Expression of a Variant Form of the Glutamate Transporter GLT1 in Neuronal Cultures and in Neurons and Astrocytes in the Rat Brain

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To identify glutamate transporters expressed in forebrain neurons, we prepared a cDNA library from rat forebrain neuronal cultures, previously shown to transport glutamate with high affinity and capacity. Using this library, we cloned two forms, varying in the C terminus, of the glutamate transporter GLT1. This transporter was previously found to be localized exclusively in astrocytes in the normal mature brain. Specific antibodies against the C-terminal peptides were used to show that forebrain neurons in culture express both GLT1a and GLT1b proteins. The pharmacological properties of glutamate transport mediated by GLT1a and GLT1b expressed in COS-7 cells and in neuronal cultures were indistinguishable. Both GLT1a and GLT1b were upregulated in astrocyte cultures by exposure to dibutyryl cAMP. We next investigated the expression of GLT1b *in vivo*. Northern blot analysis of forebrain RNA revealed

two transcripts of ~3 and 11 kb that became more plentiful with developmental age. Immunoblot analysis showed high levels of expression in the cortex, hippocampus, striatum, thalamus, and midbrain. Pre-embedding electron microscopic immunocytochemistry with silver-enhanced immunogold detection was used to localize GLT1b *in vivo*. In the rat somatosensory cortex, GLT1b was clearly expressed in neurons in presynaptic terminals and dendritic shafts, as well as in astrocytes. The presence of GLT1b in neurons may offer a partial explanation for the observed uptake of glutamate by presynaptic terminals, for the preservation of input specificity at excitatory synapses, and may play a role in the pathophysiology of excitotoxicity.

Key words: glutamate; transport; dihydrokainate; presynaptic; astrocytes; synapse; excitotoxicity

Glutamate transport is the major if not the only mechanism other than diffusion for clearing the excitatory transmitter glutamate from the extracellular space (Nicholls and Attwell, 1990; Lipton and Rosenberg, 1994; Rusakov and Kullmann, 1998; Danbolt, 2001). In addition to being the primary excitatory transmitter, glutamate is also paradoxically neurotoxic and has been implicated in important acute and chronic diseases of the nervous system (Meldrum and Garthwaite, 1990; Lipton and Rosenberg, 1994). Glutamate transport has been shown to be important in protecting neurons from the excitotoxicity of exogenous glutamate (Mangano and Schwarcz, 1983; Rosenberg and Aizenman, 1989; Rosenberg et al., 1992; Robinson et al., 1993) as well as endogenous glutamate (Rothstein et al., 1996; Tanaka et al., 1997; Wang et al., 1998b). Five glutamate transporters have been cloned to date (Kanai and Hediger, 1992; Pines et al., 1992; Storck et al.,

1992; Fairman et al., 1995; Arriza et al., 1997). The major transporter of the forebrain is GLT1, and in GLT1 knock-out mice, <6% of glutamate transport activity remains in membrane preparations derived from cerebral cortex compared with wild-type animals (Tanaka et al., 1997).

In the mature brain, GLT1 protein has been found to be associated only with astrocytes (Rothstein et al., 1994; Lehre et al., 1995). A post-embedding immunogold study using an antibody directed against the whole GLT1 protein that reacts strongly with GLT1 amino acid residues 493–508 (“ α 73 kDa”) found a low level (6–9% of the expression in astrocytes) of regionally limited expression in neuronal membranes in certain synaptic sites, for example in axospinous synapses in the stratum radiatum and in the inner third of the stratum moleculare of area dentata (Chaudhry et al., 1995). However, later review of these data revealed that the apparent expression associated with neuronal membranes was not significantly above background levels (Danbolt, 2001). A discrepancy that has emerged is that although immunocytochemical studies have failed to detect GLT1 in neurons in the brain, *in situ* hybridization studies have shown that mRNA for GLT1 is widely expressed in neurons in the brain (Torp et al., 1995; Berger and Hediger, 1998).

Working with nearly pure cultures of neurons derived from embryonic rat forebrain, we found a high-affinity glutamate uptake system that was comparable in activity to that present in synaptosomes or in astrocytes in culture, but whose pharmacology was distinct from that of the neuronal transporter EAAC1 and most closely resembled that of the putatively astrocytic transporter GLT1 in being readily inhibited by dihydrokainate (Wang

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et al., 1998a). The goal of the present study was to determine the molecular basis of transport in forebrain neurons by screening a cDNA library prepared from neuronal cultures for transporters with homology to the known glutamate transporters.

Preliminary reports of this work have appeared (Chen et al., 1998, 1999, 2000).

