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A monoclonal antibody raised against an [Na^++K^+]coupled L-glutamate transporter purified from rat brain confirms glial cell localization

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A monoclonal antibody (9C4) shows that an [Na^++K^+]coupled glutamate transporter protein purified from rat brain runs electrophoretically as a wide band and is localized in neuroglial cell bodies and processes, but not in neurons. This confirms the findings with polyclonal antibodies [Neuroscience 51 (1992) 295–310], and shows that the apparent heterogeneity in relative molecular mass is accounted for by a single antigenic epitope. By testing several synthetic peptides derived from the deduced amino acid sequences of two cloned rat brain glutamate transporters, the antigenic epitope was identified as residing within the peptide TQSVYDDTKNHRESNSNQC (residues 518–536) of one of these [Nature 360 (1992) 464–467].

Cotransport; Neurotransmitter; Monoclonal antibody; Astrocyte; Glutamate

1. INTRODUCTION

The high affinity uptake system for the neurotransmitter glutamate mediates electrogenic cotransport of this amino acid with sodium and potassium (the latter moving outwards) [1]. Since glutamate is considered to be the major excitatory transmitter in brain [2–5], this transport system is of particular importance. It appears to be the mechanism by which synaptically released excitatory amino acids are inactivated [6] and kept below toxic levels in the extracellular space [7–9]; thus impaired uptake in pathological conditions may lead to cell damage [10–13].

Recently we have reported on the purification of a glutamate transporter [14], and its identification and localization in brain by polyclonal antibodies raised against the purified protein [15]. By screening a cDNA library with these antibodies, a glutamate transporter has been cloned [16]. However, these polyclonal antibodies labelled a wide band upon electrophoresis and immunoblotting of detergent extracts of rat brain. It might therefore be argued that they recognize more than one protein. If so, the study on the localization [15] of the transporter would be equivocal.

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Abbreviations: BSA, bovine serum albumin; DEAE, diethylaminoethyl; ELISA, enzyme linked immunosorbent assay; GAT, GABA transporter; GLAST and GLT, two different glutamate transporters; GLYT, glycine transporter; GNA, Galanthus nivalis lectin; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; PROT, proline transporter; PMSF, phenylmethanesulfonyl fluoride; TBST, 10 mM Tris-HCl pH 8.0, 150 mM NaCl, 8 mM NaN_3, 0.05% Tween 20.

In this study we have raised a monoclonal antibody against the purified 73 kDa polypeptide [14,15] and we have synthesized 12 peptides based on the sequences of the two cloned rat brain glial glutamate transporters reported to date [16,17]. With these tools we demonstrate that (1) recognition of a single epitope is sufficient to account for the broad electrophoretic band characteristic of the 73 kDa polypeptide; (2) the 73 kDa polypeptide is indeed localized in glia cells, including their fine processes.

2. MATERIALS AND METHODS

2.1. *Antigen purification and polyclonal antibodies*

Purification of a glutamate transporter was done as described [14,15]. The purest fraction (the peak fraction from the DEAE-cellulose column) was further purified by preparative electrophoresis, dialysed against water, lyophilized and stored at −20°C until used as described [15]. The polyclonal antibodies [15] were obtained by immunizing a rabbit with this protein and affinity purifying the ensuing IgG on a column with immobilized deglycosylated antigen. Protein was determined as described [18], when necessary after rapid gel filtration to remove interfering substances [14]. Sodium dodecyl sulphate-extracts from whole rat brains were prepared as described [15].

2.2. *Immunization*

50 μg of the above lyophilized protein was dissolved in 0.2 ml water, emulsified with 0.3 ml complete Freund's adjuvant and injected intraperitoneally into a Balb/c male mouse. Three more immunizations with the antigen in Freund's incomplete adjuvant were performed at intervals of about one month. After the second and third immunizations, the mice were bled and the serum was tested on immunoblots containing sodium dodecyl sulphate-extracted brain proteins.

