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Cerebellar choline acetyltransferase positive mossy fibres and their granule and unipolar brush cell targets: a model for central cholinergic nicotinic neurotransmission

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Summary

A subset of cerebellar mossy fibres is rich in choline acetyltransferase, the rate-limiting enzyme for the synthesis of acetylcholine. These choline acetyltransferase-positive mossy fibres are concentrated in the vestibulocerebellum and originate predominantly from the medial vestibular nucleus. The granular layer of the vestibulocerebellum is also enriched in unipolar brush cells, an unusual type of small neuron that form giant synapses with mossy fibres. In this immunocytochemical light and electron microscopic study, we explored whether choline acetyltransferase-positive mossy fibres innervate unipolar brush cells of the rat cerebellum. We utilized monoclonal antibodies to rat choline acetyltransferase of proven specificity, and immunoperoxidase procedures with 3,3'-diaminobenzidine tetrahydrochloride as the chromogen. A high density of choline acetyltransferase-positive fibres occurred in the nodulus and ventral uvula, where they showed an uneven, zonal distribution. Immunostained mossy fibre rosettes contained high densities of round synaptic vesicles and mitochondria. They formed asymmetric synaptic junctions with dendritic profiles of both granule cells and unipolar brush cells. The synaptic contacts between choline acetyltransferase-immunoreactive mossy fibres and unipolar brush cells were very extensive, and did not differ from synapses of choline acetyltransferase-negative mossy fibres with unipolar brush cells. Analysis of a total area of 1.25 mm^2 of the nodulus from three rats revealed that 14.2% of choline acetyltransferase-immunoreactive mossy fibre rosettes formed synapses with unipolar brush cells profiles. Choline acetyltransferase-positive rosettes accounted for 21.7% of the rosettes forming synapses with unipolar brush cells. Thus, the present data demonstrate that unipolar brush cells are innervated by a heterogeneous population of mossy fibres, and that some unipolar brush cells receive cholinergic synaptic input from the medial vestibular nucleus. The ultrastructure of these synapses is compatible with the possibility that choline acetyltransferase-positive mossy fibres co-release acetylcholine and glutamate. As the granular layer of the vestibulocerebellum contains nicotinic binding sites, the choline acetyltransferase-positive mossy fibres may be a model for studying nicotinic neurotransmission in the CNS.

Introduction

The granular layer of the vestibulocerebellum contains not only granule and Golgi cells, like other regions of the cerebellar granular layer, but is also enriched with unipolar brush cells (UBCs). Unipolar brush cells are a special type of small neuron, originally identified as 'pale cells' (Altman & Bayer, 1977), with a single brush-like dendritic arbor arising from a short stalk (Hockfield, 1987; Cozzi *et al.*, 1989; Munoz, 1990; Résibois & Rogers, 1992; Braak & Braak, 1993; Harris *et al.*, 1993; Berthié & Axelrad, 1994; Floris *et al.*, 1994; Mugnaini & Floris, 1994; Mugnaini *et al.*, 1994; Jaarsma *et al.*, 1995a; Rossi *et al.*, 1995; Mugnaini *et al.*, 1997). A characteristic feature of the UBCs is that they form extraordinarily extensive synaptic contacts (Harris *et al.*, 1993; Floris *et al.*, 1994; Mugnaini *et al.*, 1994), which had formerly been identified as *en marron* synapses and hairy dendrite synapses of Golgi cells (Hámori & Szentágothai, 1966; Chan-Palay & Palay, 1971; Mugnaini, 1972; Monteiro, 1986). Subtle variations occur in the fine structure of the UBCs and the

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mossy fibre rosettes, as well as in the configuration of the mossy fibre-UBC synapses, indicating a degree of functional heterogeneity yet to be understood in detail (Mugnaini *et al.*, 1994; see also Figs 107, 110–113 & 224 of Palay & Chan-Palay, 1974).

The biophysical and pharmacological properties of the mossy fibre-UBC synapse have been studied in detail with patch-clamp recording methods in thin cerebellar slices. (Rossi *et al.*, 1994,1995; Slater *et al.*, 1996a,b). These studies have demonstrated that the excitatory synaptic currents (EPSCs) elicited in UBCs by white matter stimulation are of unusually long duration and are mediated by ionotropic glutamate receptors. As a consequence of the unique ultrastructure of the mossy fibre-UBC synapse, entrapment of glutamate in the synaptic cleft appears to play a prominent role in determining the time course of the synaptic current. Identification of the origin(s) of the mossy fibre afferents would undoubtedly represent an important step towards further clarification of the role of the UBCs in cerebellar circuitry.

Mossy fibres in the granular layer of the cerebellar cortex and cochlear nuclear complex cumulatively derive their name from the rosette-like configuration of their terminals, which are invariably provided with multiple neurotransmitter release sites and form the structural and functional core of complex synaptic fields, or glomeruli. A number of studies, however, indicate that there are several morphologically and biochemically distinct types of mossy fibres, which possibly reflect their multiple anatomical origins (Brodal & Drabløs, 1963; Palay & Chan-Palay, 1974; King *et al.*, 1992; Dunn *et al.*, 1996; Voogd *et al.*, 1996). A subset of cerebellar mossy fibres are densely stained by immunocytochemistry for choline acetyltransferase (ChAT), the rate-limiting enzyme in the synthesis of acetylcholine (ACh). These ChAT-immunoreactive mossy fibres are enriched in the vestibulocerebellum, particularly the nodulus and the ventral uvula (lobules X and IXd; Kan *et al.*, 1978, 1980; Ojima *et al.*, 1989; Barmack *et al.*, 1992a), and presumably correspond to the acetylcholinesterase-positive mossy fibres (Brown & Palay, 1962; Csillik *et al.*, 1963; Shute & Lewis, 1965). The vast majority of these fibres originate from the medial vestibular nucleus and a smaller part from the nucleus prepositus hypoglossi (Barmack *et al.*, 1992b).

In this study we have re-investigated by light microscopic immunocytochemistry the distribution of cerebellar ChAT-positive mossy fibres and we have analysed the fine structural features of their synapses by immunoelectron microscopy. We show that ChAT-positive mossy rosettes innervate granule cells with simple synaptic junctions and UBCs with extensive synaptic junctions, and argue that these contacts could be useful models for studying ACh-mediated synaptic mechanisms in the CNS.

Materials and methods

Animals

Sprague-Dawley and Wistar rats of either sex, 150–300 g in body weight (b.w.) were used for this study. The animals were housed and handled according to approved guidelines supervised by institutional animal care committees. Before perfusion fixation through the ascending aorta (Friedrich & Mugnaini, 1981), the rats were deeply anaesthetized with sodium pentobarbital (40–45 mg kg^{-1} b.w., in saline) injected intraperitoneally.

