

EAAC1, a High-affinity Glutamate Transporter, is Localized to Astrocytes and Gabaergic Neurons besides Pyramidal Cells in the Rat Cerebral Cortex

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High-affinity uptake of glutamate from the synaptic cleft plays a crucial role in regulating neuronal activity in physiological and pathological conditions. We have used affinity-purified specific polyclonal antibodies raised against a synthetic peptide corresponding to the C-terminal region of rabbit and rat EAAC1, a glutamate (Glu) transporter believed to be exclusively neuronal, to investigate its cellular and subcellular localization and whether it is expressed exclusively in glutamatergic cells of infragranular layers, as suggested by previous studies. Light microscopic studies revealed that EAAC1 immunoreactivity (ir) is localized to neurons and punctate elements in the neuropil. EAAC1-positive neurons were more numerous in layers II–III and V–VI, i.e. throughout all projection layers. Most EAAC1-positive neurons were pyramidal, although non-pyramidal cells were also observed. Some EAAC1-positive non-pyramidal neurons stained positively with an antiserum to GAD, thus demonstrating that EAAC1 is not confined to glutamatergic neurons. Non-neuronal EAAC1-positive cells were also observed in the white matter, and some of them stained positively with an antiserum to GFAP. Ultrastructural studies showed that EAAC1-ir was in neuronal cell bodies, dendrites and dendritic spines, but not in axon terminals, i.e. exclusively postsynaptic. Analysis of the type of axon terminals synapsing on EAAC1-ir profiles showed that 97% of them formed asymmetric contacts, thus indicating that EAAC1 is located at the very sites of excitatory amino acid release. Unexpectedly, EAAC1-ir was also found in a few astrocytic processes located in both the gray and the white matter. The localization of EAAC1 may explain the pathological symptoms that follow EAAC knockout (seizures and mild toxicity), as seizures could be due to the loss of EAAC1-mediated fine regulation of neuronal excitability at axodendritic and axospinous synapses, whereas the mild toxicity may be related to the functional inactivation of astrocytic EAAC1.

Introduction

Glutamate (Glu) transporters mediate high-affinity uptake of Glu from the synaptic cleft into presynaptic axon terminals and surrounding glial cells (Kanai *et al.*, 1993, 1994; Nicholls, 1993; Danbolt, 1994). Physiological studies have shown that they contribute to shaping postsynaptic responses (Barbour *et al.*, 1994; Maki *et al.*, 1994; Tong and Jahr, 1994; Mennerick and Zorumski, 1995; Takahashi *et al.*, 1995; Otis *et al.*, 1996; but see Isaacson and Nicoll, 1993; Sarantis *et al.*, 1993), and knockout experiments have revealed that Glu transporters are essential for maintaining physiological levels of Glu and for preventing excitotoxicity (Rothstein *et al.*, 1996). However, the function of Glu transporters might not be limited to terminating synaptic action. Indeed, recent studies have shown that: (i) Glu transporters are electrogenic and can directly modulate neuronal (Lester *et al.*, 1996; Sonders and Amara, 1996) or glial (Mennerick *et al.*, 1996) excitability; and (ii) Glu transporters may also release Glu into the extracellular space in a

Ca²⁺-independent, nonvesicular manner (Attwell *et al.*, 1993; Levi and Raiteri, 1993).

Five Glu transporter cDNAs have been isolated in the mammalian CNS: EAAC1 (Kanai and Hediger, 1992; Arriza *et al.*, 1994; Shashidharan *et al.*, 1994; Kanai *et al.*, 1995; Bjoras *et al.*, 1996; Nakayama *et al.*, 1996; Velaz-Faircloth *et al.*, 1996), GLT1 (Pines *et al.*, 1992; Arriza *et al.*, 1994), GLAST (Storck *et al.*, 1992; Arriza *et al.*, 1994), EAAT4 (Fairman *et al.*, 1995) and EAAT5 (Arriza *et al.*, 1997). They exhibit ~50% of sequence identity and differ significantly in tissue distribution, kinetics and pharmacological properties (Kanai *et al.*, 1993, 1994; Danbolt, 1994; Fairman *et al.*, 1995; Arriza *et al.*, 1997). Early *in situ* hybridization and immunocytochemical studies revealed different patterns of cellular localization and distribution for GLT1, GLAST, EAAC1 and EAAT4 (Danbolt *et al.*, 1992; Kanai and Hediger, 1992; Rothstein *et al.*, 1994; Torp *et al.*, 1994; Chaudry *et al.*, 1995; Kanai *et al.*, 1995; Kondo *et al.*, 1995; Lehre *et al.*, 1995; Dykes-Hoberg *et al.*, 1996; Velaz-Faircloth *et al.*, 1996). These studies have generated the notion that GLT1 and GLAST are localized exclusively to glial processes, whereas EAAC1 and EAAT4 are expressed only in neurons. Nothing is known of the cellular localization of EAAT5, a retina-specific glutamate transporter, although indirect evidence suggest that it might be neuronal (Arriza *et al.*, 1997). This notion should not, however, be interpreted dogmatically, as GLT1 appears to be expressed also by neural cells (Rauen and Kanner, 1994; Schmitt *et al.*, 1996; Torp *et al.*, 1996) and GLAST expression has been reported also in non-glial cells (Rothstein *et al.*, 1994; Schmitt *et al.*, 1997).

With respect to EAAC1, most *in situ* hybridization studies report that this transporter is expressed exclusively in neurons (Kanai and Hediger, 1992; Arriza *et al.*, 1994; Shashidharan *et al.*, 1994; Kanai *et al.*, 1995; Bjoras *et al.*, 1996; Velaz-Faircloth *et al.*, 1996). However, it has recently been suggested that it might also be expressed in non-neuronal cells (Kiryu *et al.*, 1995; Palos *et al.*, 1996). Given the poor cellular resolution of radioactive *in situ* hybridization, and that immunocytochemical data come only from a general mapping study on the localization of three Glu transporters (Rothstein *et al.*, 1994), the question of the precise cellular and subcellular localization of EAAC1 remains unanswered.