2.3. *Generation and selection of hybridoma cell lines*

The mouse with the highest titer was given a fourth injection. Four days later the spleen cells were collected. The activated lymphocytes were enriched on a two-step discontinuous Percoll gradient (35% Per-

coll in F1 and 51% Percoll in F2) to increase the rate of producing hybridomas [19,20]. Mouse myeloma cells (NS0) were fused with spleen cells at a 1:1 ratio, using polyethyleneglycol as described [21]. After fusion, the cells were cultured in Iscove's modified Dulbecco's medium supplemented with hypoxanthine, thymidine, aminopterin and 20% fetal calf serum on a feeder layer of peritoneal macrophages from Balb-c mice. Positive clones were selected after 8–10 days (using the ELISA test system described below) and subcloned in medium without aminopterin. After the second subcloning, strongly positive clones were mass cultured. Ascites was induced in Balb-c mice. Both supernatants and ascites were tested on Western blots and on ELISA. Commercial kits were used to determine immunoglobulin class (The Binding Site, Birmingham, UK; Sigma, St. Louis, MO).

2.4. *ELISA*

All incubations were performed on a horizontal shaker at room temp. Each well in 96-wells polystyrene tissue culture plates was incubated (2 h) with 25 μl of the peak fraction from the DEAE-cellulose column [14] (diluted 1 + 2 with water; protein concentration: 25–50 μg/ml) and blocked (2 h) with 300 μl of 20% newborn calf serum in TBST (150 mM NaCl, 0.05% v/v Tween 20, 10 mM Tris-HCl pH 8.0, 8 mM NaN_3) followed by incubation (1 h) with 50 μl supernatant and washing with TBST (6 cycles of 50 seconds). Bound antibodies were detected with alkaline phosphatase-conjugated rabbit anti-mouse IgG (Promega, Madison, WI) diluted 1:1000 in 20% (v/v) newborn calf serum in TBST (50 μl, 1 h). Washing in TBST (6 cycles of 50 seconds) was followed by addition of 200 μl *p*-nitrophenyl phosphate (1 mg/ml) in 0.1 M sodium glycine pH 10.4 with 1 mM $MgCl_2$ and $ZnCl_2$. The OD_{405} was measured after 20, 60 and 120 minutes.

2.5. *Electrophoresis and immunoblotting*

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis was done as described [14, 22]. After electrophoresis the proteins were either silver stained [14] or blotted onto nitrocellulose membranes as described [23] and briefly rinsed in TBST. After blocking (60 min) of unspecific binding sites with blocking solution (BSA 10 mg/ml in TBST), the nitrocellulose was incubated (overnight, room temp.) with antibodies in new blocking solution. Affinity-purified rabbit IgG anti-glutamate transporter antibodies [15] were used at 50 ng/ml while purified IgM 9C4 antibodies were used at 100 ng/ml when not stated otherwise. Cell culture supernatants were diluted 1 + 1 with 2 × blocking solution. Bound antibodies were detected with anti-rabbit IgG (200 ng/ml; Promega, Madison, WI) or anti-mouse Ig conjugated to alkaline phosphatase (200 ng/ml; Promega, Madison, WI).

2.6. *Purification of mouse IgM from ascites*

A 2 ml column (bed height 25 mm) of *Galanthus nivalis* (GNA) lectin immobilized on agarose beads (E-Y Laboratories, San Mateo, CA) was equilibrated (at 4°C) with starting buffer (150 mM NaCl, 10 mM sodium-HEPES pH 7.5). 1.2 ml ascites was applied to the column (flow rate 15 ml/h). Subsequently, the column was closed for 60 min and then washed with 12 ml starting buffer (flow rate 120 ml/h). Bound IgM was eluted with 6 ml 0.2 M α-mannose in starting buffer (flow rate 3–6 ml/h) and concentrated by ultrafiltration.

