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Unusual neurofilament composition in cerebellar unipolar brush neurons

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Summary

During antibody screening on sections of rat cerebellum, we noticed a group of small neurons which exhibited unusual staining properties. They were robustly immunopositive for the high molecular weight neurofilament protein, moderately immunostained with antibodies to the low molecular weight neurofilament protein and α-internexin, but only faintly immunoreactive (in PAP sections) or essentially immunonegative (in immunofluorescent sections) with all members of a panel of antibodies directed against the middle molecular weight neurofilament protein. Since neurons generally react equally well with phosphate-independent, (antibodies to) low, middle and high molecular weight neurofilament protein, we conclude that middle molecular weight neurofilament protein is present in these cells in an unusually low relative amount. These cells are found in the granular layer and appear concentrated in the flocculus, ventral paraflocculus, and vermis, particularly in the ventral uvula and nodulus (lobules IXd and X). Previous studies performed by Hockfield defined a population of neurons of similar appearance and distribution using the monoclonal antibody Rat-302, which recognized an uncharacterized 160 kDa protein. We show here that the cells described by Hockfield are identical to those we have found and furthermore that the Rat-302 antibody specifically recognizes the dephosphorylated form of the lysine-serine-proline repeated sequences of high molecular weight neurofilament protein. These cells were studied by pre-embedding immunoelectron microscopy. The nucleus is deeply indented and shows little condensed chromatin. The cytoplasm contains scattered microtubules and a larger number of neurofilaments than expected in a small cell. There are numerous large dense core vesicles, an unusual organelle consisting of ringlet subunits, and relatively little granular endoplasmic reticulum. A thin axon and a single stout dendritic trunk emanate from the perikaryon. Although the cell body and the dendritic shaft may form either complex contacts with mossy fibres (resembling those previously termed *en marron* synapses) or simple symmetric synapses with small boutons containing pleomorphic vesicles, most of the synaptic relations are established on the shafts of brush-like branchlets that form at the tip of the dendrite and enter one or two glomeruli. Each branchlet forms an extraordinarily extensive asymmetric synapse with the mossy fibre rosette and the subsynaptic region shows a microfibrillar web connected to the postsynaptic density. In addition to other organelles, the branchlets contain numerous mitochondria and large dense core vesicles. Short, non-synaptic appendages with few cytoplasmic organelles emanated from the cell body, dendritic shaft and branchlets. The immunoreaction products of all neurofilament antibodies were similarly distributed within the small cells, and were absent from the granular reticulum, the Golgi apparatus, the appendages and the subsynaptic region. These high molecular weight neurofilament protein rich small cells correspond to the pale cells, the calretinin and secretogranin positive small cells and the unipolar brush neurons newly described with the Golgi method. Unlike the multipolar Golgi neurons, unipolar brush cells are not immunopositive for the inhibitory neurotransmitters GABA or glycine. The unusual concentration of secretogratin and two different types of calcium-binding protein (calretinin and high molecular weight neurofilament protein) along with the high content of mitochondria suggest that these cells subserve a function that requires an unusual degree of metabolic activity, perhaps as a result of their unusually rich synaptic connections.

Introduction

Intermediate filaments are major structural components of most types of eukaryotic cell. Since different types of cell frequently express intermediate filaments composed of biochemically and immunologically distinct subunits, these cytoskeletal elements have become widely used as cell-type specific markers

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(Steinert & Roop, 1988). In neurons the predominant subunits are the so-called neurofilament triplet proteins, usually referred to as low molecular weight neurofilament protein (NF-L), middle molecular weight neurofilament protein (NF-M) and high molecular weight neurofilament protein (NF-H), although subunits such as α-internexin and peripherin may also be found (for recent reviews, see Shaw, 1991; Fliegner & Liem 1991). The cerebellar cortex represents an excellent model system to analyse the differential distribution of neurofilament proteins because it contains several well characterized neuronal cell types: stellate and basket cells, Purkinje cells, granule cells, Golgi cells, and Lugaro cells (reviewed by Cajal, 1911; Ito, 1984; Jakob, 1928; Fox *et al.*, 1967; Eccles *et al.*, 1967; Mugnaini, 1972; Palay & Chan-Palay, 1974). These neurons are classically described according to their layered distribution, shape and size. Granule, Golgi and Lugaro cells are situated in the granular layer. Granule cells are microneurons (6–8 μm in diameter) possessing 2–4 short dendrites with claw-like terminals; Golgi cells are multipolar neurons with extensive dendritic fields occupying both granular and molecular layers; and Lugaro cells are scattered, fusiform elements whose dendrites stretch out along the Purkinje cell layer. Although the identity of the major cell types and their connectivity are well established, numerous questions about the details of cellular content, microanatomy and function remain to be answered.

Recently, we have produced a panel of very specific antibodies directed against several different regions of the major neurofilament subunits (Harris *et al.*, 1991; this paper). Here, we show that these antibodies reveal a class of small neurons in the cerebellar granular layer, which are different from granule, Golgi and Lugaro cells and which show a very unusual immunoreactivity with neurofilament antibodies. These neurons, which are intermediate in size between granule cells and Golgi cells, stain very strongly with phosphate-independent NF-H antibodies, but are very weakly immunoreactive with all antibodies against NF-M we have tested. This is in contrast to the situation seen with many other neurons of the adult rat, which stain equally strongly with phosphate-independent NF-M and NF-H antibodies (e.g., Shaw *et al.*, 1991; Trojanowski *et al.*, 1986). Here, we demonstrate that the cells we have identified are the same as those described by others as pale cells (Altman & Bayer, 1977; Sturrock, 1990), Rat-302 cells (Hockfield, 1987), secretogranin II positive small cells (Cozzi *et al.*, 1989), calretinin positive small cells (Floris *et al.*,

Table 1. Antibodies used in this study

Name	*Specificity on rat*	*Immunogen*
Polyclonal sera		
R9	NF-M extreme C-terminus, P-ind	RM:677–845
R12	NF-H extreme C-terminus, P-ind	RH:846–1072
R14	NF-H, NF-M, lysine-serine-proline repeats P-ind	RH:559–794
R15	NF-M, central part of molecule, P-ind	CM:381–605
R18	α-internexin C-terminus, P-ind	RI:336–500
H297	NF-L, P-ind	rat NF-L
H298	NF-M, P-ind	rat NF-M
H301	NF-H, P-ind	rat NF-H
α-MSH	NF-M, P-ind, N-terminal peptide	α-MSH neuropeptide
Mouse monoclonal ascites		
DA2	NF-L, P-ind	dephosphorylated pig neurofilaments
NN18	NF-M (amino acids 677–732), P-ind	pig neurofilaments
5C6	NF-M (amino acids 732–761), P-ind	RM:677–845
3H11	NF-M (amino acids 761–845), P-ind	RM:677–845
N52	NF-H, lysine-serine-proline repeats, P-ind	dephosphorylated pig neurofilaments
SMI-32	NF-H, lysine-serine-proline repeats, only de-P	rat hippocampus
NAP4	NF-H phosphorylated lysine-serine-proline, P	pig neurofilaments
3G3	NF-H (amino acids 846–1022), P-ind	RH:846–1072
5B8	NF-H (amino acids 1022–1072), P-ind	RH:846–1072
3G8	rat α-internexin, P-ind	RI:336–500

Most of these antibodies have been described in previous studies (Harris *et al.*, 1991, Shaw *et al.*, 1985, 1986). P refers to phosphorylation; P-ind means that the antibody stains the protein irrespective of the level of lysine-serine-proline phosphorylation, only de-P means it only stains the protein if the lysine-serine-proline sequences are not phosphorylated and P means it only stains the protein if the lysine-serin-proline sequences are phosphorylated. Polyclonal and monoclonal antibodies to α-internexin will be described in more detail in a future publication.

1992, 1994), and unipolar brush cells (Mugnaini & Floris, 1993). In addition, we elucidate the basis for Rat-302 staining by showing that this antibody specifically recognizes the non-phosphorylated form of the lysine-serine-proline repeated sequences found in the C-terminal tail of NF-H.

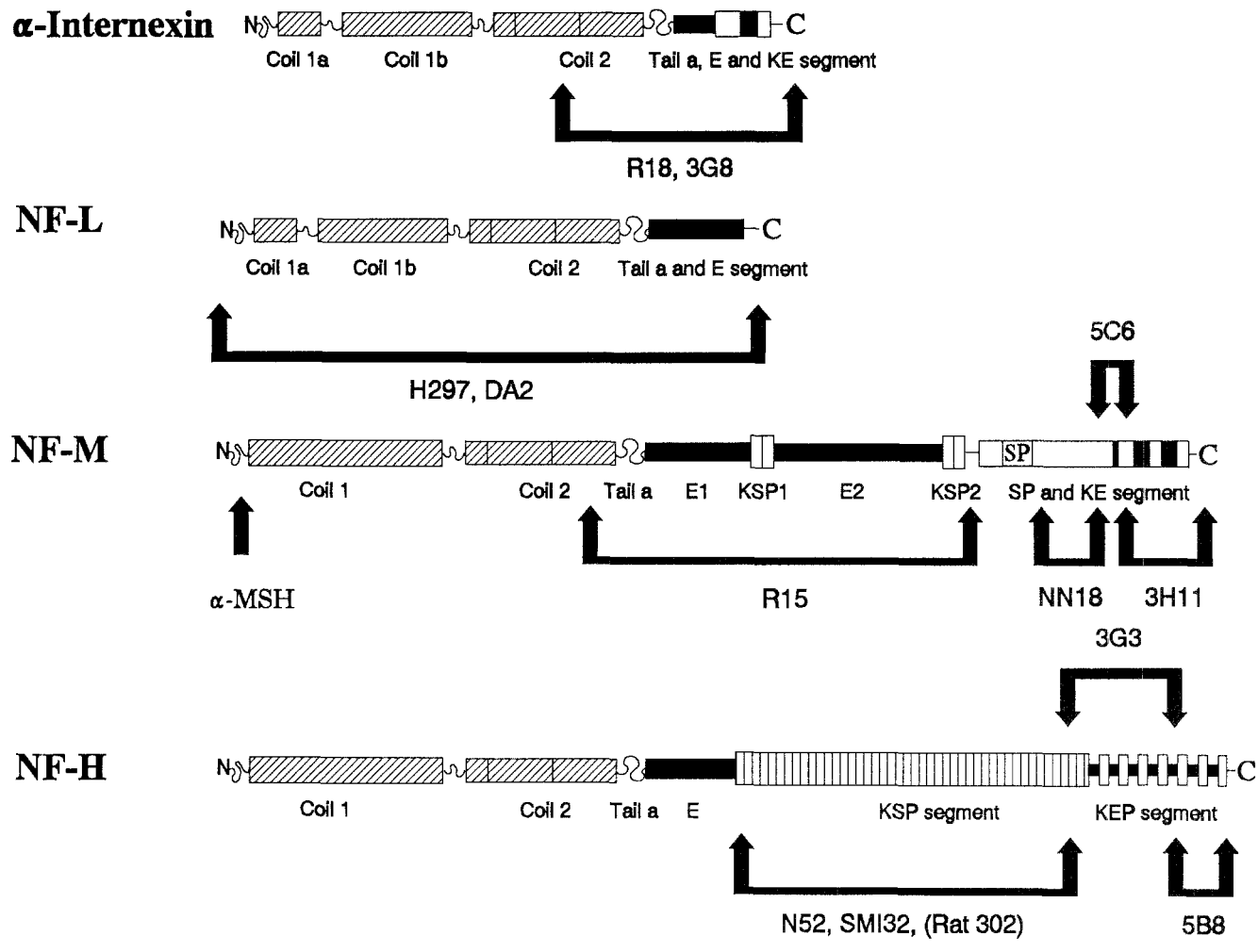
Materials and methods

Antibodies

The antibodies used in the study are listed in Table 1, and their epitope maps are diagrammatically illustrated in Table 2. Most of these antibodies have been previously characterized in detail. Briefly, the three NF-M monoclonal antibodies, NN18, 5C6 and 3H11 each map to distinct segments of the extreme C-terminus of NF-M (Harris *et al.*, 1991). The α-MSH antibody, obtained from Immunonuclear Corporation, was originally raised against the neuropeptide α-MSH, but exhibits staining for NF-M on immunoblots and stains neurofilaments immunocytochemically (Draeger *et al.*, 1983). Further studies showed that this antibody recognizes the N-terminal peptide of NF-M which is very similar to that of α-MSH (Shaw *et al.*, 1985). R15 is a new rabbit polyclonal antibody raised against a recombinant fusion protein (CM: 381–605, see Harris *et al.*, 1991) containing the central region of NF-M between the C-terminal of Coil II and the start of the extreme C terminal KE segment (see Shaw, 1991, and Fliegner & Liem, 1991, for details of neurofilament structure). On immunoblots, R15 recognizes NF-M and does not cross-react with NF-L or NF-H (not shown). H297, H298 and H301 were raised against native rat sciatic nerve neurofilament proteins and have been previously characterized (Shaw *et al.*, 1986). Monoclonal antibodies NN18 and N52 can be obtained from Boerhinger-Mannheim and Sigma, SMI-32 from Sternberger Immunocytochemicals and DA2 from Chemicon. The IgM monoclonal Rat-302 antibody was a kind gift of Dr Susan Hockfield.

