Anat Embryol (1997) 195:317–326

ORIGINAL ARTICLE

R. Torp · F. Hoover · N.C. Danbolt
J. Storm-Mathisen · O.P. Ottersen

Differential distribution of the glutamate transporters GLT1 and rEAAC1 in rat cerebral cortex and thalamus: an in situ hybridization analysis

Accepted: 27 September 1996

Abstract The distributions in rat cerebral cortex and thalamus of the mRNAs encoding the glutamate transporters GLT1 and rEAAC1 (a rat homologue of rabbit EAAC1) were investigated by nonautoradiographic in situ hybridization using digoxigenin-labelled riboprobes. The probe recognizing rEAAC1 mRNA labelled exclusively neurons while GLT1 mRNA was found in glia as well as in select neuronal populations. The neurons containing the GLT1 transcript exhibited a distribution that was different from, and at some sites complementary to, the distribution of neurons containing rEAAC1 mRNA. In the subiculum, neurons positive for GLT1 and rEAAC1 were found in the deep and superficial part of the cell layer, respectively, while in the parietal neocortex GLT1 predominated in layer VI and rEAAC1 in layer V. Very few neuronal populations, most notably cells in hippocampal subfields CA3 and CA4, and in layer II in the entorhinal cortex, appeared to be equipped with both transcripts. In the thalamus the GLT1 labelling predominated in the midline and intralaminar nuclei while rEAAC1 labelling was found throughout this structure. It was concluded that the cerebral cortex and thalamus show cellular, laminar, as well as regional heterogeneities in the expression of the two glutamate transporters.

Key words Non-autoradiographic in situ hybridization · Riboprobes · GAD 65 · Glia · Glutamate transporters

Introduction

Glutamate is thought to play a central role in cortical neurotransmission. It is a likely transmitter candidate in corticofugal, commissural, and associational projections, and has also been proposed as a transmitter in certain corticopetal fiber systems including those of thalamic origin (Fonnum 1984; Ottersen et al. 1995). Studies have suggested that a perturbation of glutamatergic neurotransmission is implicated in a series of psychiatric and neurological disorders including psychosis and epilepsy (Carlsson and Carlsson 1990; During and Spencer 1993; Riederer et al. 1993; Rothstein et al. 1995). This calls for a better understanding of how synaptically released glutamate is cleared from the extracellular space of the cerebral cortex.

Several membrane proteins with the properties expected of high affinity glutamate transporters have been cloned (review: Danbolt 1994), and three prototypical transporters have been identified in the rat. These are GLT1 (Pines et al. 1992), GLAST (Storck et al. 1992), and rEAAC1 (Bjørås et al. 1996; also see Kanai et al. 1995; Velaz-Faircloth et al. 1996). The latter is a rat homologue of EAAC1 originally cloned in rabbit (Kanai and Hediger 1992). Two of these transporters (GLT1 and rEAAC1) appear to be strongly expressed in the cerebral cortex and other parts of the forebrain, while GLAST is particularly enriched in the cerebellum (Rothstein et al. 1994; Torp et al. 1994; Kanai et al. 1995; Lehre et al. 1995; Schmitt et al. 1996).

Although several studies have examined the distribution of the different glutamate transporters and their respective mRNAs in the rat brain (for references, see above), none has addressed specifically the localization of glutamate transporters in the cerebral cortex. The allocortex, in particular, has received little attention. The aim of the present study was to resolve whether GLT1 and rEAAC1 exhibited distinct regional and laminar distributions in the cerebral cortex and thalamus, as would be expected if different fibre systems were associated with different glutamate transporters. The in situ hybridization procedure was based on digoxigenin-labelled RNA probes. This procedure, which was also used in a recent study by Schmitt et al. (1996), provides a better resolution than the autoradiographic techniques employed in most of the previous in situ hybridization analyses of the rat brain.

R. Torp (✉) · F. Hoover · N.C. Danbolt · J. Storm-Mathisen
O.P. Ottersen
Department of Anatomy, Institute of Basic Medical Sciences, University of Oslo, P.O. Box 1105, Blindern, N-0317 Oslo, Norway
Tel.: +47–2285–1270; Fax: +47–2285–1299;
e-mail: torp@basalmed.uio.no