2.7. *Synthetic peptides and coupling to ovalbumin*

Peptides were obtained 80–95% pure (as determined by HPLC) either from the Centre of Biotechnology (Oslo, Norway) or from Multiple Peptide Systems (San Diego, CA). All the peptides (see legend to Fig. 2 for details) except GLYT and GAT1 were obtained as carboxy-terminal amides. The peptides were coupled to ovalbumin as described [24]: 1 ml 2% glutaraldehyde was added to 1 ml ovalbumin (1 mg/ml) and peptide (1 mg/ml) in 0.15 M NaCl and 0.1 M sodium-HEPES pH 7.4. After incubation (60 min, 4°C, continuous stirring) sodium borohydride (10 mg/ml) was added. The mixture was incubated (60 min, 4°C), dialysed against 0.1 M sodium phosphate buffer pH 7.4, spotted on nitrocellulose, dried and incubated with antibody (see 'immunoblotting' above, legend Fig. 2 and 'Immunocytochemistry' below).

2.8. *Immunocytochemistry*

Rats were perfusion-fixed and the tissue was subsequently processed for pre-embedding light and electron microscopic immunocytochemistry largely as previously described [15], but using biotinylated sheep anti-mouse Ig and streptavidin-biotinylated horseradish peroxidase complex (Amersham) both diluted 1:100 in 100 mM Tris-HCl pH 7.4 with 0.3 M NaCl and 1% (v/v) newborn calf serum (without Triton). The antibody preparation was applied at 0.1–120 μg/ml (with 10% newborn calf serum) for 48 h at 4°C. Cellulose nitrate/acetate filters (Millipore), carrying spots (ca. 0.2 μl) of the peptides coupled to ovalbumin by glutaraldehyde (see above), were processed together with sections in order to test for crossreactivity in the immunocytochemistry conditions [2].

Staining of cell nuclei with the 9C4 antibody was seen light microscopically in tissue which had been perfusion-fixed with a formaldehyde/glutaraldehyde mixture (1%/2.5%) and stored in fixative. This staining was strongly reduced when the tissue was used shortly after fixation (the following day) or when the formaldehyde-glutaraldehyde mixture was replaced with 4% formaldehyde alone. With fresh formaldehyde/glutaraldehyde-fixed tissue, nuclear staining was not evident at concentrations of GNA-isolated IgM below 36 μg/ml. The following description is based on the use of GNA-isolated antibody (see above) below 36 μg/ml.

3. RESULTS

3.1. *Screening of hybridomas and antibody specificity*

One clone (9C4) which was strongly positive in the ELISA test system produced an IgM antibody that labelled specifically a 73 kDa band on immunoblots. Immunoblots probed with this antibody were indistinguishable from electrophoresis gels of highly purified protein stained with silver, and from immunoblots of crude or partially purified protein probed with the pol-

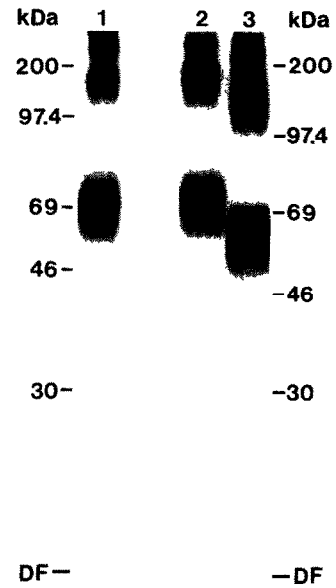

Fig. 1. Purified glutamate transporter protein (1 μg in each lane) was electrophoresed and visualized either directly with silver staining of the gel (lane 1) or, after blotting, with the 9C4 monoclonal antibody (lanes 2 and 3). The protein in lane 3 was treated with *N*-glycosidase F as described [15]. After deglycosylation, the relative molecular mass of the protein is reduced by about 10 kDa (lane 3), but the band width is not reduced.

