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RESEARCH ARTICLE

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Cerebellar unipolar brush cells are targets of primary vestibular afferents: an experimental study in the gerbil

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Abstract The unipolar brush cell (UBC) is an excitatory glutamatergic interneuron, situated in the cerebellar granular layer, that itself receives excitatory synaptic input on its dendritic brush from a single mossy fiber terminal in the form of a giant glutamatergic synapse. The UBC axon branches within the granular layer, giving rise to large terminals that synapse with both granule cell and UBC dendrites within glomeruli and resemble in morphological and functional terms those formed by extrinsic mossy fibers. So far, the only demonstrated extrinsic afferents to the UBC are the choline acetyltransferase (ChAT)-positive mossy fibers, some of which originate from the medial and descending vestibular nuclei. To ascertain whether UBCs are innervated by primary vestibular fibers, we performed a tract-tracing light and electron microscopic study of the vestibulocerebellum in gerbils. Macular and canal vestibular end-organs were individually labeled by injection of biotinylated dextran amine. After an appropriate survival time, gerbils were then processed for light and electron microscopic analysis of central vestibular projections. In the nodulus and uvula, labeled primary vestibular fibers formed mossy terminals synapsing with both granule cells and UBCs in all of the injected gerbils. Thus, innervation of UBCs by extrinsic mossy fibers carrying static and dynamic vestibular signals represents the first synapse of networks that contribute a powerful form of distributed excitation in the granular layer.

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Introduction

The cerebellar unipolar brush cell (UBC) is one of the nontraditional types of interneuron residing in the granular layer (Mugnaini and Floris 1994; Mugnaini et al. 1997; Diño et al. 2000a, 2000b). Among the neurons of the cerebellar cortex, only granule cells (Eccles et al. 1967) and UBCs (Diño et al. 2000b; Nunzi et al. 2001) are excitatory. UBCs receive excitatory synaptic input on the dendritic brush from a single mossy fiber (MF) terminal in the form of a giant glutamatergic synapse (Rossi et al. 1995; Kinney et al. 1997; Slater et al. 1997, 2000). UBC axons branch within the granular layer, giving rise to large glutamatergic terminals that synapse with both granule cell and UBC dendrites within glomeruli and resemble in morphological terms those formed by extrinsic MFs (Diño et al. 2000a, 2000b; Nunzi and Mugnaini 2000, 2001). These hitherto unappreciated UBC networks contribute a powerful form of distributed excitation within the basic circuit of the cerebellum, and it is crucial to identify the sources of their mossy fiber inputs.

The predominant distribution of UBCs in the vestibulocerebellum (Diño et al. 1999; Takács et al. 1999) suggests that UBCs may receive vestibular inputs. To date, the only known extrinsic afferents to UBCs are the choline acetyltransferase (ChAT)-positive MFs (Jaarsma et al. 1996), which originate from the medial vestibular and prepositus hypoglossal nuclei (Barmack et al. 1992). Light and electron ChAT immunocytochemistry revealed that only about one-fifth of UBCs situated in the nodulus/uvula are innervated by ChAT-positive MF rosettes (Jaarsma et al. 1996), suggesting that there may be other extrinsic sources of MF input to UBCs.

To determine if UBCs receive inputs from primary vestibular afferents, we injected the tract-tracer biotinylated dextran amine (BDA) in gerbil vestibular end-organs, and then analyzed the nodulus and uvula by elec-

tron microscopy to identify the terminals of labeled peripheral MFs and their target cells. Our results demonstrate unequivocally that UBCs receive synaptic inputs from primary vestibular afferents innervating canal and macular end-organs.

Some of the data were presented elsewhere in preliminary form (Diño et al. 1997, 2000a).

Materials and methods

Animals

Thirty adult gerbils purchased from a commercial breeder were used for these experiments. All efforts were made to minimize both the suffering and the number of animals used. All experiments conformed to protocols approved by the Northwestern University Center for Experimental Animal Resources (CEAR) and by the Institutional Animal Care and Utilization Committee of the University of Texas Medical Branch, both AAALAC-accredited facilities. Animal procedures followed guidelines issued by the National Institutes of Health and the Society for Neuroscience.