Mouse monoclonal antibodies to rat ChAT

Monoclonal antibodies (mAbs) to rat brain ChAT were obtained as indicated (Cozzari *et al.*, 1990). Fusion of spleen cells with myeloma cells was done 3 months after primary immunization, and 12 IgG mAbs to be used in immunohistochemistry were purified from ascites over a column of Sepharose/protein-A. The monoclonal property of cell lines was established by repeated single cell cloning until it was determined that all wells showing cell growth also contained antibodies against native ChAT. Competitive binding of mAbs to ChAT revealed that seven distinct epitopes were recognized. Individual epitopes were later confirmed from cross-reactivity spectra of mAbs. Two mAbs, mAb 5 and mAb 17, turned out to be directed against an epitope common to a few mammalian species (rat, mouse, calf, pig, cat) and bind native ChAT with strong affinity, and they were used for the present study. Binding affinities of mAbs were calculated from immunoprecipitation tests at antibody concentrations which produced 50% enzyme precipitation. The mAbs did not show bands on Western blots. Accordingly, each one of them stained native ChAT spotted on nitrocellulose paper, but did not react with SDS-treated ChAT. No immunohistochemical stain was present in brain sections of species known to contain enzyme that is not recognized in immunoprecipitation tests after extraction.

Procedure for light microscopic immunocytochemistry

Eight Sprague-Dawley rats, four males and four females, and 12 Wistar rats, ten males and two females, were perfused with 0.12 M sodium phosphate buffer (PB) (pH 7.3) followed by 200 mL $100g^{-1}$ b.w. of a chilled fixative, consisting of 4% freshly depolymerized paraformaldehyde. The bodies were kept refrigerated for 1 h following perfusion, after which the cerebella were dissected out and cryoprotected in 30% sucrose in saline for 2 days. The brains were sliced at 30–40 μm in the sagittal or the coronal plane with a freezing-stage sliding microtome. Free-floating sections were processed for immunocytochemistry according to protocols for the peroxidase-anti-peroxidase (PAP) or the avidin-biotinylated peroxidase-complex (ABC) procedures, with 3,3′-diaminobenzidine tetrahydrochloride (DAB) as the chromogen. Sections were first thoroughly rinsed in 50 mM Tris-buffered saline (TBS) (pH 7.6) blocked for 1 h at 4° C in TBS containing 10% normal serum of the species of the secondary antibodies (goat or horse), and then incubated for 48 h at 4° C with mouse-anti ChAT monoclonal antibody (Ab 5 or Ab 17; Cozzari *et al.*, 1990) at 1:1000 in TBS containing 2% normal serum and 0.2% Triton X-100. Subsequently, sections were repeatedly rinsed, incubated

with biotinylated (1:200) or non-biotinylated (1:100) secondary antibodies for 2 h at room temperature, rinsed, incubated with ABC-reagent (1:100) or the PAP-reagent (1:100), respectively, and further processed for the DAB reaction with or without heavy metal intensification (Adams, 1981). To assess non-specific staining due to the immunoprocedure, in every experiment some sections were run in buffer without primary antibodies.

Procedure for pre-embedding immunoelectron microscopy

Six male Sprague-Dawley rats and six Wistar rats, four males and two females, were perfused with PB (pH 7.3) followed by 200 mL $100g^{-1}$ b.w. of a chilled fixative, consisting of 4% freshly depolymerized paraformaldehyde with the addition of 0.1% glutaraldehyde, and then with 100 mL $100g^{-1}$ b.w. of the same fixative without glutaraldehyde. The cerebella were sectioned at 50 µm on a Vibratome. The Vibratome slices were immersed in 15% and 30% sucrose in PB, 15 min each, then rapidly frozen in liquid nitrogen and immediately thawed. This 'freeze-thaw' treatment was repeated 2–3 times to improve penetration of immunoreagents. Sections were immunoreacted as specified above, with TBS as the diluent and rinsing solution, without detergent. After immunoreaction, slices were rinsed in PB and postfixed in 1% osmium tetroxide in PB, rinsed in distilled water, and dehydrated in 50% and 70% ethanol, contrasted for 45 min with 1% uranyl acetate in 70% ethanol, further dehydrated in ethanol-propylene oxide, and flat embedded in Epon between acetate sheets. Portions of the granular layer of the nodulus and the ventral uvula containing immunostained fibres were cut out and re-embedded flat on prepolymerized Epon blanks. Ultrathin sections, silver-yellow in interference color, were cut from the nodulus, the ventral uvula and the transition zone between the flocculus and the paraflocculus, contrasted for 3 min with lead citrate, and analysed in a Zeiss EM10 or a Phillips CM100 electron microscope operated at 60–80 kV.

Immunochemicals and other reagents

Goat anti-mouse sera and mouse clonoPAP were purchased from Sternberger Monoclonals Inc.(Baltimore, MD); biotinylated horse anti-mouse and ABC reagents from Vector Laboratories (Burlingame, CA); DAB from Aldrich (Milwaukee, WI); osmium tetroxide from EM Corp. (Cambridge, MA); glutaraldehyde and Epon from TAAB (Marivaac, Halifax, Nova Scotia), and paraformaldehyde from Polysciences (Warrington, PA). Other chemicals were from Sigma (St. Louis, MO).

Results

Light microscopy

Similar results were obtained with two batches of monoclonal anti-ChAT antibodies (Ab5 and Ab17). The overall staining pattern throughout the brain was generally consistent with distinct labelling of all putative and proven cholinergic nerve cell bodies and processes as previously described (Armstrong *et al.*, 1983; Houser *et al.*, 1983; Woolf, 1991; Lauterborn *et al.*, 1993; Butcher, 1995). Immunostaining was absent when primary antibodies were omitted.

Our light microscopic observations in the cerebellar cortex were generally in agreement with those of Ojima and colleagues (1989) and Barmack and colleagues (1992a). Only axonal structures were labelled. In the nodulus and the ventral uvula numerous ChAT-immunoreactive mossy fibres were present (Fig. 1). Typically, labelled mossy fibres gave rise to several branches and had large rosettes, located along the course and at the endpoint of the axon. *En passant* rosettes were somewhat less ramified than terminal rosettes. The frequency of occurrence of ChAT-positive rosettes in these folia was lower than the expected total number of glomeruli. This was confirmed in 'semithin' (0.5–2 µm thick) sections (not illustrated) of immunoreacted Vibratome slices embedded in resin and in ultrathin sections (see below), indicating that only a fraction of the mossy fibres that innervate the nodulus and the ventral uvula are ChAT-positive. The density of ChAT-immunoreactive mossy fibres showed a distinct variation throughout the mediolateral extent of the nodulus and ventral uvula. Labelled mossy fibres were present at higher density in four zones, approximately 0.6 mm wide, two of which flanked the midline and two others which occupied the lateralmost portions of these lobules. These zones were separated by a bilateral, approximately 0.5 mm wide, intermediate zone of lower density (Fig. 2) and poorly delimited borders. Visual analysis of immunostaining in male and female Sprague-Dawley and Wistar rats did not reveal differences between sexes and between strains.