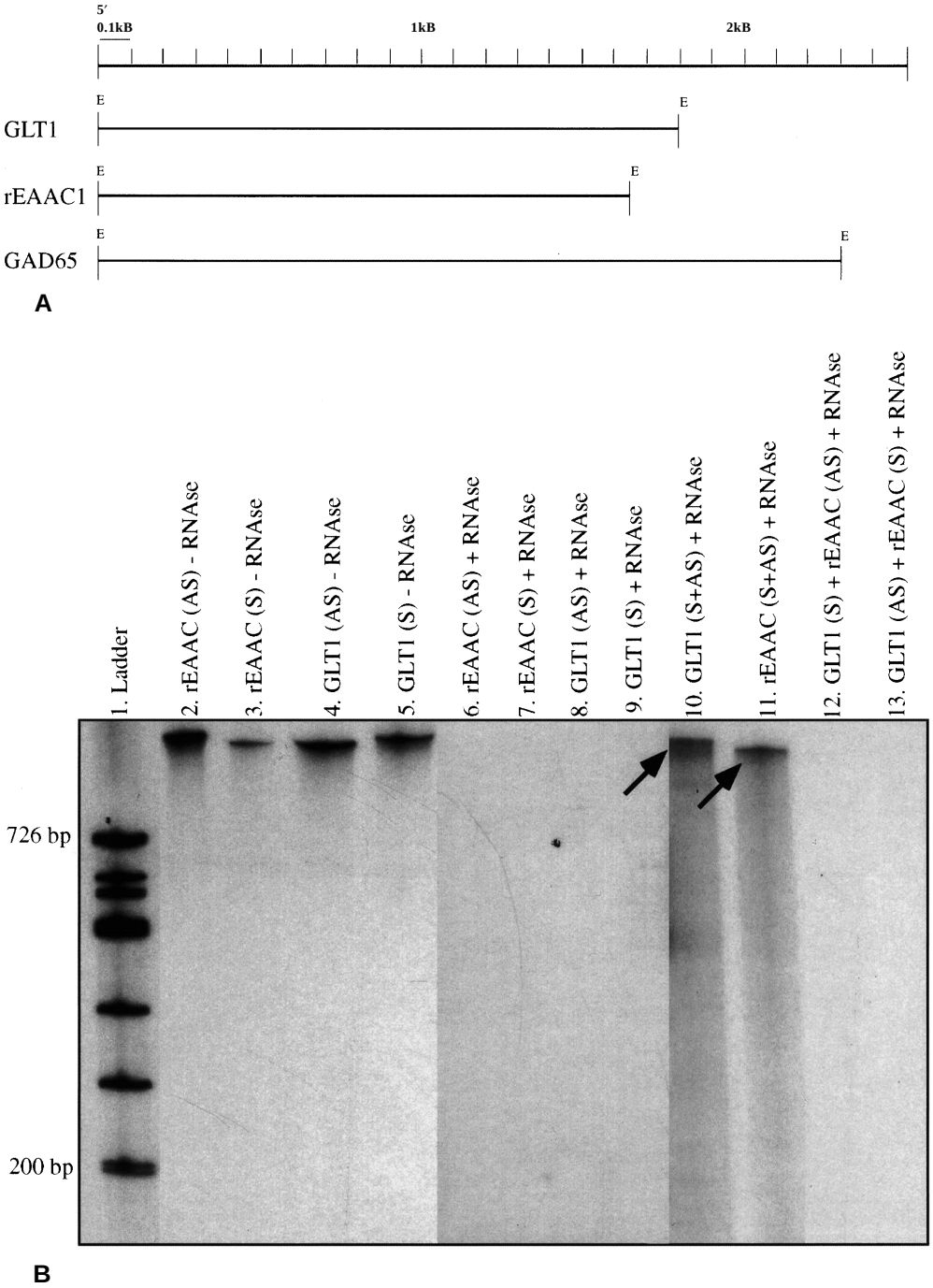

Fig. 1 **A** Line diagram of cRNAs probes used for in situ hybridizations and RNAse protection assays. The horizontal lines represent the cDNA portions of the recombinant plasmids. All inserts were cloned into the EcoR I site of the pBluescript vector. **B** RNase protection experiments demonstrate that the GLT1 and rEAAC1 probes do not cross-hybridize. Full-length radiolabelled sense (S) or antisense (AS) RNA probes encoding rat GLT1 and rEAAC1 were hybridized alone (*lanes 2–9*), with RNA from either complementary RNA (*lane 10* GLT1, *lane 11* rEAAC1) or with heterologous RNA (*lane 12* GLT1 (sense)+rEAAC1 (antisense), *lane 13* GLT1 (antisense)+rEAAC1 (sense). Following hybridization, the samples were digested with RNase One (except those labelled - RNase) and the remaining duplexes were fractionated in a denaturing acrylamide gel. The dried gel was exposed to X-ray film. *Arrows* point to the protected regions of the complementary probes (*lanes 10, 11*). Note that there was no region of the GLT1 and rEAAC1 probe that was resistant to RNAse digestion when the two probes were hybridized to one another. The radioactive ladder used as a standard (*lane 1*) is a Hinf I digest of PhiX174 DNA (Promega), and was generated by end-labelling with ^{32}P-dATP

Abbreviations for figures *APT* anterior pretectal nucleus, *AV* anteroventral thalamic nucleus, *CA1*, *CA3*, *CA4* hippocampal subfields, *CG* central gray, *CI* inferior colliculus, *CP* caudate putamen, *CS* superior colliculus, *DG* dentate gyrus, *DpG* deep gray layer of the superior colliculus, *FR* fasciculus retroflexus, *GP* globus pallidus, *HI* hilar region of the hippocampus, *InG* intermediate gray layer of the superior colliculus, *LEA* lateral entorhinal area, *LG* lateral geniculate nucleus, *LS* lateral septal nucleus, *M* molecular layer of the fascia dentata. *MD* mediodorsal thalamic nucleus, *MEA* medial entorhinal area, *MG* medial geniculate nucleus, *O* stratum oriens of the hippocampus, *P* pyramidal cell layer of the hippocampus, *PAS* parasubiculum, *PC* posterior commissure, *PO* posterior thalamic nuclear group, *PRH* perirhinal cortex, *PS* presubiculum, *PT* parataenial nucleus of the thalamus, *R* stratum radiatum of the hippocampus, *RS* retrosplenial cortex, *RT* reticular thalamic nucleus, *S* subiculum, *SG* suprageniculate nucleus, *SuG* superficial gray layer of the superior colliculus, *VL* ventrolateral thalamic nucleus, *VPM* ventral posteromedial thalamic nucleus

Materials and methods

Plasmids and probe synthesis

The GLT1 and rEAAC1 cDNA (sequences according to Pines et al. 1992 [with correction; Kanner 1993]; Bjørås et al. 1996) were contained in the EcoR I site of the pBluescript vector and linearized with Nae I (Fig. 1A). The GAD 65 cDNA (kindly provided by Dr. Allan J. Tobin, UCLA, USA, and characterized by Esclapez et al. 1993) was linearized with Xba I (Fig. 1A). To synthesize cRNA probes for in situ hybridization assays, run-off transcripts were generated in the presence of digoxigenin-labelled UTP using the T3/T7 RNA polymerase and purified as described by the manufacturer (Genius non-radioactive RNA-labelling kit; Boehringer Mannheim).

To obtain cRNA probes for the RNAse protection assay, linearized GLT1 and rEAAC1 DNA templates were transcribed in a standard mixture containing 2.5 mM ribonucleotides (rATP, rCTP, rGTP, rUTP) and 165 nM ^{32}P UTP using either the T7 (sense) or T3 (antisense) RNA polymerases. The DNA template was removed with the addition of 1 U/ml RQ1 DNAse I (Promega) for 15 min at 37°C. The RNA was extracted with phenol:chloroform:isoamyl alcohol (25:24:1) and precipitated in ethanol at –70°C until use.

RNAse protection assay

RNAse protection assays were performed using an RNase One protocol (Promega). In vitro transcribed RNAs (100 kcpm/µl) encoding GLT1 and/or rEAAC1 were mixed with hybridization buffer (0.2 M PIPES pH 6.5, 50% formamide, 3.7 M NaCl, 5.0 mM EDTA) and hybridized for 15 h at 55°C. Following hybridization, some hybrids were treated with 30 U/ml of RNase One (Promega) in digestion buffer (10 mM TRIS-HCl, pH 7.5, 5 mM EDTA, 200 mM sodium acetate) for 60 min at 37°C. Those hybrids surviving digestion were ethanol precipitated and fractionated in a 6% denaturing polyacrylamide, 8 M urea gel in Tris boric acid EDTA (TBE) buffer. The gel was transferred to filter paper, dried, and exposed to X-ray film at –70°C until the desired signal to noise ratio was obtained. The resulting autoradiogram was then scanned into a Macintosh computer and printed on a Tektronix 300 dpi dye sublimation printer.

Northern blot analysis

Northern blot containing Poly (A)+ RNA isolated from different rat tissues was obtained from Clontech Laboratories. The rEAAC1 cDNA inserted in the pBluescript vector was linearised with Nae1 and radiolabelled with (α-^{32}P)-dCTP (50 µCi/probe) utilising the Megaprime DNA labelling system (Amersham). The blot was hybridized for 20 h at 42°C (50% formamide) and the filter was washed following the manufacturer's instructions (Clontech). As a control, the blot was rehybridized with the human β-actin cDNA provided by the manufacturer.

Tissue preparation

Adult male Wistar rats (300–400 g) were deeply anaesthetised with pentobarbital and decapitated. At least ten different rats were used for each observation. The brains were removed as quickly as possible and frozen on aluminium foil placed on powdered dry ice. Horizontal and sagittal sections were cut at 12 µm on a cryostat and thaw-mounted onto coated slides. They were postfixed in 4% paraformaldehyde for 15 min and kept in ethanol at 4°C until required.

