Similar Perisynaptic Glial Localization for the Na^+,K^+-ATPase α_2 Subunit and the Glutamate Transporters GLAST and GLT-1 in the Rat Somatosensory Cortex

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Several isoenzymes of the Na^+,K^+-ATPase are expressed in brain but their specific roles are poorly understood. Recently, it was suggested that an isoenzyme of the Na^+,K^+-ATPase containing the α_2 subunit, together with the glutamate transporters GLAST and GLT-1, participate in a coupling mechanism between neuronal activity and energy metabolism taking place in astrocytes. To substantiate this hypothesis, we compared the distribution of α_2, GLAST and/or GLT-1 in the rat cerebral cortex using double immunofluorescence and confocal microscopy, and immunocytochemistry at the electron microscopic level. We also investigated the relationship between α_2, GLAST or GLT-1 and asymmetrical synaptic junctions (largely glutamatergic) and GABAergic nerve terminals. Results show that the α_2 subunit has an exclusive astroglial localization, and that it is almost completely co-distributed with GLAST and GLT-1 when evaluated by confocal microscopy. This similar distribution was confirmed at the ultrastructural level, which further showed that the vast majority of the α_2 staining (73% of all labelled elements), like that of GLAST and GLT-1, was located in glial leaflets surrounding dendritic spines and the dendritic and/or axonal elements of asymmetrical (glutamatergic) axo-dendritic synapses. Synapses ensheathed by α_2, GLAST or GLT-1 virtually never included (≤2%) GABAergic nerve terminals or synaptic junctions. However, a subset of GABAergic nerve terminals (10–14%) were directly apposed to asymmetrical axo-dendritic junctions surrounded by α_2, GLAST or GLT-1. Altogether these results demonstrate that α_2, GLAST and GLT-1 have comparable perisynaptic distribution within cortical astrocytes most likely associated with glutamatergic synapses.

Introduction

Functional activation of specific brain areas is associated with a restricted spatial and temporal increase in cerebral metabolism, a response that forms the basis of functional brain imaging techniques [for reviews see (Villringer and Dirnagl, 1995; Magistretti and Pellerin, 1996)]. While the main elements underlying this physiological process are still largely unknown, considerable progress has been made with respect to excitatory neurotransmission. For instance, glutamate released from excitatory synapses has been found to stimulate glycolysis in astrocytes, a mechanism suggested to be responsible for the coupling between neuronal activity and glucose metabolism (Pellerin and Magistretti, 1994; Magistretti and Pellerin, 1999), and consistent with glutamatergic neurotransmission being the major determinant of *in vivo* glucose oxidation in brain (Rothman *et al.*, 1999). The purported effectors of this metabolic coupling are the glial Na^+-dependent glutamate transporters GLAST and/or GLT-1, and an isozyme of the Na^+,K^+-ATPase which, upon activation, triggers a cascade of intracellular events leading to glycolytic processing of glucose, production and release of lactate by astrocytes and its subsequent uptake by neurons for further energy production (Magistretti *et al.*, 1999). Such a scheme confers a pivotal role for astrocytes in the coupling of neuronal activity to glucose metabolism, and implies the existence of an activity-dependent astrocyte–neuron lactate shuttle for the supply of energy substrates to neurons (Pellerin *et al.*, 1998a,b; Magistretti and Pellerin, 1999).

Re-uptake of glutamate from the synaptic cleft into astrocytes is mediated by the specific high-affinity glial transporters GLAST and GLT-1 which are particularly enriched in brain areas with high glutamatergic activity and are essential to maintain glutamate levels below neurotoxicity limits (Rothstein *et al.*, 1994; Lehre *et al.*, 1995; Chaudry *et al.*, 1995; Schmitt *et al.*, 1996, 1997). While these two transporters co-exist side by side in the same astrocytic cell membrane, they do not form a complex with each other (Haugeto *et al.*, 1996), and it has been suggested that they exert important functions at glutamatergic synapses other than terminating the synaptic actions of glutamate.

The Na^+,K^+-ATPase, or Na pump, is a ubiquitous plasma membrane transport ATPase responsible for maintaining Na^+ and K^+ ionic gradients essential in the CNS for generation of action potentials [for a review see (Mobasheri *et al.*, 2000)]. It is composed of two subunits, α and β: the multispanning plasma membrane catalytic α subunit that hydrolyses ATP is also the cellular receptor for cardiac glycosides, a class of specific inhibitors that includes ouabain; the β subunit is a glycosylated polypeptide that stabilizes the α subunit and modulates the K^+ and Na^+ affinity [for a review see (Blanco and Mercer, 1998)]. In the CNS, three α and two β subunits encoded by different genes are expressed and lead to the formation of different Na^+,K^+-ATPase isozymes, depending on the possible combinations of α and β subunits (Shull *et al.*, 1986; Kent *et al.*, 1987; Sweadner, 1992). Astrocytes were shown to express α_1, α_2 and β_2 subunits both *in vitro* and *in vivo* (McGrail *et al.*, 1991; Watts *et al.*, 1991; Brines and Robbins, 1993; Cameron *et al.*, 1994; Fink *et al.*, 1996; Peng *et al.*, 1998) with a predominance of the $\alpha_2\beta_2$ heterodimer in astrocytes exposed to or in contact with neurons (Gloor *et al.*, 1990). Specific roles for glial Na^+,K^+-ATPases, and specifically the $\alpha_2\beta_2$ isoenzyme, have been postulated and include a contribution to K^+ homeostasis (Watts *et al.*, 1991; Peng *et al.*, 1996, 1998) and to neurotransmitter (notably glutamate) clearing from the synaptic cleft (Pellerin and Magistretti, 1997; Abe and Saito, 2000). Interestingly, it has been shown that a glial isozyme most likely containing the α_2 subunit is recruited in response to glutamate uptake and is necessary to couple neuronal activity to glucose utilization (Pellerin and Magistretti, 1997).

To substantiate this hypothesis, namely the participation of the α_2 subunit and the astrocytic glutamate transporters GLAST and/or GLT-1 in the metabolic coupling response to glutamate, we investigated their cellular and ultrastructural localization in the rat frontoparietal somatosensory cortex, a region particularly rich in glutamatergic afferents. Furthermore, we evaluated their relationships with excitatory glutamatergic (asymmetrical)

(DeFelipe *et al.*, 1988; Conti *et al.*, 1989; Dori *et al.*, 1989; Nie and Wong-Riley, 1996) and inhibitory GABAergic (immunolabelled for the terminal-enriched GABA synthesizing enzyme glutamic acid decarboxylase, GAD_{65}) (Soghmonian and Martin, 1998) synaptic junctions, two systems that regulate their respective neurotransmitter levels in the cleft by neuronal and glial Na^+-dependent carriers (Gadea and Lopez-Colome, 2001a,b). The results show that (i) the α_2 subunit of the Na pump is located exclusively in glial cells and is co-distributed with GLAST and GLT-1, (ii) the ultrastructural distribution of the α_2 subunit is virtually identical to that obtained for GLAST and GLT-1, and (iii) the α_2-containing astrocytic processes are primarily associated with axo/dendritic appositions and asymmetrical (glutamatergic) synaptic junctions, but not with GABAergic terminals or symmetrical junctions. These findings support the participation of astrocytes in coupling cerebral metabolism to neuronal activation, whereby α_2, GLAST and/or GLT-1 would be the molecular effectors.