Peripheral tracer injections

Gerbils were anesthetized (2 mg/kg body wt. xylazine and 40 mg/kg body wt. ketamine, i.p.) and mounted on the customized head holder of a Kopf stereotaxic frame. With sterile technique, a 0.6-mm dental burr was used to approach the vestibule. A glass pipette, with its tip broken to a final diameter of 20–40 µm, was backfilled with 0.2–5 µl of a 5–25% solution of rhodamine-linked BDA (Molecular Probes, Eugene, OR) in 0.01 M phosphate buffer (PB) (pH 7.4). The pipette was lowered into the sensory neuroepithelium of the left vestibular end-organ of interest and BDA was pressure injected (30 psi) over a 5-min period. After a survival time of 6–10 days, the animals were deeply anesthetized and perfused transcardially with 0.9% saline solution followed by a fixative containing 4% freshly depolymerized paraformaldehyde and 0.1% glutaraldehyde in 0.12 M phosphate buffer, pH 7.4.

In 21 of the gerbils, the pigmented epithelium and membranous labyrinth of the cristae ampullares and the otolithic membranes of the utricular and saccular maculae were carefully teased from the sensory epithelium to allow successive visualization of the chromogen. Tissue was then incubated with ABC complex (Vector Laboratories, Burlingame, CA) using the cobalt intensified diaminobenzidine (DAB)/glucose oxidase reaction (Kevetter and Perachio 1986; Adams 1977). The tissue was then dehydrated in alcohol/propylene oxide and embedded in Epon resin. End-organs were examined to verify injection sites and identify any spread to adjacent end-organs.

In the remaining nine gerbils, the right and left temporal bones containing the exposed vestibular end-organs were bathed in fixative for an additional hour postperfusion before they were decalcified overnight (Calex; Fisher Scientific, Pittsburgh, PA). After cryoprotection in a 30% sucrose/saline solution, the temporal bones containing injected vestibular end-organs were serially sectioned (30–40 µm) in a cryostat. Some of the sections were then viewed under a fluorescence microscope, while others were treated with 10% methanol and hydrogen peroxide solutions in PB to abolish endogenous peroxidase activity, and then incubated in avidin-biotin peroxidase solution following the Elite ABC/DAB protocol (Vector Laboratories, Burlingame, CA). Sections were examined to identify the injected site and identify any spread to other end-organs.

Primary afferents in the cerebellum

To verify BDA transport to CNS targets, the brainstem and cerebellum were dissected out of the skull and either cryoprotected in 10%, 20% and 30% sucrose/PB solutions and then cut at 20 µm in a freezing microtome (brainstem), or sliced at 40 µm on a Vibratome (left half of the cerebellum). Sections were then processed for light and/or electron microscopy.

Light microscopy

To detect labeled primary vestibular afferents, we utilized the immunocytochemical procedure described by Saldaña et al. (1996), wherein BDA was detected with ABC complex using DAB as chromogen (brown reaction product). Sections then were processed for calretinin immunocytochemistry to identify UBCs, as previously described by Floris et al. (1994), except that SG substrate (Vector Laboratories, Burlingame, CA) was used as a chromogen (blue reaction product). Mouse monoclonal antibody to calretinin (1:1000) was purchased from Chemicon International (Temecula, CA).

Seven of the gerbils with extracellular injections of BDA into vestibular end-organs showed labeled fibers in the granular layer of the vestibulocerebellum and were selected for further study.

Electron microscopy

Because UBCs are easily identified under the electron microscope, ultrathin sections were single labeled to detect BDA by the ABC method (Vector) using DAB as chromogen. Immunoreacted Vibratome slices of the nodulus/uvula folia were postfixed in buffered 1% OsO_4, pH 7.3, rinsed and stained en bloc in 1% uranyl acetate in maleate buffer, pH 5.2–6.0, and then dehydrated, infiltrated and polymerized as described (Floris et al. 1994).

Series of 100 ultrathin sections (75 nm thick) from the nodulus/uvula were cut on the ultramicrotome and collected on single-hole copper grids covered with Formvar. Out of these series, ultrathin sections spaced 1 µm apart were analyzed under a Zeiss EM-10 electron microscope operated at 80 kV to identify target cells of labeled afferent fibers.