In situ hybridization

In situ hybridization was carried out using a modified version of the protocol by Hoover and Goldman (1992). In brief, postfixed sections were rehydrated through graded alcohols, rinsed in 2X SSC and pretreated by proteinase K (10 µg/ml) in 0.1 M TRIS-HCl buffer (pH 7.2) containing 0.05 M EDTA for 15 min at 37°C. The sections were acetylated with 0.25% acetic anhydride in 0.1 M triethanolamine (pH 8.0) for 10 min at room temperature, and incubated in 150 µl of hybridization solution (10 mM TRIS-HCl pH 7.4, 50% formamide, 0.3 M NaCl, 1 mM EDTA, 10% dextran sulphate, and 1% blocking solution) containing 10–15 ng digoxigenin-labelled RNA probe (full length). The slides were covered with Parafilm and placed in a humid chamber for 16–18 h at 55°C. Following hybridization, the sections were rinsed in 2X SSC at room temperature for 45 min and immersed in 2X SSC (pH 7.4) containing 50% formamide at 55°C for 30 min. To remove the formamide the sections were treated with 2X SSC for 2×10 min at room temperature. Any remaining unhybridized RNA was removed from the sections using ribonuclease A (50 µg/ml) in 10 mM TRIS-HCl (pH 7.5), 0.5 M NaCl and 1 mM EDTA. The sections were then rinsed in the same solution without ribonuclease A for 30 min at 60°C. After these washes, sections were processed for immunodetection using the nucleic acid detection kit (Boehringer, Mannheim) based on alkaline phosphatase labelled antibodies to digoxigenin. As controls, sense riboprobes identical to the mRNAs of rat GLT1, rEAAC1 and GAD 65 were used. The sense riboprobes gave no labelling of the tissue sections (inset, Fig. 6B). The sections were analysed in a Leica RBE microscope. The terminology of brain structures followed that of Paxinos and Watson (1986).

Results

Probe specificity

The RNAse protection assay was used to determine whether the GLT1 and rEAAC1 probes possessed detectable stretches of sequence homology allowing the formation of stable hybrids that would be resistant to RNase digestion. Full-length radiolabelled sense or antisense RNA probes encoding the rat GLT1 and rEAAC1 genes were hybridized alone, with complementary RNA, or to heterologous RNA (Fig. 1B). In no case did we detect any RNAse-resistant hybrids resulting from the cross-hybridization of the GLT1 and rEAAC1 probes.

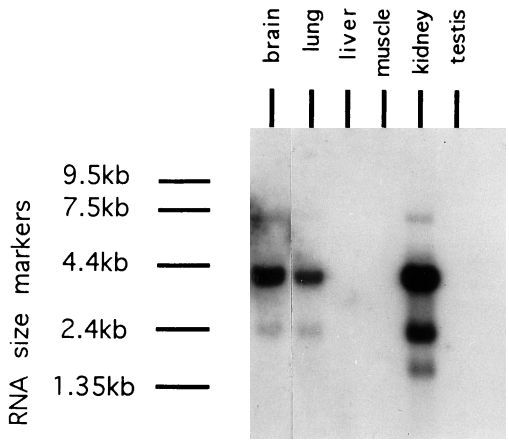

Fig. 2 Northern blot analysis using a rEAAC1 cDNA probe. A strong rEAAC1 signal of approximately 4.2 kb in size and minor signals of 2.4 and 7.0 kb were observed in the brain

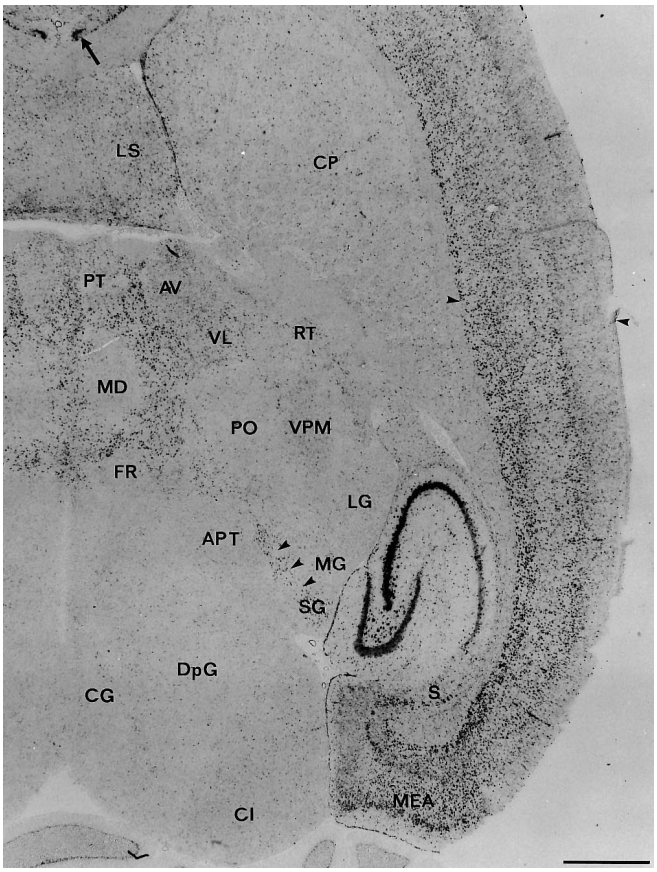

Fig. 3 Horizontal section of rat brain labelled with an RNA antisense probe to GLT1 mRNA (*arrow* indusium griseum, *arrowheads in cortex* indicate approximate border between parietal and temporal cortical areas, *arrowheads in thalamus* indicate suprageniculate nucleus). *Bar* 1 mm

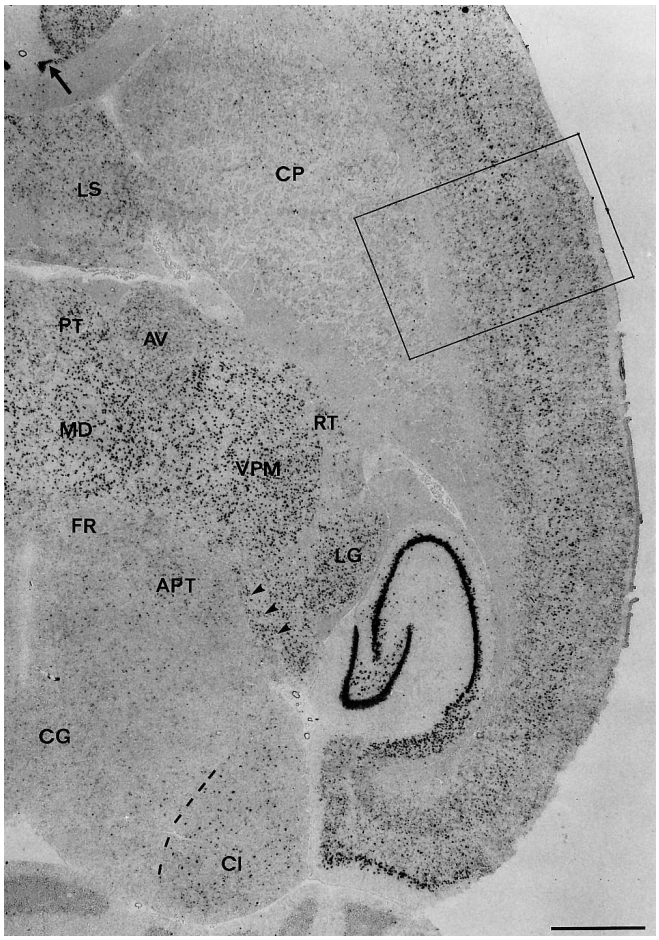

Fig. 4 Neighbouring section to that in Fig. 3, labelled with an RNA antisense probe to rEAAC1 (*broken line* indicates border of the inferior colliculus, *frame* shows area enlarged in Fig. 6B). *Arrow*, indusium griseum. *Bar* 1 mm

Northern blots

Northern blot analysis revealed a strong rEAAC1 signal at about 4.0 kb and minor signals at 2.4 and 7.0 kb in brain (Fig. 2). The blots showed similar bands in the kidney (plus an additional band at about 1.7 kb) and signals at 4.2 and 2.4 kb in the lung. No specific hybridization was observed in other rat tissues including heart, spleen, liver, muscle or testis. Rehybridizing with the human β-actin cDNA gave a prominent band at approximately 2.0 kb in all tissues (not shown).

mRNAs encoding GLT1 and rEAAC1: regional and laminar distributions

Closely spaced sections incubated with probes to GLT1-mRNA, rEAAC1-mRNA, and GAD 65-mRNA (Figs. 3–5) allowed direct comparison of the distributions of the three mRNA species. The distribution of the latter message is well-known (Esclapez et al. 1993). It served as a guide for the definition of nuclear borders (Fig. 5) and as a positive control.