Materials and Methods

Animals and Preparation of Tissue Sections

Male Sprague–Dawley rats (n = 16, 260–280 g) were housed in a temperature-controlled (21–25C) room, under natural daylight conditions. They had free access to food and water. For immunocytochemistry, rats were deeply anaesthetized with sodium pentobarbital (65 mg/kg body weight, i.p., Somnotol, MTC Pharmaceuticals, Cambridge, Ontario, Canada), and their brains fixed by perfusion through the ascending aorta with 1 litre of 4% paraformaldehyde and 0.06% glutaraldehyde in 0.12 M sodium phosphate buffer (NaPB, pH 7.4). Brains were removed and postfixed (4C, 2 h) in the same solution. They were then cut in coronal sections (50 μm thick) on an Oxford vibratome (Technical Products International, Inc., St Louis, MO, USA) at the level of the frontoparietal somatosensory cortex (layers II–V). For double immunofluorescence (see below), brains (n = 3) were cryoprotected (sucrose 30%, 2 days), frozen in isopentane (–45C) and sectioned (20 μm thick) on a freezing microtome. All sections were collected in NaPB and immunostaining was performed on free-floating sections. Experiments were approved by the Animal Ethics Committee based on guidelines of the Canadian Council on Animal Care.

Immunocytochemistry

α_2 *Subunit of the* Na^+,K^+*-ATPase*

Sections were rinsed (4 10 min) in 0.12 M NaPB (pH 7.4) followed by phosphate-buffered saline (PBS) containing 0.2% gelatin (4 10 min). They were incubated overnight at room temperature with a mouse monoclonal antibody (McB2, 1/250, kindly donated to us by Dr Kathleen J. Sweadner, Laboratory of membrane biology, Neuroscience Center, Massachusetts General Hospital, Charlestown, MA, USA) that selectively recognized an epitope (GREYSPAATTAENG) near the N-terminus of the α_2 subunit of the Na^+,K^+-ATPase from rat brain axolemma [for characterization and specificity, see (Pacholczyk and Sweadner, 1997; Peng *et al.*, 1997; Arystarkhova and Sweadner, 1996)]. The sections were then rinsed in PBS-gelatin (PBSG) and incubated (1 h 30 min) with a biotinylated horse anti-mouse IgG (1/200, Vector Laboratories, Burlingame, CA, USA) and with the avidin–biotin complex (1 h 15 min, ABC Elite kit from Vector). The immunocytochemical reaction was revealed with 0.05% of 3′-3 diaminobenzidine tetrachloride (DAB, Sigma) containing 0.005% H_2O_2. While some thick sections were directly mounted on microscopic slides for observation at the light microscopic level, most were postfixed in osmium tetroxide and flat embedded in Araldite 502 (JBEM Services, Montreal, Canada). Selected area from the frontoparietal somatosensory cortex were then trimmed and embedded in blocks of Araldite. Semithin sections (1–2 μm) were obtained from some blocks, mounted on microscopic slides and observed under light microscopy. Serial thin (pale gold 90 nm) sections were cut on a Reichert–Jung ultramicrotome, collected on copper grids, double-stained with uranyl acetate (12–16 min) and lead citrate (2–3 min), and examined under a Jeol CX100-II electron microscope.

Double Immunolabelling

As the α_2 subunit (this study) appeared localized strictly around neuronal perikarya, and in punctate elements of the neuropil in a manner reminiscent of that reported for the glutamate transporters GLAST and GLT-1 (Chaudry *et al.*, 1995; Lehre *et al.*, 1995; Lehre and Danbolt, 1998), we first compared their distribution using double immunofluorescence and confocal microscopy. Also, as these markers were exclusively astroglial and largely perijunctionally located, we then performed double immunocytochemistry at the ultrastructural level to (i) examine their relationships with cortical GABAergic axon terminals which are highly synaptic and whose effects are terminated by uptake of GABA through active, high-affinity Na^+-dependent transporters (GAT1–GAT3) which can be found on astrocytes (Minelli *et al.*, 1996), and (ii) compare the fine cortical distribution of GLAST and GLT-1 to that obtained for α_2.

Double Immunofluorescence and Confocal Microscopy

α_2*/GLAST and* α_2*/GLT-1*

Following rinses as above, the sections were simultaneously incubated (overnight) with the mouse monoclonal anti-α_2 subunit antibody (1/50) and either a rabbit anti-GLAST (A522, 1/1500) or anti-GLT-1 (B12, 1/500) antibody (generously given to us by Dr Niels C Danbolt, Anatomical Institute, University of Oslo, Oslo, Norway, and characterized in detail previously) (Lehre *et al.*, 1995; Lehre and Danbolt, 1998). Following a few rinses, the sections were then incubated (~4 h) with a goat anti-mouse Cy3- (1/600) or a goat anti-rabbit Cy2(1/400)-conjugated secondary antibody. Tissue sections were rapidly rinsed and examined under a confocal laser scanning microscope (BioRad MRC 600, Hertfordshire, UK) linked to a Nikon Optoplot epifluorescent microscope. Images were acquired through separate channels for one single focal plane (0.5 μm) using double excitation at 468 nm (Cy2) and 568 nm (Cy3). Co-localization of α_2 with either GLAST or GLT-1 was assessed by merging the two images from the same focal plane, using the confocal assistant program (version 4.02).

Double Immunocytochemistry at the Ultrastructural Level

GAD65/α_2*, GAD65/GLAST or GAD65/GLT-1*

GABA terminals were immunodetected with an antibody against the glutamic acid decarboxylase isoform 65 (GAD65, the synthesizing enzyme for GABA) which is predominantly enriched in nerve terminals (Soghomonian and Martin, 1998). The mouse anti-GAD 65 (1/3000, Boehringer Mannheim, Indianapolis, IN, USA) was simultaneously incubated overnight in 0.1 M PBSG with either a mouse anti-α_2 (1/250), a rabbit anti-GLAST (A522, 1/6000) or a rabbit anti-GLT-1 (B12, 1/2800) antibody. The secondary antibodies (horse anti-mouse 1/200, and goat anti-rabbit 1/200, Vector Laboratories) were revealed with 0.05% DAB containing 0.005% H_2O_2, and the sections processed as above.

Quantitative Analysis at the Ultrastructural Level

All quantitative analyses were performed directly under the electron microscope at a working magnification ranging from 14 000 to 40 000×. The first analysis of the cellular distribution of the α_2 subunit of the Na^+,K^+-ATPase was derived from five rats and a total of 425 immunolabelled astroglial profiles. These were assigned to the specific cellular elements that they ensheathed including dendritic spines and shafts, axonal varicosities and axon terminals, whether alone or included in axo-dendritic appositions or synaptic junctions, as well as small myelinated axons or small neuronal elements that could not be clearly identified (defined as 'neuron vicinity' in Fig. 5), and microvessels or capillaries. In double immunostained sections for GAD65/GLAST or GAD65/GLT-1, the cellular distribution of the glial markers was first established using the same categories as described above for the α_2 subunit. Then, interactions between the three glial markers and GAD-immunoreactive nerve terminals was determined. For this purpose, all GAD terminals seen in double-labelled material (establishing or not a synaptic junction) were counted and divided as either ensheathed by, adjacent to or located in the immediate vicinity of α_2 (n = 3 rats, 353 terminals), GLAST (n = 3 rats, 356 terminals) or GLT-1 (n = 2 rats, 288 terminals) immunoreactive glial