Choline acetyltransferase-positive mossy fibres similar to those of the nodulus/ventral uvula occurred in other lobules, more frequently in the vermis than in the hemispheres, but always at much lower densities than in the nodulus/ uvula. The distribution of ChAT-immunoreactive mossy fibres in our rats was similar to that depicted in Figs 6 and 7 of Ojima and colleagues (1989). Frequently, labelled rosettes occurred in small foci of increased immunostaining density in both vermis and hemispheres. In certain regions, such the paramedian lobule, the density of labelled rosettes was always very low. There was an enrichment of labelled rosettes in the flocculus and in the transitional zone between the flocculus and ventral paraflocculus, as compared to the rest of the lateral cerebellum (not shown).

Other fibres containing ChAT-like immunoreaction product were also distributed as previously observed in the rat cerebellum (Ojima *et al.*, 1989; Barmack *et al.*, 1992a). These fibres consisted of: (1) a peculiar population of small mossy fibre profiles situated in a sharply delineated portion of the granular layer in the caudo-dorsal uvula (lobules IXa-b; not illustrated, but see Barmack *et al.*, 1992a); (2) thin beaded fibres in the granular, Purkinje and molecular layers of all folia (Figs 1B and 2); and (3) a thin fibre plexus innervating the cerebellar nuclei (not shown). Only the large

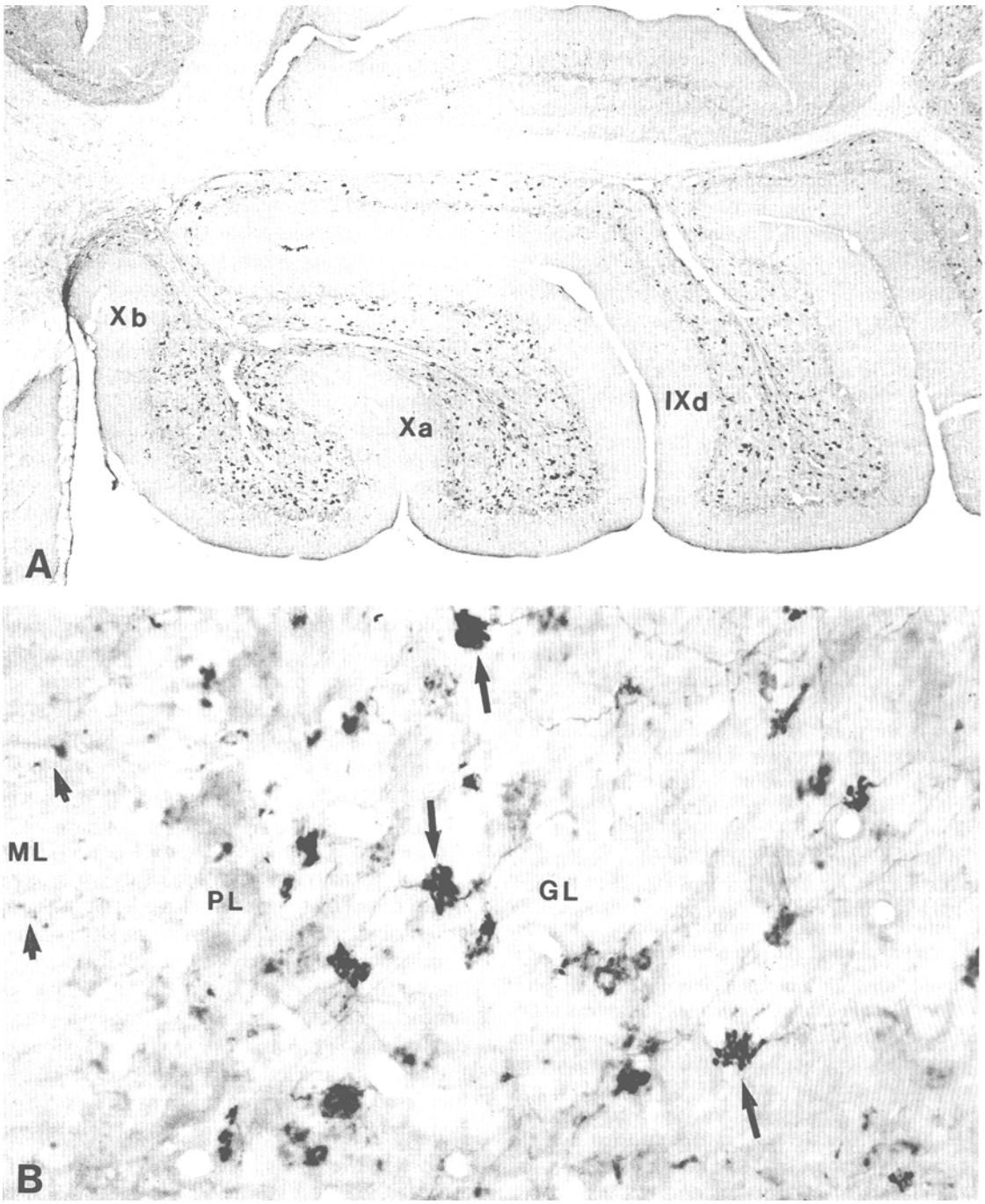

Fig. 1. Light photomicrographs from a parasagittal section of the caudal cerebellum in the rat, immunostained with monoclonal antibody to ChAT. (A) Immunostaining in the nodulus (lobules Xa and Xb) and the ventral uvula (lobule IXd) is primarily localized to missy fibres in the granular layer and their large rosettes. ×45. (B) Detail of the nodulus demonstrating ChAT-positive, large mossy rosettes (arrows) in the granular layer (GL) and small ChAT-positive endings (arrowheads) in the molecular layer (ML). PL, Purkinje cell layer. ×450.

mossy fibres of the nodulus and the ventral uvula were analysed further in this study. Other aspects of the cholinergic innervation in the cerebellum are presented elsewhere (Jaarsma *et al.*, 1996).

Electron microscopy

In ultrathin sections prepared from the surface of the immunoreacted slices, where penetration of immunoreagents was excellent, both labelled and unlabelled mossy rosettes were observed. Immunopositive and immunonegative rosettes were often situated in neighbouring glomeruli. In spite of the brief fixation, freeze-thawing, and lengthy immunoreaction protocol, the ultrastructure of the granular layer was