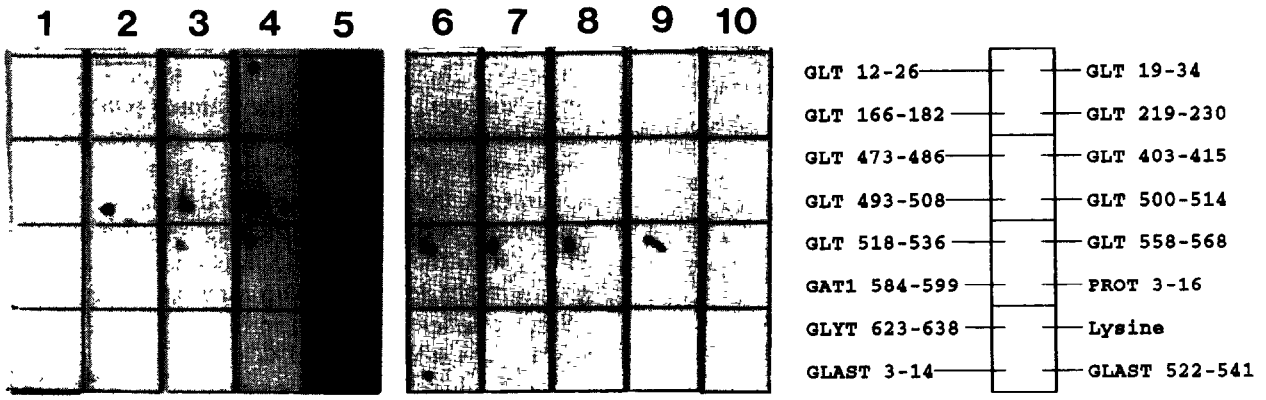

Fig. 2. Specificity of the 9C4 monoclonal antibody (nitrocellulose strips 6–11) and the polyclonal antibodies [15] (strips 1–5). About 0.1 μl of the peptide conjugates were spotted onto strips of nitrocellulose and probed with antibodies as described in section 2. The peptides were based on the sequences of the two cloned glutamate transporters (GLT [16] and GLAST [17]), the glycine transporter (GLYT) [25], the GABA transporter (GAT) [26] and the proline transporter (PROT) [27]. The numbers indicate amino acid residue number. Strips 1, 2, 3, 4 and 5 were incubated with 0, 5, 50, 500 and 5000 ng/ml of the polyclonal antibodies, respectively; while strips 10, 9, 8, 7 and 6 were incubated with 0.5, 5, 50, 500 and 5000 ng/ml of 9C4, respectively. The 9C4 antibodies react selectively with the GLT 518–536 peptide; slight crossreactivity was seen with the GLAST 3–14 peptide (concentration for equal staining intensity more than 1000× higher).

yclonal antibodies (Fig. 1). Immunoblots labelled with the 9C4 antibody were identical to those shown in Figs. 1, 3 and 5 in reference [15]. Thus, the monoclonal antibodies recognized a broad 73 kDa band together with a variable amount of high relative molecular mass material. The latter represents aggregates of the 73 kDa polypeptide and is not seen when fresh brain tissue is solubilized directly in sodium dodecyl sulphate and electrophoresed immediately [15]. The aggregates can to some extent be dissolved by boiling (2 min) the protein sample in a solution containing sodium dodecyl sulphate (20 mg/ml) and 2-mercaptoethanol (5% v/v) prior to electrophoresis (data not shown). When the purified protein was deglycosylated with *N*-glycosidase F as described [15], an equally broad band was obtained with about 10 kDa lower relative molecular mass (Fig. 1, lane 3). Variation of glycosylation is thus not the cause of the band width.

Following the cloning of two glutamate transporters, GLT [16] and GLAST [17] and several other transporters [25–27], 15 peptides encompassing parts of these proteins were synthesized. One of these peptides (GLT 518–536) did react with the 9C4 monoclonal antibody (Fig. 2). This confirms that this antibody does indeed react with the glutamate transporter. Less than 5 ng/ml of the IgM is sufficient to detect this epitope in the spottest. A more than 1000 times increase in antibody concentration is required to detect crossreactivity with GLAST 3–14. The 9C4 antibody did not inhibit glutamate transport (data not shown). The polyclonal IgG antibodies reacted strongly with several of the GLT peptides, the main reactivity being directed against epitopes different from that detected by 9C4 (Fig. 2).