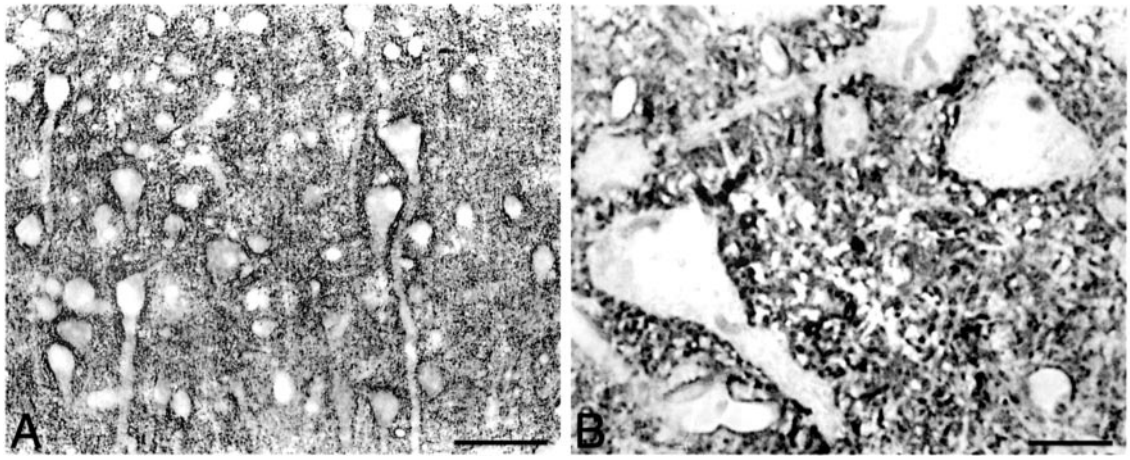

Figure 1. Immunocytochemical localization of the α_2 subunit of Na^+/K^+-ATPase in the rat frontoparietal cortex (layers III to V) in thick (*A*) and semithin (*B*) sections. Numerous immunostained profiles that appear like small puncta surrounding unstained perikarya are distributed in the neuropil. Some areas in the neuropil are more intensely labelled than others, as seen around cell soma and proximal dendrites in (*B*). Scale bars: 50 μm (*A*), 15 μm (*B*).

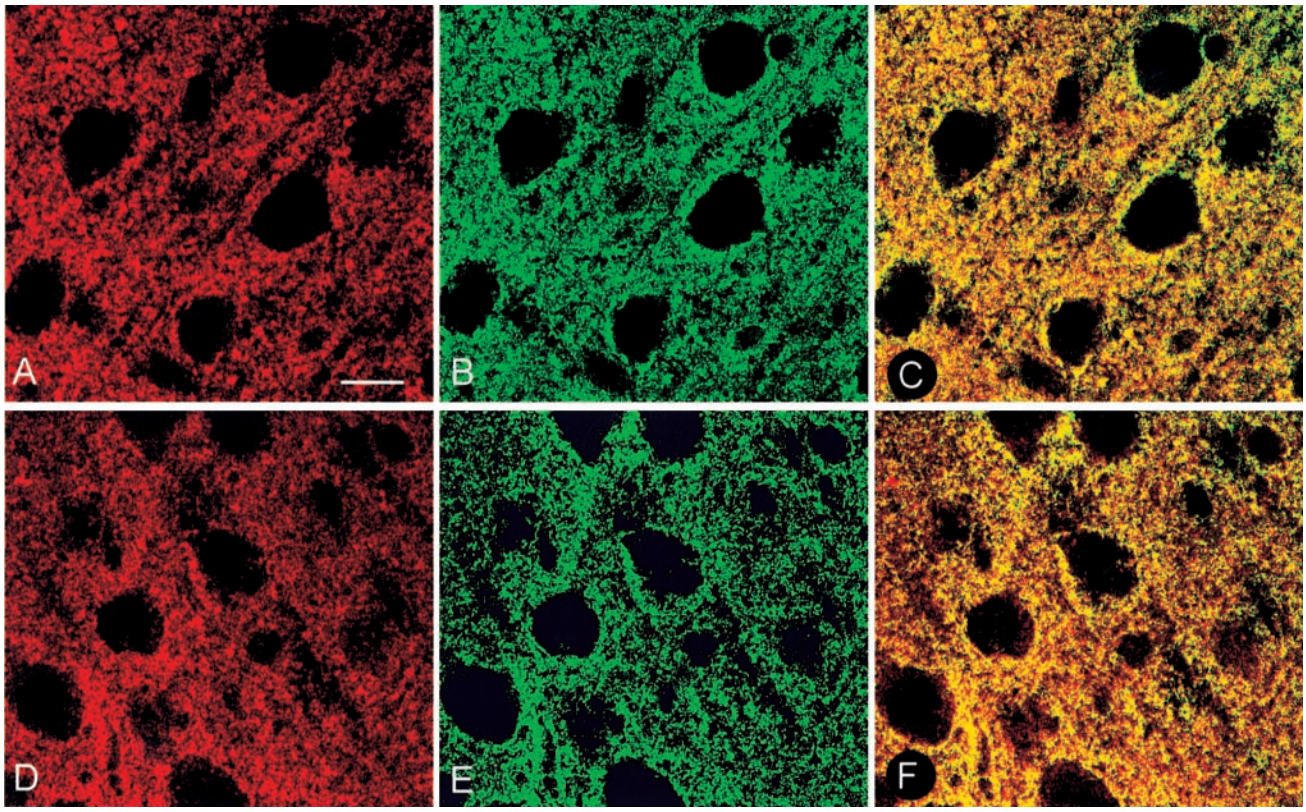

Figure 2. Double immunofluorescence labelling of the α_2 subunit of Na^+/K^+-ATPase (*A* and *D*) and GLAST (*B*) or GLT-1 (*E*), as visualized by confocal microscopy in the right (top panel, α_2/GLAST) and left (lower panel, α_2/GLT-1) frontoparietal cortex. The distribution of α_2 is highly superimposable to that of these two glial glutamate transporters as shown by the appearance of a yellow colour upon merging of their respective images (*C* and *F*, respectively for α_2/GLAST and α_2/GLT-1). Bar = 15 μm.

processes. Results are expressed as means ± SD. ANOVA was used to compare the distribution between the different glial markers and their association with GAD-positive nerve terminals. Representative photomicrographs were taken for purposes of illustration.

Results

Localization of the α_2 Subunit of the Na^+,K^+-ATPase: Comparison with GLAST and GLT-1

At the light microscopic level, α_2-immunoreactivity in the rat somatosensory cortex had a diffuse, punctate distribution in the neuropil with no apparent cellular labelling, and no delineation related to cortical layers (Fig. 1*A*). In semithin sections, the α_2-positive puncta appeared particularly dense around unstained perikarya and proximal dendrites of both pyramidal and non-pyramidal neurons, and neuropil staining was not uniform, with some puncta being more intensely stained than others within the same interneuronal area (Fig. 1*B*). When examined under confocal microscopy, α_2 immunofluorescent labelling similarly exhibited a peri-neuronal and non-uniform punctate distribution