Results

Light microscopy

Injection sites

Most of the gerbils with BDA-injected vestibular end-organs showed labeled injections sites and a varying density of labeled fibers in the vestibular nuclei, but either had sparsely labeled fibers in the cerebellar cortex or did not show labeling of cerebellar afferents at all. The seven gerbils selected for study had distinct labeling of the BDA-injected end-organ and the corresponding vestibular fibers were labeled along their course to the nodulus/uvula. Histological examination showed that two of these animals had the BDA injection site centered on the crista of the lateral canal (Fig. 1A), two on the crista of the lateral canal with some spread to the entire labyrinth (Fig. 1B), two on the utricular macula (Fig. 1C) with some spread of BDA to the lateral canal, and one on the saccular macula with some spread to the entire labyrinth. In some instances, the labeling in adjacent structures appeared to include terminals of the vestibular efferent neurons since they were characterized by relatively large areas of innervation with bouton-only endings (Purcell and Perachio 1997). Spread of the tracer outside the in-

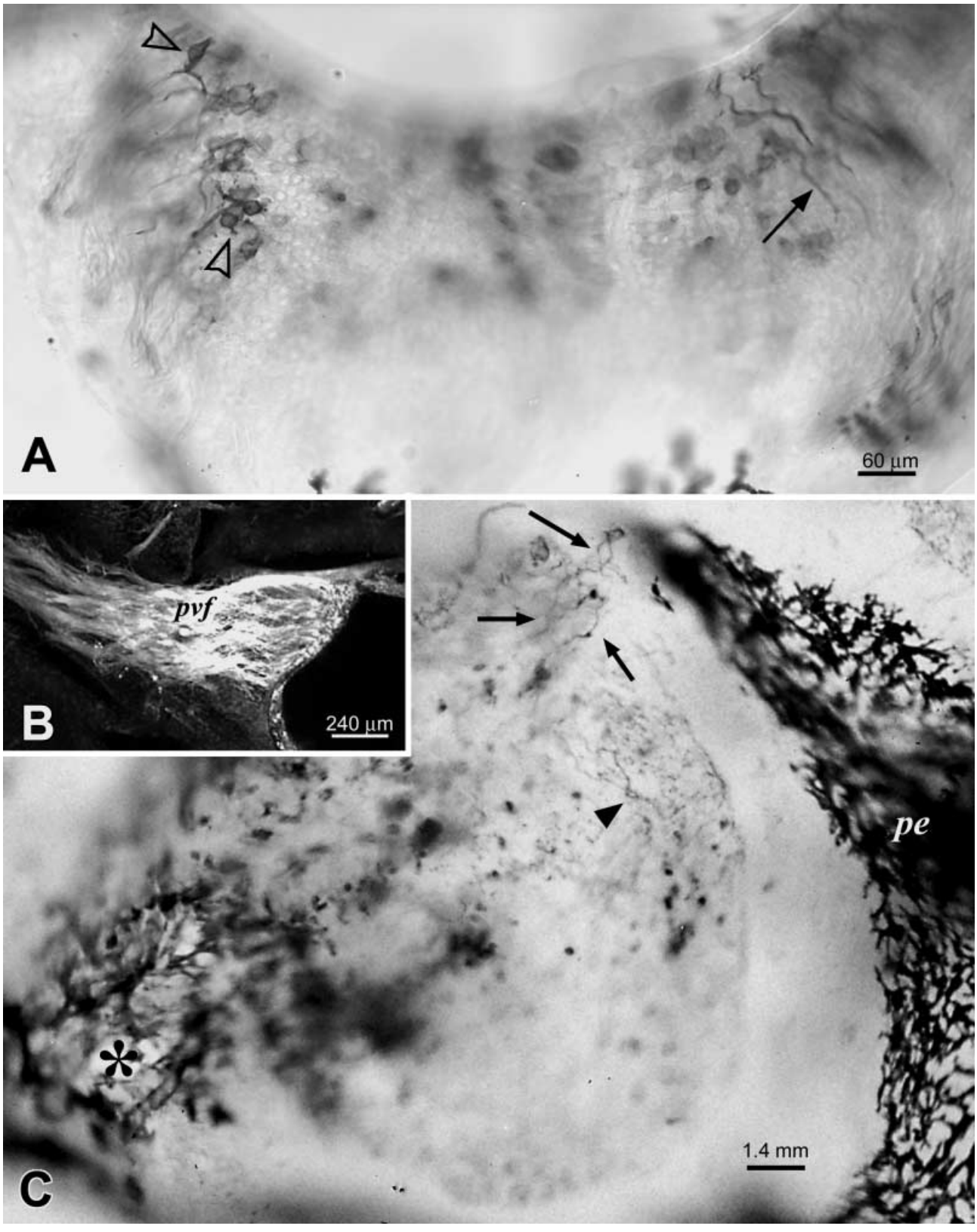

Fig. 1A–C BDA-injected vestibular end-organs. **A** Side view of lateral canal crista in ABC/DAB reacted whole-mount. *Arrowheads* point to labeled afferent terminals in the sensory epithelium. *Arrow* points to some of the underlying primary afferent fibers. **B** Fluorescence microscopy of a section of the decalcified temporal bone containing injected lateral canal crista labeled with rhodamine-conjugated BDA. (*pvf* labeled primary vestibular fibers). **C** *Top view* of utricular macula in ABC/DAB reacted whole-mount (*asterisk* injection site, *arrows* labeled primary vestibular fibers, *arrowheads* labeled efferent fibers, *pe* pigmented epithelium)