In the present study, we have investigated the cellular localization of EAAC1 in the cerebral cortex of adult rats by using light and electron microscopic immunocytochemical techniques. Our aim was to identify unequivocally the cell types expressing EAAC1 in a brain region where the role of glutamatergic synaptic transmission appears to be prominent in both physiological and pathological conditions (Conti and Hicks, 1996). A preliminary account of part of these results has been presented in abstract form (Conti *et al.*, 1996a).

Materials and Methods

Adult albino rats (Sprague-Dawley) weighing 200–300 g were used in these studies. Care and handling of animals were approved by the Animal Research Committee of the University of Ancona. Rats were anesthetized with 12% chloral hydrate and perfused through the ascending aorta with physiological saline followed by either 4% paraformaldehyde (PFA) in phosphate buffer (PB; pH 7.4) for light microscopy, or by 4% PFA and 0.5% glutaraldehyde in PB for electron microscopy. Brains were removed and postfixed in 4% PFA for 2–12 h. Sections 300 μ m thick were then cut on a Vibratome in either a coronal or parasagittal plane and collected in phosphate buffered-saline (PBS).

Antibodies

The affinity-purified polyclonal antibodies against EAAC1 used in the present study were raised in rabbits against a synthetic peptide (DKSDDTISFTQTSQF) conjugated to thyroglobulin by glutaraldehyde (Rothstein *et al.*, 1994). The synthetic peptide corresponds to the C-terminal region of rabbit (Kanai and Hediger, 1992) and rat (Kanai *et al.*, 1995; Kiryu *et al.*, 1995; Bjoras *et al.*, 1996; Velaz-Faircloth *et al.*, 1996) EAAC1, and of the human homolog EAAT3 (Arriza *et al.*, 1994). Immunoblot analysis of rat synaptic membranes and blocking experiments have shown that antibodies to EAAC1 are specific for this transporter (Rothstein *et al.*, 1994). Details on the characterization and immunocytochemical application of these antibodies in the rat brain have been published (Rothstein *et al.*, 1994). In the present material, additional controls were performed, such as substituting the primary antibody with PBS or normal sheep serum (NSS), or preadsorbing it with synthetic peptide.

For co-localization experiments (see below) polyclonal antibodies to glutamic acid decarboxylase (GAD₆₇) and monoclonal antibodies to glial fibrillary acidic protein (GFAP) were used. Anti-GAD antibodies have been purchased from Chemicon (Temecula, CA, USA; AB108, lot 54196353) and have been previously characterized (K2; Kaufman *et al.*, 1991), whereas anti-GFAP antibodies were from Sigma (St Louis, MO, USA).

Immunocytochemical Procedure

The free-floating sections used for EAAC1 immunocytochemistry were rinsed in PBS for 30 min and incubated first in NSS (10% in PBS) for 1 h at room temperature, and then in the primary antiserum for 12 h at 4°C. EAAC1 antibodies were used at concentrations of 0.06–0.15 μ g/ml. The next day, after three PBS rinses (5 min each), sections were incubated first in NSS (10% in PBS; 10 min) and then in biotinylated goat anti-rabbit IgG (bGAR; 1:100; Vector, Burlingame, CA; BA-1000) for 1 h. After further rinsing in PBS (3 \times 5 min), they were processed according to the avidin-biotin peroxidase complex procedure (Hsu *et al.*, 1981; Vector; PK-6100; 30 min). The reaction product was demonstrated by 3',3'-diaminobenzidine tetrahydrochloride (DAB; 40 mg/50 ml) with 0.03% hydrogen peroxide. Sections were then mounted on subbed slides, air-dried and coverslipped with DPX.

Co-localization Experiments

To demonstrate co-localization of EAAC1 and GAD or GFAP in the same cortical neurons, we compared the paired surfaces of adjacent 30 μ m thick sections incubated alternately with the two antibodies to identify the same perikarya bisected into two consecutive sections, as described by Kosaka *et al.* (1985a,b). This method for demonstrating two antigens within the same cell has several advantages over other methods (see Kosaka *et al.*, 1985a). Cortical regions exhibiting clear landmarks (blood vessels, artifacts, etc.) were identified using a 25 \times objective of the microscope and photographed at the same or at higher magnification. Paired photomicrographs were then used to identify immunoreactive perikarya that were present on both sections. The procedure for the visualization of GAD and GFAP immunoreactivity was as described for EAAC1; GAD and GFAP were used at dilutions ranging from 1:3000 to 1:4000 and 1:1000 to 1:2000 respectively.

Electron Microscopy

To increase the penetration of immunoreagents, Vibratome sections were pretreated by a mild ethanol treatment (10%, 25%, 10%; 5 min each).

EAAC1 antibodies were used at concentrations of 0.08–0.15 μ g/ml; Triton X-100 was not used. After completion of the ICC procedure, sections were washed in PB, postfixed for ~30 min in 2.5% glutaraldehyde in PB, washed in PB and postfixed for 1 h in 1% OsO₄. After dehydration, sections were cleared in propylene oxide, flat-embedded in Epon-Spurr between acetate sheets (Aclar; Ted Pella, Redding, CA), and polymerized at 60°C for 48 h. When polymerization was complete, the embedded sections were examined under a dissecting microscope. Small strips of tissue containing both cortical gray matter and the underlying white matter were excised with razor blades and either glued to cured resin blocks or re-embedded in Epon-Spurr. Semithin (1 μ m) sections were cut with a Reichert ultramicrotome, and collected on glass slides without counterstaining for light microscopic inspection. Ultrathin sections were cut either from the surface or from the edge (i.e. perpendicular to the plane of section), counterstained with uranyl acetate and lead citrate or with lead citrate only, and examined with a JEOL T8 electron microscope. Identification of immunolabeled and unlabeled profiles was based on standard morphological criteria (Peters *et al.*, 1991; Privat *et al.*, 1995).

