiber terminals and swellings in the superficial layers of the DCN (Figs. 4A,B, 6A,B) and in the granule cell lamina (5A,B) make synaptic contact with small dendritic profiles (Figs. 4C, 5C, 6C). These profiles have the morphology characteristic of granule cell dendrites, which are known to participate in cochlear nucleus glomeruli (Mugnaini et al., 1980). Under the light microscope, granule cell dendrites are thin, and, at the tips, there are terminal tufts with the appearance of claws with digitiform extensions (Brawer et al., 1974; Mugnaini et al., 1980; Hackney et al., 1990). Electron micrographs in the literature have revealed examples of granule cell claws that give rise to thin appendages, which penetrate the profiles of mossy fiber terminals (Mugnaini et al., 1980). The dendrites contacted by labeled mossy fibers from the cuneate nucleus are small and give rise to finger-like projections that are embedded within the swellings (Fig. 5B, stars; Fig. 6C, asterisks). These dendritic extensions are also postsynaptic to the mossy fibers. We infer that cuneate mossy fibers form synaptic glomeruli with the distal dendrites of granule cells.

Labeled boutons arising from cuneate fibers also formed synaptic contacts with proximal dendrites (Fig. 7). In two instances, these dendrites were observed in the same section arising directly from cells exhibiting the morphological characteristics of granule cells (Mugnaini et al., 1980). The cells are small (5–8 μm in diameter), and they have a low cytoplasmic-to-nucleus ratio, contain few cytoplasmic organelles, and bear nuclei with prominent patches of peripheral chromatin. In two other instances, boutons were observed making contact with more distal dendrites. These boutons were followed through serial sections, and they arose from granule cells.

The mossy fiber projections from the cuneate region make type 1 synapses (Gray 1959). The terminals contain round synaptic vesicles and form asymmetric synapses. The thickening of the membrane on the presynaptic side is diminished compared to that on the postsynaptic side, although the immunoreaction product in the terminals can obscure the presynaptic side of the synapse. Nevertheless, unambiguous examples of asymmetric synapses were plentiful (Fig. 8). All mossy fibers examined in the electron microscope contained clear, round, synaptic vesicles (e.g., Fig. 8, arrow).

Postembedding immunogold techniques were used to label the putative neurotransmitter in the cuneocochlear projection fibers. The anterogradely labeled mossy fibers were double labeled with an antibody against glutamate (Fig. 9A), but they were not labeled with antibodies directed against GABA (Fig. 9B) or against choline acetyltransferase, which is the enzyme that synthesizes acetylcholine (Fig. 9C). The immunogold staining appears to be specific, because profiles that surround the mossy fiber have a complementary pattern of labeling with the glutamate and GABA antibodies (Fig. 9A,B, asterisks and solid stars). These results indicate that cuneate mossy fibers in the cochlear nucleus are enriched in glutamate, and they may use glutamate as their neurotransmitter.

DISCUSSION

We have found that a population of mossy fiber terminals in the cochlear nucleus originates in the cuneate nucleus and the immediately surrounding region of the brainstem. The cuneate fibers project to the granule cell domains of the ipsilateral cochlear nucleus, and their mossy fiber terminals directly contact the dendrites of granule cells. These mossy terminals appear to exert excitatory postsynaptic effects, because they contain round synaptic vesicles, they make asymmetric synapses with their targets, and they are immunolabeled with an antibody to glutamate.

The existence of large terminals in the DCN had been previously reported, but their presence has been largely ignored, perhaps due to a lack of information. Lorente de Nó (1981, Fig. 6-6) described "gigantic endings" in the DCN of neonatal kittens, but their resemblance to growth cones implied a developmental curiosity more than a permanent structure. Large axonal endings that resemble mossy fiber terminals in the cerebellar granule cell layer have also been observed in the cochlear nucleus under electron microscopy (McDonald and Rasmussen, 1971; Kane, 1974; Mugnaini et al., 1980). Cochlear nucleus mossy fibers are found among granule cells, and they form the centerpiece of each glomerulus, a complex synaptic structure that can involve granule and unipolar brush cell dendrites, Golgi cell axons, and a surrounding glial capsule (Mugnaini et al., 1980; Wright and Ryugo, 1995).

The cuneate mossy fibers analyzed in this study formed synapses on the proximal dendrites of small cells that have the ultrastructural features of granule cells as well as on small glomerular dendrites that have the morphological characteristics granule cell dendritic claws (Mugnaini et al., 1980). We have also studied the dendritic claws of granule cells labeled with biocytin under light and electron microscopy (Weedman and Ryugo, unpublished observations). Mossy fiber terminals from the cuneate nucleus are associated with dendritic profiles and fine filiform hairs that closely resemble the biocytin-labeled dendrites of granule cells. Axon terminals containing pleomorphic vesicles make contact with granule cell dendrites postsynaptic to the cuneate mossy fibers (Fig. 4C). These axon terminals have been postulated to arise from Golgi cells of the cochlear

Fig. 5. Mossy fiber terminals are observed commonly in the granule cell lamina. This mossy fiber terminal (**A**, star) in the lamina is large (9–15 μm) and irregular in shape (**B**). St, strial border of the PVCN. **C:** The labeled terminal is situated among granule cells (GC) and forms numerous synapses (arrows) with surrounding dendritic profiles. In addition, pieces of dendritic fingers (stars) are embedded in the mossy fiber (M), and they also receive synapses. The unmyelinated fiber that gives rise to the mossy terminal can be observed at the bottom of the electron micrograph (asterisk).