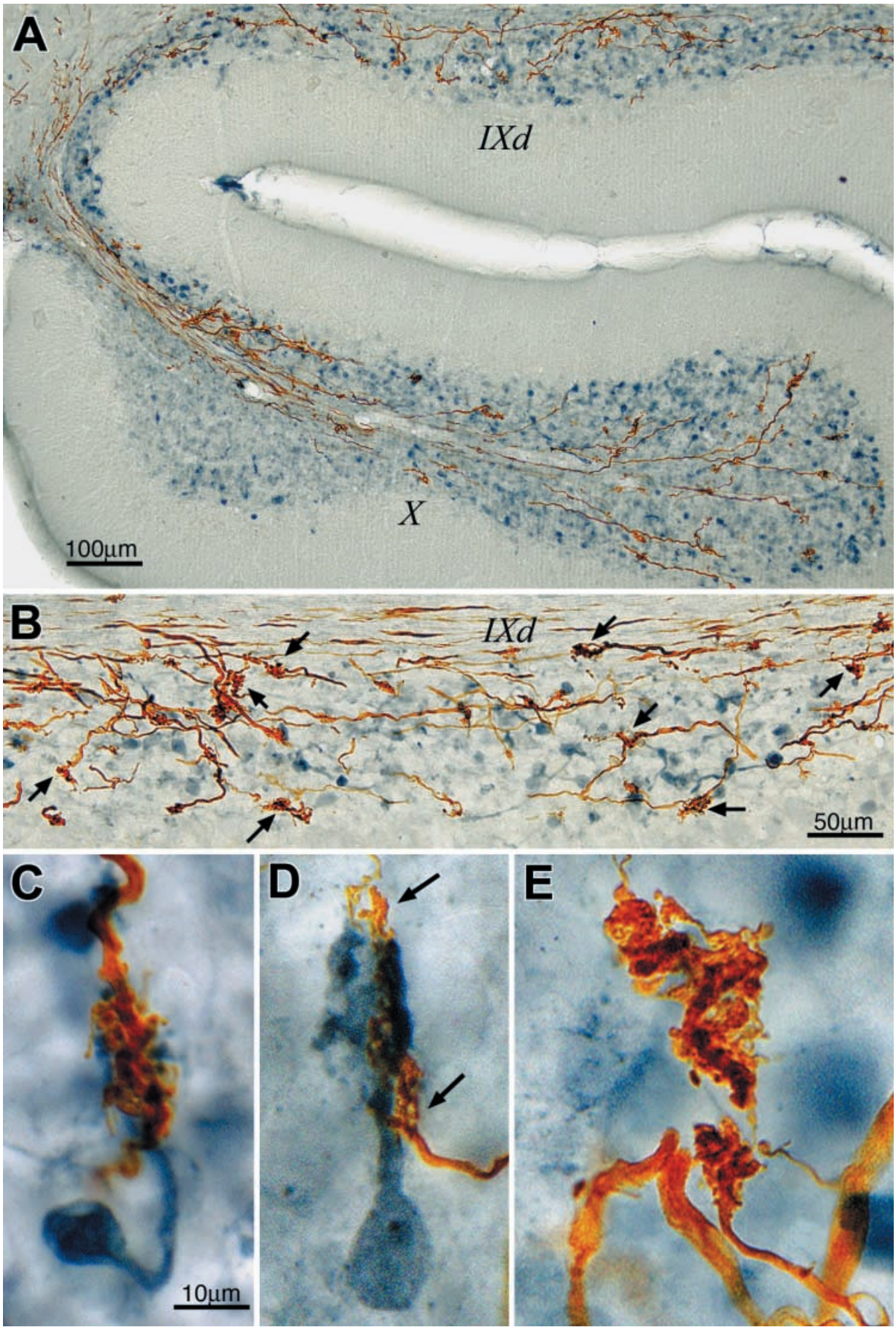

Fig. 2 Legend see page 166

jected end-organ appeared as faint staining of the surface of epithelium lining the labyrinth.