Monoclonal antibodies to the extreme C-terminus of NF-H were made by immunization of mice with RH: 846–1072 fusion protein which contains the extreme C-terminal 226

Table 2. Epitope maps of antibodies used in this study. Details of the various different segments of the four neurofilament proteins are described in previous publications (Shaw, 1991, 1992). The detailed characterization of some of these antibodies has also been described previously (Harris *et al.*, 1991).



amino acids of rat NF-H and does not include the highly ordered lysine-serine-proline repeated sequences (Harris *et al.*, 1991). These two antibodies, 3G3 and 5B8, both stain phosphorylated and dephosphorylated NF-H on blots strongly and specifically and will be described in more detail elsewhere. They bind to different epitopes within the tail segment, as can be shown following cyanogen bromide cleavage of the RH:846-1072 fusion protein, the neurofilament component of which contains a single methionine 50 amino acids from the C-terminus. 3G3 stains a large RH: 846–1072 cyanogen bromide fragment identified as rat NF-H amino acids 846–1022, unambiguously locating the epitopes for this antibody to within this segment. 5B8 fails to stain this large fragment, showing that this antibody has a different epitope and suggesting that it resides partially or wholly in the C-terminal 50 amino acids of rat NF-H. Antibodies to α-internexin were made against RI: 336-500, a recombinant fusion protein containing the last 164 amino acids of rat α-internexin (Fliegner *et al.*, 1990). A cDNA encoding the whole of rat α-internexin was the gift of Dr R. Liem. R18 is a rabbit polyclonal antibody and 3G8 is a mouse monoclonal antibody, both raised against this fusion protein. Both antibodies stain native rat α-internexin cleanly and specifically on immunoblots and will be described in more detail in a future publication.

Single and double label immunofluorescence

Twenty-one adult Sprague-Dawley rats and two adult Long Evans rats were used for this study. All animals were housed and handled according to approved guidelines and deeply anaesthetized with pentobarbital (35 mg kg^{-1} b.w., injected intraperitoneally) before rapid killing by transcardial perfusion with a strong fixative solution. Animals were perfused with 25 ml heparinized normal saline, 100 ml cold normal saline, and 800 ml cold 4% paraformaldehyde in PBS; brains were dissected out and post-fixed in the same solution for 1–12 h. Brains were then cryo-protected in cold 20% sucrose for 6–12 h and then in 30% sucrose for 12–18 h (until tissue sank). Cerebella were then quick-frozen in liquid nitrogen-cooled isopentane, mounted and sectioned (usually at 8 μm) on the cryostat. Frozen sections were collected either into PBS (for free-floating incubations) or onto gelatin-subbed slides that were then kept frozen at −20° C until staining. Some rat brains were prepared by quick freezing without fixation to check for fixation-induced staining artifacts.

Immunohistochemistry was performed either on free-floating tissue in solution or on sections mounted on glass slides. Non-specific binding was reduced by incubation with 0.1% BSA: 0.3% goat serum in PBS for 20 min at 37° C. Following a PBS rinse, primary antibody in PBS containing 0.1–0.5% Triton X-100 was applied for at least 45 min at 37° C or overnight at 4° C. Specimens were then washed three times in PBS for 10 min at room temperature and fluorescent secondary antibodies (Jackson ImmunoResearch, West Grove, PA) applied at final dilutions of 1:40 in 0.1% BSA: 0.3% goat serum in PBS plus 0.1–0.5% Triton X-100 for at least 45 min at 37° C. After washing three times in PBS for a total of 30 min specimens were mounted for immunofluorescence microscopy. Double-label applications were performed in the same manner, but with primary and secondary antibody mixtures. Sections and whole mounts were mounted in an anti-bleaching mounting medium (1 mg ml^{-1} para-phenylenediamine in 90% glycerol, 10% Tris 200 mM (pH 7.9)), sealed with nail polish and stored at 4° C. Some sections were processed using an avidin-biotin peroxidase enhancement method using diaminobenzidine chromogen with the Vectastain Elite following the manufacturers instructions (Vector Laboratories, Burlingame, CA).

Characterization of Rat-302

SDS-PAGE gels and immunostaining were performed essentially as described previously (Harris *et al.*, 1991). Tissue culture supernatant from Rat-302 hybridoma cultures were used at dilutions of 1:5 to 1:10, and nitrocellulose immunoreplicas were stained using appropriate anti-mouse IgM secondary antibodies coupled to alkaline phosphatase. Staining was performed using Nitro Blue Tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate (phosphatase conjugates and chromogens purchased from Sigma). Neurofilament preparations were obtained by axonal flotation from rat spinal cord and were enzymatically dephosphorylated overnight at room temperature with *E. coli* Type IIIS alkaline phosphatase (Sigma) as described previously (Shaw *et al.*, 1986; Harris *et al.*, 1991). Detailed epitope mapping of Rat-302 was performed on recombinant fusion proteins containing defined segments of rat NF-H sequence. These were purified from inclusion body preparations by DEAE-cellulose chromatography in 6 M urea and run out on SDS-PAGE gels as described (Harris *et al.*, 1991).

Double labelling with Acetylcholinesterase (AChE) histochemistry and neurofilament protein immunocytochemistry

Acetylcholinesterase staining was performed on free-floating sections followed by immunofluorescence labelling with NF-H antibody as described above. Acetylcholinesterase staining involved pre-incubation in 0.5 M Na citrate, 0.3 M $CuSO_4$, 0.05 M $K_3Fe(CN)_6$, 0.1% Triton-X-100 in 0.1 M Acetate buffer (pH 6.0) for 20 min at room temperature, followed by a 15 min incubation in 20 ml of pre-incubation solution containing 1.4 mg ethopropazine and 14 mg acetylthiocholine iodide, a 5 min wash in 0.05 M TRIS-HCl (pH 7.6) and development in 0.5% DAB in PBS with 0.1% Triton X-100 (Geneser-Jensen & Blackstad, 1973).

PAP-immunocytochemistry: light microscopy

Animals were perfused with saline followed by a zinc-formol fixative consisting of 4% commercial formaldehyde, 0.5% zinc dichromate and 0.9% sodium chloride (pH 5.1, adjusted with 1 N NaOH), at room temperature. One hour after perfusion the brains were removed and the cerebella were either cryoprotected and then sectioned on a freezing microtome at 20–25 μm, or immediately sliced on the Vibratome at 20–30 μm without cryoprotection or freezing. In both variants of the procedure, the sections were immunoreacted free floating. Sections were incubated for 1 h in 5% dry milk in 0.5 M Tris buffer (pH 7.6) with or without 0.1–0.5% Triton-X100 and then for 48 h with the primary antibody (R14 serum, diluted 1:1000; R9 serum, diluted 1:500; DA2 ascites, diluted 1:500; 3G8 ascites, diluted 1:200) in 0.5 M Tris-HCl, 1% dry milk, with or without 0.1–0.5% Triton-X100, at 6° C, under gentle agitation. After rinsing in the buffer, sections were processed according to the standard PAP procedure, utilizing secondary antibody and PAP

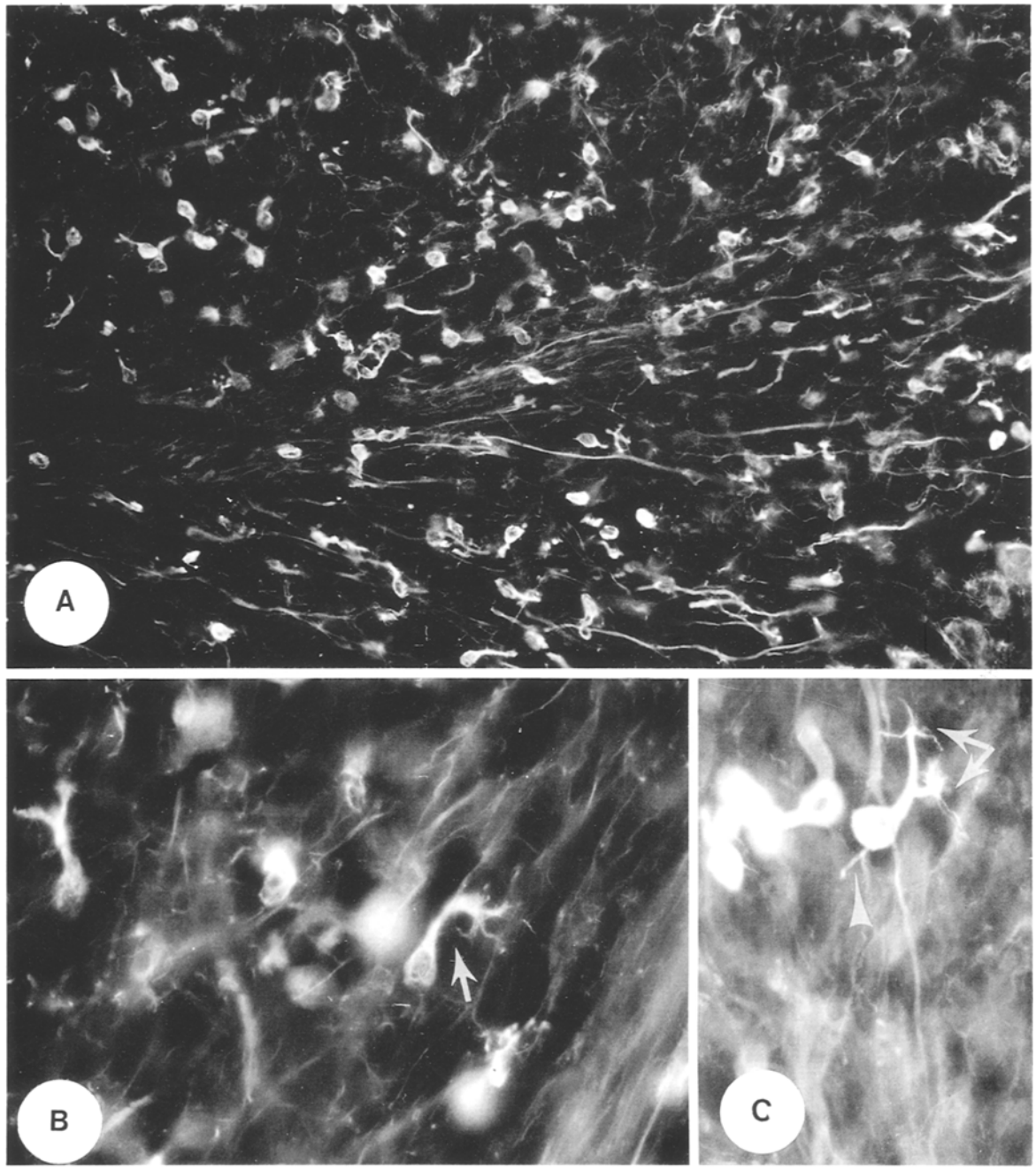


Fig. 1. Distribution of NF-H in the granular layer of the cerebellar nodulus shown by immunofluorescence. (A) phosphate-independent NF-H antibody (R14) reveals immunopositive fibres and a high concentration of unipolar brush cells, which are heavily immunoreactive, while neither Golgi cells nor granule cells appear stained. (B) and (C) Higher magnifications of NF-H-immunoreactive unipolar brush cells; the positivity is found in the cell body, in the dendrite and, although weaker, in the initial axon segment (arrowhead in C). In the neuron illustrated in (B, arrow), the dendritic trunk continues undivided until it terminates with a spray of short branchlets. In (C) is shown a cell with a single dendritic trunk that branches once (split arrow) before it terminates with brush-like branchlets, presumably entering two separate glomeruli. A = 100 ×, B and C = 300 ×.