The GLT1 message was particularly abundant in the hippocampus and neocortex, in agreement with previous data obtained by autoradiographic hybridization techniques (Torp et al. 1994). Compared with the latter techniques, the digoxigenin-based procedure used here provided a better discrimination between the individual cortical layers and cell types [also see Schmitt et al. (1996), who used a similar procedure].

Neighbouring sections revealed pronounced differences in the laminar distribution of the two glutamate transporter mRNAs. The GLT1 signal was most conspicuous in layer VI of the neocortex (Figs. 3, 6A). The labelling was strong throughout this layer in temporal neocortical areas but predominated in the deep part of this layer in the parietal cortex (rostral to arrowheads in Fig. 3). In contrast, the rEAAC1 message was concentrated in layer V and in scattered cells in layer III and IV and superficial part of layer VI (Figs. 4, 6B, 9A).

A particularly differentiated labelling pattern was found in the entorhinal area (Figs. 7, 8). As in the parietal neocortex there was a distinct band of GLT1-labelled cells in the deep part of layer VI; this extended into the

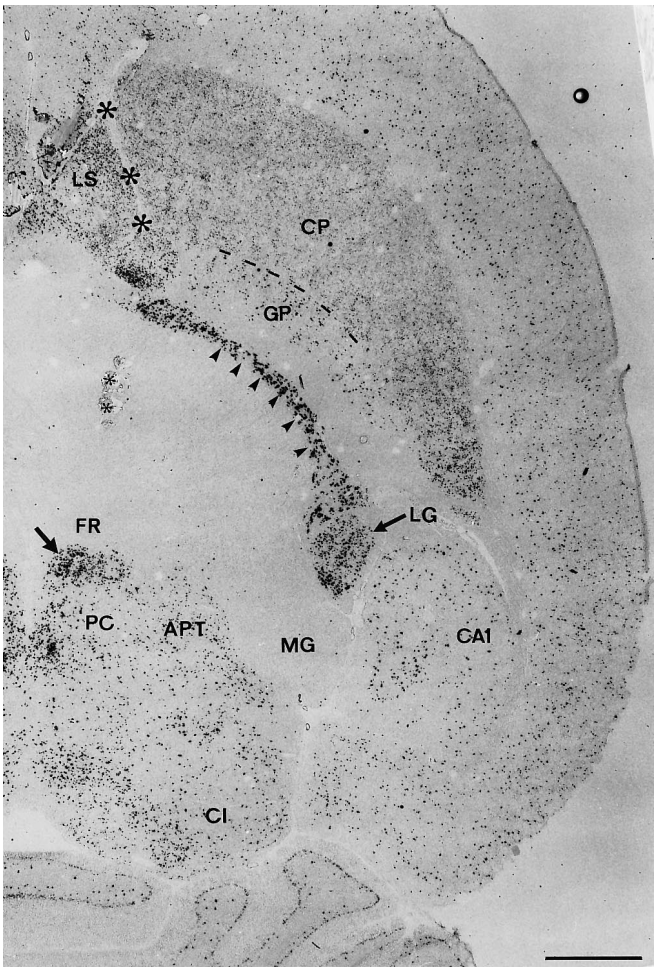

Fig. 5 Section close to those in Figs. 3 and 4, labelled with an RNA antisense probe to GAD 65 (*left arrow* precommissural nucleus, *large asterisks* lateral ventricle, *small asterisks* cutting artefact, *arrowheads* reticular thalamic nucleus). *Bar* 1 mm

deep part of the subiculum (Fig. 7A). Relatively strong labelling also occurred in layers II and III. The rEAAC1 message, in contrast, was clearly most prevalent in layer II (the labelling in this layer was less pronounced in the lateral than in the medial entorhinal area) and in scattered cells in layer III, as well as in the superficial part of the subiculum (Fig. 7B). Thus the latter area showed a complementary distribution of the two mRNA species. The para- and presubiculum contained scattered cells with high levels of mRNA for rEAAC1 (Figs. 7B, 9B) but were relatively weakly labelled with the probes to GLT1 mRNA, except for distinct bands of cells in layer IV and in the deep part of layer VI (Fig. 7A). The latter cells were in register with labelled cells in the medial entorhinal area and subiculum (see above).

The labelling pattern in the hippocampus will be described in the next section (“Cellular distribution”).

At low magnification the labelling for GLT1 appeared relatively weak in most subcortical areas (Fig. 3). The thalamic lateral geniculate and reticular nuclei, which could easily be delineated by reference to the adjacent section incubated with probes to GAD 65 mRNA (Fig. 5), were particularly weakly labelled. The same was true for the posterior complex and the mediodorsal nucleus of the thalamus, the pretectal area, the periaqueductal grey substance, and the superior and inferior colliculi.

The rostral border of the pretectal area was distinct in sections labelled by probes to GAD 65 mRNA (Fig. 5). A large number of cells anterior to this border displayed an intermediate staining intensity for GLT1 mRNA (Fig. 3). The areas of labelling, which outlined the mediodorsal and parataenial nuclei of the thalamus and bordered on the medial aspect of the posterior complex and the anteroventral and ventrolateral nuclei, corresponded to the midline and intralaminar nuclei. More specifically, a careful examination of horizontal as well as sagittal sections revealed numerous positive cells in the anterior and posterior paraventricular nuclei, the centrolateral and central medial nuclei, and the intermediodorsal nucleus (compare Fig. 3 with Plate 103 in Paxinos and Watson 1986). Some labelling also occurred more caudally, in the suprageniculate nucleus (arrowheads, Fig. 3).

The rEAAC1 labelling in the subcortical areas dealt with above was more prominent than the GLT1 labelling and showed a strikingly different distribution (Fig. 4). Cells with staining intensities similar to those of layer V of the neocortex filled most of the thalamus, including the intralaminar nuclei and the medial and lateral geniculate bodies. The reticular, anteroventral and part of the midline nuclei (medial to PT in Fig. 4) were relatively weakly stained.

mRNAs encoding GLT1 and rEAAC1: cellular distribution

The cellular distribution could be examined most conveniently in the hippocampus, where the different cell types are easily identified due to their spatial segregation. In the hippocampus rEAAC1 was found exclusively in neurons (Figs. 7B, 8C, D). Pyramidal and granule cells were strongly labelled, as were subpopulations of neurons in the deep hilar region. Scattered weakly stained cells occurred in the dendritic layers. GLT1, in contrast, was present in glial cells throughout the dendritic and cellular layers (Figs. 7A, 8A, B). The difference in the cellular distribution of the two transporters is particularly evident in Fig. 7, which shows the dentate molecular layer containing a high density of cells positive for GLT1 mRNA, but no cells positive for rEAAC1 mRNA.