Data Analysis

All data were collected from a region of the parietal cortex characterized by the presence of a conspicuous layer IV, with intermingled dysgranular regions, densely packed layers II and III, and a relatively cell-free layer Va. This area corresponds to the first somatic sensory cortex (SI; Chapin and Lin, 1990).

For a more detailed analysis of the immunoreactive profiles, grids containing thin sections were collected from ten blocks trimmed out from five Epon-embedded Vibratome sections and only one section for each grid was examined. Sections were scanned at the electron microscope and all immunostained profiles identified as dendritic shafts or spines were recorded at a magnification of 20 000 \times to examine their synaptic association with asymmetric or symmetric synaptic terminals. Labeled profiles containing reaction product that obscured the postsynaptic site were not counted. A terminal was considered to be synaptic when it showed a junctional complex, and a restricted zone of parallel membrane apposition with slight enlargement of the intercellular space that was always devoid of reaction product. Terminals forming asymmetric synapses were identified by the presence of round vesicles and thick postsynaptic densities, whereas those forming symmetric synapses contained pleomorphic vesicles and lacked marked postsynaptic densities. When the synaptic specialization was not recognizable in the plane of the section, the terminal was considered 'unclassifiable'.

Results

Specific EAAC1 immunoreactivity (ir) has been observed in the CNS of all animals, with highest expression in the hippocampus, cerebellum and basal ganglia, followed by the cerebral cortex, olfactory bulb and superior colliculus, in agreement with previous *in situ* hybridization and immunocytochemical studies in rats (Kanai and Hediger, 1992; Rothstein *et al.*, 1994; Kanai *et al.*, 1995; Kiryu *et al.*, 1995; Bjoras *et al.*, 1996; Velaz-Faircloth *et al.*, 1996). In all brain regions, EAAC1-ir was localized to cell bodies, proximal processes (but not nuclei) and punctate elements in the neuropil.

In the SI cortex, EAAC1-positive (+) neurons were numerous in all cortical layers (Fig. 1A,B). Specific immunostaining was not observed in control sections incubated with EAAC1 antibodies preadsorbed with 50 μ M EAAC1 peptide (Fig. 1C), or in sections processed without the primary antibodies (not shown). Comparable immunostaining patterns were observed in all cortical areas. EAAC1+ neurons were more numerous in layers II–III and V–VI, and most intensely stained in layer V (Fig. 2A–C). Analysis of the morphological features of immunoreactive cells (see Conti *et al.*, 1992, for criteria) revealed that most EAAC1+ neurons were pyramidal (Fig. 3A,B), and that some were non-pyramidal (Fig. 3C–E). EAAC1-ir was also present in punctate structures in

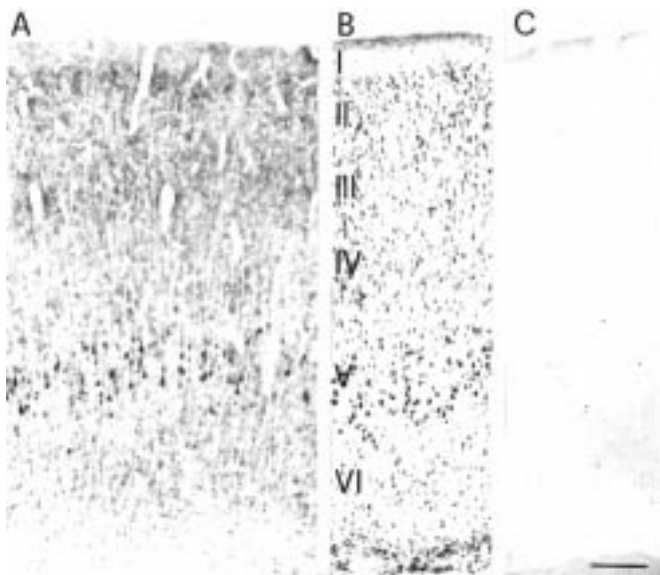


Figure 1. (A) EAAC1-ir in the first somatic sensory cortex of an adult rat. Note the presence of EAAC1+ cells in all cortical layers, and the strong immunostaining in layer V neurons. (B) A thionin-stained section adjacent to that shown in A. Roman numerals indicate cortical layers. (C) EAAC1-ir is completely abolished following incubation in EAAC1 antibody preadsorbed with 50 μ M synthetic peptide. Scale bar: 200 μ m.

the neuropil that were more numerous in supragranular layers, in particular in the inner portion of layer I.

Since in the cerebral cortex the large majority of non-pyramidal neurons are inhibitory and use GABA as neurotransmitter (Hendry, 1996), the observation that some non-pyramidal neurons express EAAC1 suggests that EAAC1 and GAD (or GABA) may be co-expressed. Analysis of EAAC1+ and GAD+ neurons at the cut surface of paired sections (Kosaka *et al.*, 1985a,b) incubated alternately with antibodies directed against EAAC1 and GAD was therefore performed to evaluate this hypothesis. More than 50 paired low-power photomicrographs were studied in detail. In all cases, a large number of EAAC1+ non-pyramidal cells could be identified also on the paired surface incubated with the GAD antibody (Fig. 4), thus indicating that the same neurons co-express EAAC1 and GAD. Given that the results were unequivocal, and that a numerical estimate of the population of GAD+ non-pyramidal neurons expressing EAAC1 was beyond the goals of this study, we did not perform a quantitative analysis.

Lightly stained non-neuronal cells were also observed in the underlying white matter and in the corpus callosum; they were morphologically heterogeneous, had variable dimensions (major diameter 5.5–13 μ m; mean 9.2), and exhibited several processes radiating in all directions (Fig. 5A). To verify whether these cells were astrocytes, we studied the cut surface of paired sections incubated alternately with antibodies directed against EAAC1 and GFAP. Analysis of 50 paired low-power photomicrographs revealed that few EAAC1+ cells of the white matter could be identified also on the paired surface incubated with the GFAP antibody (Fig. 5B,C), thus indicating that the same cells co-express EAAC1 and GFAP.