Trajectory and cerebellar distribution of labeled fibers

Upon entering the vestibular nuclear complex, the primary vestibular fibers divided, one branch innervating the vestibular nuclei and the other coursing through the superior vestibular nucleus and the dorsal division of the lateral vestibular nucleus before entering the cerebellum. In the cerebellum, labeled primary afferents arched medially and caudally through the medial cerebellar nucleus and passed into the folial white matter of the nodulus/uvula. Cerebellar branches formed collaterals innervating the medial cerebellar nucleus and terminated in the granular layer of the nodulus/uvula (Fig. 2A, B). Labeled fibers also terminated in lobules I, V, and VI, but to a much lesser degree (not shown). Neurons of the vestibular nuclei, medial cerebellar nucleus, and nodulus/uvula were invariably BDA negative.

Mossy terminals of labeled primary afferents

Within the granular layer, all labeled primary vestibular afferents formed MF rosettes (Fig. 2C–E). Some of the labeled terminals intimately interdigitated with (Fig. 2C) or were apposed on one side (Fig. 2D) to the brushes of calretinin-positive UBCs. These terminals presumably occupied glomeruli containing dendrioles of the UBC brush (Fig. 2C) or brush dendrioles mixed with granule cell dendrites (Fig. 2D). Other labeled terminals occupied neuropil areas free of blue reaction product (Fig. 2E), presumably representing glomeruli containing dendrites belonging exclusively to granule cells.

Electron microscopy

To determine if the labeled afferents innervating ampullary and otoliths organs differed in their targets, we first analyzed the postsynaptic targets of labeled MFs in two cases in which the injection site was centered either on the crista of the lateral canal or on the saccular macula. Because the same MF terminal may contact exclusively granule or UBC dendrites, or mixtures of granule and UBC dendrites (Mugnaini et al. 1997), we chose to analyze ultrathin sections spaced 1 µm apart. This spacing allowed repeated sampling from the same glomerulus as well as sampling from different glomeruli. We examined 17 MF terminals from gerbils with distinct BDA injection in the crista of the lateral canal, and 14 MF terminals in a gerbil with distinct BDA injection in the sacculus. Labeled ampullary MFs terminals formed synapses with both granule cell dendrites and UBC brush dendrioles (Fig. 3) in 71% (12/17) of the glomeruli, and exclusively with granule cell dendrites (Fig. 4) in 29% (5/17) of the glomeruli. Labeled saccular MF terminals formed synapses with both UBCs and granule cells or UBCs only in 71% (10/14) of the glomeruli, and with granule cells only in 29% (4/14) of the glomeruli. All of the synaptic junctions formed by labeled terminals were of the asymmetric category, which is typical of excitatory terminals. Synaptic junctions on granule cell dendrites measured <0.5 µm in length, while those on UBC dendrioles ranged in length from <0.5 µm to 1 µm or more, in accordance with previous data (Mugnaini et al. 1994, 1997).

In the remaining five gerbils processed for electron microscopy, ultrathin sections were analyzed at random. In all of these cases, we observed densely BDA-labeled mossy terminals that occupied the glomerular centers and formed synapses with UBCs, granule cells or both.

◀ **Fig. 2A–E** Panels show microphotographs from double-labeled cerebellar sections cut in parasagittal planes. BDA-labeled primary vestibular fibers are stained brown (DAB reaction) and calretinin-positive UBCs are stained blue (SG reaction). **A** Primary afferents to the nodulus (*X*) and ventral uvula (*IXd*) after tracer injection in the lateral canal crista. **B** Primary afferents to the ventral uvula (*IXd*) after tracer injection in the saccular macula. **C** The mossy terminal of a primary afferent from the saccular macula penetrates into the dendritic brush of a calretinin-positive UBC. **D** The mossy terminal of a primary afferent from the lateral canal crista adjoins the dendritic brush of a calretinin-positive UBC. The aspects of the mossy terminal unrelated to the brush (*arrows*) are free of blue reaction product, suggesting that, at these sites, the terminal is in contact with calretinin-negative granule cell dendrites. **E** The mossy terminals of two primary afferents from the lateral canal crista terminate in glomeruli free of blue reaction product, indicating that they are in contact with calretinin-negative granule cell dendrites. *Scale bar in* **C** applies also to **D** and **E**

Discussion

This study unequivocally demonstrates that UBCs receive synaptic inputs from primary vestibular fibers in gerbils. Although primary vestibular fibers and their terminals in the vestibular nuclei were labeled by the procedure, none of the neurons in the vestibular nuclei and medial cerebellar nucleus, some of which provide secondary vestibular fibers to the cerebellum, was BDA positive. This confirms previous data with similar injections (Kevetter and Perachio 1986). The trajectories and distribution of primary vestibular fibers we observed are essentially similar to those previously published (Korte and Mugnaini 1979). Thus, BDA extracellularly injected directly into vestibular end-organs should label exclusively vestibular fibers originating in the periphery, because BDA is not transported transynaptically (see also Kevetter and Perachio 1986).