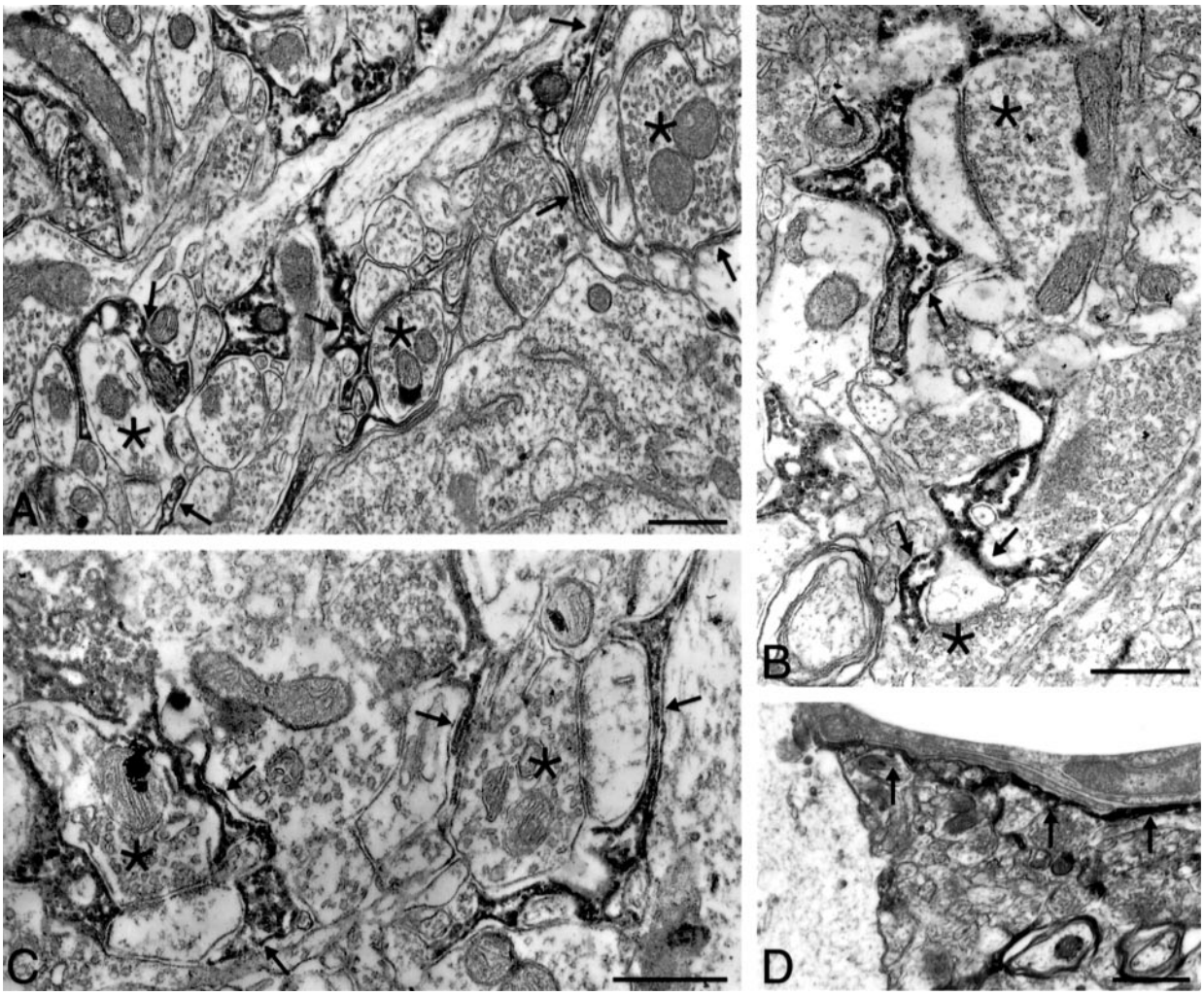

Figure 3. Electron microscopic immunocytochemistry of the α_2 subunit of Na^+/K^+-ATPase in the rat frontoparietal cortex (layers III to V). Immunoreactive astrocytic processes (small arrows) adjacent to axo-dendritic asymmetrical junctions (∗ on the axon terminals in *A*, *B* and *C*) or the basal lamina of a microvessel (*D*). The synapses surrounded by α_2 staining have the typical morphology of glutamatergic synapses with asymmetrical post-synaptic specializations. The staining is restricted to the inside of astrocytic cell processes. Scale bars: 0.5 μm.

in the neuropil (Fig. 2*A*,*D*). This localization was virtually identical to that of the two glial glutamate transporters GLAST and GLT-1 labelled in the same tissue sections (Fig. 2*B* and *E*, respectively). As indicated by the appearance of a yellow colour upon merging of the two confocal images taken at the same focal plane, there was an almost complete co-distribution of α_2/GLAST (Fig. 2*C*) and α_2/GLT-1 (Fig. 2*F*) immunofluorescent elements of the neuropil. The glial transporters GLAST and GLT-1 appeared slightly more enriched around unstained cortical perikarya than the α_2 subunit staining which was comparatively more homogeneous in 0.5 μm thick focal plane (compare Fig. 2*A*,*D* and Fig. 2*B*,*E*). At the ultrastructural level, α_2-immunoreactivity was exclusively localized to glial elements, mostly in thin glial leaflets (Fig. 3) although large processes (Fig. 4*B*) and rare perikarya (data not shown) were also apparent. The vast majority (~73%) of α_2-immunostained glial processes targeted dendritic spines whether alone (~30.5 ± 4.6%), or as part of axo-dendritic asymmetrical synaptic junctions (~38.9 ± 4.8%) or appositions (~4.0 ± 2.6%). In fact, the α_2-staining was particularly intense and frequent (45.8 ± 7.5% of all labelled glial elements) in small leaflets that surrounded axon terminals (6.9 ± 2.3%), small dendritic spines (21.6 ± 5.1%) or both elements of axo-dendritic asymmetrical synaptic junctions (17.3 ± 4.6%) (Fig. 5). However, α_2-stained glia also surrounded small axonal varicosities, neuronal cell bodies, dendritic shafts and all capillaries seen in our material (Figs 3 and 5). Neuronal cell bodies, dendrites or nerve terminals, including those surrounded by α_2-labelled glial sheaths, were never immunostained.

When the distribution of GLAST and GLT-1 was similarly analysed at the electron microscopic level in tissue double-stained with GAD65, virtually superimposable results were obtained (Fig. 5). In agreement with previous studies using the same antibodies (Lehre *et al.*, 1995; Lehre and Danbolt, 1998), multiple astrocytic processes were immunostained with a particularly dense labelling in small leaflets surrounding asymmetrical synaptic junctions (Figs 6 and 7). The distribution patterns for GLAST and GLT-1 (Fig. 5) corresponded very closely to that described above for the α_2 subunit of the Na^+,K^+-ATPase. Their primary targets were the dendritic spines (~68% and ~73% for GLAST and GLT-1, respectively), either alone (30.3 ± 6.3% and 28.8 ± 1.8%, respectively) or included in axo-dendritic asymmetrical synaptic junctions (35.3 ± 5.9% and 42.6 ± 1.9%, respectively) or appositions (2.2 ± 1.6% and 1.9 ± 0.1%, respectively) (Fig. 5). GLAST and GLT-1 stainings were also frequently

