ULTRASTRUCTURAL LOCALIZATION OF GLIAL FIBRILLARY ACIDIC PROTEIN IN MOUSE CEREBELLUM BY IMMUNOPEROXIDASE LABELING

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ABSTRACT

Glial fibrillary acidic protein was localized at the electron microscope level in the cerebellum of adult mice by indirect immunoperoxidase histology. In confirmation of previous studies at the light microscope level, the antigen was detectable in astrocytes and their processes, but not in neurons or their processes, or in oligodendroglia. Astrocytic processes were stained in white matter, in the granular layer surrounding synaptic glomerular complexes, and in the molecular layer in the form of radially oriented fibers and of sheaths surrounding Purkinje cell dendrites. Astrocytic endfeet impinging on meninges and perivascular membranes were also antigen positive. In astrocytic perikarya and processes, the immunohistochemical reaction product appears both as a diffuse cytoplasmic label and as elongated strands, which by their distribution and frequency could be considered glial filaments.

KEY WORDS glial fibrillary acidic protein · immunoperoxidase · electron microscope

The glial fibrillary acidic (GFA) protein is specific to the nervous system of vertebrates (2, 4, 9). It is thought to be the protein subunit of glial filaments since it is found predominantly in tissues rich in fibrous astrocytes, such as leucotomy scars, multiple sclerosis plaques, and the subependymal layer of the cerebral ventricles (9), and since it is found in fibrous astrocytes by immunofluorescence studies on histological sections (2, 4).

Since GFA protein has been found to be structurally related to neurofilament protein according to biochemical and immunological criteria (6, 17, 19), it was also of interest to investigate whether the GFA protein is limited to glial cells by a method which permits a higher resolution of fine structural detail. In addition, it seemed pertinent to study the subcellular distribution of GFA protein at the ultrastructural level and, as part of a study on the localization of various nervous system antigens, to obtain improved tissue preservation.

MATERIALS AND METHODS

IMMUNOLOGICAL REAGENTS: Antiserum to GFA protein from normal human spinal cord was prepared in rabbits as described previously (5). The protein preparation migrated as a single band in sodium dodecyl sulfate (SDS) polyacrylamide electrophoresis and showed a single precipitin line in double immunodiffu-

sion and immunoelectrophoresis with the antiserum to GFA protein. The antiserum did not react with tubulin (up to 113 μg per well) nor actin (up to 164 μg per well) by immunodiffusion. It also gave only one precipitin line with brain homogenate by immunoelectrophoresis (5). Antiserum controls included normal rabbit serum and rabbit anti-GFA protein antiserum absorbed several times with human GFA protein until no more precipitin line was detectable by double immunodiffusion.

Fab' fragments (11) of goat antirabbit immunoglobulin (GARIgG) were coupled to horseradish peroxidase (Type VI, Sigma Chemical Co., St. Louis, Mo.) according to the method of Nakane and Kawaoi (13). The purified conjugate had an absorption ratio (280 and 403 nm) of 1:4 and was stored in phosphate-buffered saline (PBS), pH 7.2, containng 2% fetal bovine serum at −70°C.

PREPARATION OF TISSUE FOR IMMUNOHISTOLOGY: C57BL/6J mice (21–28 days of age) were anesthetized by intraperitoneal injection of avertin (0.02 ml/g body wt) and perfused through the heart with freshly prepared 4% paraformaldehyde. For some experiments, solutions containing 4% paraformaldehyde and 0.1% or 0.25% glutaraldehyde were used. After perfusion, the brains were removed and immersed in the fixative at 4°C for 2–4 h. Mid-sagittal slices of cerebellum 60 μm in thickness were cut on an Oxford Vibratome (Ted Pella, Inc., Tuscin, Calif.) and rinsed overnight with six changes of PBS at 4°C. The slices were then treated with 0.1 M $NaIO_4$ in PBS for 15 min, rinsed with three changes of PBS over 15 min and incubated with a solution of $NaBH_4$ (10 mg/ml PBS) for 10 min, all at room temperature. After four changes of PBS, sections were further incubated at room temperature with 5% dimethylsulfoxide (DMSO) in PBS for 10 min. After two further changes of PBS, the slices were ready for the immunoperoxidase reaction.