Furthermore, our results indicate that both ampullary and otolith end-organs provide input to UBC networks in the nodulus and uvula. This is consistent with recent findings of retrograde labeling studies that demonstrated both canal and otolith organ-related inputs to the nodulus and uvula (Purcell and Perachio 2001). However, this conclusion is subject to the caveat that spillage of the tracer from individually injected end-organs may actual-

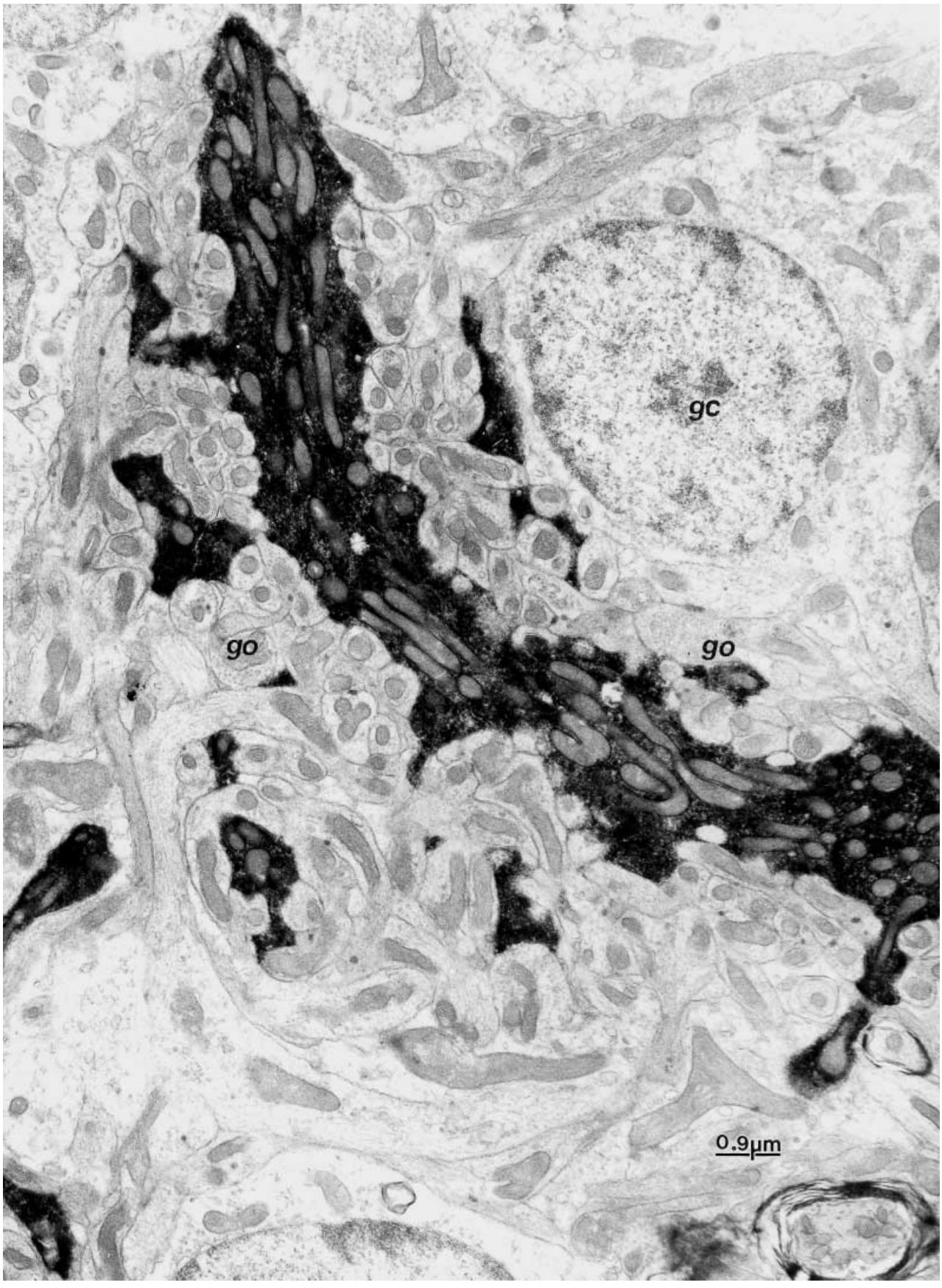

Fig. 3 Electron micrograph of the mossy terminal (electron-dense profiles) of a primary vestibular fiber labeled by BDA injected in the lateral canal