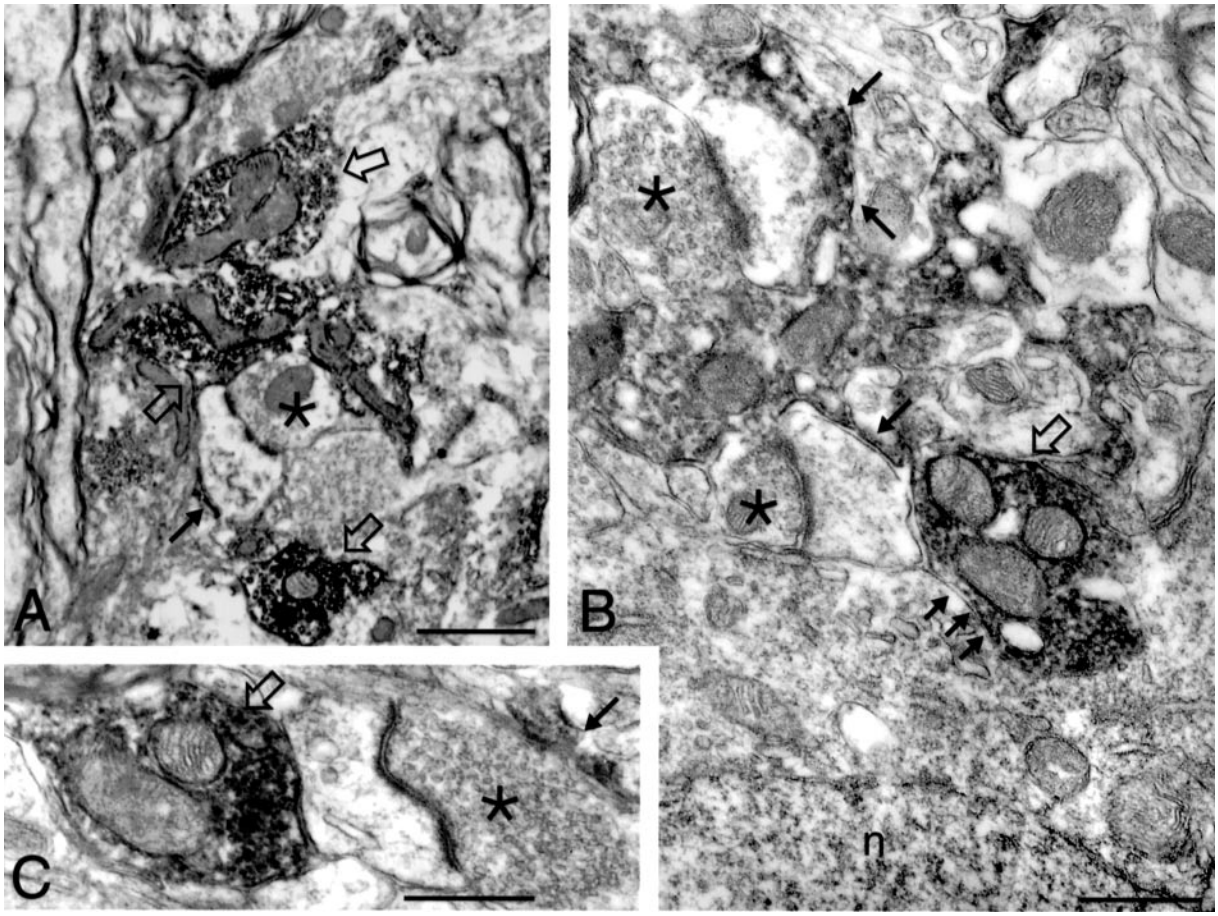

Figure 4. Double electron microscopic immunocytochemistry of the α_2 subunit of Na^+/K^+-ATPase and GAD nerve terminals in the rat frontoparietal cortex (layers III to V). Astrocytic processes immunoreactive for α_2 (black arrow) are adjacent to asymmetrical axo-dendritic synapses (* on the axon terminals in *A*, *B* and *C*). Most GAD terminals (open arrow) are not surrounded by α_2-immunostained astrocytic processes (*C*) but are frequently localized in proximity of α_2-ensheathed asymmetrical synapses (*A*, *B*). In (*B*), a large α_2-immunostained glial process is seen extending to at least two asymmetrical synapses in the plan of the section. The GAD terminal in (*B*) is apposed to a neuronal cell body (triple arrows), the nucleus (n) of the cell is indicated. Scale bars: 0.5 μm.

observed (overall frequency of 47.4 ± 5.6% and 50.3 ± 9.8%, respectively) in small glial leaflets surrounding the axon terminals, small dendritic spines, or both elements of axo-dendritic asymmetrical synaptic junctions (Fig. 5). Similar to α_2 staining, proximal dendritic shafts, neuronal perikarya and capillaries were also encompassed by a thin glial leaflet immunoreactive for GLAST or GLT-1 (Fig. 5).

Interactions Between GAD Terminals and Glial Elements Containing α_2, GLAST or GLT-1

As expected [for a review, see (Soghomonian and Martin, 1998)], GAD-immunostained nerve terminals were very numerous in the somatosensory cortex and, at the electron microscopic level, they contacted cell soma (Fig. 4*B*), proximal dendrites and dendritic spines (Figs 6 and 7). When visible in the plane of the section, synaptic junctions were symmetrical and were either axosomatic, or axo-dendritic with dendritic shafts or, primarily, dendritic spines (Fig. 7*A*). Most (66–71%) GAD terminals were not accompanied by any glial leaflet immuno- positive for α_2, GLAST or GLT-1 (Fig. 8). Also, in contrast to asymmetrical axo-dendritic synapses that were often completely ensheathed by α_2- (Figs 3 and 4), GLAST- or GLT-1-immuno- positive glia (Figs 6 and 7), none of the GAD-immunolabelled synaptic junctions were completely encompassed by any of these glial markers (Figs 4, 6–8). On rare occasions (1–2% of all GAD-positive terminals examined; Fig. 8), the dendritic or axonal element of the axo-dendritic or axosomatic GABAergic synaptic junctions or appositions were contacted by glial processes immunoreactive for α_2 (Fig. 4*B*), GLAST (Fig. 6*A*,*C*) or GLT-1 (Fig. 7*A*). However, a relatively important population of GAD-labelled terminals were adjacent (13–19%) to asymmetrical synaptic junctions ensheathed by glial leaflets immunostained for α_2, GLAST or GLT-1 (Figs 4*C*, 6*A*,*C* or 7*B*), or located in the vicinity (10–14%) of such immunostained glial processes (Figs 4*A*, 6*B*,*C* or 7*C*,*D*).

Discussion

It has recently been suggested that a glial Na^+,K^+-ATPase, and more specifically an isoform highly sensitive to ouabain akin to the α_2 subunit, was involved in the glycolytic response induced by glutamate transport into astrocytes (Pellerin and Magistretti, 1997). In support of this hypothesis, our results clearly demonstrate, for the first time at the ultrastructural level, that the α_2 subunit of the Na^+,K^+-ATPase in the rat frontoparietal somatosensory cortex is preferentially localized in astrocytic processes around axonal and/or dendritic elements of asymmetrical (glutamatergic) axo-dendritic synaptic junctions or appositions. This distribution was found by double immunofluorescence and quantitative electron microscopy immunocytochemistry to be virtually identical to that of GLAST and GLT-1, two glial

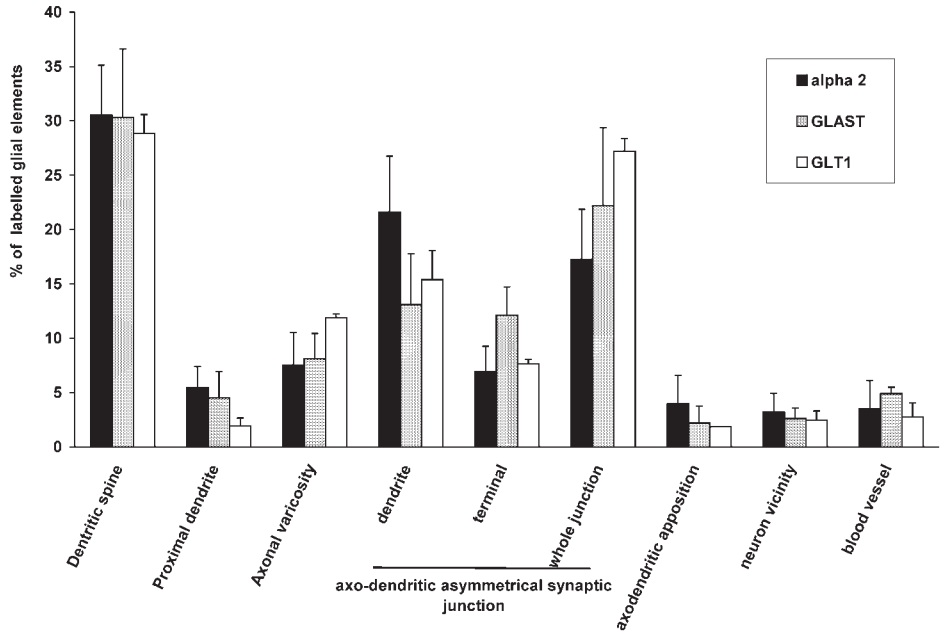

Figure 5. Histogram of the distribution of the cellular elements ensheathed by glial leaflets immunostained for the α_2 subunit, GLAST or GLT-1 in the rat frontoparietal cortex. Note the similar distribution of these three proteins in glial leaflets. They all prominently encircle dendritic spines and axo-dendritic asymmetrical synaptic junctions ($n = 5$ rats for α_2-labelled profiles; three rats for GLAST profiles counted around 356 GAD terminals, and two rats for GLT-1 profiles found around 288 GAD terminals). See Materials and Methods for a more complete description of the categories. Results are expressed as means $\pm$ SD.