3.2. *Immunocytochemical localization*

Both at the light (Fig. 3) and electron (Fig. 4) microscopic level, the staining was very similar to that obtained with the polyclonal antibodies [15]. The labelling was (apart from what is mentioned in section 2.8) restricted to astroglia-like elements at all concentrations of antibody. The cells were distributed in grey as well as in white matter in all regions investigated: neocortex, hippocampus, basal ganglia, cerebellum, diencephalon, mesencephalon and medulla oblongata. At the light microscopic level, the glial elements were clearly detectable in hippocampus at an IgM concentration of about 0.3 μg/ml. At the electron microscopic level, about 10 times higher concentrations were necessary to obtain convincing contrast between immunoreactive and non-immunoreactive structures in uranyl and lead treated ultrathin sections. At antibody concentrations of 12 μg/ml and higher, the staining penetrated more deeply into the tissue, but was not qualitatively different (Fig. 4).

To ascertain that staining was specific under the immunocytochemical conditions (higher 9C4 antibody concentration and different secondary antibodies than in Fig. 2), filters with spots of the peptide–ovalbumin conjugates were processed together with the sections (see section 2). Tested under these conditions, the crossreactivity with the peptide GLAST 3–14 was only detectable at concentrations of antibody at 36 μg/ml and higher. Even at 120 μg/ml the staining was weaker than the staining of GLT 518–536 at an antibody concentration of 1 μg/ml. None of the other conjugates were stained.

At concentrations of 36 μg/ml and below of the 9C4 antibody, the light microscopic staining of cerebellum was clearly weaker than the staining of hippocampus, other parts of the cerebrum (including neocortex) being intermediate. The differences in staining intensity disappeared at higher concentrations of the 9C4 antibody.

Light microscopically, delicate structures compatible

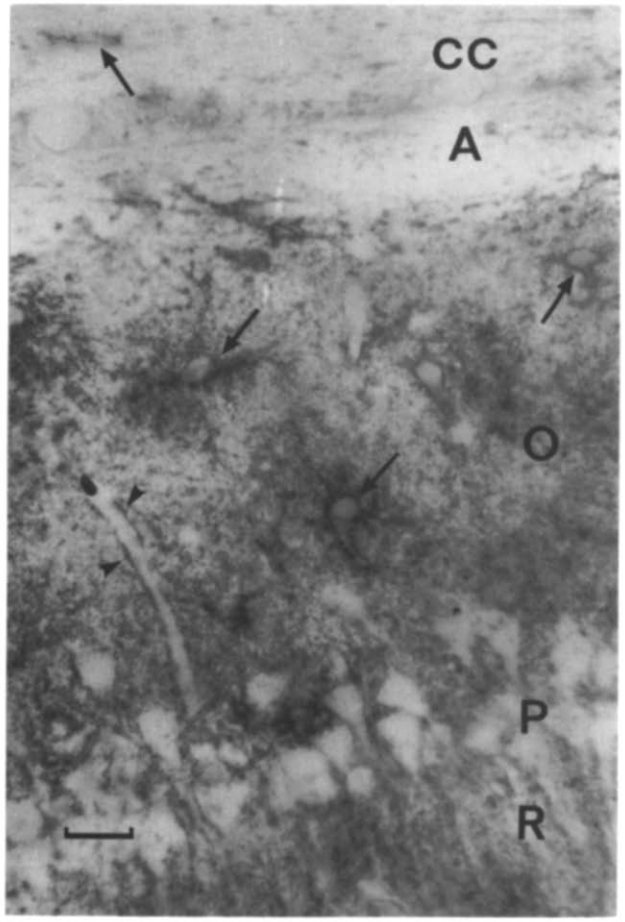

Fig. 3. Light microscopic localization of glutamate transporter in rat brain (hippocampus CA1). Note staining of astrocytes (arrows) and their numerous delicate processes; in the cell bodies and larger processes, the staining is seen to follow the plasma membrane. Cell bodies that have been cut open by the knife at the surface of the section have more strongly stained processes than those situated deeper in the section. Symbols: white matter of the alveus (A) and corpus callosum (CC), stratum oriens (O), stratum pyramidale (P), stratum radiatum (R), perivascular processes (arrowheads) surrounding a capillary with an erythrocyte (stained due to endogenous peroxidase). Formaldehyde/glutaraldehyde fixation. Purified IgM 12 μg/ml. Objective ×40. Scale bar 20 μm.