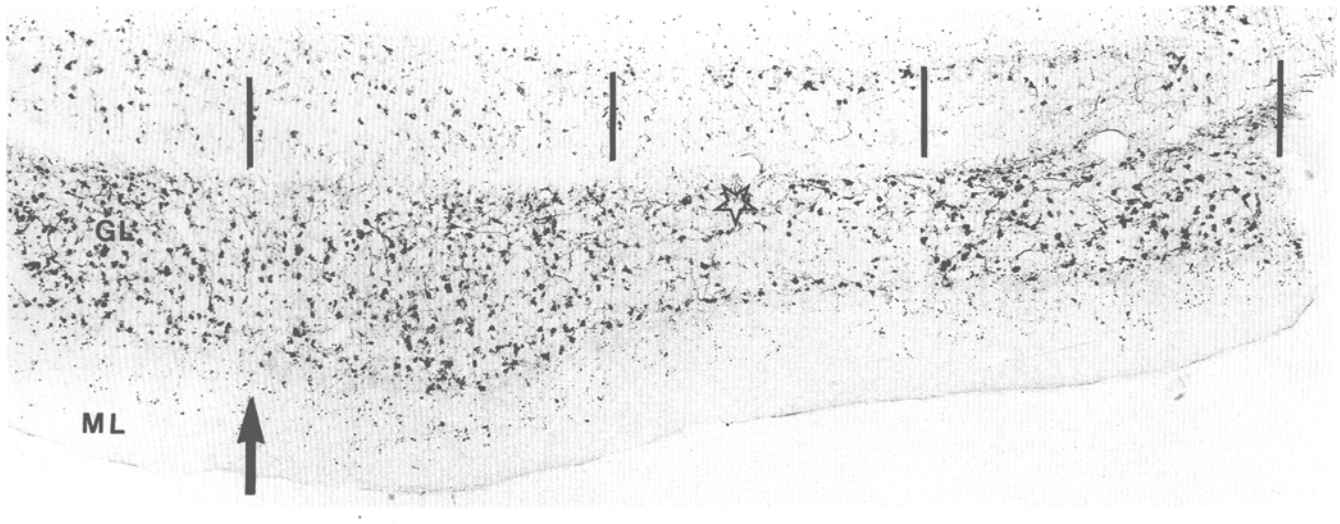

Fig. 2. Light micrograph from a coronal section of the nodulus showing that ChAT-positive mossy fibres occur along the entire extent of the folium, but at varying densities. In the granular layer (GL), labelled mossy fibres form two high density zones flanking the midline (arrow), and two other high density zones in the lateral most portions of the lobule (between lines). The high density zones on each side are separated by an intermediate zone of lower labelling density (star). Note the small ChAT-positive boutons in the molecular layer (ML). ×85.

reasonably well preserved, which allowed characterization of the internal structure of both the rosettes and their postsynaptic elements. Identification of cell bodies and dendrites of granule cells and UBCs was done according to previously established criteria (Mugnaini *et al.*, 1994).

The form of the immunostained mossy rosettes varied considerably. Figure 3 shows glomeruli containing rosettes, which are ramified and whose profiles are marked by immunoreaction product. In all the glomeruli with immunopositive rosettes there were unlabelled peripheral small boutons which contained pleomorphic synaptic vesicles and were identified as varicosities of the Golgi cell axonal plexus (Fig. 3A). In spite of their varying configuration, all profiles of immunolabelled mossy rosettes contained numerous round clear-core synaptic vesicles and mitochondria, and few, if any, large dense-core vesicles (Fig. 3B). On the basis of the packing density of synaptic vesicles, Palay and Chan-Palay (1974) categorized mossy fibre endings into rosettes of the 'dispersed' and the 'clustered' type, having 'loosely scattered' or 'fairly tightly packed' synaptic vesicles, respectively. All ChAT-immunoreactive rosettes were of the 'clustered' type. Non-labelled rosettes belonged to either category.

Glomeruli with immunolabelled rosettes were classified into glomeruli containing dendritic profiles belonging exclusively to granule cells (Fig. 3), and glomeruli containing dendritic profiles of both granule and UBC dendrites (Figs 4, 5). In some of the glomeruli of the second category the UBC dendrites prevailed. Criteria for the identification of dendritic profiles of the granule cells and UBCs have been extensively demonstrated elsewhere (Mugnaini *et al.*, 1994; reviewed by Mugnaini *et al.*, 1997), and will be outlined here only briefly. Granule cells dendrites are thin, have smooth contour, contain several microtubules, lack neurofilaments, form clusters and are bounded to each other by *puncta adherentia*; near their digitiform tips they lose most of their microtubules, contain some tubular or cisternal smooth endoplasmic reticulum and one or two mitochondria. They form frequent symmetric synapses with the Golgi axonal plexus and distinct, small, asymmetric synapses with the mossy fibre rosettes. The UBC dendrite emerges from the cell body with a thick stem, which after an usually short course brakes up into numerous dendrioles of varying diameter, forming a brush-like structure. The dendritic stem and the dendrioles bear numerous appendages that do not form synaptic junctions. Only the appendages contain scarce organelles; all other UBC dendritic profiles contain numerous neurofilaments, microtubules, mitochondria, tubular or saccular smooth endoplasmic reticulum, polyribosomes, large dense-core vesicles, some subsurface cisterns, and occasionally a characteristic assembly of ringlet subunits. Symmetric synapses with Golgi boutons are infrequently encountered on the UBC dendrite, while asymmetric synapses with a mossy fibre rosette are common and may occur on the shafts of the dendrioles or on the stem dendrite. A minority of the immunolabelled mossy rosettes formed giant synapses with UBC somata. These are not illustrated, but a similar arrangement, where a mossy rosette synapses on the emerging dendritic shaft of an UBC, is shown in Fig. 4. We have obtained a single electron