transporters for glutamate that are characteristically located around glutamatergic synapses and concomitantly expressed by astrocytes (Chaudry *et al.*, 1995; Lehre *et al.*, 1995; Lehre and Danbolt, 1998). Additionally, the results demonstrate that symmetrical GABAergic synaptic junctions, at which the action of GABA is terminated by high-affinity Na^+-dependent transporters partly located in glial cells [(Minelli *et al.*, 1996); for a review, see (Gadea and Lopez-Colome, 2001b)], are not encircled by glial elements stained for the α_2 subunit, GLAST or GLT-1. However, a relatively important number of GABA terminals were located in close proximity of asymmetrical (glutamatergic) synaptic junctions ensheathed by one of these glial markers. The present data suggest that the α_2 subunit could be a functional effector in the coupling of neuronal activity to glucose metabolism following activation of glutamate neurotransmission, and that both GLAST and GLT-1 are possible co-effectors in this pathway.

Subcellular Localization of the α_2 Subunit

The astrocytic expression of the α_2 subunit has been demonstrated previously in primary cultures and in tissue sections by immunocytochemistry (McGrail *et al.*, 1991; Brines and Robbins, 1993; Juhaszova and Blaustein, 1997; Peng *et al.*, 1997), immunoblotting (Brines and Robbins, 1993; Cameron *et al.*, 1994), and *in situ* hybridization (Watts *et al.*, 1991). In addition, these studies reported that the α_2 subunit was located in astrocytes in proximity to neurons and blood vessels, and that it was particularly enriched in astrocytes in contact with neurons. Our results not only confirm, in the rat somatosensory cortex, the exclusive localization of the $28a_2$ subunit to astrocytes, but further demonstrate that it is co-distributed with glutamate transporters GLAST and GLT-1, and also provide the first ultrastructural evidence that it is particularly enriched in thin leaflets around axonal and/or dendritic elements of axo-dendritic asymmetrical synaptic junctions. Although the chemospecificity of the junctions was not determined in the present study, their features and characteristics are reminiscent of those containing excitatory amino acids, and more specifically glutamate (DeFelipe *et al.*, 1988; Conti *et al.*, 1989; Dori *et al.*, 1989). Indeed, glutamate is contained in the vast majority (>80%) of axon terminals forming asymmetrical synapses in the cerebral cortex (Nie and Wong-Riley, 1996). Moreover, the two glial glutamate transporters GLAST and GLT-1, which share the same distribution as the α_2 (see below), have been shown to selectively encircle glutamatergic synapses identified both by the type of junctions they form and by immunocytochemistry to glutamate (Chaudry *et al.*, 1995; Lehre *et al.*, 1995; Lehre and Danbolt, 1998). Thus, our results clearly indicate a close association between glial processes expressing an isozyme of the Na^+,K^+ ATPase containing the α_2 subunit and glutamatergic terminals.

α_2 Subunit and the Glutamatergic System

Our results agree and extend the previously described astroglial localization of GLAST and GLT-1 and, more so, in thin leaflets around glutamatergic synaptic junctions (Rothstein *et al.*, 1994; Lehre *et al.*, 1995; Ullensvang *et al.*, 1997; Lehre and Danbolt, 1998). This is also in agreement with the observation that the highest intensity of expression of the two glial glutamate transporters was positively correlated to both high glutamatergic activity and astrocytic glutamate metabolism (Schmitt *et al.*, 1996, 1997). But more importantly, the most striking observation of the present study was the strictly superimposable distribution between the α_2 subunit of the Na^+,K^+-ATPase and the glutamate transporters GLAST and GLT-1. These findings indicate that the uptake of glutamate into astroglial cells is tightly coupled to a Na^+,K^+-ATPase isozyme which involves the α_2 subunit, and implies a functional relationship between glutamate neurotransmission and an α_2-isoform of the Na^+,K^+-ATPase. A specific role for an α_2-containing isoform has been ascribed