(Sternberger Monoclonals), and DAB as the chromogen. For controls, normal serum was substituted for the primary antiserum, or the primary antibody incubation step was omitted from the procedure. Control sections were free of immunoreaction product.

PAP-immunocytochemistry: electron microscopy

Animals were perfused with 100 ml calcium-free, oxygenated Ringer variant followed by 250 ml of a fixative consisting of 4% formaldehyde and 0.1% glutaraldehyde in 0.12 M

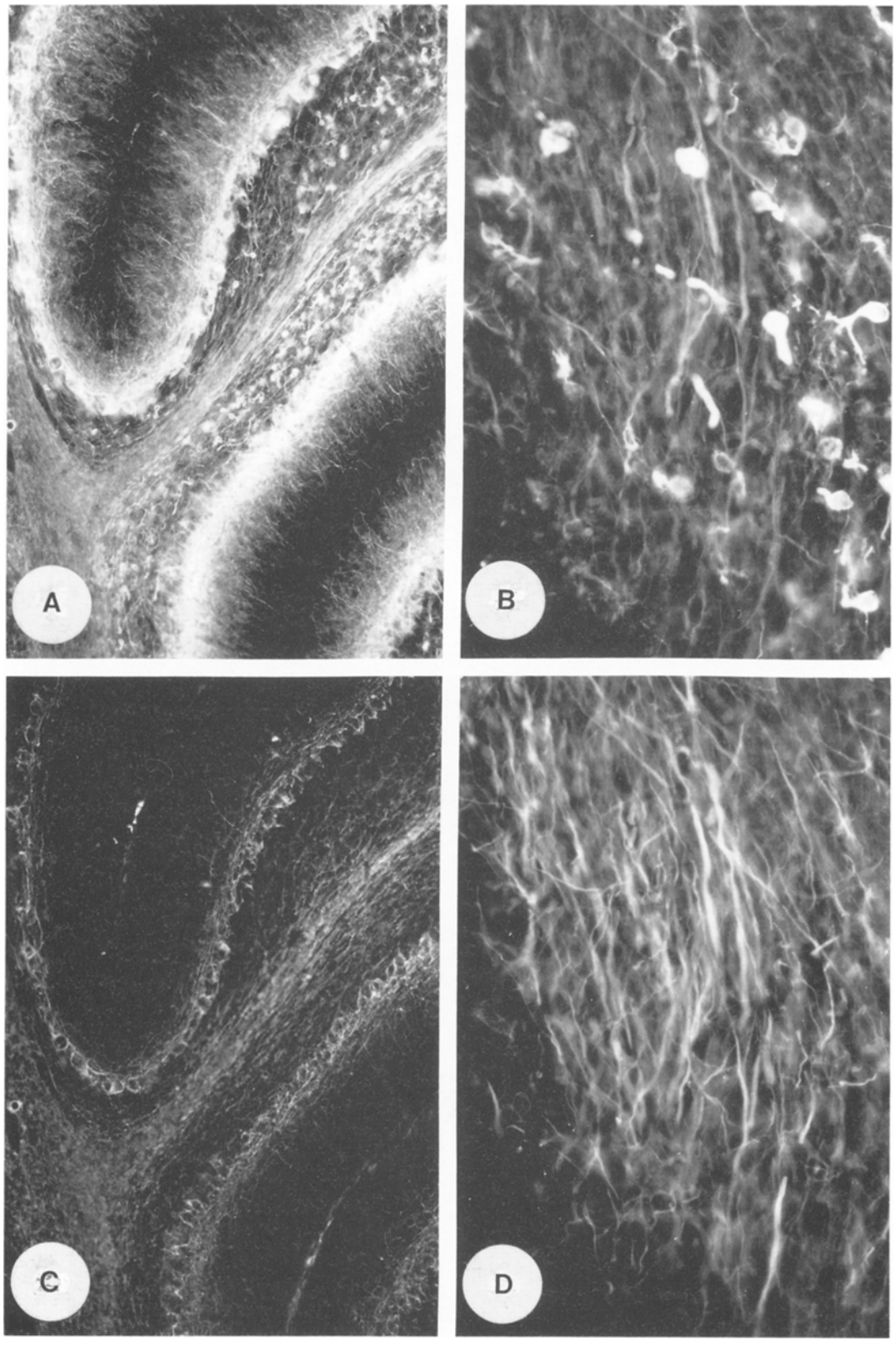


Fig. 2. Lobule IXc of the cerebellum. Double-label immunofluorescence with antibodies raised against NF-H (R14, fluorescein conjugated) (A,B) and NF-M (NN18, rhodamine conjugated) (C,D). (A) Unipolar brush cells are strongly immunoreactive to anti-NF-H. Purkinje neurons, axons of basket and stellate cells and other nerve fibres also show an intense staining with this antibody. (B) Higher magnification of a portion of the granular layer of lobule IXb represented in (A), showing intense NF-H-immunoreactivity in cell bodies and dendrites of unipolar brush cells. (C) the same section as (A) stained with NF-M antibody. (D) The same section as (B) stained to reveal NF-M staining. Unipolar brush cells do not appear immunoreactive to NF-M antibody, while the surrounded axonal processes are equivalently stained in both panels. (A) and (C) = 30 ×; (B) and (D) = 150 ×.

phosphate buffer, and 350 ml of the same fixative, glutaraldehyde omitted. One hour after the perfusion, the cerebella were removed and sliced at 40 μm on a Vibratome. The slices were immunoreacted as specified above, with or without 0.1–0.2% Triton-X100 and using 0.12 M phosphate buffer as diluent for all steps and as rinsing solution. After the immunoreaction, slices were postfixed in OsO_4 and uranyl acetate, dehydrated, flat embedded in Epon, remounted on Epon blanks, and sectioned on the ultramicrotome. Ultrathin sections were contrasted for 3 min with lead citrate and photographed in an electron microscope operated at 80 kV.

Results

Single and double label immunofluoresence microscopy

Sections of adult rat cerebellum, reacted with phosphate-independent antibodies to NF-H by the indirect immunofluorescence method, showed distinct immunostaining of cell bodies, dendrites and axons of Purkinje neurons, basket and stellate cell axons, and nerve fibres in the granular layer and the white matter, as previously reported (e.g., Sternberger & Sternberger, 1983). In addition, we noticed well immunostained small axons in the lower third of the molecular layer which run parallel to the course of the folia and were presumed to be parallel fibres, as well as a population of small neurons located at all levels of the granular layer and in the folial white matter that were heavily concentrated in vermal lobules IXd (the ventral uvula) and X (the nodulus), and in the flocculus and ventral paraflocculus (see Fig. 5a for overview). Cells of this type could be found in the other vermal lobules, but were rarely seen in the cerebellar hemispheres. All phosphate-independent NF-H antibodies used gave strong and clear staining of these cells. Since two of these antibodies (3G3 and 5B8, see Materials and Methods) do not stain the multiply repeated lysine-serine-proline sequences, we can be sure that the heavy staining is a result of a relatively large amount of NF-H and is not exaggerated by the multiple presentation of lysine-serine-proline sequences recognized by many NF-H antibodies. The stained neurons had round or oval cell bodies measuring 9–12 μm in diameter (Figs 1A,B & 3A,B). The cell body usually gave rise to a single, densely stained dendritic trunk, 2–3 μm thick, which terminated in a brush-like spray of short branchlets (Fig. 1C, arrows). Cells with two such dendrites, or with a single stem dividing to form two brush tips, were only occasionally observed. We became interested in these cells because they were evidently identical with the unipolar brush cells recently described with the Golgi method by Mugnaini and Floris (1993) and because in double-label immunofluorescence they showed marked differences in the level of triplet protein staining. Both NF-H and NF-L antibodies gave strong staining (e.g., Fig. 2A,B) although all NF-M antibodies appeared negative or almost negative (Fig. 2C,D). The panel of NF-M antibodies utilized in this study covers a minimum of five different epitopes ranging from the extreme N-terminus to the extreme C-terminus (see Table 1 and 2). Other neurons (e.g. in the cerebellar nuclei and the brainstem motor nuclei) in the same sections showed roughly equivalent perikaryal staining with phosphate independent NF-H, NF-M, and NF-L antibodies, so that this gross reduction of NF-M staining relative to the other proteins is an unusual feature of this cell type, although, as discussed below, Purkinje cells also show unusually strong staining with NF-H antibodies. With double-label immunofluorescence, the unipolar brush cells were also immunoreactive to calretinin antibodies but appeared immunonegative for GABA and glycine (not shown). Cerebellar sections from Sprague-Dawley and Long-Evans rats did not differ in immunostaining pattern, suggesting that the unusual neurofilament staining pattern of these neurons is not a strain specific phenomenon. Antibodies which were specific only for phosphorylated forms of NF-H showed no staining of these neurons, whereas SMI 32, which only stains dephosphorylated NF-H, stains them strongly and clearly. The unipolar brush cells also showed weak but convincing staining with α-internexin antibodies in immunofluorescence microscopy, a finding confirmed by PAP labelling (see below). Interestingly, and in contrast to previous reports (Chiu *et al.*, 1989; Kaplan *et al.*, 1990), we saw weak but convincing staining of Purkinje perikarya with all α-internexin antibodies used here, a finding also seen with PAP immunostaining (see below).

Comparison between Rat-302 and NF-H antibody staining

In 1987 Hockfield described a population of rat cerebellar neurons with very similar features and distribution to those described here. These neurons were defined by their reactivity to an IgM monoclonal antibody Rat-302, which reacts with a doublet of proteins bands running at approximately 160 kDa on SDS-PAGE of brain homogenates. The identity of the protein bands was not determined so that the basis for Rat-302 staining was unknown. When we performed double-label immunofluorescence microscopy with Rat-302 and R14, a polyclonal antibody to NF-H, it was absolutely clear that all Rat-302 positive cells were identically labelled with R14 (Fig. 3A,B). In particular, the fine details of cell body staining on these cells were exactly superimposable, showing that Rat-302 recognizes a cytoplasmic epitope which colocalizes with NF-H. In other regions of the nervous system, R14 staining was much more extensive than Rat-302. We saw Rat-302 staining only in cell bodies of certain

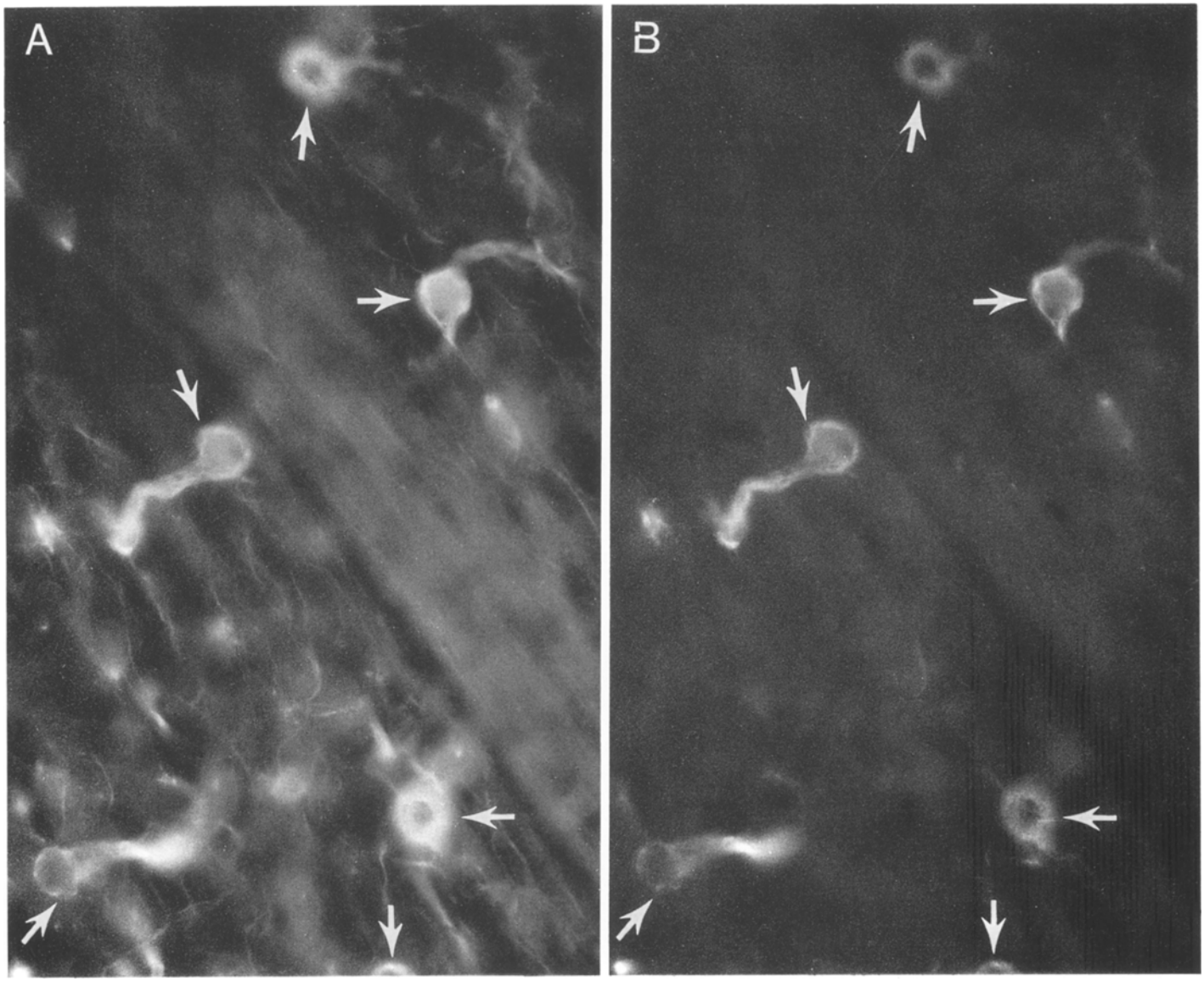


Fig. 3. (A) and (B) show double labelling of the same unipolar brush cells (arrows) with polyclonal antibody R14 to NF-H (A, fluorescein conjugated) and Rat-302 (B, rhodamine conjugated). Within the Rat-302 stained cells there is an exact overlap of staining with R14. 660 ×.

neuron types and a few processes which appear to be dendritic in origin.