Electron Microscopy

Granular electron-dense reaction product indicated that EAAC1-ir was present throughout the cortical layers in several

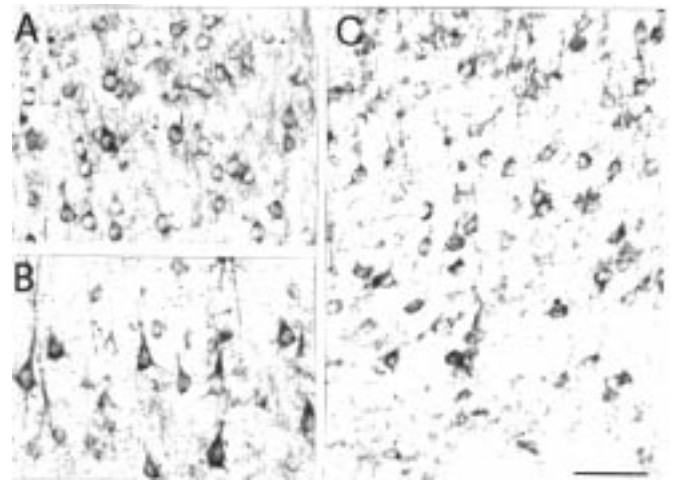


Figure 2. EAAC1+ neurons in layers II–III (A), V (B) and VI (C). EAAC1+ neurons in layer V are more intensely stained than those in layers III and VI. Scale bar: 50 μ m.

neuronal cell bodies and dendrites and in a few astrocytic processes, but not in axon terminals (Fig. 6).

In the perikarya, labeling was in cisterns of the rough endoplasmic reticulum and Golgi apparatus; clumps of EAAC1-ir were also scattered in the cytoplasm or close to areas of the plasma membrane not associated with axon terminals (Fig. 6A). In dendrites, labeling was present in the cytoplasm throughout their extension. Labeled proximal dendrites were often contacted in the plane of section by several unlabeled axon terminals (Fig. 6B), whereas labeled distal dendrites and dendritic spines were generally contacted by a single unlabeled axon terminal (Fig. 6C). The analysis of the type of terminals synapsing on EAAC1+ profiles showed that the vast majority (97%) of them were asymmetric. Of the 295 terminals synapsing on dendritic shafts, 286 showed a thick postsynaptic density and were therefore identified as asymmetric, eight were unclassifiable and only one was symmetric. Similarly, of the 240 axon terminals synapsing on dendritic spines, 234 were identified as asymmetric, six were considered unclassifiable and none was symmetric.

EAAC1-ir was also present in some small, distal, astrocytic processes with an irregular profile located among unlabeled dendrites and axon terminals (Fig. 6C–E), whereas astrocytic somata and their proximal processes were only rarely stained. EAAC1+ distal astrocytic processes were mainly close to axon terminals, forming asymmetric synaptic contacts. EAAC1-ir was never found in perivascular astrocytic processes. EAAC1-ir was also present in astrocytic processes of the white matter and the corpus callosum (not illustrated).

Discussion

The distribution of EAAC1-ir described here is in basic agreement with previous *in situ* hybridization and immunocytochemical studies (see below), which have described the prominent expression of EAAC1 in neurons of the mammalian neocortex. In addition, the present study shows that in the cerebral cortex of adult rats EAAC1 is also localized to some astrocytic processes, and that in neurons EAAC1 is preferentially localized to dendrites and spines postsynaptic to excitatory axon terminals.

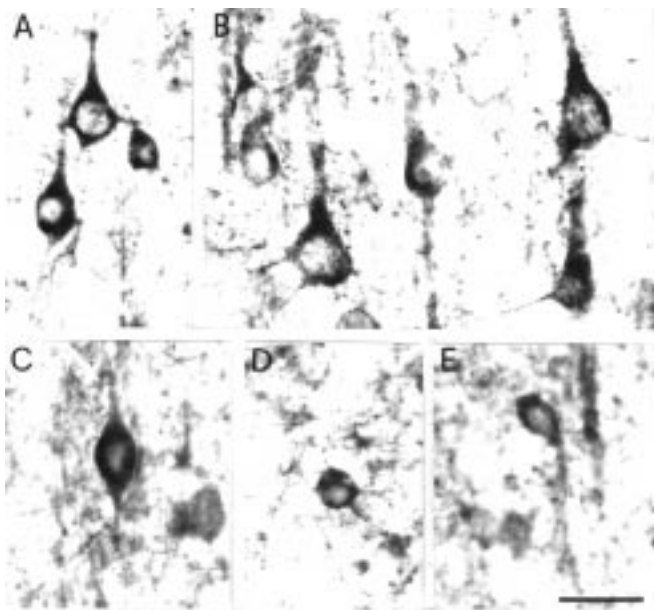


Figure 3. EAAC1+ neurons in the SI cortex are both pyramidal (A,B), and non-pyramidal (C–E). Scale bar: 30 μ m.

EAAC1 Expression in Cortical Astrocytes

To visualize the cellular localization of EAAC1, we used affinity-purified polyclonal antipeptide antibodies raised against a synthetic peptide corresponding to the predicted C-terminal domain of EAAC1 (Rothstein *et al.*, 1994). Immunoblot and immunocytochemical studies have shown that these antibodies specifically detect EAAC1 protein (Rothstein *et al.*, 1994; present results). Given the specificity of the antibodies used in the present investigation, it is unlikely that the EAAC1-ir observed in astrocytic processes could reflect cross-reactivity with other Glu transporters, although the possibility that EAAC1 antibodies might have cross-reacted with other unidentified membrane proteins cannot be ruled out. The use of techniques for achieving good preservation of nervous tissue has allowed the definition of the electron microscopic features of astrocytes and astrocytic processes (Peters *et al.*, 1991), which can now be identified unambiguously (Peters *et al.*, 1991; Privat *et al.*, 1995; see also Minelli *et al.*, 1995, 1996; Conti *et al.*, 1996b, for recent data from our laboratory). The specificity of the antibodies used here and the conservative criteria used to identify astrocytic processes support the conclusion that some astrocytic processes in the neocortex of adult rats do express EAAC1, and the results of the co-localization study performed on the cells of the white matter are in line with this hypothesis.