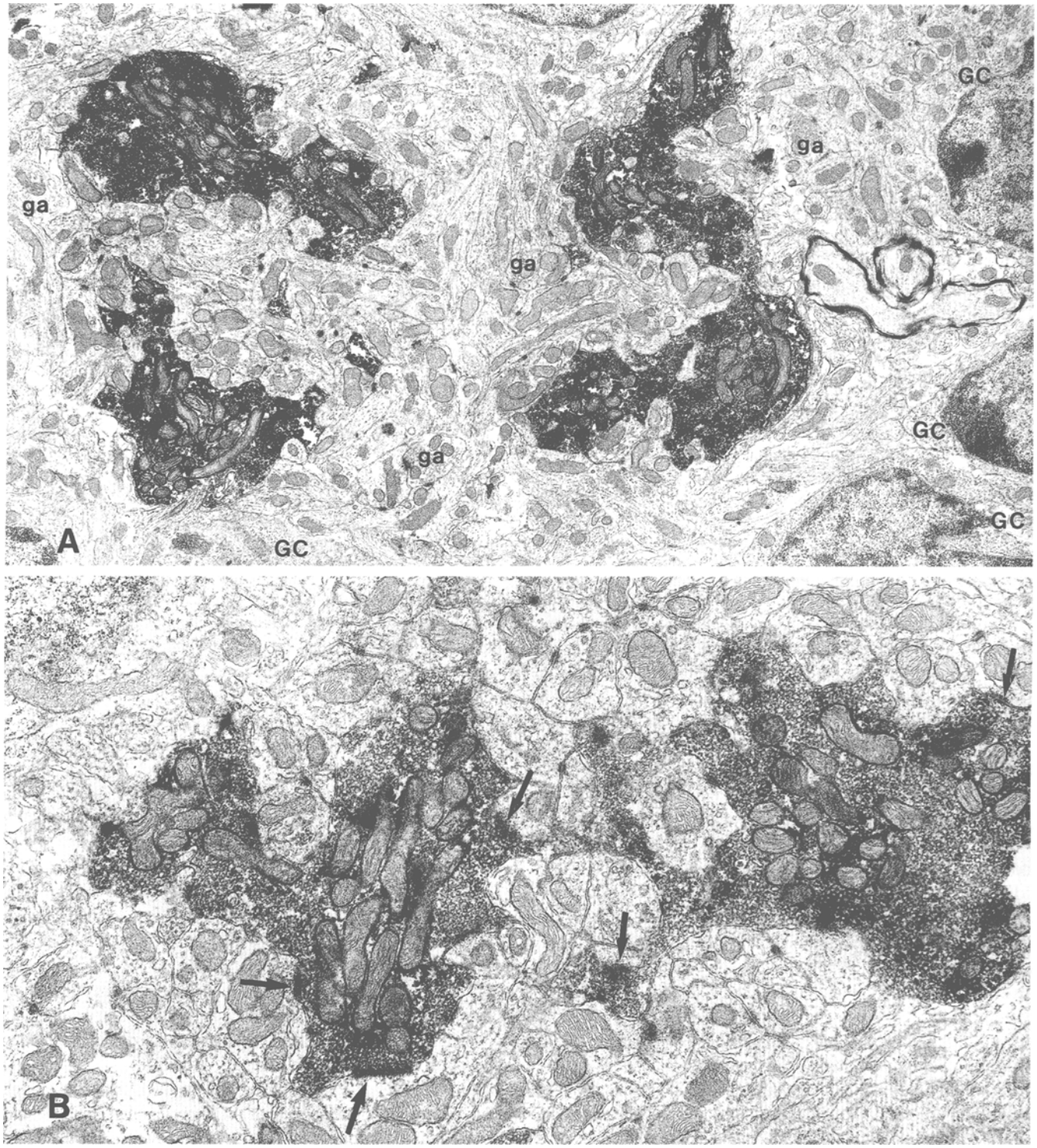

Fig. 3. (A) Low power immunoelectron micrograph of the granular layer in the nodulus showing a glomerulus with multiple profiles of a branched mossy rosette, which was made electron dense by the ChAT-like immunoperoxidase labelling. Note the high densities of mitochondria and synaptic vesicles. GC, granule cell bodies, ga, ending of Golti cell axons at the periphery of the glomerulus. ×9100. (B) Immunoelectron micrograph of a branched ChAT-positive mossy rosette (electron dense profile) in synaptic contact with granule cell dendrites. Arrows indicate short postsynaptic densities. ×22 000.

micrograph where an UBC dendrite formed contacts with both a labelled and an unlabelled mossy rosette (not shown).

Synaptic junctions between labelled mossy rosettes and granule cell dendrites appeared distinctly asymmetric and usually measured 0.1–0.3 μm in length (Fig. 4B). Also the synaptic junctions between labelled mossy rosettes and UBCs were asymmetric; they

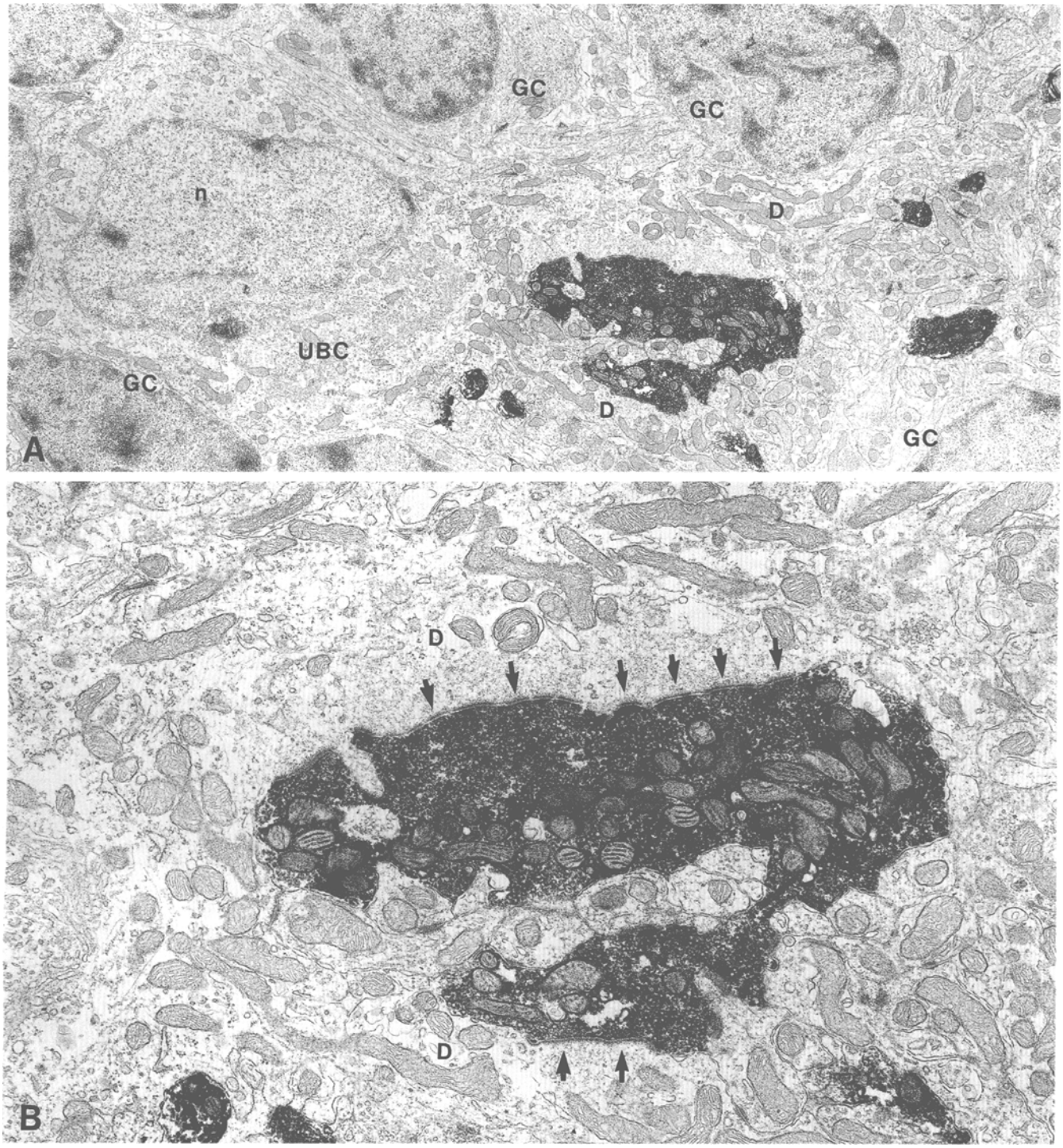

Fig. 4. Immunoelectron micrographs of a UBC that receives contact from a ChAT-positive, electron dense, mossy ending. (A) This low power micrograph shows a UBC with a nucleus (n) and the emerging dendritic trunk which splits into two branches (D) immediately after emanating from the soma (UBC). GC, granule cell bodies. ×7100. (B) Detail from (A) showing the long, asymmetric synaptic junctions (arrowheads) between the UBC dendrites (D) and the mossy ending. ×19 300.