Characterization of Rat-302 monoclonal antibody

The superimposition of Rat-302 and NF-H staining suggests that Rat-302 recognizes an epitope associated with neurofilaments, and therefore possibly situated on one or other of the neurofilament subunits. Immunoblots of rat spinal cord intermediate filament preparations were performed, both with and without extensive enzymatic dephosphorylation. The appropriate preparations showed the characteristic increase in SDS-PAGE gel mobility of rat NF-H observed following enzymatic dephosphorylation (compare Fig. 4A,B). Immunoblotting of untreated neurofilament preparations with Rat-302 shows weak and indistinct staining of protein bands about 160 kDa in apparent molecular weight in agreement with Hockfield (1987) (Fig. 4C). In contrast, the enzymatically dephosphorylated material shows a prominent stained band at 160 kDa, the position expected for enzymatically dephosphorylated rat NF-H (e.g., Carden *et al.*, 1987). Immunoblots of purified RH:559–794, a recombinant fusion protein containing 37 of the lysine-serine-proline repeats of rat NF-H (Harris *et al.*, 1991) are stained strongly and specifically by Rat-302 (Fig. 4E,F). Since mammalian phosphorylation sites are not substrates for *E. coli* protein kinases, this fusion

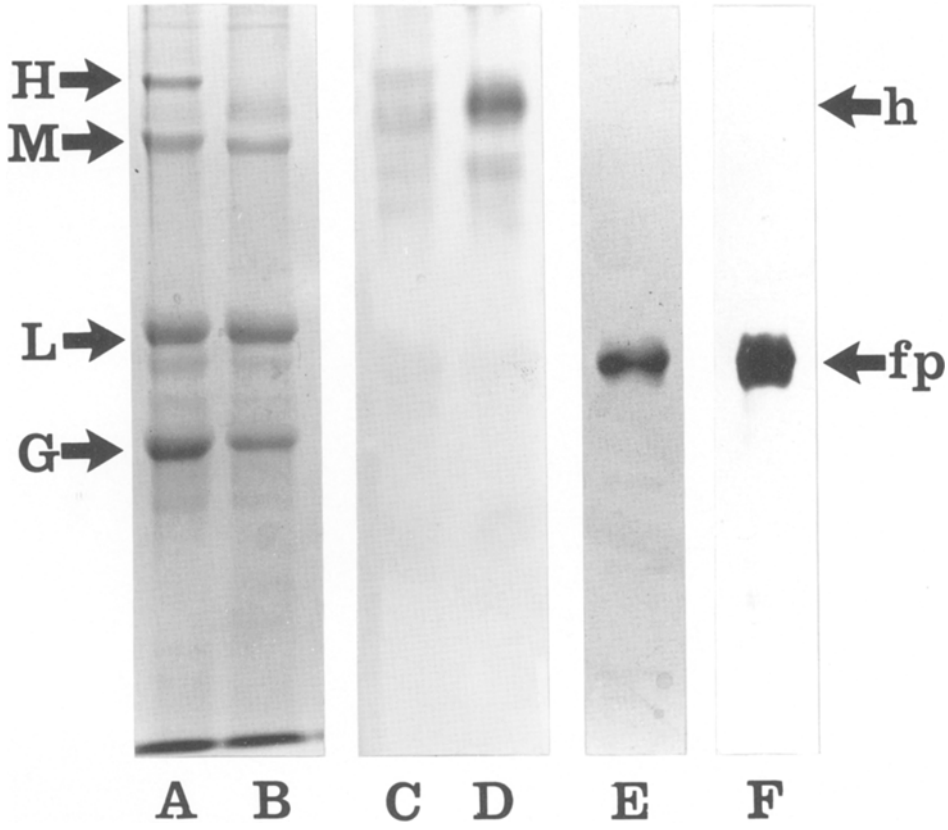


Fig. 4. Characterization of the Rat-302 MAB. Lanes A and B show SDS-PAGE of cytoskeletal material from rat spinal cord stained with Coomassie Brilliant Blue before (A) and after (B) extensive treatment with *E. coli* alkaline phosphatase. H, M, L, and G indicate the major protein bands NF-H, NF-M, NF-L and GFAP respectively. In lane B, phosphatase-treated NF-H, the position of which is labelled h on the right, runs at about 160 kDa. Lanes C and D show an immunoblot of the same two preparations stained with mAb Rat-302. While the antibody shows some weak staining in both preparations, a prominent band identified as dephosphorylated NF-H is seen only in the enzyme treated material. Lane E shows a Coomassie Brilliant Blue stained gel of purified rat NF-H KSP fusion protein, indicated by fp. Lane F shows an immunoblot of a similar preparation stained with Rat-302, revealing strong and clear staining.

protein cannot be phosphorylated. All lines of evidence are therefore consistent with the conclusion that Rat-302 stains the lysine-serine-proline repeats of NF-H but only in their dephosphorylated form.

Double label fluorescence microscopy for AChE and anti-NF-H

As previously reported (Osen & Roth, 1969), AChE staining revealed numerous patches in the granular layer of the flocculus, nodulus and ventral uvula, presumably corresponding to the terminals of cholinergic secondary vestibular fibres (Barmack *et al.*, 1992a,b) (Fig. 5B,C). In sections double stained for AChE and NF-H, the AChE reaction product often appeared to be situated close to NF-H immunopositive unipolar brush cells (Fig. 5D). As a result, the green NF-H signal is usually rendered as orange due to the addition of the brown AChE reaction product (Fig. 5D).

Light microscope PAP staining

PAP-immunoreacted sections confirmed and extended the immunofluorescence findings. After immunostaining with the different phosphate-independent neurofilament antibodies, the Purkinje cells and the unipolar brush cells were immunolabelled to unequal degrees (Fig. 6). In detergent treated sections, Purkinje cells were densely stained by antibody to NF-H (Fig. 6A), but were weakly stained by antibodies to NF-M and NF-L (Fig. 6B,C) and α-internexin (Fig. 6D). Under identical staining conditions, unipolar brush cells stained heavily with NF-H antibody (Fig. 6A), moderately with NF-L antibody (Fig. 6B) and α-internexin antibody (Fig. 6D), and only weakly with NF-M antibody (Fig. 6C). By contrast, other cells were hardly apparent even with the PAP procedure, indicating a generally low level of neurofilament expression in most neurons of the cerebellar cortex. Golgi cells were not revealed by any

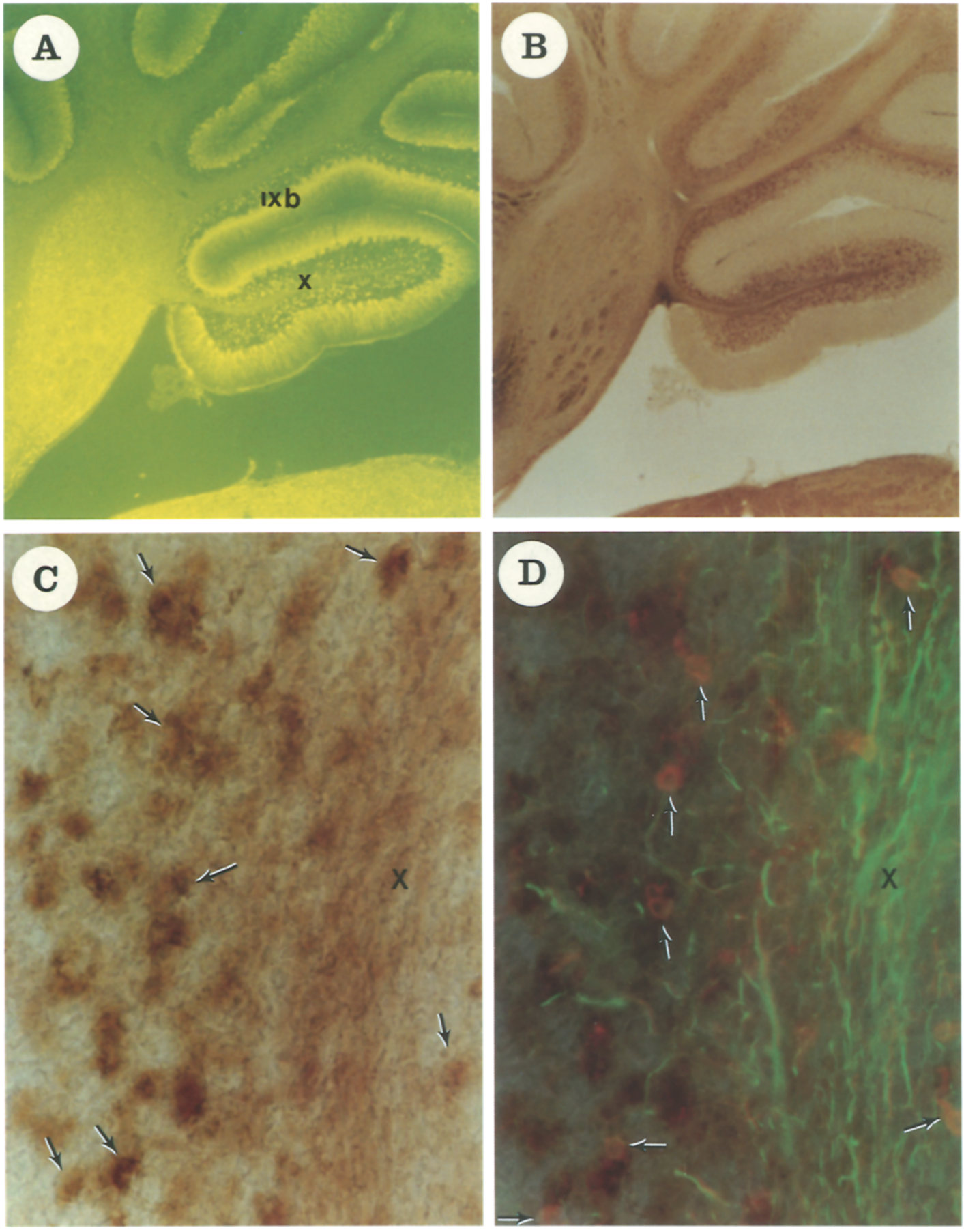


Fig. 5. Double labelling with antibody to NF-H (R14) with immunofluorescence and AChE histochemistry. (A) In the granular layer of lobules X and IXc numerous small cells are intensely immunopositive to NF-H antibody. (B) the same section shows patches of AChE-positivity in the granular layer. (C,D) Higher magnifications of part of the nodulus (X), showing that most of the brown spots of AChE-positivity (arrows in C) are situated in the vicinity of NF-H-positive perikarya of unipolar brush cells (arrows in D). (A) and (B) = 40 ×, (C) and (D) = 400 ×.

of the neurofilament antibodies in sections permeabilized with detergent (Fig. 6A–C). In Vibratome sections immunoreacted without detergent, however, they appeared faintly immunopositive (data not shown) with antibodies to NF-H and α-internexin, although they remained unstained with antibodies to NF-M and NF-L. The somata of Lugaro cells could not be identified in our sections, suggesting that they lack a substantial complement of neurofilament proteins. The cell bodies of stellate and basket neurons were weakly immunostained only in non-permeabilized Vibratome sections that were immunoreacted with anti-NF-H, NF-M or α-internexin, but they were hardly discernible with antibody to NF-L. This indicates that the weak immunoreactivity of Golgi, basket and stellate cell bodies is easily masked when structural preservation is not optimized. Granule cells were faintly stained by antibody to α-internexin (Fig. 6D, arrowheads), but appeared always unstained with antibodies to the triplet neurofilament proteins (Fig. 6A–C). The axons of stellate and basket cells (Fig. 6, unmarked fibres in ML), the Purkinje axons, as well as the mossy fibres, were distinctly stained with all antibodies (Fig. 6). As previously reported (Chiu *et al.*, 1989; Kaplan *et al.*, 1990), α-internexin antibody stained parallel fibres in the molecular layer (Fig. 6D, small, unmarked dots in ML).