IMMUNOHISTOLOGICAL PROCEDURES: The 60-μm slices were incubated for 1 h at room temperature in 0.3 ml of a 1:100 dilution (in PBS) of either rabbit anti-GFA protein antiserum, or normal rabbit serum, or rabbit anti-GFA protein antiserum exhaustively absorbed with GFA protein. Slices were rinsed with six changes of PBS at room temperature over a 2-h period and then incubated with Fab' fragments of goat antirabbit immunoglobulin coupled with horseradish peroxidase (0.17 mg protein/ml) for 1 h at room temperature. Slices were again rinsed for 2 h with six changes of PBS at room temperature. Some slices were postfixed for 30 min in 0.1%, 0.5%, or 1.0% glutaraldehyde in 0.01 M phosphate buffer pH 7.2. After two 10-min rinses with PBS, slices were incubated with 3,3'-diaminobenzidine 4 HCl (DAB) (Sigma Chemical Co.) at a concentration of 0.2 mg/ml in Tris buffer, pH 7.6 for 30 min in the dark at room temperature with constant agitation (10). Slices were then further treated with the DAB solution containing 0.005% H_2O_2 for 15 min, also in the dark with constant agitation. The reaction was terminated by two changes of PBS, 5 min each.

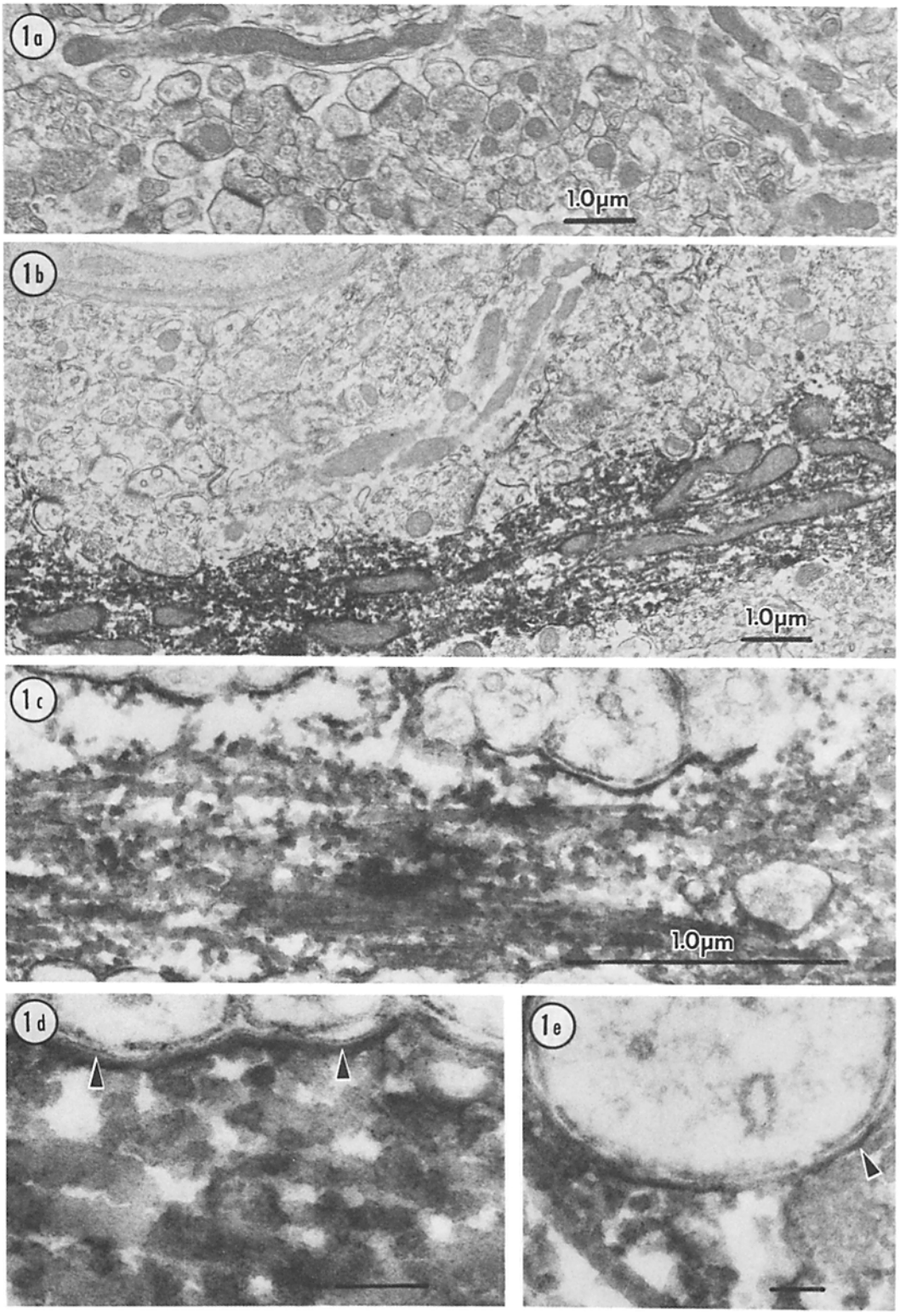
Slices were postfixed for 45 min at room temperature, either in 2% OsO_4 in 0.12 M phosphate buffer, or 1% Dalton's chrome-osmium pH 7.2, rinsed and dehydrated in acetone and propylene oxide and embedded in Epon, or an Epon-Araldite mixture. One micrometer sections were examined for localization of reaction product with the light microscope. Sections 500–600 Å in thickness were then cut with a diamond knife parallel and perpendicular to the sagittal block face, from areas with reaction product, and collected on grids. Some sections were counterstained with lead citrate (15). Sections were examined and micrographs obtained with a Philips EM 300 or a JEOL 100S electron microscope.