varied in configuration from extensive specialized appositions that measured 2–7 μm in length and had postsynaptic densities that were uninterrupted or showed only short interruptions (Fig. 4), to a series of individual short junctions, ranging from 0.2 μm to 1 μm in length (Fig. 5). The same UBC dendrite could form contacts with multiple immunolabelled axonal profiles presumably belonging to the same rosette (Fig. 5B). Unipolar brush cell dendrites synapsing with immunolabelled rosettes were occasionally also

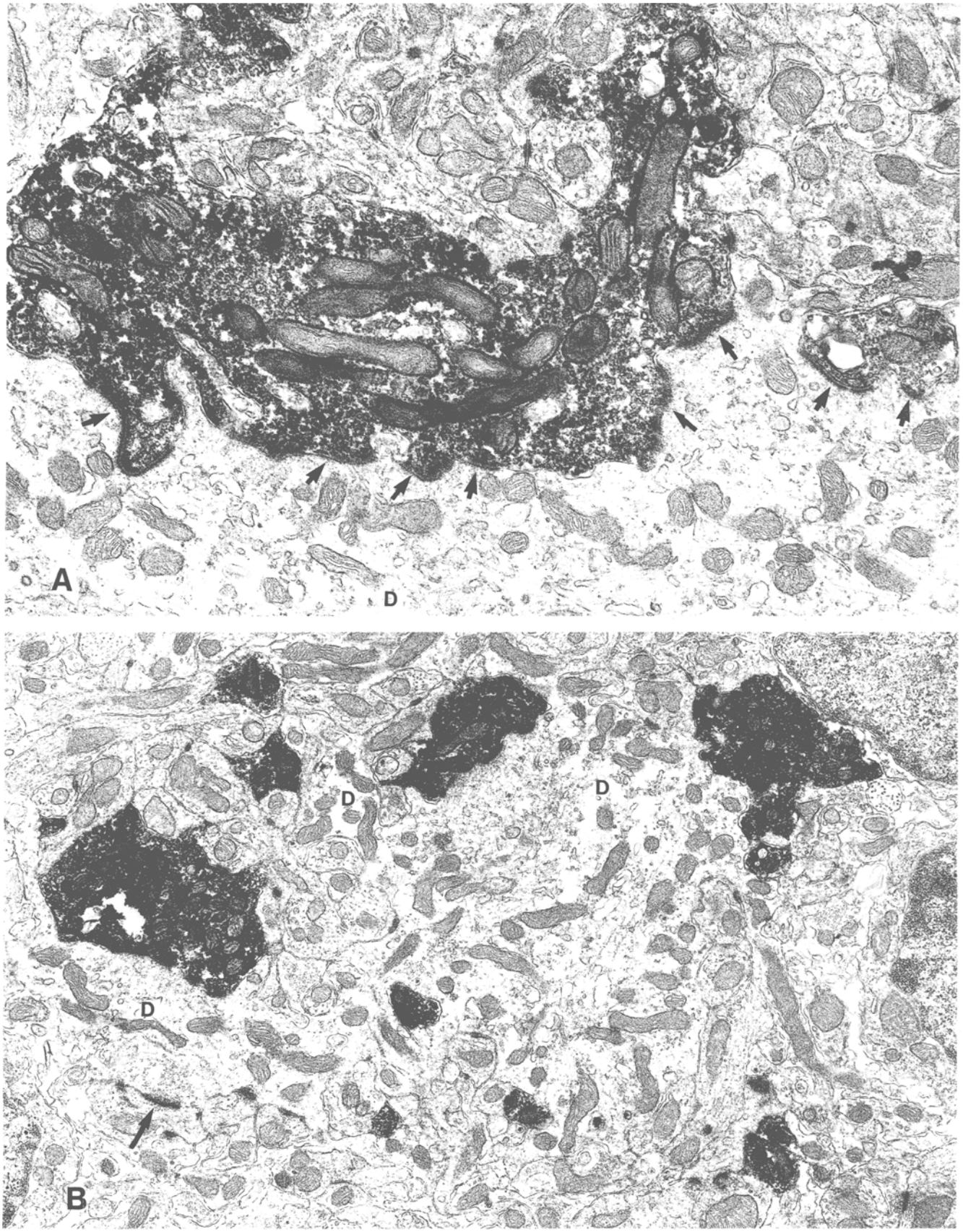
A
D
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Table 1. Estimates of ChAT-positive mossy fibre rosettes innervating UBCs, and UBCs innervated by ChAT-positive mossy fibres in the rat nodulus mm^{-2}.

Rat	*Total area examined (mm^2)*	*Total glomeruli with ChAT+ MG (mm^{-2})*	*Total glomeruli with UBC (mm^{-2})*	*Total glomeruli with ChAT+ MF & UBC (mm^{-2})*	*Total glomeruli with ChAT+ MF & UBC/ glomeruli with ChAT + MF*	*Total glomeruli with ChAT+ MF & UBC/ glomeruli with UBC*
I	0.528	435	296	63	14.5 %	21.3 %
II	0.349	381	307	69	18.1 %	22.5 %
III	0.372	414	196	42	10.1 %	21.4 %
Mean ± SE		410 ± 19	266 ± 43	58 ± 10	14.2 % ± 2.8	21.7 % ± 0.5

Glomeruli containing ChAT-immunoreactive mossy rosettes (ChAT + MF), giant UBC synapses (UBC), or both (ChAT + MF & UBC), were systematically counted, and the percentages of ChAT + MF were calculated. Values for each rat represent means from countings of three thin sections. Differences between animals were attributed to distance of the sampled area from the zone of lower density of immunostaining. Animals I–III were adult male Sprague-Dawley rats.

presynaptic to granule cell dendrites, with which they formed asymmetric synaptic junctions (Fig. 5B). These synapses are identical to those previously illustrated by Mugnaini and colleagues (1994).

Semi-quantitative analysis

In ultrathin sections from the nodulus of three different male Sprague-Dawley rats, we systematically counted the number of glomeruli containing ChAT-positive rosettes and those containing UBC synapses to obtain a rough estimate of the percentage of ChAT-positive rosettes innervating UBCs and the percentage of UBCs innervated by ChAT-positive rosettes. Three blocks from each of the three animals were selected from the paramedian zone of granular layer in the rostral nodulus (folium Xb), which is rich in ChAT-positive mossy rosettes. These blocks were prepared from parasagittal slices, ~0.1–0.7 mm from the midline, that were sufficiently flat after embedment to allow cutting of relatively large ultrathin sections, 0.1–0.2 mm^2, comprising the whole depth of the granular layer from Purkinje cell layer to white matter. Flatness is a variable independent from immunostaining. Countings from three thin sections per rat were pooled. We did not carry out countings of labelled mossy fibres in the different zones of the vestibulocerebellar folia displaying higher and lower density of immunostaining. Adequate penetration of the immunoreagents was verified in semithin (0.5 μm thick) sections cut before and after the ultrathin sections. Apparent variability between animals was tentatively attributed to distance of the sampled site from the zone of lower density immunostaining. The results, which are displayed in Table 1, indicate that only a small percentage (14.2%) of ChAT-positive rosettes synapse with UBCs, and that ChAT-positive rosettes account for approximately one fifth (21.7%) of the rosettes forming synapses with UBCs.