Astrocytic EAAC1 expression has not been reported in previous *in situ* hybridization (Kanai and Hediger, 1992; Arriza *et al.*, 1994; Shashidharan *et al.*, 1994; Kanai *et al.*, 1995; Bjoras *et al.*, 1996; Velaz-Faircloth *et al.*, 1996) and immunocytochemical (Rothstein *et al.*, 1994) studies devoted to the analysis of EAAC1 expression. Given that astrocytic EAAC1 expression, although consistent, is not quantitatively comparable to neuronal EAAC1 expression, and that only general mapping studies of the entire CNS have been performed to date, the minor astrocytic expression reported here in the neocortex is likely to have passed unnoticed. It is worth noting, however, that Palos *et al.* (1996) isolated from rat C6 glioma cells – a well-characterized astrocytoma line – a partial cDNA clone with a sequence of the EAAC1 transporter and, although they could

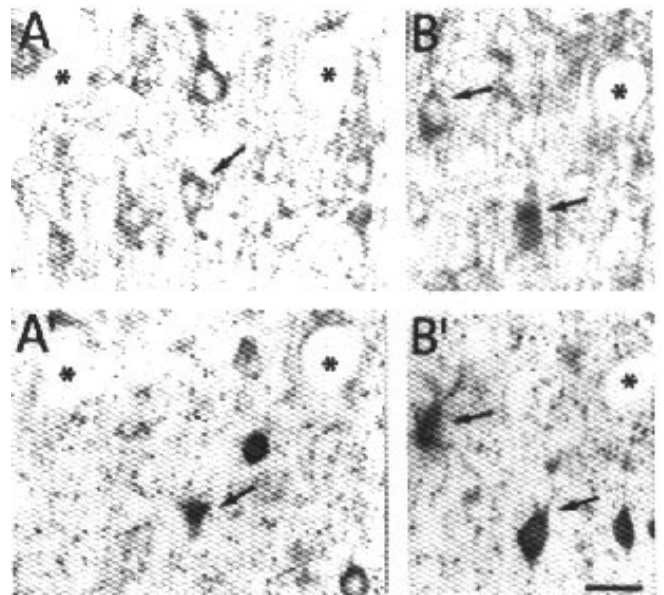


Figure 4. Co-localization of EAAC1 and GAD. Paired photomicrographs from confronting planes of two consecutive sections processed with EAAC1 (A,B) and GAD (A',B'). Asterisks indicate profiles of blood vessels that were used as landmarks. Arrows point to neurons expressing both antigens. Scale bar: 40 μ m.

not rule out the possibility that the 'unusual expression of the EAAC1 transporter by C6 cells results from change in the expression and/or activity of some oncogene(s) or oncogene product(s)' or that EAAC1 expression by C6 cells resulted from *in vitro* culturing, they hypothesized that some astrocytes might express EAAC1 (Palos *et al.*, 1996). The present results provide evidence that astrocytes do indeed express EAAC1 *in vivo*. Future studies focusing on other brain regions will help to determine whether astrocytic EAAC1 expression occurs also in other brain regions. The results of preliminary studies on the localization of EAAC1 in the hippocampus and in the thalamus, which showed that some astrocytes are EAAC1+ (S. DeBiasi, M. Melone, A. Minelli, J.D. Rothstein and F. Conti, unpublished observations), suggest that astrocytic EAAC1 expression is not restricted to the cerebral cortex.

The finding that astrocytes also express EAAC1 is not surprising *per se*, considering the major role astrocytes play in Glu uptake (Rothstein *et al.*, 1996) and in Glu-mediated synaptic transmission (Conti *et al.*, 1994, 1996b; Blankenfeld *et al.*, 1995; Hansson and Ronnback, 1995; Kirchhoff and Kettenmann, 1996). Rather, the results presented here are unexpected because so far EAAC1 has been considered a 'neuronal' transporter, and GLT1 and GLAST 'glial' transporters. However, the present evidence, together with the results of investigations on the cellular localization of GLT1 (Rauen and Kanner, 1994; Schmitt *et al.*, 1996; Torp *et al.*, 1996) and GLAST (Rothstein *et al.*, 1994; Schmitt *et al.*, 1997) suggest that the subdivision of Glu transporters into 'neuronal' and 'glial' transporters is no longer tenable. The evolution of our knowledge of the cellular localization of EAAC1 exhibits some interesting analogies with that of the localization of GAT-1, a high-affinity plasma membrane GABA transporter, in the cerebral cortex. Based on the results of pharmacological studies and on early mapping studies, GAT-1 has long been considered the 'neuronal' GABA transporter, until detailed immunocytochemical analysis revealed that a consistent, although minor, population of GAT-1+