The GLT1 mRNA was not restricted to glial cells. Confirming the results obtained with radiolabelled oligonucleotide probes (Torp et al. 1994) there was a GLT1 signal in CA3 pyramidal cells and in a subpopulation of deep hilar neurons. The latter population of cells showed a distribution similar to that of the rEAAC1 positive neurons. The dentate granule cells, and the CA1 pyramidal cells in particular (Fig. 8B), showed weak GLT1 label-

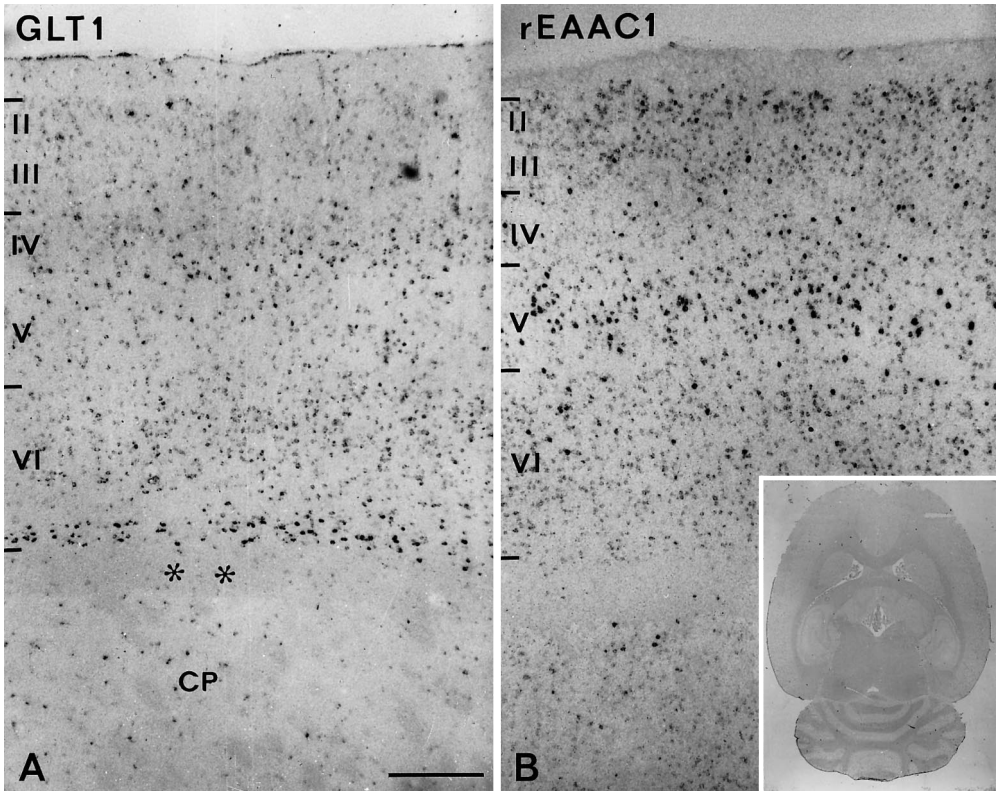

Fig. 6 Distribution of GLT1 mRNA (**A**) and rEAAC1 mRNA (**B**) in the parietal cortex (area 2). Horizontal sections. Area shown in **B** is indicated in Fig. 4. The micrograph in **A** is from a corresponding part of the cortex (neighbouring section to that in Fig. 3, but less intensely stained). *Asterisk* external capsule. Cortical layers are indicated. *Inset in* **B** horizontal section incubated with a sense probe to rEAAC1 (same concentration as antisense probe). Note complete absence of labelling. *Bar* 0.3 mm

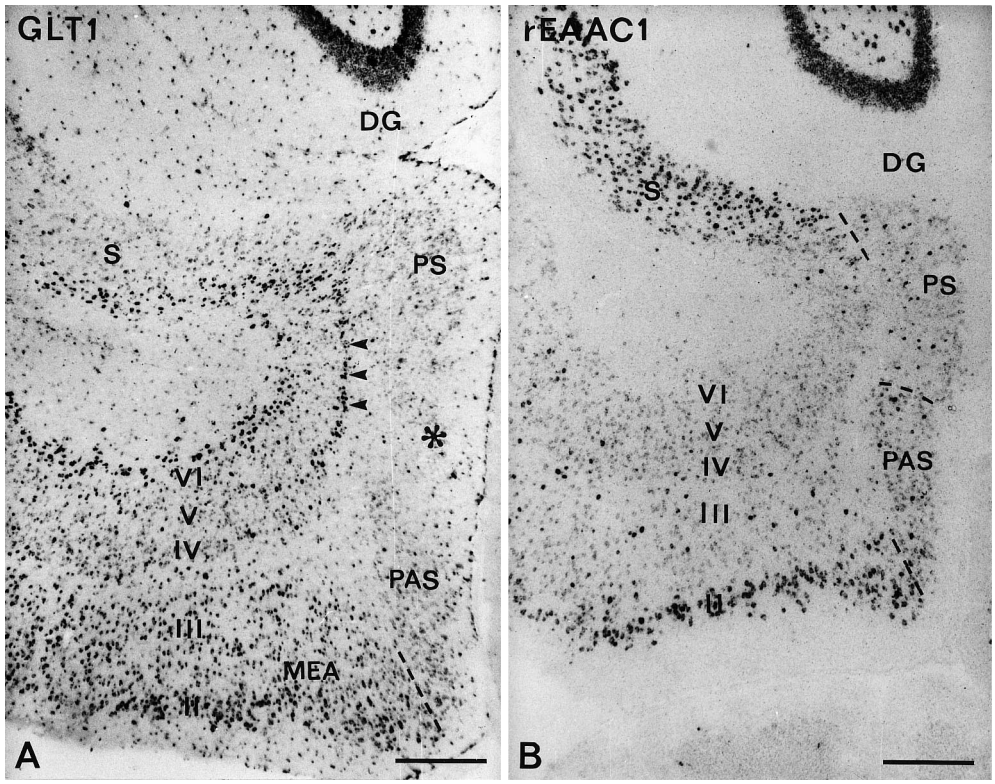

Fig. 7 Distribution of GLT1 mRNA (**A**) and rEAAC1 mRNA (**B**) in the parahippocampal region. Horizontal sections. Cortical layers are indicated (*broken lines* show approximate borders between cortical subdivisions, *asterisk* area retrosplenialis (Haug 1976), *arrowheads* band of labelled cells in layer IV). *Bar* 0.3 mm