Discussion

This study confirms that the ChAT-like immunoreactive rosettes in the rat vestibulocerebellum are large, convoluted and often ramified endings, that presumably belong to the population of the larger type of mossy fibres described by Brodal and Drabløs (1963). We have obtained the novel findings that ChAT-positive rosettes form synapses not only with granule cells, but also with UBCs, and that approximately one fifth of the UBCs situated in the nodulus are innervated by ChAT-positive rosettes. Single UBCs synapsing with two mossy rosettes, one ChAT-positive and the other ChAT-negative also exist, but they occur rarely. We have also shown that ChAT-positive mossy rosettes form synaptic junctions of varying configurations with the UBCs, indicating that variations in the form of the mossy fibre-UBC synapses are not necessarily linked with the type of neurotransmitter released at these cell junctions; thus, these variations must reflect a structural-functional aspect of this synapse still to be elucidated. Furthermore, we have shown that UBCs contacted by ChAT-positive mossy fibres may be presynaptic to granule cell dendrites. This feature, which had previously been described by standard electron microscopy and by calretinin immunocytochemistry (Floris *et al.*, 1994; Mugnaini *et al.*, 1994), may be shared by UBCs synapsing with mossy fibres of different origins.

Fig. 5. Immunoelectron micrographs of UBC dendrites (D) that receive contacts from ChAT-positive mossy endings. (A) Arrowheads indicate that this mossy fibre-UBC synapse consists of a series of individual junctions of varying lengths. ×26 300. (B) The picture illustrates dendritic profiles (D) of an individual UBC that are contacted at several points by branches of a ChAT-positive mossy fibre rosette. Arrow points to an asymmetric synapse between the UBC dendrite and a granule cell dendrite. ×16 800.

Methodological considerations and comparison with previous studies

Our data were obtained with monoclonal antisera to rat ChAT of proven specificity (Cozzari *et al.*, 1990). The distribution of the ChAT-immunolabelling in the cerebellum is similar to that observed in other studies with different antisera to ChAT (Ojima *et al.*, 1989; Barmack *et al.*, 1992a), except for minor details. The main difference between results in our sections and the rat sections of Barmack and colleagues (1992a) is that we did not observe an enrichment of labelled mossy fibres in the vermal lobules I–III, compared to other vermal lobules. Ojima and colleagues (1989), reported results similar to ours. On the other hand, Ojima and colleagues (1989) did not describe the high density of small immunopositive profiles in a sharply delineated portion of the granular layer in the caudal part of lobules IXa–b, which was present in our material and was also described by Barmack and colleagues (1992a). These discrepancies most probably arise from minor methodological variations or from the use of different rat strains and sublines. An observation that was not emphasized by either Ojima and colleagues (1989) or Barmack and colleagues (1992a) was the presence, in coronal sections of the nodulus and ventral uvula, of poorly delineated granular layer zones with higher and lower densities of ChAT-positive mossy fibres (Fig. 2). The relations of these zones with other compartments determined by physiological or anatomical methods (Boegman *et al.*, 1988; Hawkes & Turner, 1994; Wylie *et al.*, 1994; Voogd *et al.*, 1996) remain to be established.

The cholinergic nature of a population of mossy rosettes in the vestibulocerebellum is in accord with studies utilizing acetylcholinesterase (AChE) histochemistry, which show that enzyme activity is particularly high in the nodulus and ventral uvula (Csillik *et al.*, 1963; Shute & Lewis, 1965; Silver *et al.*, 1967). Brown and Palay (1972) extended at the electron microscopic level previous investigations on the localization of AChE activity in these lobules and showed that reaction product was present in the extracellular space surrounding a proportion of mossy rosettes, which contained numerous densely packed synaptic vesicles and mitochondria, but few large dense-core vesicles. These rosettes resemble those labelled by ChAT antiserum. Harris and colleagues (1993), in their study on the UBC's neurofilament proteins, showed that AChE-positive mossy fibres are situated in close proximity of the UBCs, at least judging from light microscopic criteria. Recently, the cholinergic nature of a subset of mossy fibres in the nodulus/ventral uvula was further supported by the demonstration that these lobules are enriched in sodium-dependent [^{3}H]hemicholinium binding sites (Jaarsma *et al.*, 1995b, 1997), which selectively labels the cytoplasmic choline transporter and is viewed as a reliable marker for cholinergic nerve terminals (Bekenstein & Wooten, 1989).

Relations of ChAT-positive mossy fibres and UBC

As shown in Table 1, ~15% of all ChAT-positive rosettes formed giant synapses with UBCs in regions of the nodulus most densely innervated by immunoreactive mossy fibres. Pilot estimates of ChAT-positive rosettes innervating UBCs in other vestibulocerebellar folia indicated that this proportion remains similar in zones with lower density of labelled mossy fibres (18.2% in the ventral uvula and 13.3% in the transitional zone between flocculus and ventral paraflocculus; unpublished observations). Since mossy fibres give rise to multiple rosettes, it is possible that most, if not all, ChAT-positive mossy fibres innervate one or more of the UBCs. This possibility was not tested directly in this study, because it would ideally require examination of individual mossy fibres along their entire course. In densely ChAT-immunoreactive portions of the nodulus, approximately one fifth of UBCs were innervated by ChAT-positive rosettes. In zones of lower density of ChAT-immunoreactive fibres, but with similar or higher densities of UBCs, the percentage of ChAT rosettes contacting UBCs was the same as in ChAT-rich zones, and consequently the proportion of UBCs innervated by ChAT-immunoreactive rosettes was much lower than 20 % (11.4% in the ventral uvula and 4.5% in the flocculus/ventral paraflocculus border region; unpublished observations).

In 6-μm thick paraffin sections of the mouse nodulus stained with hematoxylin/eosin, Altman and Bayer (1977) estimated the density of 'pale cells' (presumed UBCs) with nuclei displaying nucleoli to be 188 mm^{-2}. This is consistent with the density of glomeruli containing giant mossy fibre-UBC synapses determined in this study (266 mm^{-2}). With the caveats that these two measurements are derived from different species without strict stereological procedures, and that glomeruli are usually larger than UBC cell bodies, these numbers are compatible with the notion that a single mossy rosette usually contacts an individual UBC (Mugnaini & Floris, 1994; Mugnaini *et al.*, 1994).