RESULTS

Light Microscope Observations

Cerebellar slices reacted with anti-GFA protein serum show the typical pattern of immunohistological staining previously observed with immunofluorescence and immunoperoxidase techniques (2, 4, 8, 12). The GFA protein reaction product is in astrocytes of the white matter and Bergmann glial cells extending radially through the molecular

FIGURE 1 Molecular layer of a mouse cerebellum incubated with nonspecific rabbit serum (*a*) and rabbit anti-GFA protein (*b–e*) followed by incubation with horseradish peroxidase-labeled serum GARIgG. Specific reaction product is limited to astroglial processes in the cerebellar slices incubated with rabbit anti-GFA protein (*b–e*). In both Fig. 1 *a* and *b* the postsynaptic densities are very electron dense (perfusion with 4% paraformaldehyde and tissue fixed after immunocytochemical procedures in osmium tetroxide, Pb citrate contrasted, (*a*) × 11,000; (*b*) × 11,000. (*c*) Higher magnification of an astroglial process showing the globular and linear nature of the reaction product and the cell membrane labeling (perfusion with 4% paraformaldehyde and 0.25% glutaraldehyde and postfixed after incubation in osmium tetroxide. (*c*) × 40,000. (*d* and *e*) Two Bergmann astroglial processes in the molecular layer of cerebellum. Note the disposition of reaction product along the inner (cytoplasmic) layer of the plasma membrane and the outer (cytoplasmic) layer of the mitochondrial membrane (arrows) in addition to the globular diffuse or linear cytoplasmic reaction product. Tissue preparation same as in Fig. 1 *c*. (*d*) × 145,000; (*e*) × 88,000.


1a
1.0μm
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1d
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layer to form astrocytic endfeet at the meningeal surface. Astrocytic processes are also antigen positive around blood vessels. GFA antigen is not detectable in neurons or oligodendroglia. No specific reaction product is seen in slices treated with non-immune control serum.

Electron Microscope Observations

Control sections treated with non-immune serum are devoid of reaction product (Fig. 1*a*) even in the most superficial sections which are cut parallel to the original surface of the tissue slices. In slices treated with anti-GFA protein serum which was exhaustively absorbed with GFA protein, specific labeling of astrocytes is no longer detectable. Nonspecific peroxidase deposition was markedly decreased by treating tissue slices with $NaIO_4$ and $NaBH_4$ before antibody reactions. Sections treated with anti-GFA protein serum show deposits of reaction product up to 5–10 μm from the exposed surfaces of the slice. Reaction product up to only 5 μm is observed when the treatment with DMSO is omitted. Within a given section, most but not all cellular structures identifiable as astrocytic processes contain reaction product.