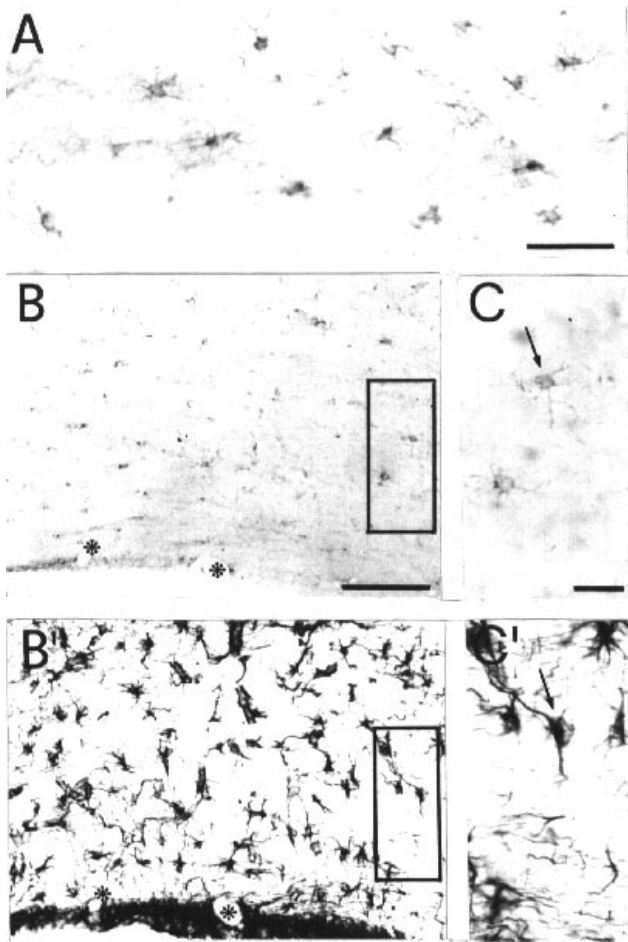


Figure 5. (A) Non-neuronal EAAC1+ cells of variable morphology and dimension in the white matter underlying the parietal cortex. (B,B') Paired low-power photomicrographs from confronting planes of two consecutive sections processed with EAAC1 (B) and GFAP (B'). Asterisks indicate profiles of blood vessels that were used as landmarks. Framed regions are shown enlarged in C and C'. (C,C') High-power photomicrographs of the framed regions in (B,B') showing an example of an EAAC1+ cell that is also immunoreactive to GFAP (arrows). Scale bars: 50 μm for A; 20 μm for B and C.

elements are astrocytic processes (Minelli *et al.*, 1995; Conti *et al.*, 1997). This analogy emphasizes the scope for detailed localization studies in shaping our understanding of transporter systems (see also Minelli *et al.*, 1996).

Electron microscopic analysis shows that in the gray matter EAAC1-ir is observed in distal astrocytic processes, but not in cell bodies or proximal processes. This observation raises two major points. First, it makes it unlikely that double-labeling immunocytochemical techniques can define whether astrocytes that express EAAC1 belong to astrocytic subpopulations expressing glial fibrillary acidic protein (GFAP; Eng *et al.*, 1971; Bignami and Dahl, 1973; Dahl and Bignami, 1973) or S-100 (Moore, 1965; Matus and Mughal, 1975), because distal profiles

do not contain gliofilaments (Peters *et al.*, 1991; Privat *et al.*, 1995). Second, it suggests that EAAC1 is a protein that is anterogradely transported, and may provide some clues to the function of EAAC1+ astrocytic processes. Indeed, it is conceivable that small, distal astrocytic processes that are in close contact with synaptic terminals and other neuronal profiles are more likely to play an active role in Glu uptake than large, proximal processes.