Origins of ChAT-positive and ChAT-negative mossy rosettes of the vestibulocerebellum

By double labelling light microscopy with a retrograde tracer and ChAT- immunocytochemistry, Barmack and colleagues (1992b) demonstrated that ChAT-positive mossy fibres of the nodulus/ventral uvula originate primarily from the medial vestibular nucleus and to some extent from the nucleus prepositus hypoglossi. Their data in rat, however, also suggest that not all neurons retrogradely labelled from the nodulus/ventral uvula are immunoreactive for ChAT.

Another strong input to the vestibulocerebellum, particularly the nodulus and ventral uvula, derives from the vestibular ganglion (Dow, 1936; Korte & Mugnaini, 1979; Barmack *et al.*, 1993; Rubertone *et al.*, 1995). The primary vestibular fibres include a subpopulation that is strongly immunopositive for calretinin (Arai *et al.*, 1991; Résibois & Rogers, 1992; Floris *et al.*, 1994), and the calretinin-positive mossy rosettes have also been shown to synapse with both UBCs and granule cells (Floris *et al.*, 1994). Although some of the calretinin-positive rosettes might be UBC axon collaterals (Berthié & Axelrad, 1994; Rossi *et al.*, 1994,1995), the high density of primary vestibular fibres in the nodulus and the ventral uvula strongly suggests that their rosettes also synapse with the UBCs. This conclusion is also supported by the observation that the transition between the ventral uvula innervated by primary vestibular fibres and the rest of the uvula is suggestively depicted in calretinin-stained sections (Floris *et al.*, 1994), while the UBCs have a wider distribution in the uvula of the rat and other mammals (Floris *et al.* 1994; Jaarsma *et al.*, 1995a; Mugnaini *et al.*, 1996; Diño *et al.*, in preparation).

Neurotransmitter-receptor interaction

In view of our findings, it is interesting to speculate about the nature of the ACh receptors that may mediate neurotransmission at synapses of the ChAT-positive mossy fibres. Both muscarinic and nicotinic receptors occur in the rat cerebellar cortex. Ligand binding autoradiography (Rotter *et al.*, 1979; Spencer *et al.*, 1986; Neustadt *et al.*, 1988; Aubert *et al.*, 1992) and *in situ* hybridization (Vilaró *et al.*, 1992) have shown that cerebellar muscarinic receptors, which are primarily of the M2-type, are enriched over the nodulus/ventral uvula. Messenger RNA coding for the M2 receptors was localized over the granule cell layer (Vilaró *et al.*, 1992), whereas ligand binding sites primarily occur over the molecular layer and are presumably localized in the parallel fibres, as also demonstrated immunocytochemically with a polyclonal antibody specific for the M2 receptor protein (Jaarsma *et al.*, 1996). Whether M2 receptors also occur in granule cell dendrites remains to be established by immunoelectron microscopy. M2 receptors and other types of muscarinic receptors (M1, M3, and M4), however, are absent in UBCs (Levey *et al.*, 1991; Jaarsma *et al.*, 1995b, 1996).

The rat cerebellar granular layer displays low levels of high-affinity [^{3}H]nicotine binding sites (Clarke *et al.*, 1985) and concordantly expresses low levels of the principal neuronal nicotinic receptor subunits, α3, α4, and β2 (Swanson *et al.*, 1987; Wada *et al.*, 1989; Hill *et al.*, 1993); there was no enrichment of binding sites or subunit expression in the nodulus/ventral uvula. In contrast, high levels of another type of neuronal nicotinic receptor, which is labelled by [^{125}I]α-bungarotoxin, is selectively present at high levels in glomeruli of the nodulus, ventral uvula, and flocculus (Hunt & Schmidt, 1978; Frostholm & Rotter, 1986), consistent with the distribution of ChAT-positive mossy fibres or UBCs. Like UBCs – but unlike ChAT-positive mossy fibres – the [^{125}I]α-bungarotoxin-labelled glomeruli showed their highest density in the transition zone between the flocculus and ventral paraflocculus, thus suggesting that these receptors are associated with UBCs. Frostholm and Rotter (1986) suggested that these binding sites were situated on mossy fibre endings, on the basis of the observation that, during cerebellar development, the appearance of binding sites corresponded with the appearance of mossy fibres, but this does not exclude the possibility that the binding sites may actually be postsynaptic. The presence of functional ACh receptors associated with UBCs is compatible with recent electrophysiological investigations on fresh cerebellar slices, which shows that UBCs respond to bath-applied ACh with an inward current (Rossi *et al.*, 1995).

Whether cholinergic mechanisms play a role in the vestibulo-cerebellar mossy fibre system remains to be established with physiological methods. Physiological data so far invalidate the possibility that ACh mediates fast excitatory neurotransmission of a subset of mossy fibres in the nodulus/ventral uvula: First, Crepel and Dhanjal (1982) using slice preparations of rat nodulus showed that both nicotinic and muscarinic antagonists do not have any detectable effect on potentials of Purkinje cells evoked by mossy fibre activation. They concluded that "...the presence of a massive contingent of mossy fibres using ACh as a neurotransmitter is unlikely in the cerebellar lobules IX and X of the rat...." Second, synaptic currents in UBCs and granule cells evoked by mossy fibre stimulation in the nodulus/ventral uvula slice preparations were always mediated by glutamate receptors (e. g. Rossi *et al.*, 1995; Slater *et al.*, 1996a,b). It can not be excluded, however, that cholinergic mossy fibre responses may have been missed because only a minority of mossy fibres in the nodulus/ventral uvula are cholinergic. On the other hand there are only a few examples that ACh mediates fast excitatory transmission in the brain, and generally a modulatory role has been assigned to ACh (e.g. see Sivilotti & Colquhoun, 1995).

There have been various reports suggesting that ACh is co-localized with amino acid neurotransmitters (e.g. Kosaka *et al.*, 1988; Lavoie & Parent, 1994; Caffé *et al.*, 1996). The possibility that ACh and glutamate are co-distributed in ChAT-immunoreactive mossy fibres seems to be realistic, in view of the solid anatomical and physiological evidence showing that most if not all cerebellar mossy fibres use glutamate for fast excitatory transmission (Somogyi *et al.*, 1986; Garthwaite *et al.*, 1990; Ottersen *et al.*, 1990; Silver *et al.*, 1992; Rossi *et al.*, 1995; Slater *et al.*, 1996a,b). At

present, one may only speculate about the significance of the presumed ACh/glutamate co-release at the giant mossy fibre-UBC synapse and at the multiple, small synapses between mossy fibres and granule cell dendrites. A possible of scenario of how ACh could interact with fast glutamatergic transmission has been recently uncovered by McGehee and colleagues (1995), showing that presynaptic nicotinic receptor enhance fast, ionotropic glutamate receptor-mediated EPSCs at the synapses between medial habenular fibres and interpeduncular nucleus neurons. Considered their relatively large number, the synapses between granule cells and ChAT-positive mossy fibres may be amenable to similar studies. Moreover, because UBCs are related in a nearly one-to-one fashion to mossy fibres, and because of the large size of their synapses with the rosettes, the UBCs may represent a better suited model of ACh/glutamate co-release at central synapses.

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