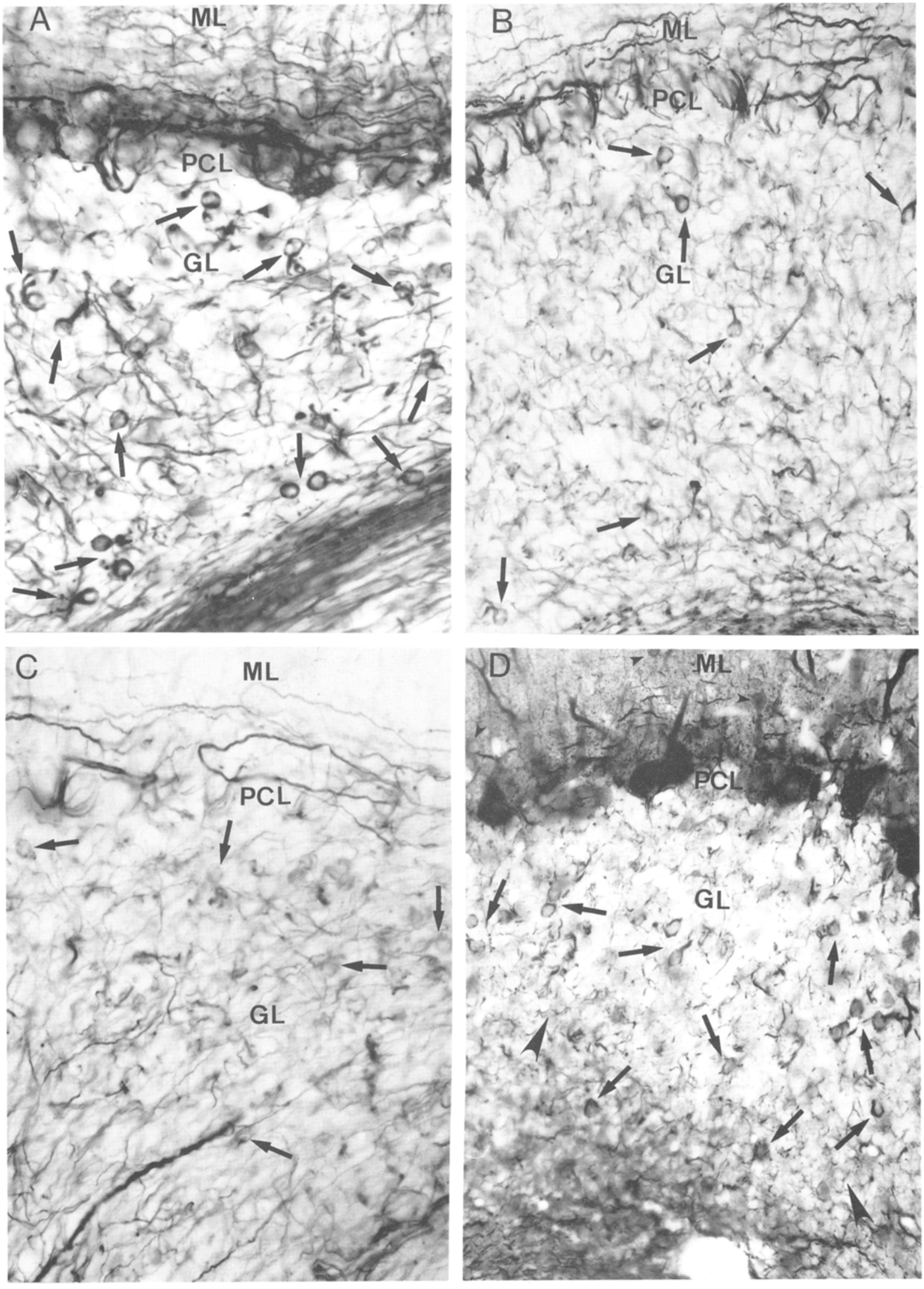
Immunoelectron microscopy with neurofilament protein antibodies

Antibodies to NF-H distinctly immunolabelled Purkinje neurons (PC in Fig. 7A), the unipolar brush neurons (Fig. 8), as well as most of the nerve fibres in both the grey and white matter. The immunoreaction product was evenly dispersed in the nerve fibres, while in cell bodies and dendrites it was patchily distributed (Figs 7,8A). The immunopositive patches, which presumably represent neurofilament rich zones, occupied a larger proportion of the cytoplasm in unipolar brush cells than in Purkinje cells. The immunonegative zones contained cisterns of the endoplasmic reticulum, polyribosomes, the Golgi apparatus, and some microtubules (open circles in Figs 7B & 8A). Many terminal boutons were unstained, but the fibrous cores of *en passant* boutons, where neurofilaments are known to be concentrated, were densely stained (white triangles in Figs 9B & 10).

The unipolar brush cells of the granular layer (Fig. 8) were clearly distinguished from the unstained granule cells because, besides being immunopositive, they had slightly larger cell bodies, and their deeply indented nuclei showed very little condensed chromatin. The cytoplasm contained several large dense core vesicles (Fig. 9A, curved arrows), as well as an unusual organelle consisting of ringlet subunits (labelled 'rs' in Fig. 9A, described elsewhere with high resolution by Mugnaini & Floris, 1994), isolated cisterns of granular endoplasmic reticulum (labelled 'ger' in Fig. 8A) and a larger number of mitochondria than expected in a small cell (Fig. 8A, unlabelled). From the perikaryon emanated a thin axon and a single stout dendritic trunk. Although the cell body and the emerging dendritic trunk occasionally formed complex contacts with mossy fibres (Fig. 10, arrows), resembling those previously termed *en marron* synapses (Palay & Chan-Palay, 1974), as well as simple symmetric synapses with small boutons containing pleomorphic vesicles (not shown, but see Fig. 11B,

Fig. 6. PAP immunolabelling of the cerebellar nodulus with antibodies to neurofilament proteins. ML, molecular layer; PCL, Purkinje cell layer; GL, granular layer. (A) Antibody to NF-H intensely stains the soma and the dendrite of the unipolar brush cells (arrows). Basket cell axons, as well as Purkinje cell bodies and dendrites, also show a strong positivity to NF-H. (B) Antibody to NF-L stains the unipolar brush cells moderately. Some of these cells (arrows) appear more densely stained than others (unlabelled). Basket cell axons are heavily immunoreactive, while Purkinje cell bodies are faintly stained. (C) Antibody to NF-M stains relatively few unipolar brush cells (arrows) and the immunoreaction is weak. Basket cell axons show rather strong immunoreactivity. (D) Antibody to α-internexin produces moderate, but distinct, immunostaining of unipolar brush cells (arrows), without detergent permeabilization. Purkinje cell bodies and dendrites are well stained; a moderate reaction is seen in the perikarya of basket neurons (small arrowheads) and their axons. Large arrowheads point to delicate rims of immunoreactivity around the clear nuclei of granule cells. The parallel fibres (unlabelled) appear as small dots in the molecular layer. 320 ×.