MOLECULAR LAYER: In the molecular layer, radially oriented Bergmann glial fibers are heavily labeled (Fig. 1*b*). Specific globular reaction product can be seen resembling beads on strings and coursing in parallel to the extending glial processes (Fig. 1*c*). However, labeling is not confined to these filamentous structures, but is also present throughout the cytoplasm (Figs. 1 and 2). Mitochondria are not stained inside but have reaction product at their outer membranes (Fig. 1*e*). Globular reaction product also accompanies the plasma membrane (Fig. 1*c–e*). Because of the size of the globular deposit (approx. range 20–40 nm), it is generally difficult to define the exact localization of the antigenic site in respect to the external or cytoplasmic surfaces of the plasma membrane; but whenever it is possible to distinguish the outer or inner leaflet of the lipid bilayer, the layer of reaction product seems to be associated with the cytoplasmic side (Fig. 1*c–e*).

Heavily stained astrocytic processes also extend to blood vessels and pia where they form endfeet abutting against the basal lamina (Fig. 2*a* and *b*). Pericytes and endothelial cells are antigen negative.

Antigen-positive structures resembling glial cell processes are also seen along Purkinje cell dendrites (Fig. 3). These dendrites contain, in addition to microtubules, 90–100 Å neurofilaments and are completely devoid of reaction product. Likewise, granule cell axons do not carry detectable GFA protein, nor are postsynaptic thickenings more intensely labeled than in the corresponding control sections (Figs. 1*a*, *b*, and 3). The postsynaptic density has a slightly increased electron density in both lead citrate counterstained and non-counterstained sections when compared to tissue which has not been subjected to the immunocytochemical procedures (Fig. 1*a*). The reaction product at the postsynaptic thickenings is not globular in character, in contrast to the specific histochemical product of the peroxidase reaction, and therefore is considered nonspecific.

GRANULAR CELL LAYER: Cell bodies of astrocytes contain reaction product in the cytoplasm of the perikaryon, but not in the nucleus (Fig. 2*c*). Nucleus, perikaryal cytoplasm, and dendritic processes of granule cells are devoid of antigen. Synaptic glomeruli are surrounded by antigen-positive astrocytic processes. Mossy fiber terminals and other neuronal elements of the synaptic glomeruli are devoid of reaction product.

WHITE MATTER: In the white matter, myelinated and unmyelinated tracts are interspersed with densely stained astrocytic processes. No label is seen associated with oligodendroglial structures or with axonal profiles. However, penetration of antibody and/or DAB into myelinated regions is less complete than into other layers, so that absence of labeling may not only be due to absence of antigen.

DISCUSSION

GFA protein is present only in astrocytes and their processes. Oligodendroglia and neurons do not contain antigen detectable by the indirect immunoperoxidase method.

These observations extend the previous immunohistological studies on the cellular localization of GFA protein (2, 4, 8, 12) to the subcellular level with preservation of the fine structural detail. Our observations concur with those in a preliminary report in abstract form by Eng and Kosek (8).

Within astrocytes, we find that GFA protein is not limited to intracellular filamentous structures, but is also diffusely present in the cytoplasm without any recognizable association with subcellular organelles. However, because of the size of the immunohistochemical reaction product which is formed in globular deposits, it is mostly impossible