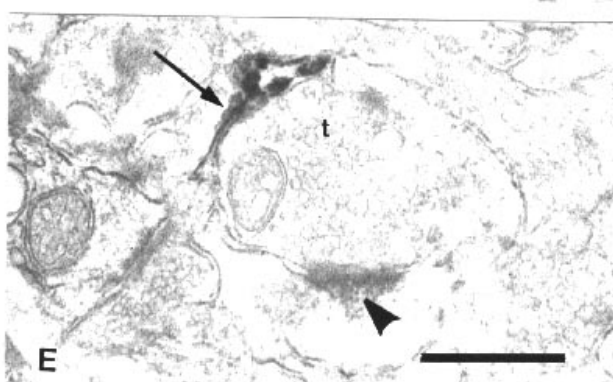
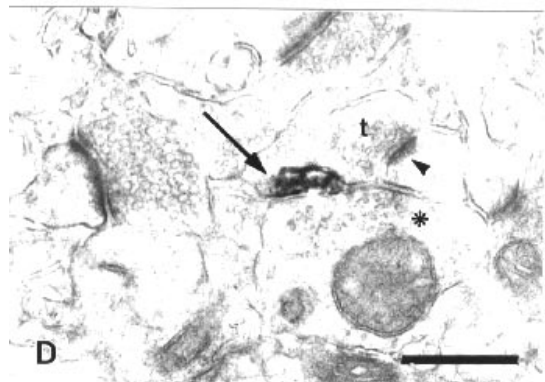
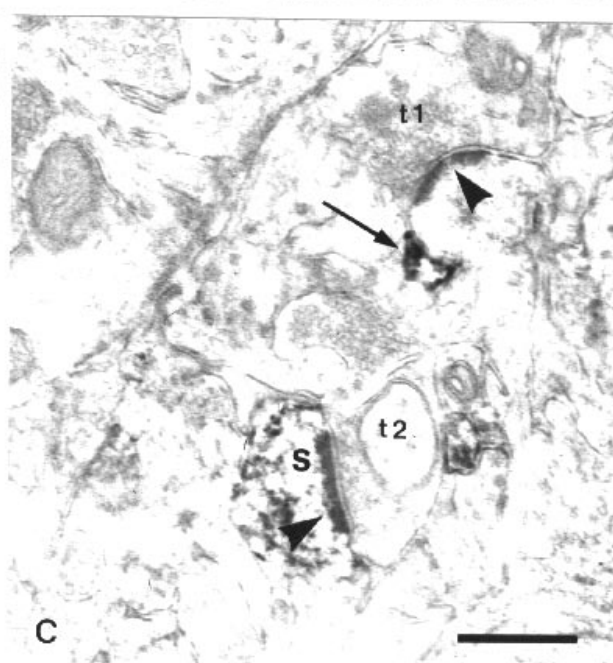
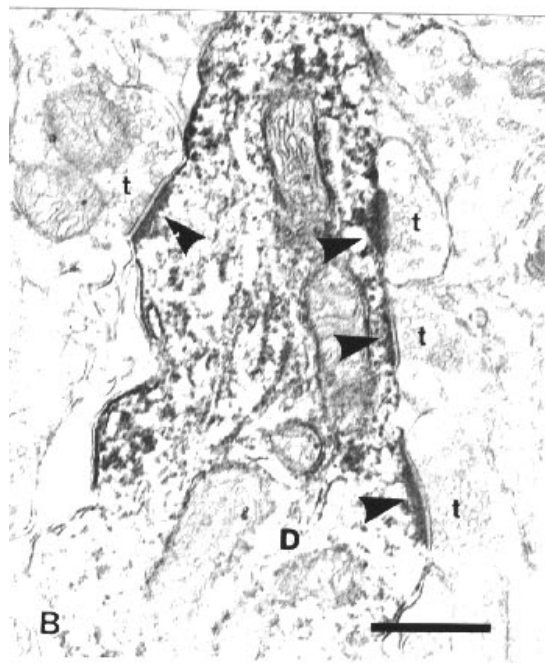
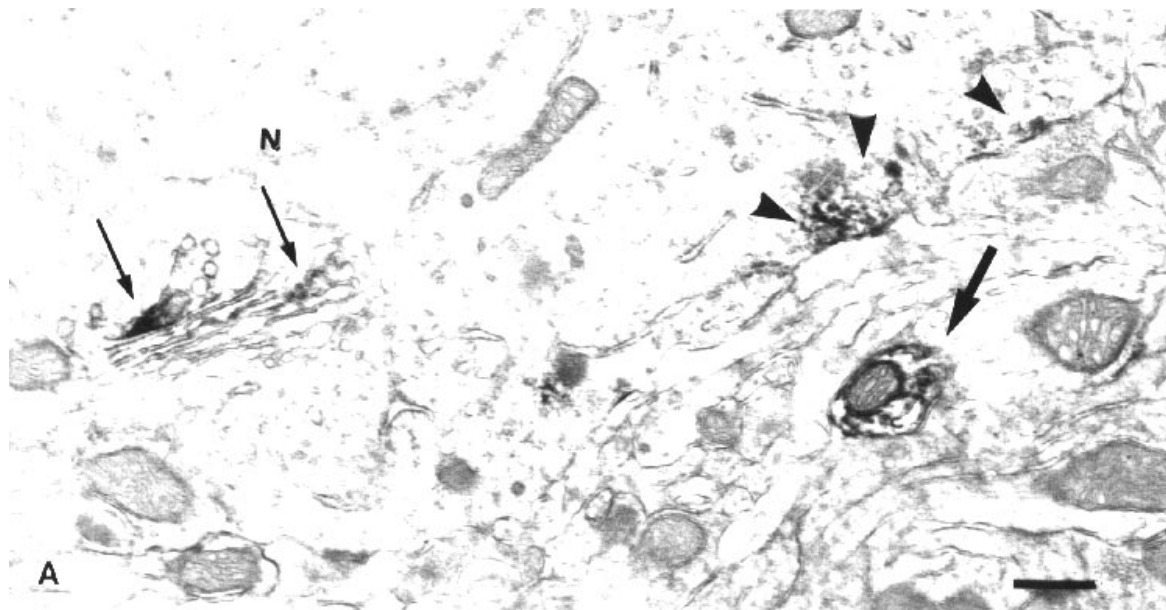
The results of the EAAC1/GFAP co-localization study in the white matter showed that some non-neuronal cells in the white matter are GFAP+ and that most of them are GFAP-. Although we cannot rule out the possibility that EAAC1+/GFAP- cells in the white matter are GFAP- astrocytes, this observation suggests that glial cells other than astrocytes express EAAC1. This possibility is supported by the variable morphology of EAAC1+ cells of the white matter and by the observations of Kiryu *et al.* (1995), who reported the presence of glial cells expressing EAAC1 mRNA in the corpus callosum of rats and interpreted these cells as oligodendrocytes.

EAAC1 Expression in Cortical Neurons

Overall, the distribution pattern of the neurons expressing EAAC1 observed in the present study is in line with that reported in previous *in situ* hybridization studies (Kanai and Hediger, 1992; Arriza *et al.*, 1994; Shashidharan *et al.*, 1994; Kanai *et al.*, 1995; Bjoras *et al.*, 1996; Velaz-Faircloth *et al.*, 1996), and is similar to that described in previous immunocytochemical investigations (Rothstein *et al.*, 1994).

Rothstein *et al.* (1994) showed that EAAC1+ neurons are mostly in infragranular layers, whereas we show here that the apparent density of EAAC1+ neurons in layers II and III is comparable to that of layers V and VI, even though positive cells in the latter layers are more intensely stained. We also studied the morphology of EAAC1+ neurons, and observed that although most EAAC1+ neurons are pyramidal neurons, there is a non-negligible population of neurons which, according to generally accepted light microscopic criteria (see Conti *et al.*, 1992), can safely be considered non-pyramidal neurons. Since few cortical non-pyramidal neurons are glutamatergic (Conti *et al.*, 1987, 1989; Conti and Minelli, 1996), the observation that some non-pyramidal neurons are EAAC1+ strongly suggests that EAAC1 is expressed in non-glutamatergic neurons, thus adding new evidence for the notion, based on studies in other brain regions, that EAAC1 is not confined to glutamatergic neurons (Rothstein *et al.*, 1994; Kanai *et al.*, 1995; Bjoras *et al.*, 1996). Again, the localization of EAAC1 is analogous to that of the major cortical GABA transporter, GAT-1, which is also expressed in non-GABAergic neurons (Minelli *et al.*, 1995; Conti *et al.*, 1997). Here, we have shown that some of the EAAC1+ non-pyramidal neurons co-express GAD by using a double-labeling technique that allows the identification of two antigens in the same neuron (Kosaka *et al.*, 1985a,b), thus elucidating, at least in part, the transmitter phenotype of non-glutamatergic EAAC1+ neurons in the cerebral cortex. The present observations on EAAC1 expression in GABAergic cortical neurons are in line with previous findings in the cerebellar cortex (Rothstein *et al.*,