MATERIALS AND METHODS

Tissue culture. Neuronal cultures were prepared from embryonic day 16 Sprague Dawley rat fetuses using methods similar to those previously described (Rosenberg, 1991) but with modifications to facilitate the production of nearly pure neuronal cultures (Wang et al., 1998a,b). Although these cultures are primarily derived from cerebral cortex, they also are derived from hippocampus and deep gray structures and are more accurately referred to as forebrain cultures. Cultures were initially plated on poly-L-lysine coated 24-well plastic plates (Costar, Cambridge, MA) using an 80:10:10 (v/v) mixture of DMEM (catalog #11960-010; Invitrogen, Grand Island, NY), Ham's F-12 (catalog #N-4888; Sigma, St. Louis, MO), and heat-inactivated iron-supplemented calf serum (catalog #A2151; HyClone, Logan, UT), containing 2 mM glutamine, 25 mM HEPES, 24 U/ml penicillin, and 24 μ g/ml streptomycin in a 5% CO₂ (balance air) incubator at 36°C. Cell proliferation was inhibited by exposure to 5 μ M cytosine arabinoside at 24 hr *in vitro* for 72 hr. On the fourth day of culture, the medium was completely removed and replaced with 90% MEM, 10% NuSerum IV (Collaborative Research, Bedford, MA), 2 mM glutamine, 5 mM HEPES, containing 10 μ g/ml superoxide dismutase (Roche Molecular Biochemicals, Indianapolis, IN) 1 μ g/ml catalase (Sigma CV-40), total glucose 11 mM, and total sodium bicarbonate 9.3 mM, plus 2% B27 supplement (Invitrogen 17504-036). Medium was not subsequently changed. To minimize evaporation, culture dishes were kept on "wet dishes" containing a filter paper pad that was always saturated with water. The immunochemical characterization of these cultures has been described previously (Wang et al., 1998a). Contamination by astrocytes was determined by immunochemical labeling with anti-glial fibrillary acidic protein antibody and was found to be <0.2% of total cells.

Library screening and RT-PCR. Total RNA (2.1 mg) was extracted from 21 d *in vitro* neuronal cultures using Tri-Reagent (Molecular Research Center, Inc., Cincinnati, OH). From this RNA, 11 μ g of poly(A) RNA was isolated using the Message Maker System (Invitrogen, Rockville, MD). The SuperScript Plasmid System (Invitrogen) was used to generate a cDNA library from 6 μ g of this mRNA (3 μ g/reaction). From two bulk ligations (300 ng of pCMVSPORT 2 vector, *NotI-SalI* cut, and 48 ng of cDNA per 120 μ l of ligation), followed by electroporation into the ElectroMAX DH10B cells, 7×10^7 primary clones were produced. Slightly $>10^7$ primary clones were expanded by bacterial culture in a semisolid agarose solution to 10^{12} . A portion of this bacterial stock was grown at 30°C in Terrific Broth containing ampicillin. The cDNA inserts from 14 randomly picked clones were sized using PCR and the SP6 and T7 primers. The average insert size of these 14 clones was 1.4 kb.

We used a 5' primer in the 5'-untranslated region (UTR) of the GLT1 cDNA (CGCCATGGCATCAACCGAGGG) to perform RT-PCR reactions with different 3' primers according to the sequences of 3'-UTRs of GLT1a (CCTTTTGTAAGGAAGCCTGTTT) and GLT1b (AGCT-TGGGTGACATGATTCCTTAC) on mRNAs from neuronal cultures. Bands of 1.8 kb were obtained in both reactions, purified, and cloned into the TOPO-TA vector (Invitrogen), sequenced, and found to be GLT1 clones with the same 5' end and different 3' ends. The longest clones obtained from RT-PCR were then subcloned into pcDNA3 vector (Invitrogen) for expression studies in COS-7 cells (Gluzman, 1981).

Polyclonal antibodies. To differentiate the expression of GLT1a and GLT1b proteins, a polyclonal antibody against the synthetic peptide ECKVPFPLDIETCI corresponding to the last 15 amino acids of GLT1b conjugated to keyhole limpet hemocyanin was generated in rabbits (Research Genetics, Huntsville, AL). N-terminal directed antibody was also generated against the peptide MASTEGANNMPKQVE (amino acids 1–15 of GLT1) conjugated at its C terminus. Before being used in immunocytochemistry and immunoblot analysis the antisera were affinity-purified using peptide-binding columns. Polyclonal antibody against the C terminus of GLT1a protein based on the published sequence (amino acid 559–573 of GLT1) was generously provided by Dr. J. Rothstein (Johns Hopkins University) and has been previously characterized with respect to its specificity and localization in brain (Rothstein

et al., 1994). We hereafter refer to these antibodies as anti-cGLT1b, anti-nGLT1, and anti-cGLT1a antibodies, respectively.