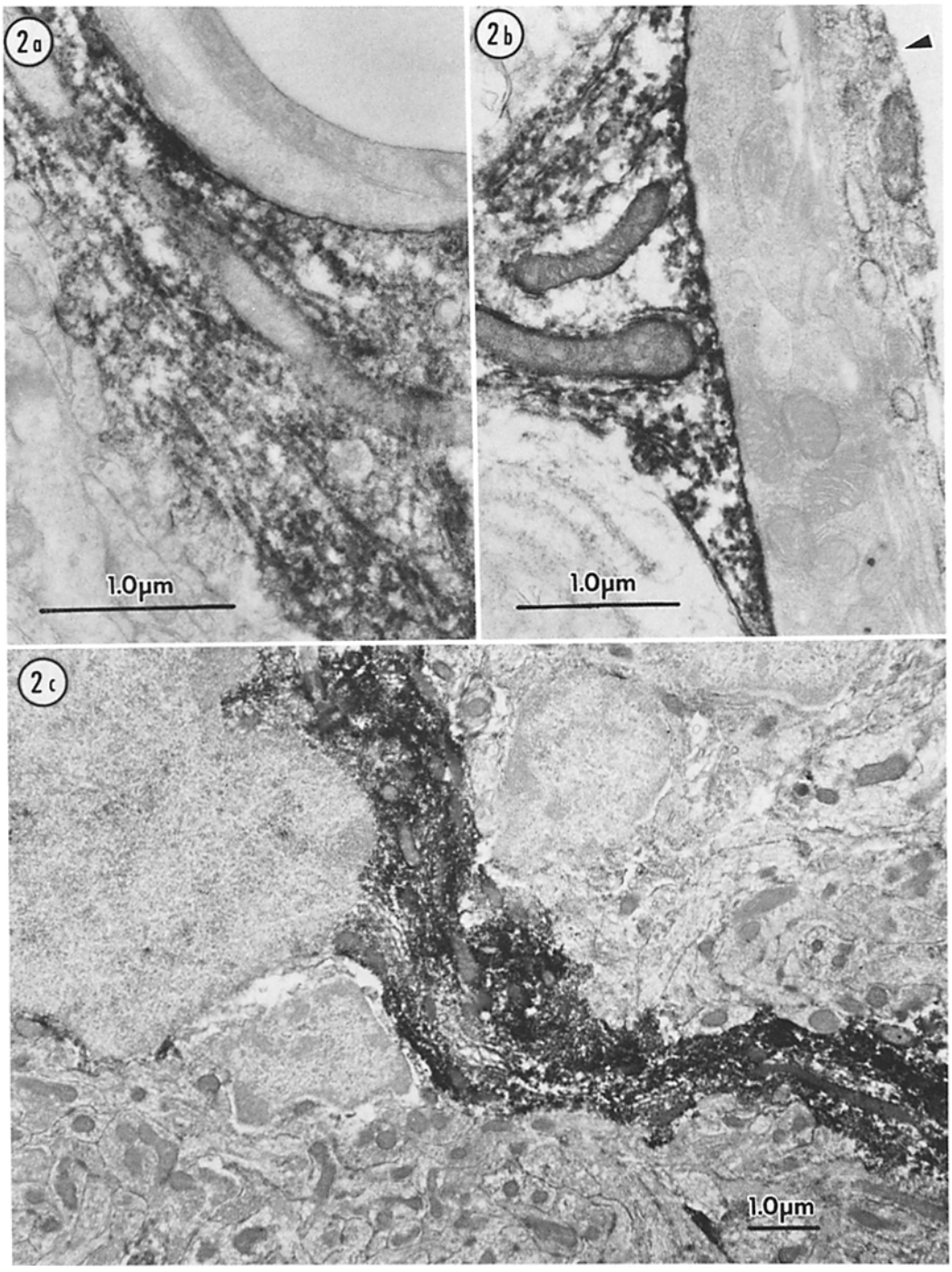

FIGURE 2 Two astrocytic endfeet abutting a capillary (*a*) and the pial surface (*b*). Note the linear distribution of the reaction product and the diffuse deposition in the cytoplasm. Note the lack of reaction product in the adjacent cell processes. Electron-dense deposits in the meninges (arrow) are also observed at the cut surface of sections treated with control sera. In contrast to the globular specific peroxidase reaction product, these deposits are weak and fuzzy in appearance. (*a*) Tissue preparation same as Fig. 1*c*. X 28,800. (*b*) Perfused as for Fig. 1*c* and postfixed before DAB in 0.5% glutaraldehyde. × 24,000. (*c*) An astrocyte cell body in the granular cell layer is filled with reaction product. Note that the nucleus and adjoining granular neurons are devoid of reaction product. 4% Paraformaldehyde perfusion and postfixed before DAB reaction with 1% glutaraldehyde. × 9,900.