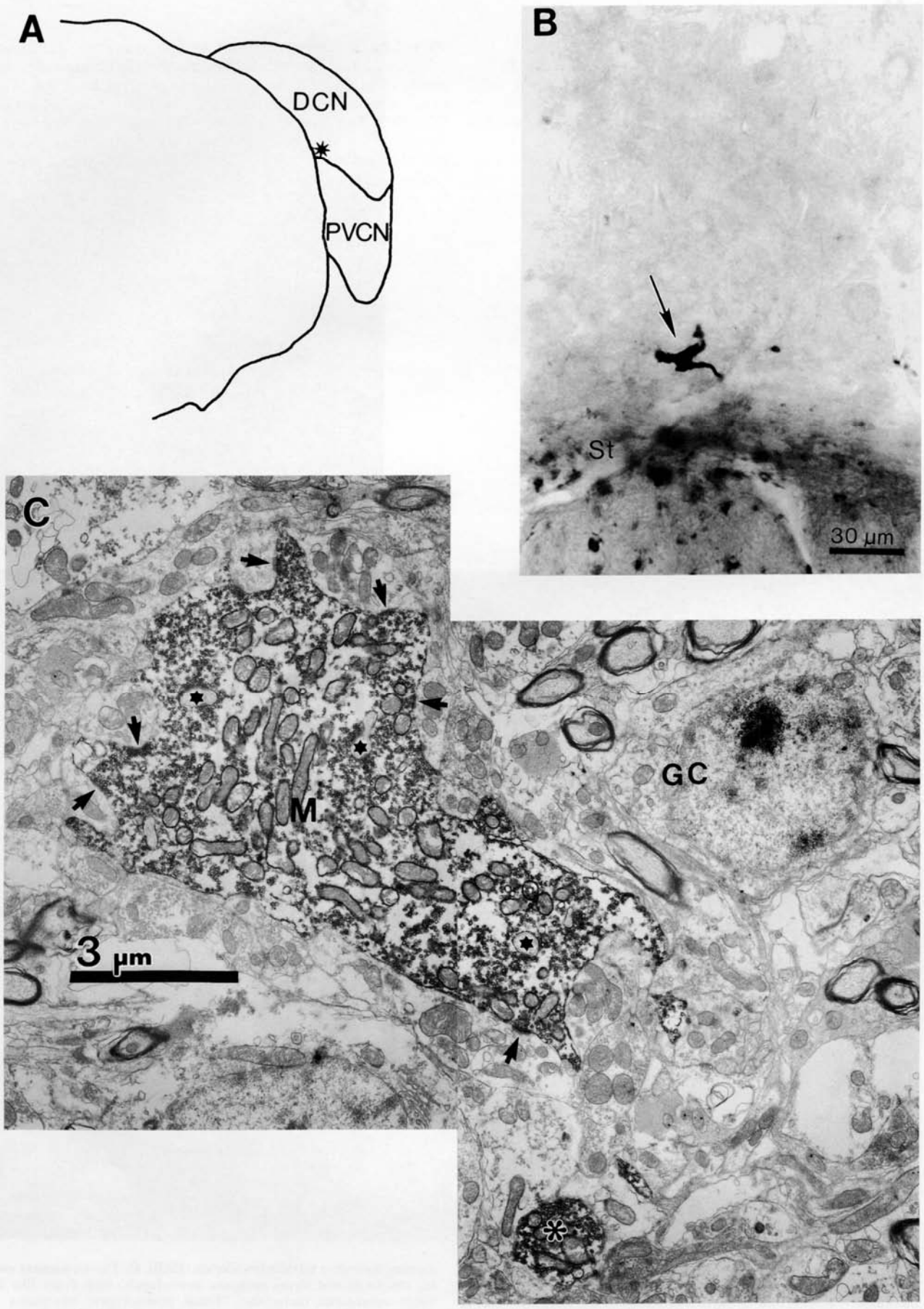

Figure 5

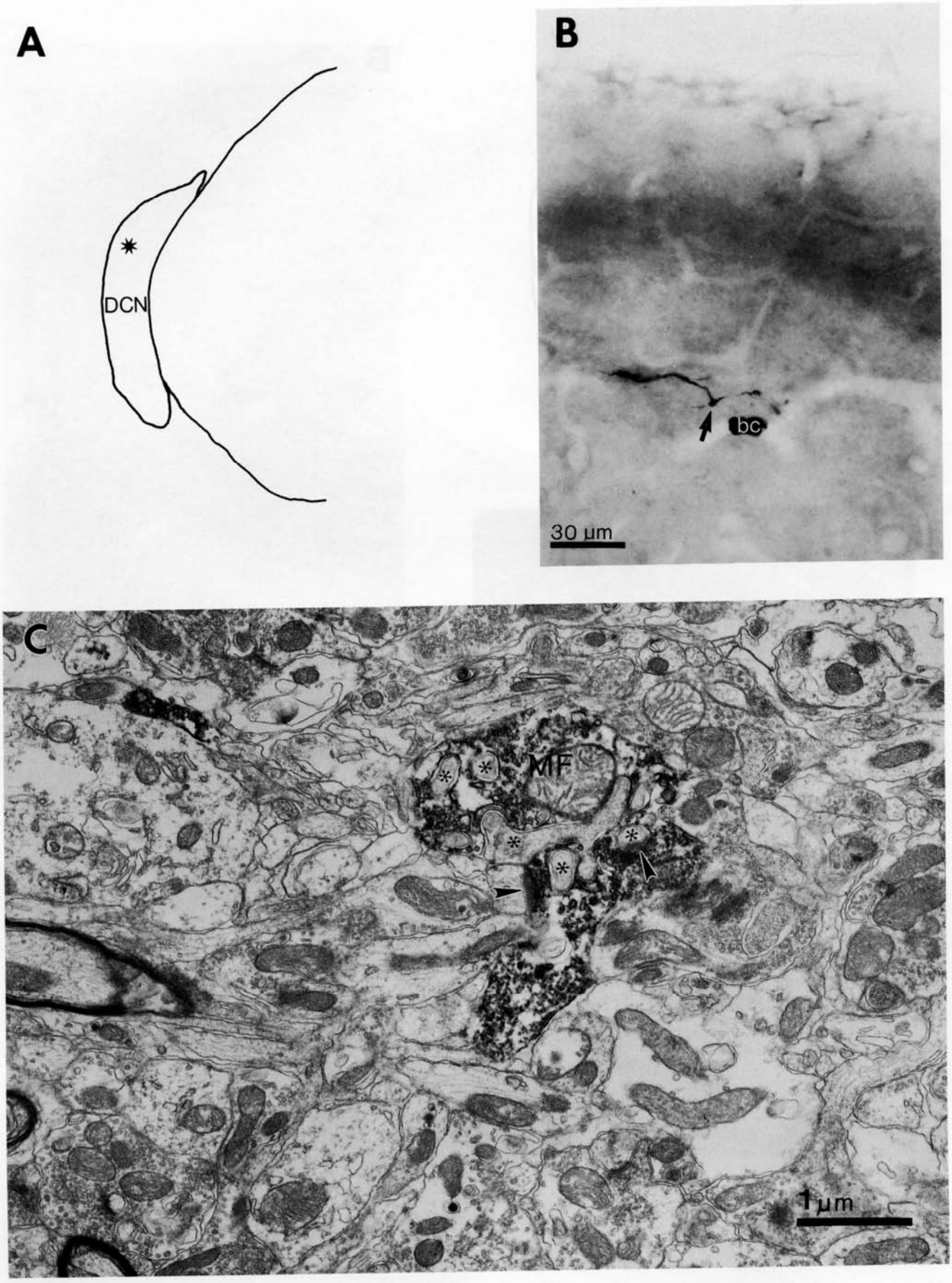

Fig. 6. The en passant swellings of cuneate fibers are smaller, but they have an ultrastructural appearance similar to the terminal swellings. A labeled fiber in layer 2 of the DCN (**A**, star) gives rise to several swellings, one of which is clearly en passant (**B**, arrow). Endogenous peroxidase in blood cells (bc) has reacted with 3,3′-diaminobenzidine tetrahydrochloride (DAB). **C:** The en passant swelling envelopes and forms synapses (arrowheads) with finger-like dendritic extensions (asterisks). These postsynaptic structures are characteristic of granule cell dendritic claws. A stretch of the labeled fiber is visible in the upper left corner of the micrograph.