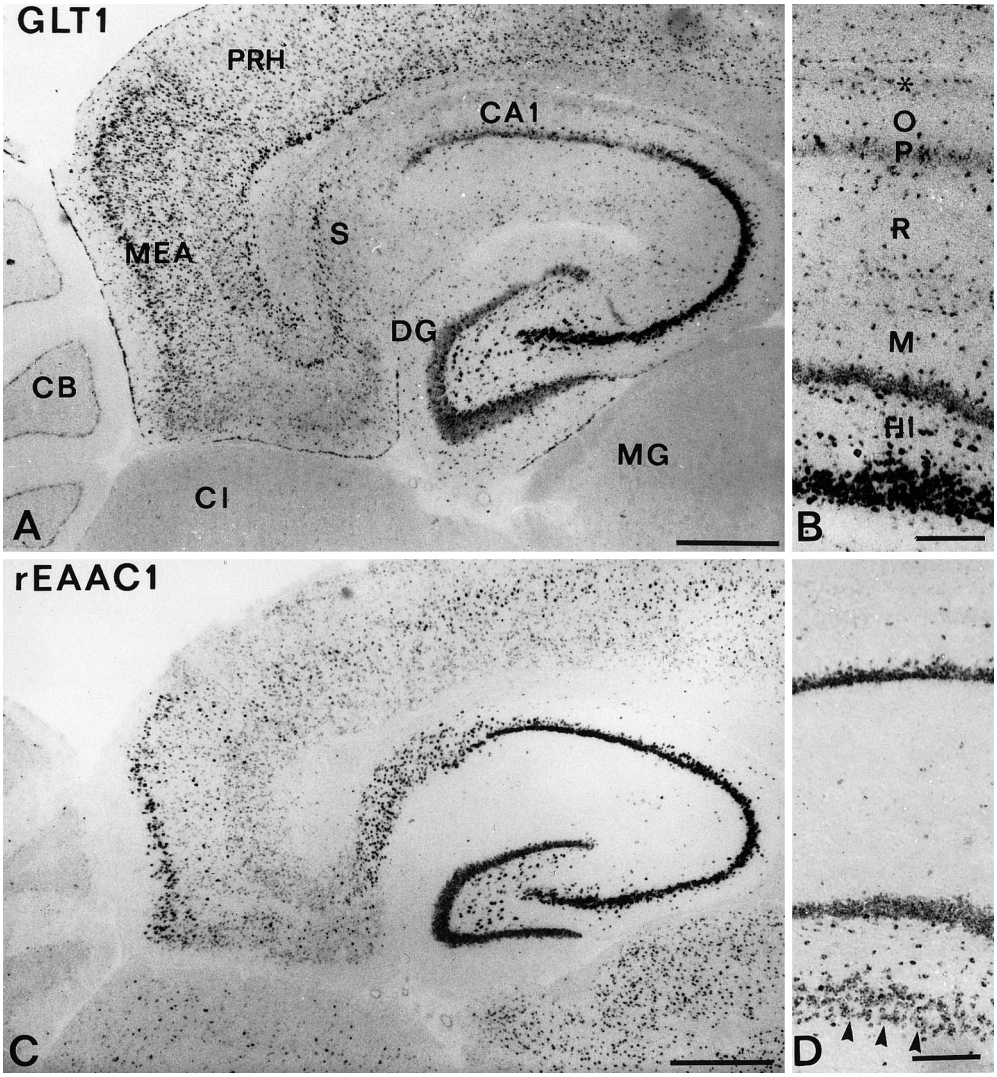

Fig. 8 Distribution of GLT mRNA (**A, B**) and rEAAC1 mRNA (**C, D**) in the hippocampus and parahippocampal region. Horizontal sections. Part of section in **C** is shown at larger magnification in Fig. 7B. **B, D** are from neighbouring sections to **A, C** and show an enlarged view of the CA1 and fascia dentata (*asterisk* alveus, *arrowheads* CA3 pyramidal cells). *Bars* **A, C** 0.6 mm; **B, D** 0.2 mm

ling but the respective layers stood out at low magnification due to their high cell density and the abundant glial labelling associated with them (Figs. 3, 8A).

The paleo- and neocortex resembled the hippocampus inasmuch as GLT1 mRNA occurred in both glia and neurons. The glial labelling was particularly evident in layer I (Figs. 6A, 7A), which like the dentate molecular layer was devoid of cells positive for rEAAC1. GLT1 mRNA-containing cells of the same size as those in layer I pervaded the remaining layers of the cortex as well as the underlying white matter (Fig. 7A). However, superimposed on this glial labelling were positive cells that according to size and shape could be identified as neurons. Neuronal labelling was most pronounced in layer II of the medial entorhinal cortex (Fig. 7A) and in the deep part of layer VI of several neocortical and allocortical areas (Figs. 6A, 7A). As mentioned above, this band of cells extended into the subiculum to complement the neuronal labelling produced by the probe to rEAAC1 (Fig. 7).

Discussion

Methodological comments

The probes used here were transcribed from cDNAs that when expressed in HeLa cells were found to encode transporters with substrate selectivities and ion dependencies typical of high affinity glutamate uptake in the brain (Pines et al. 1992; Bjørås et al. 1996). Radiolabelled cDNA probes to GLT1 (Pines et al. 1992) and rEAAC1 (present report) hybridize selectively to different bands in Northern blots (major band at approximately 11 kb and 4.0 kb, respectively). Further, the RNase protection assay showed that sense GLT1 mRNA (transcribed in vitro) did not protect the rEAAC1 probe from degradation, and vice versa. Taken together these results suggest that rEAAC1 and GLT1 do not form crosshybridization products that survive the RNAse treatment following the in situ hybridization, and that the probes selectively signal the presence of the respective mRNAs. This was borne out by the in situ hybridization experi-

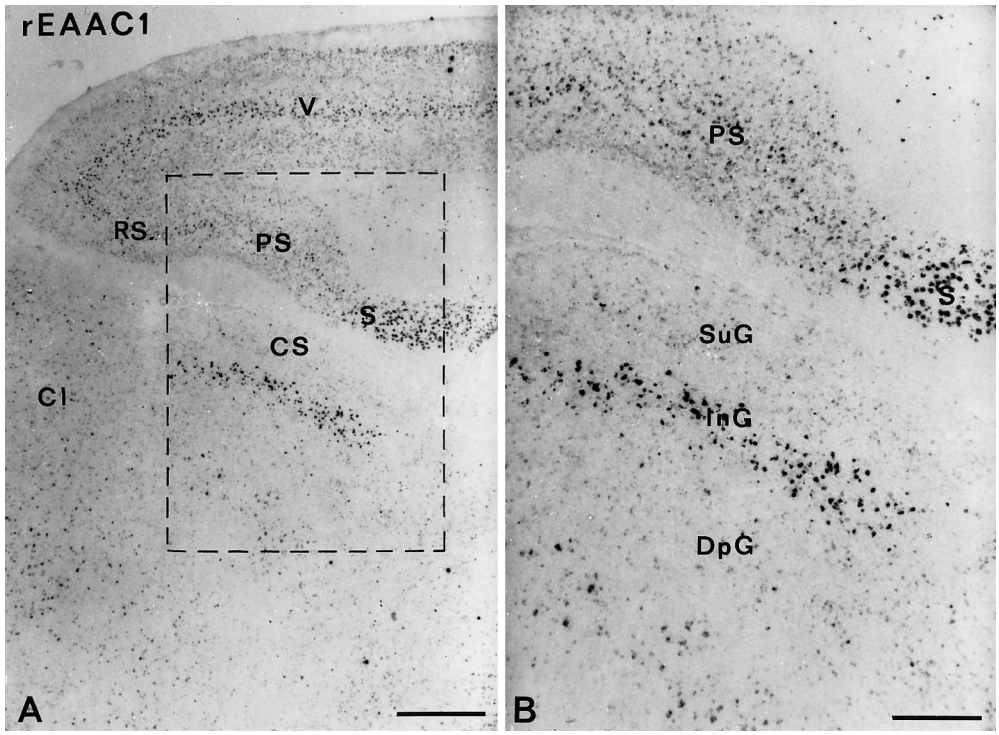

Fig. 9 Sagittal section showing the distribution of rEAAC1 mRNA in the dorsal mesencephalon and posterior cortical areas; *Frame* indicates area enlarged in **B**. Note strong labelling in the intermediate gray layer (*InG*) of the superior colliculus (*V* cortical layer V). *Bars* **A** 0.6 mm; **B** 0.3 mm

ments, which showed that the two probes yielded distinct expression patterns.