Fig. 7. Pre-embedding PAP immunoreaction with anti-NF-H antibody on Purkinje cells. Cell nuclei are labelled n. (A) Electron micrograph of Purkinje cell bodies (PC) surrounded by basket cell axons (ba). The micrograph is obliquely orientated; the molecular layer (ML) is at the bottom left hand side and the granular layer (GL) is at the top right. In the cytoplasm of the Purkinje cell, the immunoreaction is intense but unevenly distributed, although the penetration of immunoreagent is maximal, as the section was obtained from the very superficial part of the immunoreacted block. Asterisks mark embedding resin still visible within the nuclear territories of a Purkinje cell and a granule cell (GC). By contrast, the neurofilament rich basket axons display an even staining for the antibody. 6 000 ×. (B) Higher magnification of a cytoplasmic portion of a Purkinje cell stained with anti-NF-H antibody. Positivity appears patchy, since no reaction product is found on the Golgi apparatus (ga), nor on the regions occupied by granular endoplasmic reticulum (ger) or profiles which are possibly single microtubules (inside the open circles). At right, a basket fibre (ba) shows dense immunoreaction product outlining and partly obscuring bundles of axoplasmic neurofilaments. 19 100 ×.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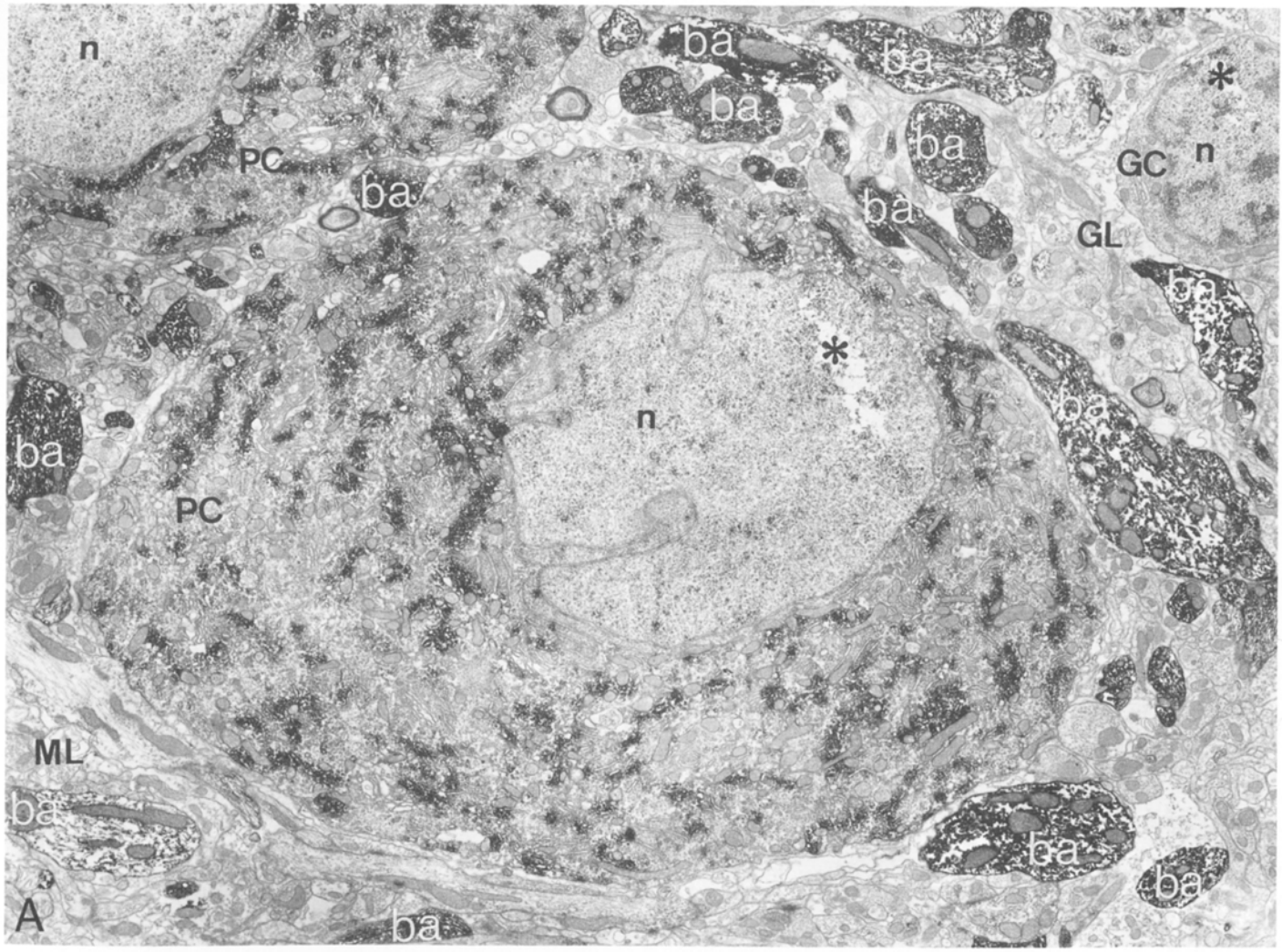
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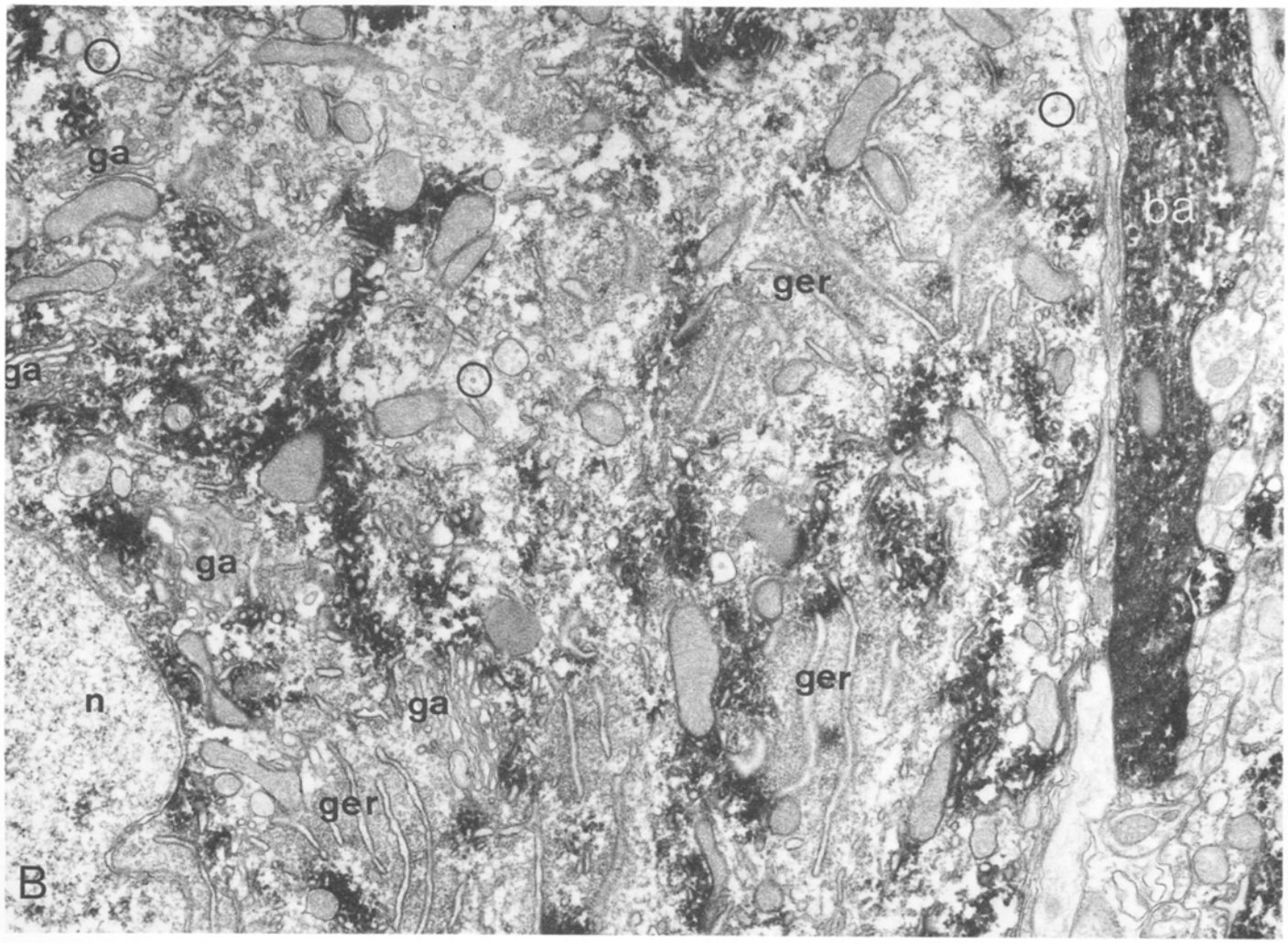
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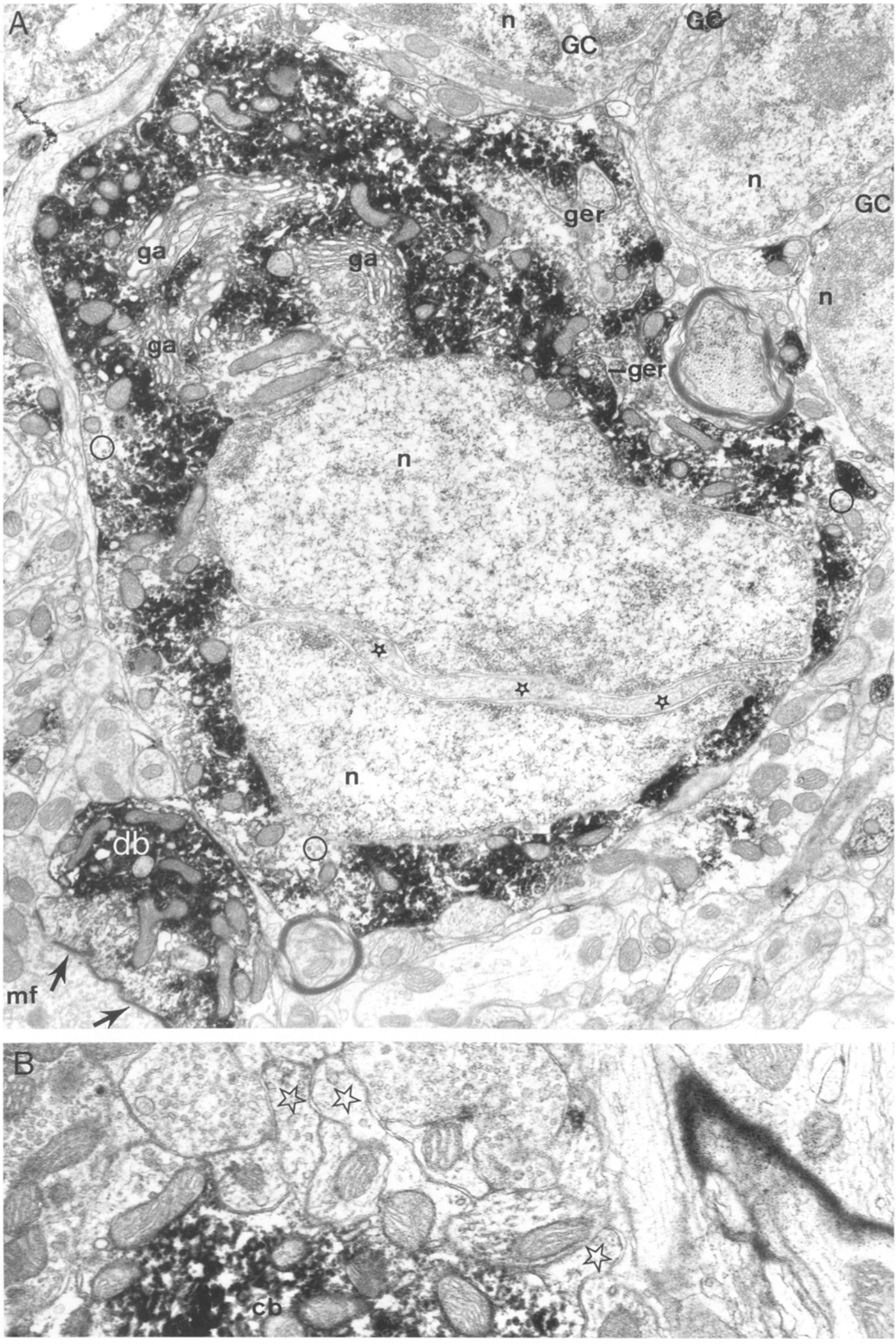
crossed arrow, after immunostaining with α-internexin), most of the synaptic relations were established on the shafts of the branchlets (Figs 8A & 9B) which form at the brush-like tip of the dendrite of the unipolar brush cell and enter one or two glomeruli. Each branchlet was engaged in an extraordinarily extensive asymmetric synapse with the mossy fibre rosette. The branchlets contained abundant immunoreaction product in most of their cytoplasm, except at the subsynaptic region, which showed a microfibrillar web connected to the postsynaptic density (arrows in Figs 8A & 9B). This peculiar mode of distribution of the immunoreaction product was also observed in the slices permeabilized with detergent (Fig. 10), thus excluding the possibility that immunonegativity of the postsynaptic webs depended on insufficient penetration of immunoreagents into the unipolar brush cells. In addition to other organelles, the branchlets contained numerous mitochondria and large dense core vesicles. Short, unstained, non-synaptic appendages, lacking neurofilaments and containing few other cytoplasmic organelles (stars in Fig. 8B), emanated from the cell bodies of many of the unipolar brush neurons, and from their dendritic trunks and branchlets (star in Fig. 9B).

After immunostaining with antibodies to NF-L and NF-M, unipolar brush cells of the granular layer and other neuronal components of the cortex showed patterns of immunoreactivity (data not shown) that were identical to those described above, although the immunoreaction product was less abundant. After immunostaining with antibody to α-internexin, the unipolar brush cells of the granular layer were more evenly stained in their perikaryal cytoplasm (Fig. 11A) than after immunostaining with antibodies to the triplet neurofilament proteins. The postsynaptic filamentous webs in the dendritic branchlets, however, remained unstained or showed only a faint electron density (region occupied by arrow in Fig. 11B). Mossy fibre terminals (Fig. 11, mf) were stained not only in their filamentous core, but also in the cytosol surrounding synaptic vesicles. In the granule cell bodies (Fig. 11A, GC), the cytoplasmic rim was moderately positive, except for the Golgi apparatus, thus confirming the light microscopic immunostaining shown in Fig. 6D. The pattern of immunoreactivity to Rat-302 antibody in the cell bodies and dendrites of the unipolar brush cells (not shown by immuno-electron microscopy, but see Fig. 3) was identical to that obtained with other antibodies to NF-H.

Fig. 8. Electron micrographs showing strong immunoreactivity to anti-NF-H in unipolar brush cells of the cerebellar nodulus. (A) Unipolar brush cell with its clear, euchromatic nucleus (n). A narrow cytoplasmic region (stars with open centres, corresponding to a deep nuclear indentation) is occupied by polyribosomes and seems to divide the nucleus into two territories. Although the immunoreaction is extremely heavy, some areas of the unipolar cell cytoplasm are completely devoid of staining. These areas contain the Golgi complex (ga), isolated cisterns of granular endoplasmic reticulum (ger), microtubules (inside the open circles) and polyribosomes. On the lower left, the intensely immunoreactive dendritic branchlet (db) of a unipolar brush cell establishes a long synapse (arrows) with a mossy fibre terminal (mf). The region in close proximity to the synapse is almost devoid of immunoreaction product. 17 100 ×. (B) Portion of the cell body (cb) of an immunoreactive unipolar brush cell with typical non-synaptic appendages (open stars), free of neurofilaments and immunoreaction product. 41 500 ×.

Fig. 9. Pre-embedding immunolabelling of unipolar brush cells with antibody to NF-H. (A) Electron micrograph of portion of a unipolar brush cell, revealing an intense and uneven cytoplasmic immunoreactivity. The unique cytoplasmic organelle consisting of ringlet subunits is labelled rs. This organelle appears immunonegative. Many dense core vesicles (curved arrows) are present. At n, portion of the clear cell nucleus. 39 000 ×. (B) Extensive synaptic junctions (arrows) between two dendritic branchlets (db) of a unipolar brush cell and a mossy fibre terminal (mf). In the terminal, the immunostained territory (white triangle) corresponds to the neurofilament-rich core. Both dendritic branchlets appear intensely positive, except for the subsynaptic regions, which are completely devoid of immunostaining and contain a microfilamentous web in continuity with the postsynaptic densities. The immunoreaction product absorbed on the outer membrane of the numerous mitochondria, which are present in the cores of the branchlets and the mossy fibre terminal, has presumably diffused from the surface of the neighbouring neurofilaments. Mitochondria (m) that are peripherally situated in the synaptic profiles appear free of absorbed immunoreaction product. Arrowhead indicates a punctum adherens between two dendritic profiles presumably belonging to granule cells. 36 000 ×.