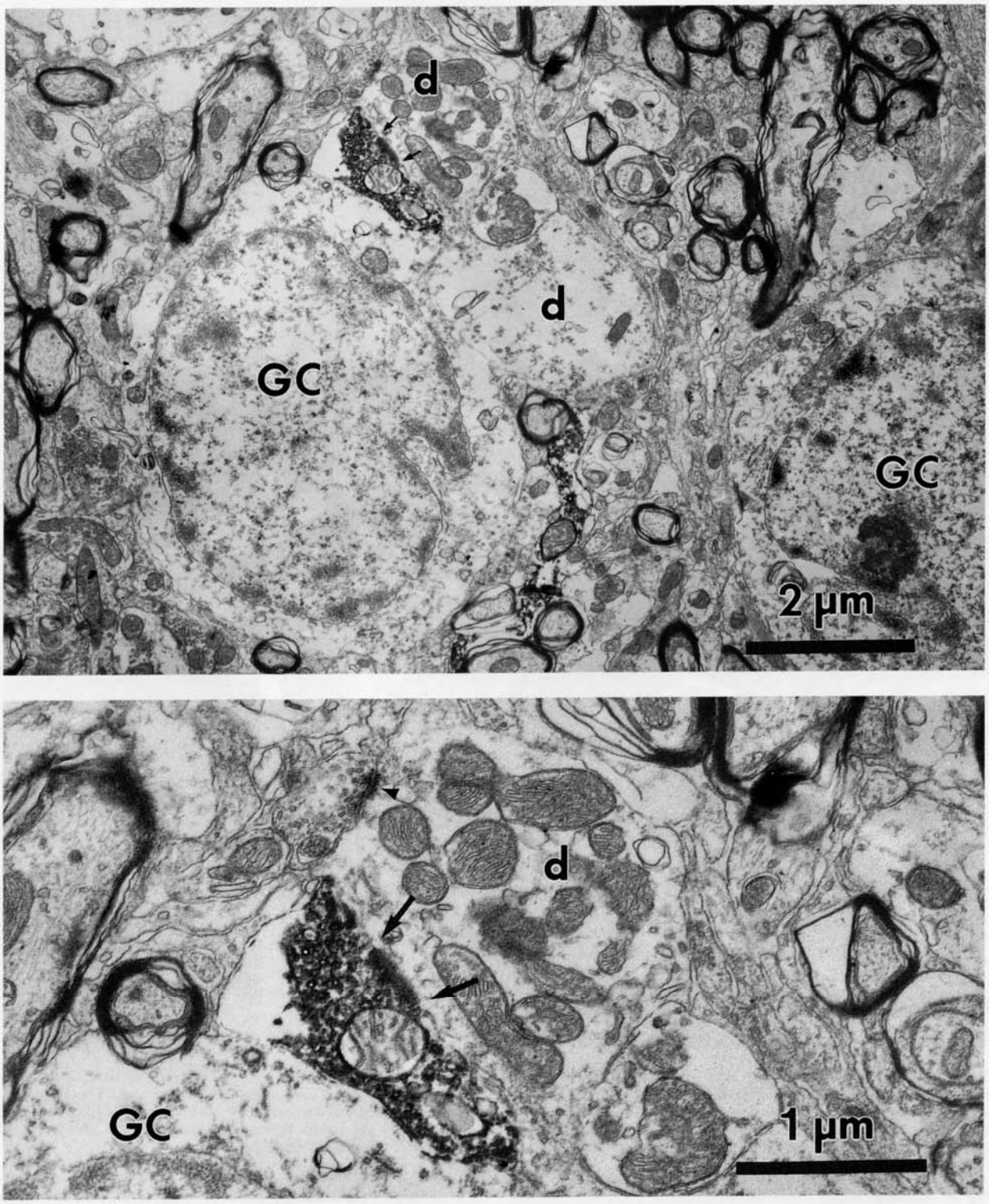

Fig. 7. Cuneate mossy fiber contacts the proximal dendrite of a granule cell. **A:** A labeled mossy fiber bouton forms a synapse (flanked by arrows) with the proximal dendrite (d) of a granule cell (GC) in the DCN. A second granule cell (GC) is visible on the right. **B:** A higher magnification electron micrograph of the same labeled bouton shown in A illustrating its prominent postsynaptic density (flanked by arrows). A terminal containing flattened synaptic vesicles makes a symmetric synapse (arrowhead) on the dendrite just above the mossy fiber terminal.