Differential distribution of GLT1 mRNA and rEAAC1 mRNA

This is the first comparative in situ hybridization analysis of the distributions of GLT1 and rEAAC1, the predominant glutamate transporters in the forebrain (Rothstein et al. 1996). Based on analysis of neighbouring sections it could be shown that the two transporters are differentially expressed in the cortex and thalamus, at the cellular, laminar, as well as regional level. The expression of rEAAC1 is similar to that of its rabbit homologue EAAC1 (Kanai and Hediger 1992) in that it is restricted to neurons. In contrast, GLT1 mRNA is found in glia as well as in neurons, with a glial predominance in most areas (see Schmitt et al. 1996, for a recent double-labelling study). In areas showing a neuronal expression of both rEAAC1 and GLT1 the two transcripts occur in the same cell populations, as is the case in the CA3 of the hippocampus, or in separate populations, as is the case in the subiculum, where the labelling patterns display a striking complementarity.

The present data thus confirm and extend previous findings (Torp et al. 1994; Schmitt et al. 1996) that mRNA encoding the “glial” transporter GLT1 is expressed in certain neuronal populations. This is in contrast to the GLT1 protein, which according to pre-embedding immunocytochemical analyses is enriched exclusively in glial cells (Rothstein et al. 1994; Lehre et al. 1995). The possibility remains that the level of the GLT1 protein that is expressed in neurons is below the detection limit of standard immunocytochemical techniques. Support for this notion was derived from a recent quantitative analysis of postembedding immunogold-labelling for GLT1, which revealed that the spine synapses in the stratum radiatum of CA1 (most of which are established by axons of CA3 pyramidal cells) were associated with particle counts that were slightly higher than background levels (Chaudhry et al. 1995). If this reflects the presence of a small pool of GLT1 protein in CA3 axon terminals it implies that the discrepancy alluded to above relates to quantity rather than quality. At any rate the discordant observations suggest that there may be regulatory mechanisms at play that control the posttranscriptional processing of GLT1.

Kanai et al. (1995) and Velaz-Faircloth et al. (1996) recently published in situ hybridization data based on radiolabelled riboprobes directed to rat homologues of EAAC1 with sequences very similar to that of rEAAC1 of Bjørås et al. (1996). The published sequences differed from that of rEAAC1 by three amino acids or a single amino acid, respectively. The autoradiograms showed labelling patterns that were consistent with the more detailed pattern obtained by the present non-autoradiographic procedure.

Obviously the present data on rEAAC1 mRNA do not allow conclusions as to the subcellular compartmentation of this transporter. However, correlations can be made with the immunocytochemical data of Rothstein et al. (1994) that were obtained in the rat with an antiserum directed to a C-terminal peptide of rabbit EAAC1. An identical peptide sequence is found in rEAAC1 (Bjørås et al. 1996). The immunocytochemical analysis suggest-

ed that EAAC1 was mainly expressed postsynaptically. That postsynaptic glutamate uptake is of biological importance is in line with recent observations of Takahashi et al. (1995a), who showed that inhibition of postsynaptic uptake slows the decay of the AMPA receptor-mediated synaptic current in the climbing fibre synapses within the cerebellum. Similar studies have not been performed in the cerebral cortex.

However, there is unequivocal evidence for the existence also of presynaptic uptake sites. The ability of putative glutamatergic terminals to accumulate glutamate or the metabolically inert glutamate analogue D-aspartate has been demonstrated in the hippocampus by autoradiography (Storm-Mathisen and Iversen 1979; Taxt and Storm-Mathisen 1984) and, at higher anatomical resolution, by immunogold cytochemistry of exogenous D-aspartate (Gundersen et al. 1993). The retrograde axonal transport of radiolabelled D-aspartate in putative glutamatergic neurons constitutes additional evidence for presynaptic uptake in hippocampus (Fischer et al. 1986) and other brain regions (Streit 1980). The available data can be reconciled by postulating the existence of several neuronal glutamate transporters, some still unknown, with distinct distributions along the cell membrane. One must also consider the possibility that preembedding immunocytochemistry may fail to reveal transporter molecules at the presynaptic membrane, due, e.g., to high protein concentrations and poor accessibility of antigens at this site. Postembedding immunogold cytochemistry is required to resolve the exact distribution of rEAAC1 along the neuronal membrane.

Many of the neuronal populations shown to express rEAAC1 mRNA in the present material are thought to use glutamate or a related excitatory amino acid as transmitter. This is true for the layer V pyramidal neurons in the neocortex and the sequentially arranged neuronal populations that funnel information through the hippocampal formation: layer II neurons of the entorhinal cortex, granule cells of the fascia dentata, CA3 and CA1 pyramidal cells of the hippocampus, and neurons of the subiculum (review: Ottersen et al. 1995). Glutamate has also been proposed as a transmitter in a number of cortical afferents from the thalamus (Ottersen et al. 1983; Tsumoto 1990; Johnson and Burkhalter 1992), which contained a high density of labelled cells in the present material. Although this pattern of distribution would be compatible with a presynaptic localization of the rEAAC1 protein it must be emphasized that all of these populations are themselves targets of glutamatergic projections. As such they could be equipped with postsynaptic uptake sites in line with the data of Rothstein et al. (1994) reviewed above.

It is premature to discuss the detailed patterns of EAAC1 and GLT1 distributions in a physiological or hodological context. Suffice it to conclude that the different fibre systems in the cortex seem to be associated with different types and levels of glutamate transporter. On a general note the existence of several different glutamate transporters with highly specific expression patterns suggests that the functional role of this family of molecules extends beyond that of maintaining a low concentration of glutamate in the extracellular space. Evidence is accumulating that glutamate transporters are involved in the control of synaptic excitation (Barbour et al. 1994; Mennerick and Zorumski 1994; Takahashi et al. 1995b; Tong and Jahr 1994) and that their activity is subject to regulation (Casado et al. 1993; Levy et al. 1995; Trotti et al. 1995). The information provided here on the detailed distribution of rEAAC1 and GLT1 may become useful in future attempts to manipulate cortical neurotransmission by specific drugs, and will serve as a platform for studies of factors that regulate the expression of these transporters at the transcriptional level.

Acknowledgements We thank Dr. Allan J. Tobin at the Brain Research and Molecular Biology Institutes, Los Angeles, California for providing the GAD 65 clones. Thanks are also due to Jorunn Knutsen for her expert technical assistance. This work was supported by the Norwegian Research Council and by the EU BIOMED2 grant no. BMH4-CT960851.