with having fine processes of astrocytes were seen to pervade the neuropil in all regions. This was confirmed electron microscopically: the predominant site of immunoreactivity was astrocytic processes (Fig. 4), while neurons were unstained. The unlabelled nerve terminals include those of glutamatergic neurons such as the terminals of hippocampal Schaffer collaterals and mossy fibres, which have been found to contain high affinity glutamate uptake sites [28]. As noted previously [15,30], at places of heavy labelling the diaminobenzidine reaction product tends to fill the cytoplasmic space of the processes probably due to diffusion from its site of formation. However, it was never seen extracellularly and appeared concentrated at the inner aspect of the cell membrane (Fig. 4).

4. DISCUSSION

The 9C4 monoclonal antibody shows that one epitope is sufficient to explain the staining pattern seen on the immunoblots. Further, when the immunoblot is compared to a silver stained gel of the same purified preparation, the protein recognized seems to be a major component of the purified preparation. Finally, the reactivity with the GLT 518–536 peptide shows that the 9C4 antibody does react with a glutamate transporter.

Since purified neurotransmitter transporters [31–33] and the in vitro translated GABA transporter [27] as well as the glutamate transporter [16], run as wide bands upon electrophoresis, this migration pattern appears to be a molecular property of these proteins. Proteolysis contributes to the wideness of the glutamate transporter containing band. When dilute fractions of purified glutamate transporter protein are stored, there is a gradual shift from 73 kDa towards about 65 kDa relative molecular mass (data not shown). If the fractions are stored longer, the immunoreactivity gradually disappears.

Based on the above, the report that a different monoclonal antibody (Z8E9) [34], in contrast to the present 9C4, detected a sharp, narrow band within the purified preparation was somewhat surprising. Unfortunately, the two antibodies cannot be compared under identical conditions because the Z8E9 antibody is no longer available. However, the immunocytochemical pictures obtained by Z8E9 and 9C4 and by our polyclonal antibodies are in agreement in that only glial cells are stained. (Antibody producing clone was lost after freezing – H. Koepsell, personal communication.)

The finding that the 9C4 antibody causes plasma membrane associated labelling restricted to the cytoplasmic side supports the proposed model for the glutamate transporter [16], in which the GLT amino acid residues 518–536 and 493–508 are located on one of the intracellular domains of the transporter.

The GLAST protein is predominantly expressed in cerebellum [17] as determined by in situ hybridization with radioactive probes. In contrast, the finding that the 9C4 antibody stains cerebellum less strongly than other parts of the brain at low to moderate concentrations indicates that the GLT protein is preferentially expressed in the cerebrum, hippocampus in particular.

Our findings suggest that (1) the electrophoretic band wideness of the purified preparation of the glutamate transporter is not a sign of impurities, and (2) the transporter, which has been isolated [14,15] and cloned [16] is located in glial cells and is different from neuronal subtype(s). A related, but different glutamate transporter assumed to be localized in neurons has recently been cloned [35].