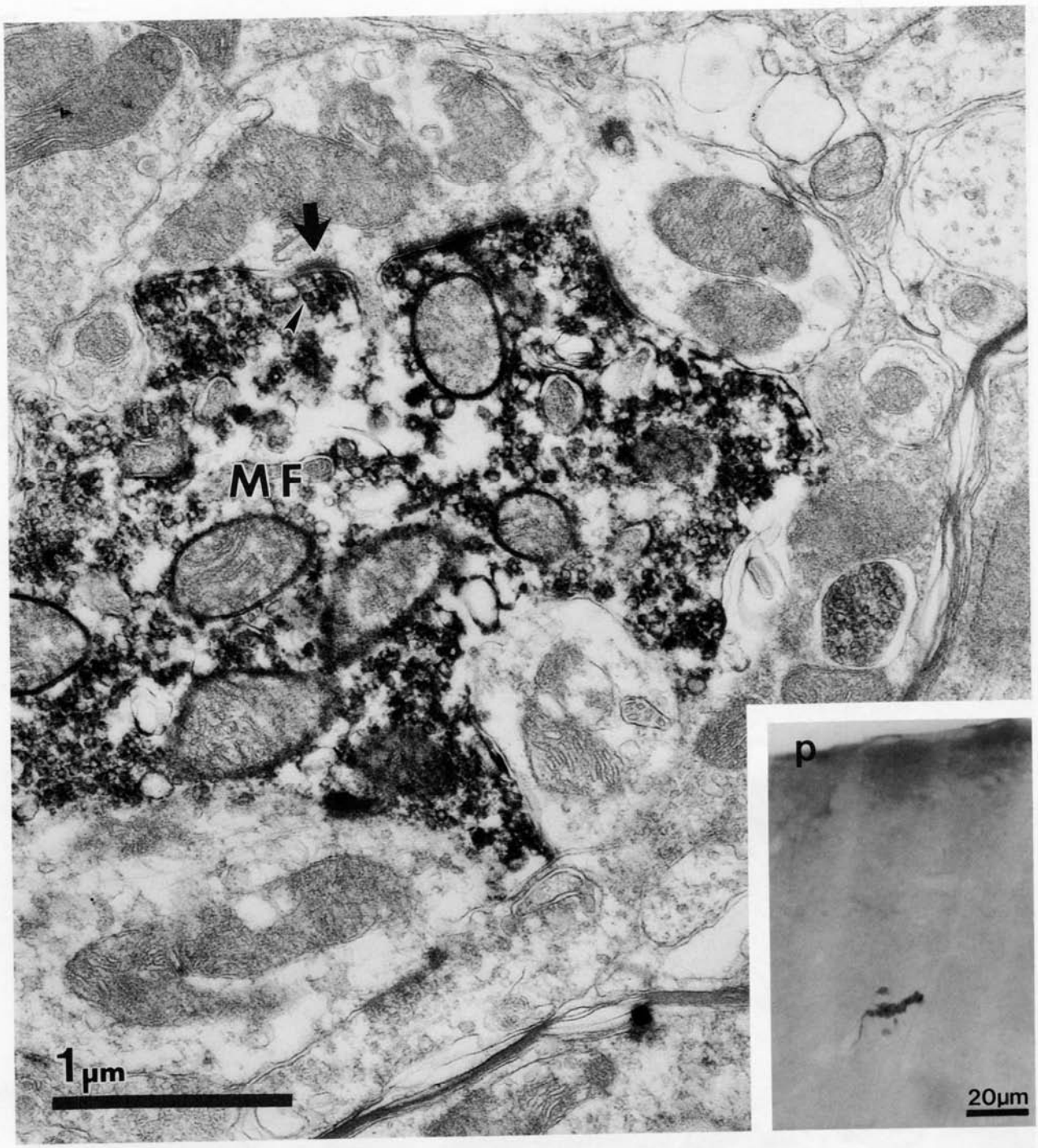

Fig. 8. Cuneate mossy fibers form type 1 synapses. Round synaptic vesicles and asymmetric synapses are characteristic of type 1 synapses that presumably exert an excitatory postsynaptic effect. This electron micrograph of a mossy fiber terminal (MF) clearly demonstrates an asymmetric synapse, where the synaptic density is most prominent on the postsynaptic side (arrow). The labeled terminal is filled with round synaptic vesicles (one is indicated with an arrowhead). This thin section was taken from a large mossy fiber located in layer 2 of the DCN. A light micrograph of the entire terminal is shown in the **inset**. The pial surface (p) of the DCN is indicated.

Fig. 9. Cuneate mossy fibers are enriched in glutamate but not γ-aminobutyric acid (GABA) or choline acetyltransferase. Electron micrographs (A–D) of serial sections through an anterogradely labeled terminal in the ventral cochlear nucleus. Ultrathin sections of the labeled terminal were processed with postembedding immunogold techniques using antibodies against glutamate (A), GABA (B), choline acetyltransferase (C), and without primary antibody (D). **A:** The section stained for glutamate labels the PHA-L-immunopositive mossy fiber terminal in addition to several neighboring processes (three are indicated by asterisks). Other processes are relatively unstained by the glutamate antibody (solid stars). **B:** The PHA-L-labeled terminal is not immunoreactive for GABA. Several of the neighboring processes stained for GABA correspond to those in A that are not enriched in glutamate (solid stars), whereas some processes devoid of GABA staining correspond to those that are heavily labeled with the glutamate antibody (asterisks). **C:** An antibody against choline acetyltransferase does not label the PHA-L-containing cuneate fiber. One process in the electron micrograph is lightly labeled for choline acetyltransferase (open star). **D:** A section where the primary antibody was omitted in the postembedding processing demonstrates a very low, nonspecific staining from the gold-conjugated secondary antibodies. Open stars in A, B, and D mark the same dendrite that is stained in C. Scale bar applies to A–D.