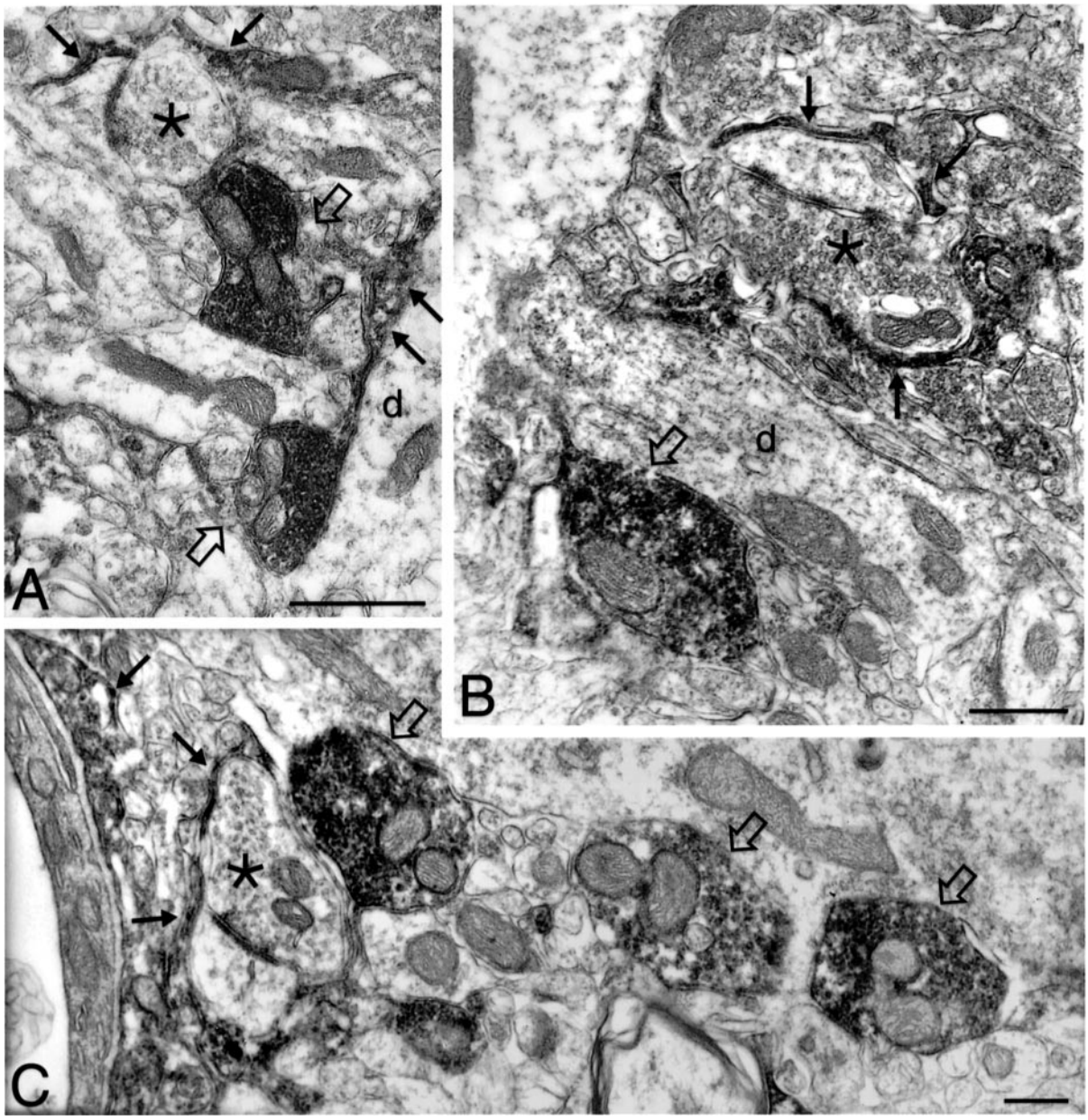

Figure 6. Double electron microscopic immunocytochemistry of GLAST and GAD nerve terminals in the rat frontoparietal cortex (layers III to V). GLAST immunoreactive astrocytic processes (small arrows) adjacent to a dendritic branch (d in *A*, *B*) or asymmetrical axo-dendritic synapses (* on the axon terminals in *A*, *B* and *C*). The perivascular astroglial leaflet in (*C*) is also immunostained for GLAST. Most GAD nerve terminals (open arrows) are not surrounded by GLAST astrocytic processes (*B*, *C*), but some are apposed to the pre- or post-synaptic elements of asymmetrical synapses encircled by a GLAST astroglial leaflet (*A*, *C*). In rare cases, GAD terminals were adjacent to a GLAST astroglial process, as shown in (*A*) for the GAD terminal apposed to the dendritic (d) branch. Scale bars: 0.5 μm.

previously to the maintenance of the electrochemical Na^+ gradient required for an adequate glutamate re-uptake in glial cells (Pellerin and Magistretti, 1997; Abe and Saito, 2000). Indeed, a quantitative analysis has revealed a tight coupling between changes in intracellular Na^+ concentration induced by glutamate uptake and the activity of the Na^+,K^+-ATPase in astrocytes (Chatton *et al.*, 2000).

Astrocytes are strategically positioned, with processes around glutamatergic synapses and others on brain capillaries, to both sense increased neuronal excitation and promote glucose uptake. This represents an ideal structural organization to insure spatial and temporal coupling between neuronal activity and energy metabolism. The molecular mechanism suggested to account for such a coupling involves, following glutamate uptake by astrocytes, an increased astroglial uptake and glycolytic transformation of glucose into lactate which will then be released to be used by neurons as a fuel during periods of activation (Pellerin and Magistretti, 1994; Magistretti *et al.*, 1999). Such conclusion was recently supported by the observation that local *in vivo* downregulation of GLAST expression in the rat frontoparietal somatosensory cortex, using antisense microinjection, markedly diminished the metabolic response to sensory stimulation (Cholet *et al.*, 2001). Similar results were obtained in young knockout mice for GLAST in which the cortical metabolic response to whisker stimulation was suppressed (Voutsinos-Porche *et al.*, 2000). Moreover, the recent demonstration that astroglial and neuronal cells possess different monocarboxylate transporters (MCT) and different isoforms of

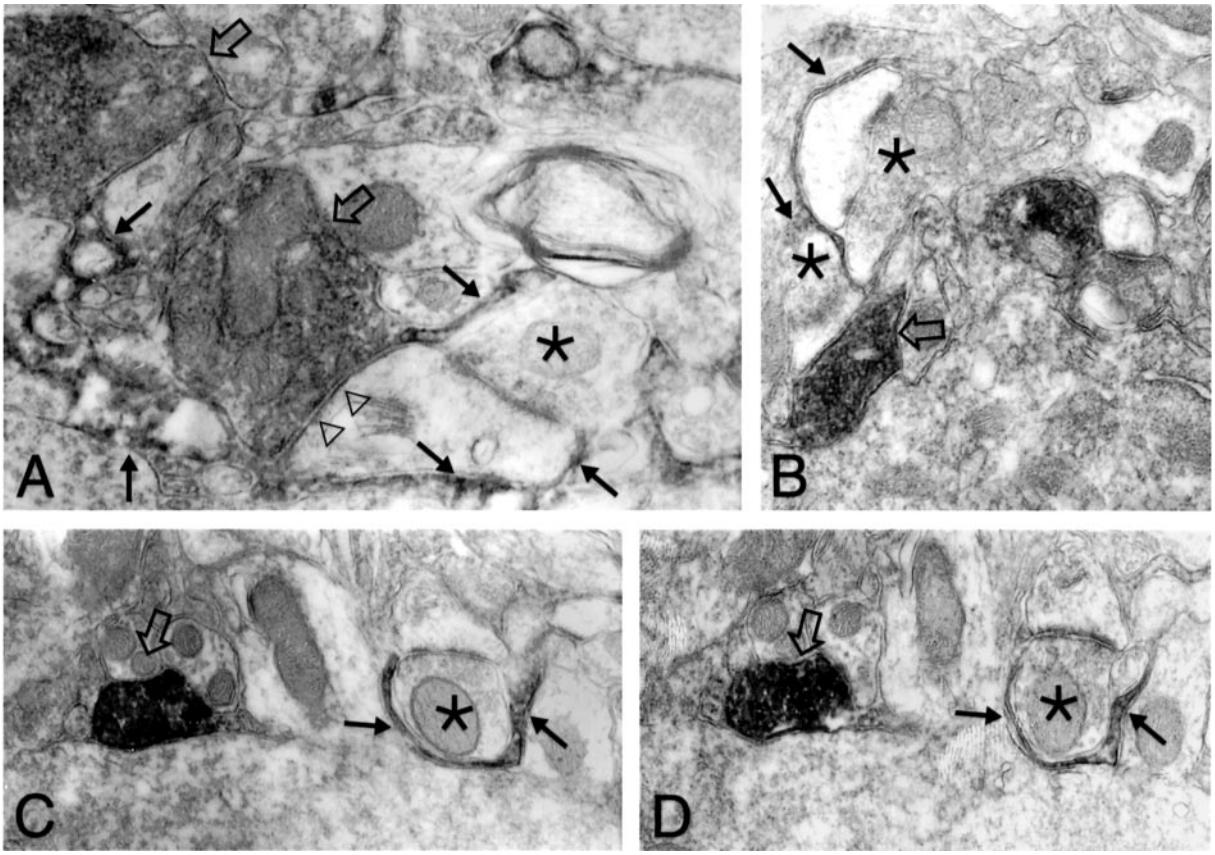

Figure 7. Double electron microscopic immunocytochemistry of GLT-1 and GAD nerve terminals in the rat frontoparietal cortex (layers III to V). GLT-1 immunoreactive astrocytic processes are frequently associated with asymmetrical axo-dendritic synaptic junctions (∗ on the axon terminals, *A–D*). Most GAD terminals (open arrows) are not surrounded by GLT-1 astrocytic processes (*B–D*), but were occasionally apposed to GLT-1 astroglial leaflet ensheathing axo-dendritic asymmetrical synapses (*A*). Note the symmetrical (△△) synaptic junction between the GAD terminal and the dendritic spine also involved in the asymmetrical synapse (*A*). Scale bars: 0.5 μm.