crista. The main body of the rosette and its branches form synaptic contacts exclusively with small profiles representing granule cell dendrites (*go* Golgi cell axon terminal, *gc* granule cell nucleus)

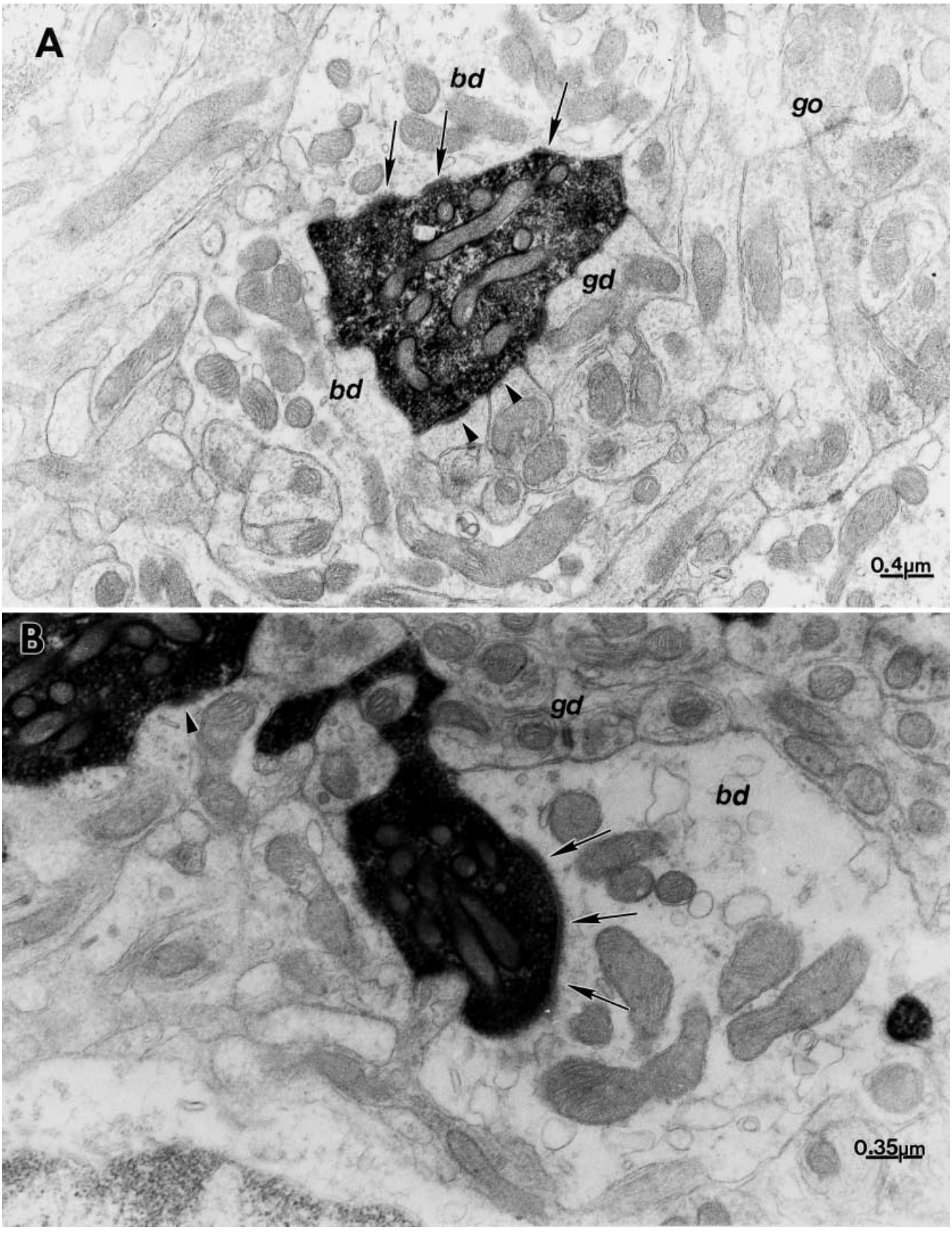

Fig. 4 Electron micrograph of the mossy terminal (electron-dense profiles) of a primary vestibular fiber labeled by BDA injected in the lateral canal crista. Branches of the mossy rosette form synaptic junctions with thick PSDs (asymmetric synapses) with UBC brush dendrioles (*bd*) and granule cell dendrites (*gd*) (*arrows* MF-UBC synapses, *arrowheads* MF-GC synapses, *go* Golgi cell axon terminal)