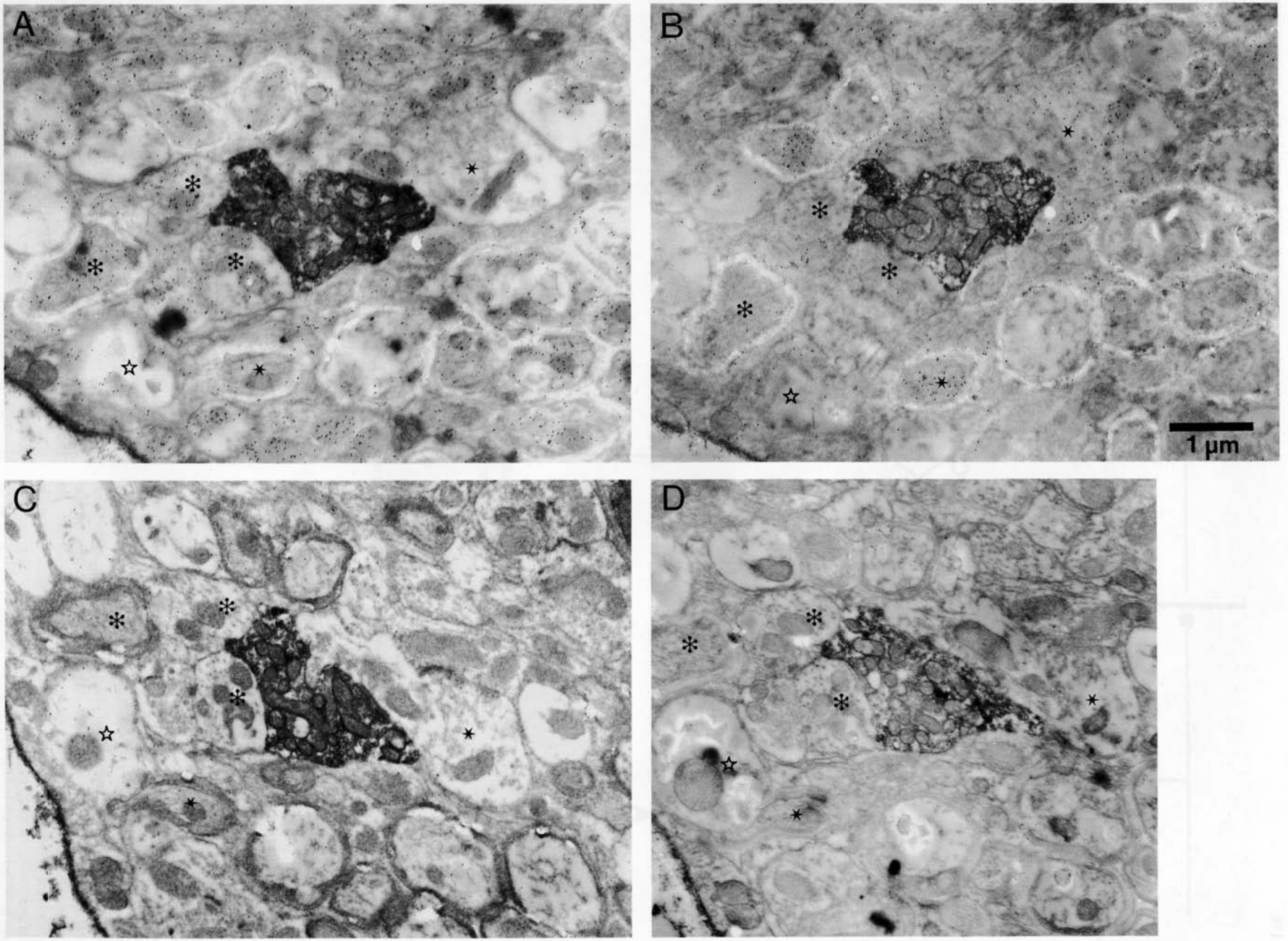

Figure 9

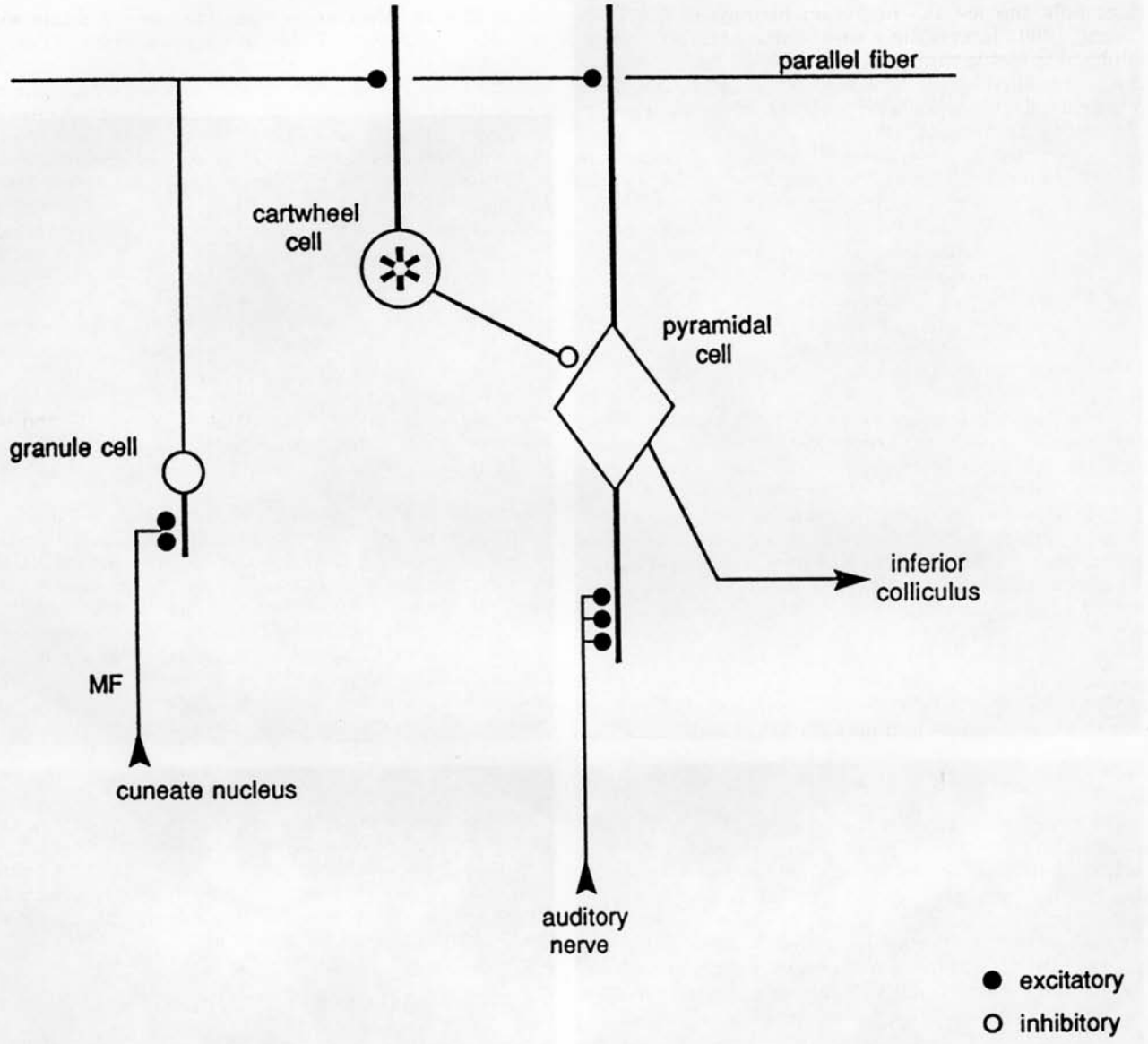

Fig. 10. Schematic illustration of somatosensory input to part of the granule cell circuit in the cochlear nucleus. MF, mossy fiber.

nucleus, and the resulting structural arrangement is reminiscent of previous descriptions of cochlear nucleus glomeruli (Mugnaini et al., 1980).

The origin of mossy fibers in the cochlear nucleus was not known until the present study. The cuneate nucleus region contributes one mossy fiber population that immunostains with antibodies for glutamate. In addition to these cuneate mossy fibers, there is evidence for a separate population of mossy fibers that contact the unipolar brush cells in the cochlear granule cell domains. These mossy fibers are enveloped by a single thick dendrite with long nonsynaptic appendages, characteristic of the unipolar brush cells (Floris et al., 1994; Wright and Ryugo, 1995). None of the cuneate mossy fibers that we analyzed with serial-section electron microscopy were observed to contact unipolar brush cells; rather, cuneate mossy fibers were surrounded by multiple small dendritic profiles. There is still a remote possibility for a population of mossy fibers that, on the basis of acetylcholinesterase histochemistry, may be cholinergic (McDonald and Rasmussen, 1971; Osen et al., 1984). Acetylcholinesterase staining, however, has proven to be somewhat ambiguous in the cochlear nucleus, because it fails to correspond to staining produced by antibodies directed against choline acetyltransferase (Godfrey and Yao, 1995; Wright and Ryugo, unpublished observations).

Although cuneate mossy fibers exhibit the morphological characteristics of excitatory synapses onto their targets in the cochlear nucleus, the overall effect of mossy fiber activation may be to inhibit some of the output by the pyramidal cells of the DCN. Electrophysiological studies of the cuneocochlear pathway in the cat have demonstrated that stimulation of the dorsal column/spinal trigeminal areas strongly inhibits type IV units in the DCN (Young et al., 1995), where type IV units correspond to pyramidal and

giant cells and are the projection neurons of the DCN (Young, 1980). Interestingly, these units were also strongly inhibited by tactile stimuli around the head, and rotation of the pinna elicited an inhibitory response in the DCN principal cells that exceeded stimulation of any other region of the body (Young et al., 1995).