Fig. 10. Pre-embedding immunoreaction with anti-NF-H on unipolar brush cells after permeabilization with detergent. Because of this treatment, the membranes are less conspicuous than in the previous illustrations. (A) The cell body and the emerging dendrite (dt) of a unipolar brush cell form extensive synapses (arrows) with the same mossy fibre terminal (mf), resembling the synapse *en marron* of Palay & Chan-Palay (1974). The terminal shows immunoreaction product corresponding only to its fibrous core (white triangle). The cell body appears patchily immunoreactive, in spite of detergent permeabilization; the Golgi apparatus (ga) and the subsynaptic region are devoid of immunoreaction product. Open arrow indicates an isolated GER cistern. The nucleus (n), with two unstained indentations (stars with open centres) and the nucleolus (nl), occupies a large part of the cell body. GCn, immunegative granule cell nuclei. 15 500 ×. (B) Higher magnification of the cell illustrated in (A), showing that immunostaining suddenly stops before the synaptic web, along the whole extent of the synaptic apposition. The labels are as in (A) 31 500 ×.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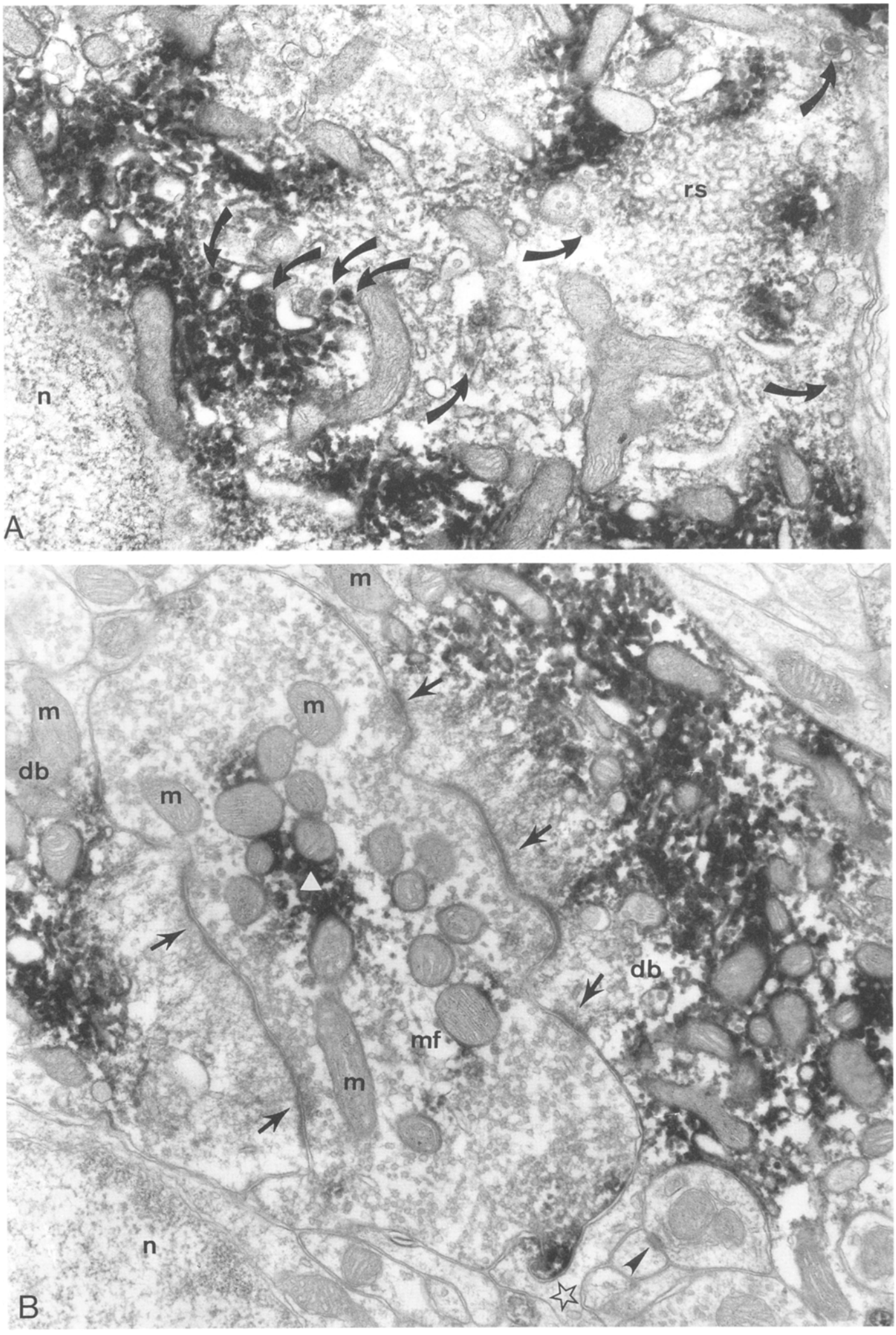
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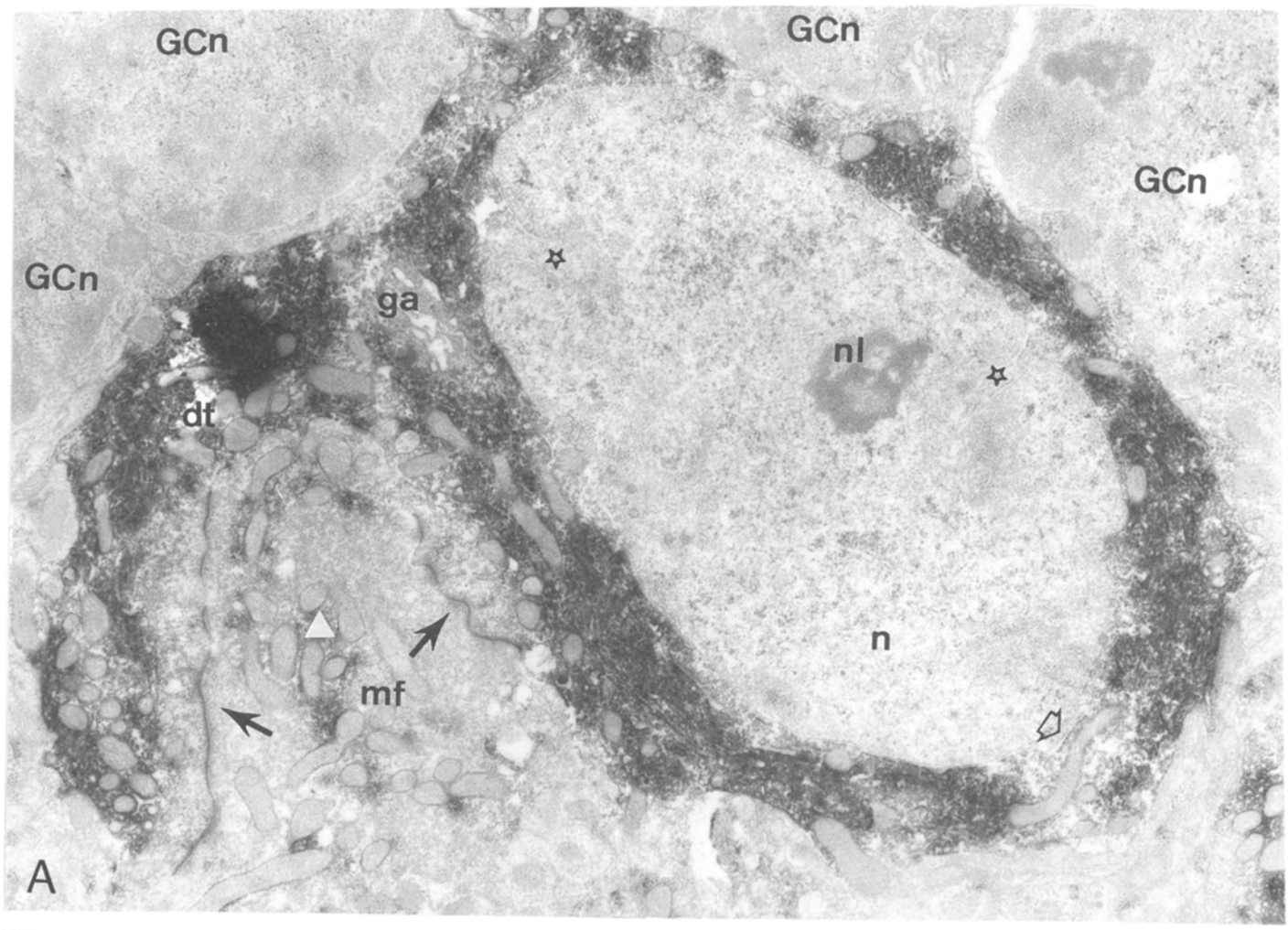
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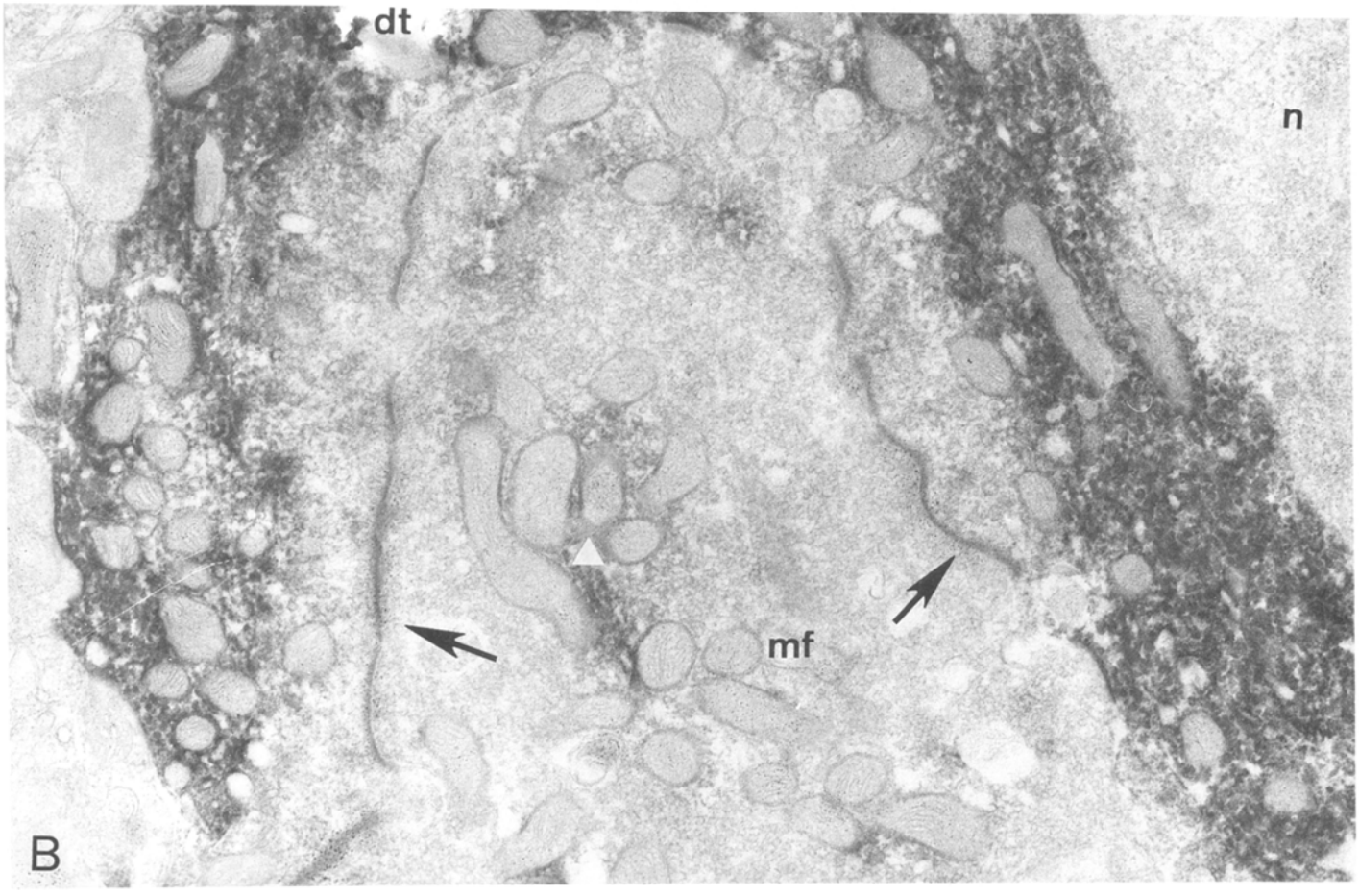
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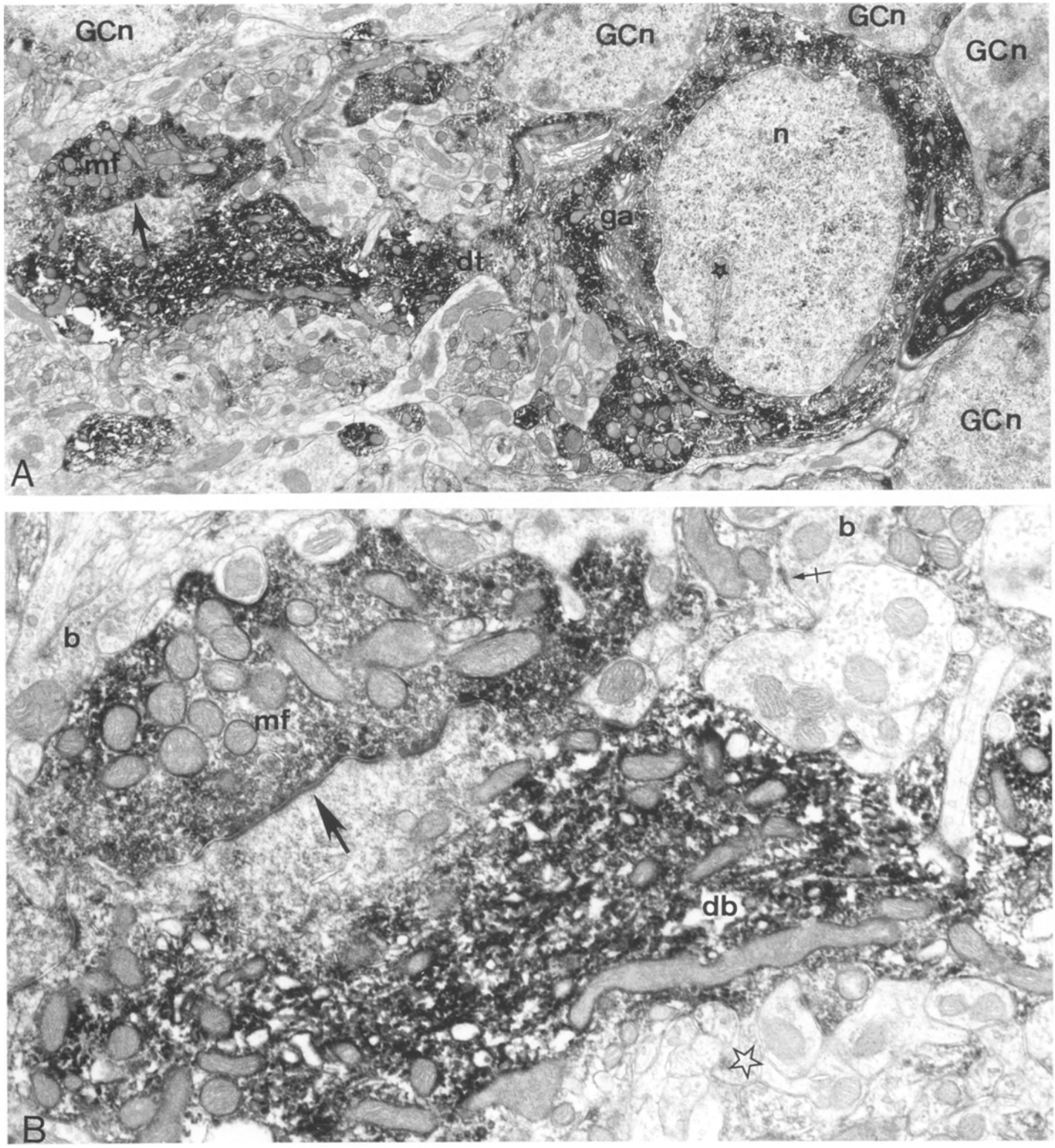