Light microscopic immunocytochemistry of cultured neurons. Neuronal cultures were grown on glass coverslips for 15–21 d. Cells were fixed with 4% paraformaldehyde in HBSS for 10 min at room temperature and then rinsed three times with Tris-buffered saline (TBS buffer) containing 50 mM Tris-HCl, pH 7.4, and 150 mM NaCl. 4% normal goat serum in TBS buffer with 0.1% Triton X-100 (TBS-T) was used to block nonspecific protein binding sites and to permeabilize cells. Antibodies were used at 140 ng/ml (anti-cGLT1a), 3.2 μ g/ml (anti-cGLT1b), and 1.5 μ g/ml (anti-nGLT1), respectively, in 2% normal goat serum in TBS-T. Cells were incubated in primary antibody solution at 4°C overnight, washed three times with TBS-T, and detected with goat anti-rabbit IgG conjugated with Oregon Green (Molecular Probes, Eugene, OR) at 1:500 dilution. Coverslips were then mounted with Fluoromount G (Fisher Scientific, Pittsburgh, PA) and examined by fluorescent microscopy or confocal microscopy. Controls were performed for each antibody by blocking with 50 μ M peptide against which the antibody was generated, by using preimmune serum at 1:500 dilution, as well as by omitting primary antibodies.

Light and electron microscopic immunocytochemistry on tissue sections. Sprague Dawley rats of postnatal days 24 and older were anesthetized deeply, using Nembutal (50 mg/kg), then transcardially perfused with a mixture of aldehydes. Aldehyde mixtures consisted of 4% paraformaldehyde alone or combined either with acrolein (3%) or glutaraldehyde (0.1–2%) and buffered using 0.1 M phosphate (PB) or cacodylate. A total of eight neocortices and hippocampi were sectioned at 40 μ m using a vibratome within 1 d after transcardial fixation. Sections were treated for 30 min with 1% sodium borohydride/PB, to terminate the cross-linking actions of the aldehydes, then rinsed repeatedly using 0.1 M PB, and stored in 0.01 M PB containing 0.9% sodium chloride (saline) (PBS) and 0.05% sodium azide.

The pre-embedding silver-intensified colloidal gold (SIG) procedure was done as described previously (Chan et al., 1990; Aoki et al., 1999), using the same dilutions of the primary antisera, biotinylated secondary antibodies directed against rabbit IgGs, 1.4 nm colloidal gold-conjugated anti-biotin IgG (Goldmark, Phillipsburg, NJ) and a silver-intensification kit (IntensEM; Amersham Biosciences, Arlington Heights, IL). Endogenous zinc was chelated by injecting sodium diethyldithiocarbamate (1 gm/kg, i.p.), 15 min before transcardial perfusion of the animal (Veznedaroglu and Milner, 1992).

The above immunocytochemical procedure was assessed for specificity by using three controls: incubating sections with preimmune serum or simultaneously with the antibody and with 50 μ M of the 15 amino acid antigen peptide or with the unique 11 amino acid sequence of GLT1b.

Tissue processing for electron microscopy was done as previously described (Chan et al., 1990; Aoki et al., 1999), using EMBED812 or Epon-Spurr (Electron Microscopy Sciences, Fort Washington, PA) as the embedding medium. Fixation with osmium tetroxide also was omitted, to minimize loss of SIG and also to minimize electron density of PSDs, caused by the accumulation of osmium over the plasma membrane. In lieu of osmium tetroxide, ultrastructural preservation and visualization of membranes was achieved by the osmium-free procedure developed by Phend et al. (1995) and Matsubara et al. (1996). In brief, 40-mm-thick sections were post-fixed and counterstained with a series of solutions, consisting of the following: tannic acid, uranyl acetate, iridium tetrabromide, and *para*-phenylenediamine-HCl, each made in a maleate buffer. These sections were infiltrated with Epon, flat-embedded between two sheets of Aclar plastic, then capsule-embedded for ultrathin sectioning. Ultrathin sections were collected serially onto Formvar-coated grids (50 or 200 mesh, thin bars). These sections were counterstained minimally with lead citrate (30 sec, using Reynold's lead citrate), to facilitate identification of processes and synapses without loss of SIG particles.

Sampling was performed strictly from the tissue-resin interface, where penetration by immunoreagents could be expected to be optimal. In addition, sampling was chosen to be from the neuropil residing between perikarya and blood vessels of layer 1, because this layer contains the highest density of synapses, most of which are glutamatergic (Aoki et al., 1994).