The idea that cuneate mossy fibers serve to inhibit the output of some DCN projection neurons is consistent with our current view of how the cuneate projections might participate in the granule cell circuit. A simplified schematic diagram of cuneate mossy fiber input to part of the granule cell circuit is shown in Figure 10. Excitatory inputs from the cuneate nucleus activate groups of granule cells in the cochlear nucleus (Wright et al., 1994) that are known to innervate both cartwheel cells (Wouterlood and Mugnaini, 1984) and pyramidal cells (Mugnaini et al., 1980; Hirsch and Oertel, 1988; Manis, 1989) by way of parallel fibers. The granule cells appear to be excitatory based on their generation of excitatory postsynaptic potentials in postsynaptic neurons (Manis, 1989), whereas the cartwheel cells are considered to be inhibitory. Terminals of cartwheel cells contain pleomorphic synaptic vesicles, form symmetric synaptic junctions (Berrebi and Mugnaini, 1991), and are immunoreactive for glutamic acid decarboxylase, which is the enzyme involved in GABA synthesis (Mugnaini, 1985), and for glycine (Osen et al., 1990). Therefore, a somatosensory stimulus could initiate granule cell activation of cartwheel cells, which, in turn, would mediate inhibition of some pyramidal cells. The consequence of this circuit might render some pyramidal cells less responsive to auditory nerve activity. Because granule cells also provide excitation to the pyramidal cells, however, it is not clear how the excitatory and inhibitory inputs onto these cells would be balanced.

The large size of some of the labeled terminals and the extent of the projections from the cuneate region into the cochlear nucleus indicate that somatosensory cues are important for auditory information processing at a very early stage in the auditory pathway. The type of somatosensory information carried by these mossy fiber projections, however, is not entirely clear. Previous studies in rat have shown that injections of HRP into the cochlear nucleus retrogradely labeled cells mainly on the lateral edge of the cuneate nucleus, but a few labeled cells were also seen scattered around the cuneate and gracile nuclei and in the spinal trigeminal nucleus (Weinberg and Rustioni, 1987). The lateral cuneate nucleus is the site of termination of cutaneous primary afferent fibers arising from the neck and distal ear (Maslany et al., 1991) as well as proprioceptive primary afferent fibers arising from suboccipital muscles (Prihoda et al., 1991). In fact, direct projections from the C2 dorsal root ganglion have been shown to have a small terminal field in the medial edge of the ventral cochlear nucleus (Pfaller and Arvidsson, 1988). The kinds of somatosensory information provided by these projections imply that cues relating head and pinna position are used for processing acoustic information, most likely in terms of orienting to a sound source.

In addition to the normal binaural (e.g., intensity and time difference) and monaural (e.g., spectral) acoustic cues, sound localization also should involve knowledge of head and pinna position with respect to body axis. Vestibular inputs to the cochlear nucleus also might be used in animals such as cats and rodents, where the pinna can move independently of the head. Thus, the system would be endowed with information regarding sound location with respect to relative and absolute body position. There is some support for this notion from reports that primary vestibular afferents project to the cochlear nucleus in the gerbil (Kevetter and Perachio, 1989). One could imagine that even tactile sensations from the face, particularly from the whiskers or vibrissae, might provide additional cues for orienting in space (see, e.g., Drager and Hubel, 1976). These functions might also require inputs from other regions, including auditory cortex, inferior colliculus, type II auditory nerve fibers, and medial olivocochlear efferents. All of these structures send projections to the granule cell domains of the cochlear nucleus (Brown et al., 1988a,b; Benson and Brown, 1990; Feliciano et al., 1993; Saldaña, 1993; Weedman et al., 1994). In a parallel fashion, there are multimodal nonauditory inputs to the auditory pathway at the level of the inferior colliculus (Schroeder and Jane, 1975; RoBards, 1979; Willard and Ryugo, 1983) and the medial division of the medial geniculate nucleus (Wepsic, 1966; Ryugo and Weinberger, 1978; Winer and Morest, 1983).

These multimodal mossy fiber inputs to the DCN resemble very closely those of the cerebellum. The similarities in cell typing and cell stratification within the two structures raises an implicit suggestion that the DCN may have a cerebellar-like function (Mugnaini and Morgan, 1987). If the cerebellum is involved in motor learning, where neural templates for anticipated vs. executed movements are compared, then an "acoustic" cerebellum may be necessary to encode consecutive temporal signals for stabilizing the location of a sound source when there are movements of the head and/or pinna. Sequential acoustic templates conceivably could be used for any number of other processing tasks requiring an acoustic cerebellum, including, for example, the acquisition of conspecific communication (or language in the case of humans), where mimicry plays such an important role. Whatever the case, the multimodal nature of inputs to the DCN reflects not only the highly integrative demands of auditory behavior but also the greatly redundant nature of the auditory pathway.

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