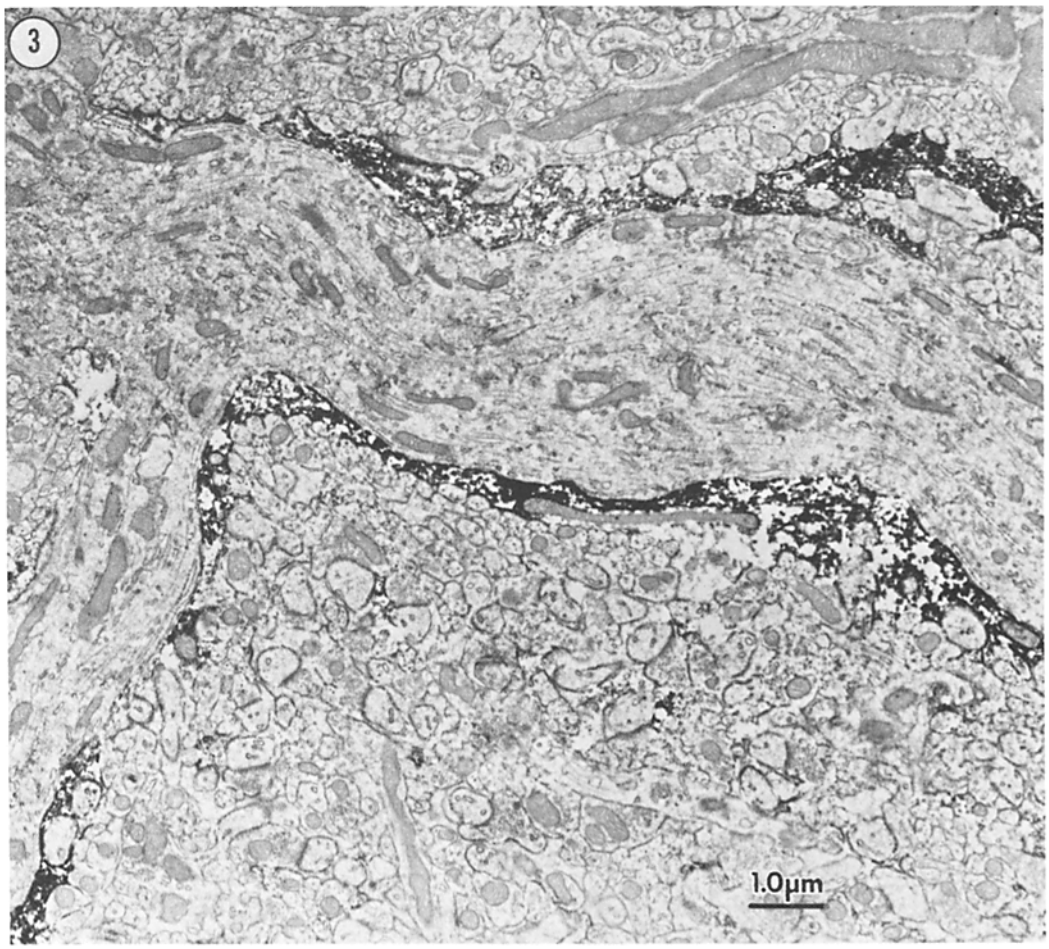

FIGURE 3 The Purkinje cell dendrite is surrounded by astroglial processes filled with reaction product. The intradendritic organelles are devoid of reaction product. Tissue preparation same as for Fig. 2. × 10,200.

to distinguish the underlying morphological features.

Whether this diffuse reaction corresponds to the water-soluble form of GFA protein (3) and whether staining of mitochondrial and plasma membranes is real or an artifact caused by the fixation and immunocytochemical procedures remain to be seen. It was found that with paraformaldehyde fixation alone, some "bleeding" of reaction product could occur, spilling over from very heavily labeled sites to adjacent, apparently unlabeled ones as suggested by Novikoff et al. (14). It seemed as though postsynaptic densities were particularly prone to stain in the close vicinity of densely labeled astrocytic processes. It cannot be decided at which step during the histological procedure this process occurs. However, perfusion with small concentrations of glutaraldehyde (0.1 or 0.25%), in addition to paraformaldehyde, and postfixation with glutaraldehyde before the diaminobenzidine reaction resulted not only in better preservation of structure, but also in a more localized, crisper appearance of reaction product. In addition, pretreatment of tissue with $NaIO_4$ which oxidatively splits carbon bonds between adjacent cishydroxyl groups and subsequent reduction of the aldehyde groups with $NaBH_4$ (1) markedly decreases nonspecific peroxidase deposition, especially at the cut edge of the section. This increase in reaction specificity may result from destruction of the negatively charged sialic acid residues. DMSO was used to improve the penetration of the various reagents.

Under all experimental conditions, no labeling

of neurons or their processes has been observed. Therefore, neurofilaments in mouse brain do not react detectably with the anti-human GFA protein antibodies in tissue sections, despite the structural similarities between the protein subunits of glial and neuronal filaments (6, 17–19). That immunocytological differences between neurofilament and GFA proteins are possible to detect has recently been shown by other studies (6, 7, 16 and our unpublished observations). In our study, a clear-cut immunological distinction between glial and neuronal filaments is evidenced at the subcellular level.

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