Figure 6. EAAC1 is expressed postsynaptically in neurons and in distal astrocytic processes. (A) In neuronal cell bodies EAAC1-ir is found in the Golgi complex (thin arrow) and in clumps along the plasma membrane (arrowheads); N, nucleus; thick arrow, labeled dendrite. (B) A heavily labeled dendrite (D) forms asymmetric synaptic contacts (arrowheads) with four unlabeled axon terminals (t). (C) A labeled distal astrocytic process (arrow) is close to an unlabeled axon terminal (t1) with asymmetric specialization (arrowhead); a second unlabeled axon terminal (t2) makes an asymmetric synapse (arrowhead) on a labeled dendritic spine (S). (D) A labeled distal astrocytic process (arrow) is present between an unlabeled axon terminal (t) with asymmetric specialization (arrowhead) and an unlabeled axon terminal with symmetric specialization (asterisk). (E) A labeled distal astrocytic process (arrow) is close to an unlabeled axon terminal (t) with asymmetric specialization (arrowhead). Scale bar: 0.5 μm .



1994), and suggest that EAAC1 expression in GABAergic neurons may occur in many brain regions.

Electron microscopic analysis of sections processed for the immunocytochemical visualization of EAAC1 indicates that in the cerebral cortex EAAC1-ir is localized to perikarya, dendrites and dendritic spines, and is not detectable in axon terminals (see Rothstein *et al.*, 1994, for a discussion on the physiological significance of this latter finding). The somatodendritic localization of EAAC1 observed in the present study in the cerebral cortex is in line with previous findings (Rothstein *et al.*, 1994), although Rothstein and colleagues (1994) reported that, at least in some brain regions, EAAC1 is found at presynaptic boutons. These findings are not contradictory; rather they suggest that there is a regional heterogeneity in EAAC1 cellular expression, as shown for GABA transporters (e.g. Minelli *et al.*, 1995, 1996; De Biasi *et al.*, 1996). The exclusively postsynaptic localization of EAAC1 in cortical neurons is clearly not compatible with the classical notion, according to which a neurotransmitter transporter is localized to the axon terminal and/or to neighboring glial cells (Nicholls, 1994). Other proposed functions include defending neurons against the build-up of toxic levels of Glu and contributing to Glu and/or GABA metabolism (e.g. Kanai *et al.*, 1995; Bjoras *et al.*, 1996). The recent demonstration that EAAC1 knockout produces only a mild neurotoxic effect (Rothstein *et al.*, 1996) seems to argue against a pivotal role of EAAC1 in preventing Glu-induced toxicity, whereas the second hypothesis, which is substantiated by the recent observation that EAAC1 antisense oligonucleotide administration produces a 10–20% decrease in cortical GABA levels (Eccles *et al.*, 1996), holds only for GABAergic neurons.

The function of EAAC1 in glutamatergic neurons, however, remains unclear. In this context, the present electron microscopic analysis of the subcellular localization of EAAC1 in cortical neurons may have disclosed a hitherto unknown but significant feature: in all the sections examined, virtually all labeled dendrites and dendritic spines were contacted by axon terminals forming asymmetric synapses. Since axon terminals forming asymmetric synapses contain, and presumably release, Glu (DeFelipe *et al.*, 1988; Conti *et al.*, 1989; Dori *et al.*, 1989) or Asp (Dori *et al.*, 1989), it follows that EAAC1 is located at the very sites of excitatory amino acid release. Interestingly, in their postembedding double-labeling immunocytochemical studies of Glu-positive thalamocortical axons, Kharazia and Weinberg (1993, 1994) noticed that Glu levels were high also in the postsynaptic region of axospinous cortical synapses, and suggested that Glu released from terminals may be taken up by postsynaptic membranes. The evidence reported here supports this view.

On these bases, we propose that the primary physiological function of EAAC1 at cortical axospinous and axodendritic synapses (of glutamatergic neurons) is to control excitatory amino acid levels within the microenvironment of single axospinous and axodendritic synapses, and thereby to regulate the excitability of postsynaptic neurons. The presence of EAAC1, a high-affinity transporter capable of taking up Glu or Asp at relatively low K_m values (Kanai and Hediger, 1992; Kanai *et al.*, 1995; Velaz-Faircloth *et al.*, 1996), in the very membrane regions where excitatory amino acid levels may be elevated [but below levels producing excitotoxicity, which appear to be regulated in the extracellular macroenvironment by glial transporters (Rothstein *et al.*, 1996)], seems particularly apposite for preventing non-physiological discharges by postsynaptic cells. The observation that EAAC1 knockout in rats produces seizures

(Rothstein *et al.*, 1996) supports this view. It is therefore conceivable that alterations in EAAC1 expression may be sufficient to initiate epilepsy, in which an rise in Glu levels occurs prior to seizure onset (During and Spencer, 1993), and that research on mechanisms that regulate EAAC1 expression (e.g. Zerangue *et al.*, 1995; Dowd and Robinson, 1996; Trotti *et al.*, 1997) might lead to the development of new antiepileptic drugs.

Conclusion

This study shows a dual localization of EAAC1 in the cerebral cortex of adult rats: a major localization to the postsynaptic region of axodendritic and axospinous synapses, and a minor one to astrocytic processes. This dual localization pattern may be related to the dual pathological symptoms that follow EAAC1 knockout, i.e. seizures and mild toxicity, and, notwithstanding the possible contribution of a reduction of GABA levels (Eccles *et al.*, 1996), it could be hypothesized that seizures are due to the loss of EAAC1-mediated fine regulation of neuronal excitability at axodendritic and axospinous synapses, and that the mild toxicity is related to the functional inactivation of astrocytic EAAC1.

Notes

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