ly have reached other ampullary and otoliths organs at some distance from the injection site. However, this possibility appears of secondary relevance, because tracer diffusing from the injection site would be diluted in the endolymph, thus hardly reaching concentrations sufficient to effectively label other primary fibers than those ending within the injected end-organ. This assumption is supported by the observation that few of the injected animals had distinctly labeled fibers in the cerebellum, although they showed at least some labeled primary fibers in the vestibular nuclei. Furthermore, all of the canal and otolith afferents and terminals that we studied by electron microscopy had an intensely electron dense axoplasm. We concluded that effective BDA uptake and transport in primary afferents to the nodulus/uvula requires high concentration of the tracer at the peripheral terminal of bipolar vestibular ganglion cells, a condition that is met only at the injection site. Thus, the innervation of UBCs by primary afferents from both canal and otoliths organs rests on fairly solid anatomical grounds.

Our light and electron microscopic data show that primary vestibular afferents to the nodulus/uvula constitute the central elements of glomeruli in which the postsynaptic targets are represented exclusively by either granule cells or UBCs, or by mixtures of granule cells and UBCs. Primary vestibular fibers (this study), ChAT-positive secondary vestibular fibers (Jaarsma et al. 1996), and UBC axons (Diño et al. 2000a; Nunzi and Mugnaini 2000, 2001), therefore, have identical targets in the cerebellar cortex. This lack of target selectivity by MF terminals in the nodulus/uvula suggests that they share adhesion molecules with granule cells and UBCs that promote the formation of synaptic contacts. However, Diño et al. (1999) reported that pontine MFs seem to avoid UBCs, which suggests that categories of MFs may repel, or be synaptically incompatible with, UBCs, while favoring contact with granule cells.

In our sample, the percentages of granule cell and granule cell/UBC glomerular fields innervated by canal afferents and otolith afferents differed. This could suggest that primary vestibular fibers differ in their target selectivity, which may reflect their morphological, chemical and functional heterogeneity. It has been shown that primary afferent fibers innervating the lateral canal and sacculus course and have differential distributions in the vestibular nuclei, although afferents from both end-organs have overlapping projections in the nodulus and ventral uvula. Moreover, three types of primary vestibular afferents, i.e., calyx, dimorphic, and bouton types, have been identified, based on anatomic and physiologic criteria (Lysakowski et al. 1995; Fernandez et al. 1988, 1990, 1995; Baird et al. 1988). Desmadryl and Dechesne (1992) and Lysakowski et al. (1999) have also shown that calyx and bouton fibers have different chemical phenotypes, as they are specifically labeled by calretinin and the intermediate filament protein peripherin, respectively. However, our electron microscopic data do not allow any definitive conclusion in this regard, because we analyzed a small number of MF terminals and the spacing of ultrathin sections was not in accordance to unbiased, stereological sampling methods that ensure sampling from different glomeruli.

In conclusion, mossy fiber terminals of primary vestibular fibers connected to canal and otolith organs synapse with granule cells and also provide input to UBCs in the gerbil nodulus/uvula, as do ChAT-positive secondary vestibular fibers. Because first order UBCs are connected to other (second order) UBCs in the granular layer, their axons form a network contributing a powerful form of distributed excitation within the basic circuit of the vestibulocerebellum (Yan and Garey 1996; Diño et al. 2000b; Nunzi et al. 2001). There is evidence that the vestibular inputs and UBCs are functionally related. A recent study in the cat has shown that both granule cells and UBCs in the nodulus/uvula express c-*fos*, an immediate early gene activated by sustained stimulation, after continuous long-term horizontal axis rotation (Mugnaini et al. 2000). The vestibulo-ocular reflex eye movements evoked by this continuous rotation depend on an intact nodulus/uvula (Angelaki and Hess 1995), and are believed to require convergence of vestibular afferent signals with diverse response dynamics and spatial sensitivities. Thus, the distributed excitatory UBC networks may play a major role in the cerebellar contribution to reflex eye movements.

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