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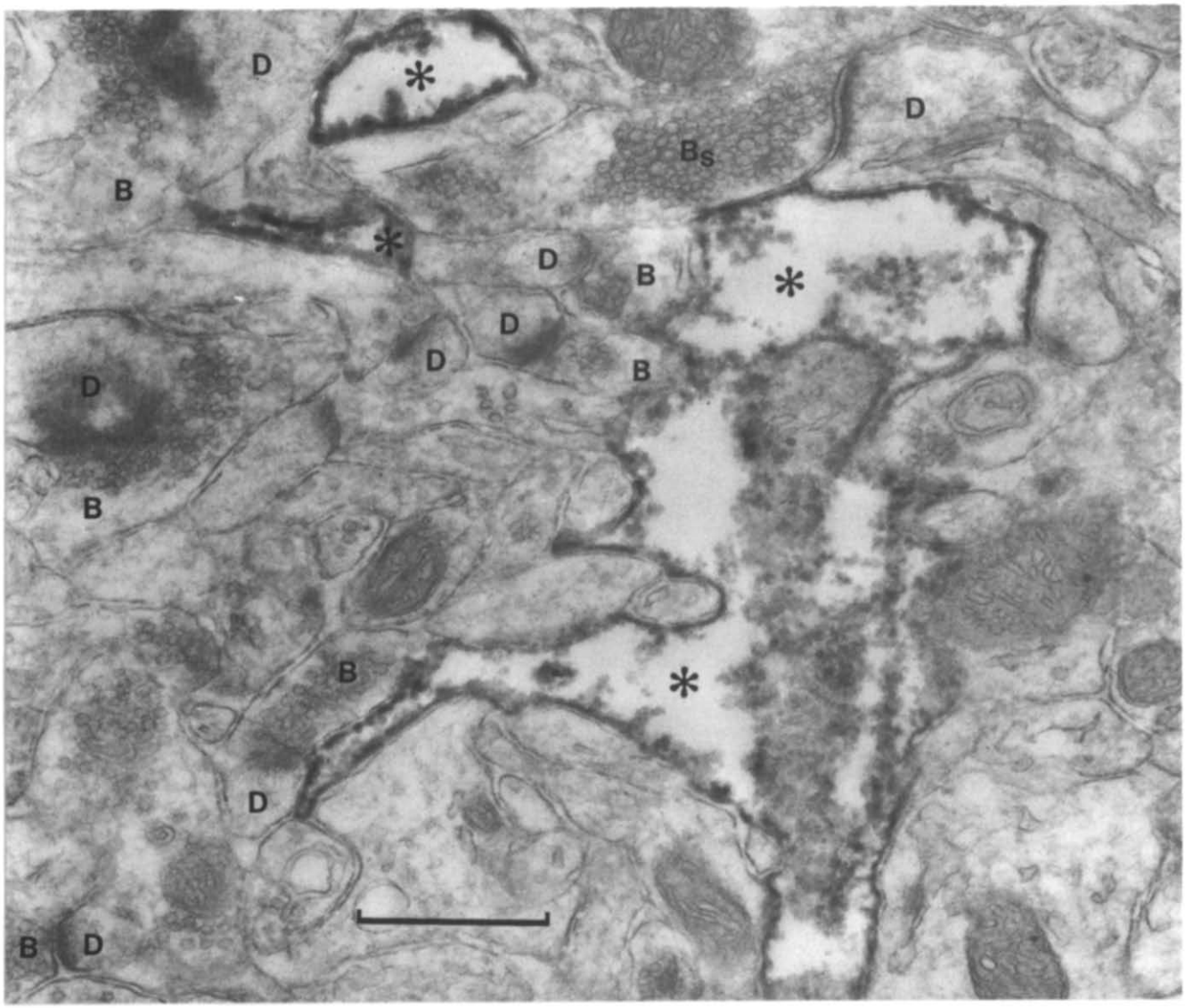

Fig. 4. Electron microscopic localization of glutamate transporter in rat brain (stratum radiatum of hippocampus CA1). Processes (extensions) of astrocytes, recognized by their characteristic irregular outlines, are heavily labelled (asterisks). The ultrastructure of the cytoplasm of labelled cellular components is poorly preserved due to the diaminobenzidine reaction product. This can diffuse from the place of formation before it precipitates, but note that it is confined to the interior of the cells. Synaptic boutons are unstained, including those cut open by the knife at the surface of the sections (not shown). The bouton labelled Bs and, less distinctly, several others (B) show the typical morphology of the glutamatergic commissural and Schaffer collateral terminals (which constitute the majority of boutons in this region [29]), forming asymmetric synaptic specializations on dendritic spines (D). Formaldehyde/glutaraldehyde fixation. Purified IgM 12 μg/ml. Scale bar 0.5 μm.

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