References

Barbour B, Keller BU, Llano I, Marty A (1994) Prolonged presence of glutamate during excitatory synaptic transmission to cerebellar Purkinje cells. Neuron 12: 1331–1343

Bjørås M, Gjesdal O, Erickson JD, Torp R, Levy LM, Ottersen OP, Degree M, Storm-Mathisen J, Seeberg E, Danbolt NC (1996) Cloning and expression of neuronal rat brain glutamate transporter. Brain Res Mol Brain Res 36: 163–168

Carlsson M, Carlsson A (1990) Interactions between glutamatergic and monoaminergic systems within the basal ganglia – implications for schizophrenia and Parkinson's disease. Trends Neurosci 13: 272–276

Casado M, Bendahan A, Zafra F, Danbolt NC, Aragon C, Gimenez C, Kanner BI (1993) Phosphorylation and modulation of brain glutamate transporters by protein kinase C. J Biol Chem 268: 27313–27317

Chaudhry FA, Lehre KP, Lookeren Campagne M van, Ottersen OP, Danbolt NC Storm Mathisen J (1995) Glutamate transporters in glial plasma membranes: highly differentiated localizations revealed by quantitative ultrastructural immunocytochemistry. Neuron 15: 711–720

Danbolt NC (1994) The high affinity uptake system for excitatory amino acids in the brain. Prog Neurobiol 44: 377–396

During MJ, Spencer DD (1993) Extracellular hippocampal glutamate and spontaneous seizure in the conscious human brain. Lancet 341: 1607–10

Esclapez M, Tillakaratne NJ, Tobin AJ, Houser CR (1993) Comparative localization of mRNAs encoding two forms of glutamic acid decarboxylase with nonradioactive in situ hybridization methods. J Comp Neurol 331: 339–362

Fischer BO, Ottersen OP, Storm-Mathisen J (1986) Implantation of D-[^{3}H]aspartate loaded gel particles permits restricted uptake sites for transmitter-selective axonal transport. Exp Brain Res 63: 620–626

Fonnum F (1984) Glutamate: a neurotransmitter in mammalian brain. J Neurochem 42: 1–11

Gundersen V, Danbolt NC, Ottersen OP, Storm-Mathisen J (1993) Demonstration of glutamate/aspartate uptake activity in nerve endings by use of antibodies recognizing exogenous D-aspartate. Neuroscience 57: 97–111

Haug FM (1976) Sulphide silver pattern and cytoarchitectonics of parahippocampal areas in the rat. Special reference to the subdivision of area entorhinalis (area 28) and its demarcation from the pyriform cortex. Adv Anat Embryol Cell Biol 52: 3–73

Hoover F, Goldman D (1992) Temporally correlated expression of the nAChR genes during development of the mammalian retina. Exp Eye Res 54: 561–571

Johnson RR, Burkhalter A (1992) Evidence for excitatory amino acid neurotransmitters in the geniculo-cortical pathway and local projections within rat primary visual cortex. Exp Brain Res 89: 20–30

Kanai Y, Hediger A (1992) Primary structure and functional characterization of a high-affinity glutamate transporter. Nature 360: 467–471

Kanai Y, Bhide PG, Difiglia M Hediger MA (1995) Neuronal high-affinity glutamate transport in the rat central nervous system. Neuroreport 6: 2357–2362

Kanner BI (1993) Glutamate transporters from brain. A novel neurotransmitter transporter family. FEBS Lett 325:95–9

Lehre KP, Levy LM, Ottersen OP, Storm-Mathisen J, Danbolt NC (1995) Differential expression of two glial glutamate transporters in the rat brain: quantitative and immunocytochemical observations, J Neurosci 15: 1835–1853

Levy LM, Lehre KP, Walaas SI, Storm-Mathisen J, Danbolt NC (1995) Down-regulation of glial glutamate transporters after glutamatergic denervation in the rat brain. Eur J Neurosci 7: 2036–2041

Mennerick S, Zorumski CF (1994) Glial contributions to excitatory neurotransmission in cultured hippocampal cells. Nature 368: 59–62

Ottersen OP, Fischer BO, Storm-Mathisen J (1983) Retrograde transport of D-[^{3}H] aspartate in thalamocortical neurones. Neurosci Lett 42: 19–24

Ottersen OP, Hjelle OP, Osen KK Laake J (1995) Amino acid transmitters, In: Paxinos G (ed) The rat nervous system, 2nd edn. Academic Press, New York London pp 1017–1037

Paxinos G, Watson C (1986) The rat brain in stereotaxic coordinates. 2nd edn. Academic Press, San Diego

Pines G, Danbolt NC, Bjørås M, Zhang Y, Bendahan A, Eide L, Koepsell H, Storm-Mathisen J, Seeberg E, Kanner BI (1992) Cloning and expression of a rat brain L-glutamate transporter. Nature 360: 464–467

Riederer P, Lange KW, Youdim MB (1993) Recent advances in pharmacological therapy of Parkinson's disease. Adv Neurol 60: 626–35

Rothstein JD, Martin L, Levey AI, Dykes-Hoberg M, Jin L, Wu D, Nash N, Kuncl RW (1994) Localization of neuronal and glial glutamate transporters. Neuron 13: 713–725

Rothstein JD, Van Kammen M, Levey AI Martin LJ, Kuncl RW (1995) Selective loss of glial glutamate transporter GLT-1 in amyotrophic lateral sclerosis. Ann Neurol 38: 73–84

Rothstein JD, Dykes-Hoberg M, Pardo CA, Bristol LA, Jin L, Kuncl RW, Kanai Y, Hediger MA, Wang Y, Schielke JP, Welty DF (1996) Knockout of glutamate transporters reveals a major role for astroglial transport in excitotoxicity and clearance of glutamate. Neuron 16: 675–686

Schmitt A, Asan E, Püschel B, Jöns T, Kugler P (1996)Expression of the glutamate transporter GLT1 in neural cells of the rat central nervous system: non-radioactive in situ hybridization and comparative immunocytochemistry. Neuroscience 71: 989–1004

Storck T, Schulte S, Hofmann K, Stoffel W (1992) Structure, expression, and functional analysis of a Na^+-dependent glutamate/aspartate transporter from rat brain. Proc Natl Acad Sci USA 89: 10955–10959

Storm-Mathisen J, Iversen LL (1979) Uptake of [^{3}H]glutamic acid in excitatory nerve endings: light and electronmicroscopic observations in the hippocampal formation of the rat. Neuroscience 4: 1237–1253

Streit P (1980) Selective retrograde labeling indicating the transmitter of neuronal pathways. J Comp Neurol 191: 429–463

Takahashi M, Billups B, Sarantis M, Attwell D (1995a) EPSC Shaping, and glutamate release in ischaemia, by glutamate uptake carriers. Soc Neurosci 21: 730.11

Takahashi M, Kovalchuk Y Attwell D (1995b) Pre- and postsynaptic determinants of EPSC waveform at cerebellar climbing fiber and parallel fiber to Purkinje cell synapses. J Neurosci 15: 5693–5702

Taxt T, Storm-Mathisen J (1984) Uptake of D-aspartate and L-glutamate in excitatory axon terminals in hippocampus: autoradiographic and biochemical comparison with gamma-aminobutyrate and other amino acids in normal rats and in rats with lesions. Neuroscience 11: 79–100

Tong G, Jahr CE (1994) Block of glutamate transporters potentiates postsynaptic excitation. Neuron 13: 1195–1203

Torp R, Danbolt NC, Babaie E, Bjørås M, Seeberg E, Storm-Mathisen J, Ottersen OP (1994) Differential expression of two glial glutamate transporters in the rat brain: an in situ hybridization study. Eur J Neurosci 6: 936–942

Trotti D, Volterra A, Lehre KP, Rossi D, Gjesdal O, Racagni G, Danbolt NC (1995) Arachidonic acid inhibits a purified and reconstituted glutamate transporter directly from the water phase and not via the phospholipid membrane. J Biol Chem 270: 9890–9895

Tsumoto T (1990) Excitatory amino acid transmitters and their receptors in neural circuits of the cerebral neocortex. Neurosci Res 9: 79–102

Velaz-Faircloth M, McGraw TS, Malandro MS, Fremeau RT, Kilberg MS, Anderson KJ (1996) Characterization and distribution of the neuronal glutamate transporter EAAC1 in rat brain. Am J Physiol Cell Physiol 39: C67–C75