Fig. 11. Pre-embedding immunoreaction with antibody to α-internexin. (A) This electron micrograph was taken near the surface of the tissue slice and reveals a strong and rather even immunoreactivity of a unipolar brush cell. The section plane passes through the cell body, with its clear indented nucleus (n) and an unstained cytoplasmic invagination (star with open centre), the emerging dendritic trunk (dt) and the initial portion of a branchlet, which forms a synapse (arrow, enlarged in B) with a mossy fibre terminal (mf). In the cell body, only the regions of the Golgi apparatus (ga) are completely free of immunoreaction product. Granule cells (labelled GCn over their nuclei) show a weak cytoplasmic immunoreactivity. 7950 ×. (B) The unipolar brush cell dendrite (db) is densely immunoreactive except for the subsynaptic region (arrow), which appears devoid of immunostaining. The mossy fibre terminal (mf) contains immunoreaction product not only in the core, but also around the synaptic vesicles (compare to the mossy fibre terminal shown in Fig. 7, that was immunoreacted with anti-NF-H). A non-synaptic dendritic appendage (open star) is very faintly immunostained. Note that the mossy fibre ending contains round synaptic vesicles and forms assymetric synaptic junctions. A small bouton (b), which contains pleomorphic synaptic vesicles, forms a symmetric synapse (crossed arrow) with a dendritic branchlet of the unipolar brush cell. The small bouton, which may belong to the axonal plexus of Golgi Type II cells, presumably provides modulatory inhibition to the unipolar brush cells. 24 250 ×.

Discussion

A prominent, small neuron type in the granular layer of the rat vestibulocerebellum was conspicuously immunoreactive with NF-H antibodies, but had progressively less reactivity against NF-L, α-internexin, and NF-M antibodies. The differences in the immunoreactivities to the various antibodies were particularly evident in immunofluorescence sections, where, unlike PAP-immunoreacted sections, the signal is not extensively amplified. Previous studies have indicated that most neurons in adult mammals stain equivalently with phosphate-independent antibodies to each of three major neurofilament subunits (Shaw *et al.*, 1981; Trojanowski *et al.*, 1986). There are some exceptions to this rule, but some of these are due to masking of specific epitopes (for example, see Eaker *et al.*, 1991). The situation concerning the cerebellar small neuron is not likely to be due to epitope masking, since antibodies to the N-terminus, the central region, and three distinct regions of the C-terminus all showed similarly reduced staining intensity, even though the same antibodies showed strong staining of NF-M in fibre tracts and cell bodies of deep cerebellar and brainstem nuclei in the same sections. It seems reasonable to conclude that the peculiar small neurons of the vestibulocerebellum contain an unusually small amount of NF-M in relation to NF-L and NF-H. The stoichiometry of the neurofilament triplet proteins *in vivo* has been determined by several groups, but with rather different results (discussed in Scott *et al.*, 1985). Scott and colleagues (1985) reported a molar ratio of NF-L:NF-M:NF-H of 4:2:1 for bovine spinal cord white matter neurofilaments, but noted significant divergence from this ratio across species boundaries and between regions of the nervous system. Numerous studies have shown that the three proteins are expressed differentially during development, with NF-H being essentially absent from NF-L and NF-M-containing neurofilaments prior to neuronal maturation (e.g., Shaw & Weber, 1982; Carden *et al.*, 1987). Since all neurofilament subunits have an α-helical region capable of being incorporated into the intermediate filament backbone, a wide range of triplet protein stoichiometries appear to be possible. The cells described appear to be a unique example of this potential variability, which is presumably of some functional significance. The additional presence of α-internexin in these cells is interesting and may rationalize the very low content of NF-M. Previous studies have shown significant sequence homologies between all regions of α-internexin and NF-M, so that α-internexin could be regarded as a truncated form of NF-M (Fliegner *et al.*, 1990; Shaw, 1992). Therefore, α-internexin may be the functional substitute for NF-M in these cells.

We have demonstrated here that these small NF-H rich neurons are identical to the Rat-302 cells of Hockfield (1987), they have the same distribution as the 'pale cells' of Altman & Bayer (1977) and Sturrock (1990), and show the same unusual morphology as the secretogranin II positive small cells of Cozzi and colleagues (1989), the calretinin-positive small cells of Floris and colleagues (1992), and the unipolar brush cells of Mugnaini and Floris (1993, 1994). Hockfield (1987) described the targeted generation of mAbs against neuronal markers by immunizing mice, which had already been made tolerant to rat spinal cord white matter antigens with rat spinal cord grey matter. This method not only produced the Rat-302 mAb studied here, but also created other mAbs that recognized Golgi II cells (mAb Rat-303) and Lugaro cells (mAbs Cat-301 and Cat-304) (Sahin & Hockfield, 1990). The present study, therefore, introduces another means to further characterize the distinct morphological, distributional and molecular features of Rat-302 neurons. We also show directly that Rat-302 recognizes the lysine-serine-proline repeats of rat NF-H. Hockfield originally noted that Rat-302 stains a doublet of SDS-PAGE bands of apparent molecular weight about 160 kDa (Hockfield, 1987). Since the dephosphorylated form of rat NF-H runs on SDS-PAGE at 160 kDa, all lines of evidence are consistent with the suggestion that Rat-302 recognizes specifically the dephosphorylated lysine-serine-proline repeats of NF-H. Presumably, the protocol used to produce Rat-302 suppressed B-cell responses to either phosphorylated or phosphate-independent neurofilament epitopes, which are both very highly expressed in white matter. The dendritic/perikaryal non-phosphorylated forms of NF-H are specific to grey matter and were, therefore, not suppressed; the production of Rat-302 seems to have been the result. Middle molecular weight neurofilament protein also contains lysine-serine-proline repeated sequences which are, however, surrounded by slightly different amino acids from those in NF-H. Although several monoclonal antibodies have been described which recognize lysine-serine-proline sequences in both NF-H and NF-M, Rat-302 recognizes only the NF-H type sequences. Finally, since Rat-302 is an IgM, it will be possible to use this in future light microscopic double-label experiments in combination with appropriate IgG mAbs, and also for immunoelectron microscopy.

Because of their proximity to Golgi II cells, we believe that Rat-302 cells have also been a confounding variable in studies by several investigators (Jakob, 1928; Brodal & Drablös, 1963; Hamori & Szentágothai, 1966; Eccles *et al.*, 1967; Mugnaini, 1972; Palay & Chan-Palay, 1972; Lange, 1974). In fact, our immunoelectron micrographs suggest that the features of the NF-H rich small cell, its dendritic and synaptic relation to the mossy rosette, are identical to those previously

attributed to Golgi cells. Studies in progress indicate that the NF-H positive small cells are immunonegative for GABA and glycine, and thus must differ from the classical Golgi cells, which are known to be positive for GABA or for GABA and glycine (Gabbott *et al.*, 1986; Ottersen *et al.*, 1988). Both large and small Golgi Type II cells are multipolar and contain a rich complement of granular endoplasmic reticulum and polyribosomes, with few neurofilaments in their cell bodies and dendrites (Golgi, 1882, 1883; Retzius, 1892; Cajal, 1911; Eccles *et al.*, 1967; Mugnaini, 1972; Palay & Chan-Palay, 1974; Mugnaini & Floris, 1994). Their dendrites clearly differ from those of the unipolar brush cells; they display ordinary features, bear few spinous appendages and form simple synaptic contacts not only with mossy fibre endings, but also with varicosities of the ascending granule cell axons (Mugnaini & Floris, 1994).

The unusual neurofilament expression pattern of the rat unipolar brush cells, accompanied by their distinct distribution and unknown function and connectivity in the cerebellar circuitry, make these neurons worthy of further attention. Their extensive synaptic connections with the terminals of mossy fibres indicates that the unipolar brush neurons may be the key elements of a synaptically secure pathway. It is quite intriguing that the same cell type would be rich in NF-H and calretinin, both of which have been implicated in calcium binding and buffering. It is also of interest that both the perikarya and the dendrites of these cells are very rich in secretogranin II, a protein involved with neurosecretion; this may suggest an unusual degree of paracrine activity. Taken together with the very large number of postsynaptic mitochondria, these findings point to an unusually high level of metabolic and electrical activity for the unipolar brush cells, hinting at an important function.

Acknowledgements

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