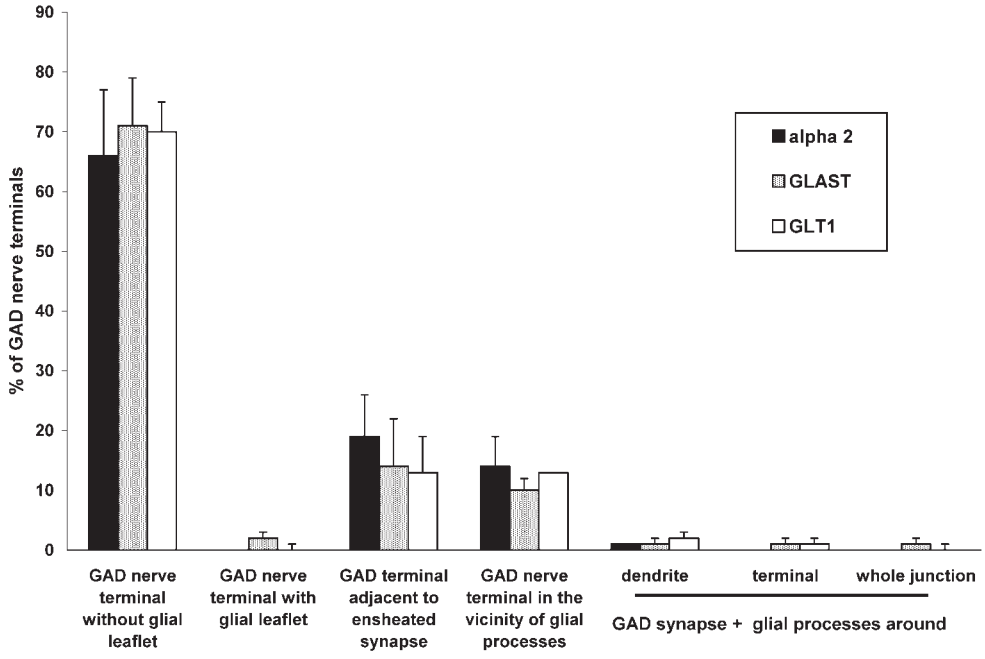

Figure 8. Relationship between GAD terminals and glial elements immunostained for the α_2 subunit, GLAST or GLT-1 in the rat frontoparietal cortex. It is apparent that the vast majority (65–70%) of GAD terminals were not surrounded by any immunoreactive glial leaflets for either of these proteins. About 25% of the GAD terminals engaged in symmetrical synaptic junctions were located either adjacent to asymmetrical synapses surrounded by a stained glial leaflet, or in the vicinity of immunoreactive glial processes ($n = 353$ GAD terminals for α_2, 356 for GLAST and 288 for GLT-1, as described in Materials and Methods). Results are expressed as means ± SD.

the lactate dehydrogenase (LDH), MCT_1 and LDH_5 being expressed in astrocytes and MCT-2 and LDH_1 in neurons (Bittar *et al.*, 1996; Pellerin *et al.*, 1998b; Pierre *et al.*, 2000) further supports the notion that astrocytes act as a lactate 'source' while neurons may be a lactate 'sink', thus forming what was proposed as an activity-dependent, astrocyte–neuron lactate shuttle (Pellerin *et al.*, 1998a). The highly localized glial Na^+,K^+-ATPase containing the α_2 subunit would constitute an additional compartmentalized element participating in a regulated metabolic cooperation process between neurons and astrocytes.

In the present study and in previous reports (Ullensvang *et al.*, 1997; Lehre and Danbolt, 1998), glial leaflets immunoreactive for GLAST, GLT-1 and the α_2 subunit of the Na^+,K^+-ATPase were concentrated around asymmetrical (glutamatergic) junctions but also directly apposed to most, if not all, capillaries located in immunostained areas. It was obvious that the perivascular glial leaflets extended to ensheath all or a large part of the capillary basal membrane, indicating that the vessels were a target and not just arbitrarily contacted by neighbouring glial processes. This organization would favour a rapid clearance of glutamate in the perivascular space. Whether this represents a protective mechanism against glutamate influx into the brain parenchyma from the circulation remains to be determined. Also, although less pronounced than that for GLAST and GLT-1 (Schmitt *et al.*, 1996, 1997; Danbolt, 2001), the apparent increased density of punctate perisomatic α_2 labelling of pyramidal cells at the light microscopic level, a characteristic not clearly detected at the ultrastructural level, most likely corresponded to α_2-ensheathed asymmetrical axo-dendritic synaptic junctions located in proximity to pyramidal cell soma, as seen in Figure *4B*. Alternatively, as suggested by Danbolt (Danbolt 2001) for the glutamate transporters, such localization of the α_2 subunit could be involved in a glutamate 'sink', possibly related to the glial uptake of glutamate for glutamine synthesis as a precursor for GABA synthesis by perisomatic GABAergic terminals.

Interactions Between GABA Terminals and α_2-, GLAST- or GLT-1-stained Glial Processes

Our results show that GABAergic terminals and those establishing axo-dendritic synaptic (symmetrical) junctions represented a negligible target for α_2-, GLAST- or GLT-1-immunoreactive glia. This observation clearly suggests that the Na^+-dependent uptake of GABA into astrocytes (Conti *et al.*, 1998; Schousboe, 2000; Gadea and Lopez-Colome, 2001b) is not coupled to a specific Na^+,K^+-ATPase isoenzyme that is composed of, or enriched in, the α_2 subunit. Such a conclusion would extend to glia the suggestion that different Na^+,K^+-ATPase isoforms restore the ionic gradients at excitatory (Na^+ and Ca^{2+} fluxes involved) and inhibitory (Na^+/Cl^- conductance) synapses [see Peng *et al.* (Peng *et al.*, 1997)]. It might also be kept in mind that in contrast to glutamate uptake, a major portion of GABA uptake takes place in presynaptic terminals (Cherubini and Conti, 2001). Thus, there might be no need for a specific glial Na^+,K^+-ATPase isoform to drive GABA uptake in astrocytes. There is, however, a minor (1–2%) population of GABAergic axon terminals forming axo-dendritic synaptic junctions that are enclosed by α_2-, GLAST- or GLT-1-immunostained glial processes. Some of these astrocytic processes adjacent to axon terminals having either symmetrical or asymmetrical specializations in the cerebral cortex may also contain GABA transporters and might participate not only to limit the spread of GABA at the synapse but also to control the overall GABA levels in the neuropil (Minelli *et al.*, 1996).

Most striking, however, was the finding that 13–19% of GABA terminals were directly apposed to axo-dendritic asymmetrical (glutamatergic) synapses ensheathed by either α_2, GLAST or GLT-1 glial processes. Such associations could, possibly, underlie the functional inhibition of glutamate release exerted by GABA in the cerebral cortex, and mediated by pre-synaptic $GABA_A$ and/or $GABA_B$ receptors on glutamate nerve terminals (Pende *et al.*, 1993; Torimitsu and Niwa, 1997; Perkinton and Sihra, 1998; Buggy *et al.*, 2000). These interactions may provide a mean to fine-tune excitatory neurotransmitter release and may explain the close association of astrocytic processes containing the GABA transporter GAT-3 with asymmetrical (excitatory/glutamatergic) synapses (Minelli *et al.*, 1996). Such interplay between inhibitory and excitatory neurotransmission may assure an adequate level of excitation at the cortical level.

In conclusion, our findings provide a morphological substrate for the coupling between Na^+-dependent transport of glutamate into astroglial cells by GLAST and/or GLT-1, and concurrent activation of an α_2-containing Na^+,K^+-ATPase isozyme most likely localized in the same glial leaflets as those accumulating glutamate. As proposed by Pellerin and Magistretti (Pellerin and Magistretti, 1994, 1997), increased neuronal activity leading to glutamate release would trigger an increase in glycolysis in astrocytes. The mechanism involves glutamate uptake via glutamate transporters, an increase in the intracellular concentration of Na^+ followed by activation of a specific glial Na^+,K^+-ATPase isozyme. Based on the suggestion that the typical glial isoform of the Na^+,K^+-ATPase is composed of $\alpha_2\beta_2$ subunits (Gloor *et al.*, 1990; Corthesy-Theulaz *et al.*, 1990) together with previous (Pellerin and Magistretti, 1997) and present (this work) evidence, it can be suggested that it is this specific isoenzyme that is mobilized by glutamate in response to increased excitatory neurotransmission. These data provide further support for the existence of a glutamate-mediated increase in astroglial glycolysis during neuronal activation and thus in the coupling between neuronal activity and glucose metabolism. Finally, the results also suggest that this physiological process might be under the control of GABAergic inputs.

Notes

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