Immunoblot analysis. Freshly obtained adult rat brain tissue was homogenized with a Brinkman Polytron in ice-cold homogenization buffer containing 5 mM MgCl₂, 5 mM EGTA, 50 mM KCl, 17 μ g/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride, and 5 mM dithiothreitol in 20 mM Tris-HCl, pH 7.4. Neuronal cultures of 21 d *in vitro* and GLT1-transfected COS-7 cells were washed twice with HBSS before adding

RESULTS

Double-stranded plasmid DNA was isolated from a cDNA library made from nearly pure cultures of rat forebrain neurons and used in a solution-based hybridization method (Gene Trapper; Invitrogen) in an attempt to isolate known and novel glutamate transporters. Probes based on the highly conserved sequences NLVQACF and AAIFIAQ were used. These probes had been used previously to discover a new transporter, EAAT4, whose existence was inferred from discrepancies between the pharmacology of cerebellar synaptosomes and the known glutamate transporters (Fairman et al., 1995). Colony hybridization was performed with a mixture of full-length EAAC1 or GLT1 cDNA probes. Southern hybridization was performed on the 22 positive clones with whole EAAC1 and GLT1 cDNA probes. Nineteen were EAAC1 (seven were full-length clones), and three were partial GLT1 clones. Sequencing of these clones revealed no variant form or novel transporter. Another round of GeneTrapper technology was applied to enrich for GLT1 clones using a sequence close to the break point of the cDNA obtained in the first screen (GTCTTAGGTCTGATTGGATTTC). Colony hybridization using a GLT1 cDNA probe yielded ten additional positive clones, all of which were partial GLT1 clones. Two clones were identical to the three GLT1 clones found in the first round of screening, and each contained bases starting with 699 of the published coding sequence (GenBank accession number U15098) (Pines et al., 1992; Kanner et al., 1993) and extending to base 2542 in the 3'-UTR before the poly(A) region. The other eight clones were identical to each other and represented a GLT1 variant with an altered sequence in the region coding for the C-terminal end of the protein. These clones had the same nucleotide sequence from 518 to 1653 of the previously described GLT1 cDNA, whereas the sequence of the last 36 bases in the coding region was different from the last 69 bases of the original GLT1 cDNA and was similar to a previously published sequence derived from the mouse (GenBank accession number AB007812) (Utsunomiya-Tate et al., 1997). The 3'-UTR region of the variant form GLT1 was also different from that of the originally reported GLT1 and unlike the original form contained a poly(A) sequence (GLT1b; GenBank accession number AF451299). We here designate the original GLT1 form as "GLT1a," and the variant GLT1 form as "GLT1b." The sequence of GLT1a we have cloned from the rat neuronal culture library contains a longer 3'-UTR than the originally published GLT1 and extends to a poly(A) sequence (GLT1a; GenBank accession number AY069978).

The two different GLT1 clones are shown in Figure 1*A*. We were not able to obtain clones of either GLT1a or GLT1b containing the full-length coding sequence from the neuronal library. We therefore obtained the full-length coding sequence by RT-PCR using specific C-terminal primers and a common N-terminal directed primer (Fig. 1*B*). A variant N terminus of GLT1 has also been cloned from mouse liver (Utsunomiya-Tate et al., 1997). Primers directed against the mouse N-terminal variant and the rat C-terminal variants failed to produce PCR products from rat neuronal culture transcripts. RT-PCR was only successful when we used primers directed against either of the rat C-terminal regions obtained by cloning and a primer against the N-terminal region contained in the published sequence for rat. In addition, we performed 5'-rapid amplification of cDNA ends (RACE) using a set of primers in the 5' region of the GLT1 coding sequence on rat neuronal mRNA to find possible 5' variant forms. We sequenced over 50 5'-RACE clones, and all were the same

Figure 2. Immunoblot characterization of GLT1 antibodies. *A*, Membrane proteins from adult rat brain were analyzed by immunoblot as described (Materials and Methods) using anti-cGLT1a, anti-cGLT1b, and anti-nGLT1 antibodies (*cGLT1a*, *cGLT1b*, and *nGLT1*, respectively). All the anti-GLT1 antibodies recognized bands at ~66 kDa and also at ~130 kDa. Immunoreactivity was completely abolished when each antibody was preabsorbed with 50 μ M of the peptide antigen (*pep*). Control experiments were done with heterologous peptides (anti-nGLT1 antibody preabsorbed with cGLT1a peptide, anti-cGLT1a antibody preabsorbed with cGLT1b peptide, and anti-cGLT1b antibody preabsorbed with cGLT1a), which had no effect on the immunoreactivity in brain membrane immunoblots. *B*, Immunoblot analysis showed that anti-cGLT1a and anti-cGLT1b antibodies have no cross-reactivity. Membrane proteins from cultured neurons, rat brain tissue, and COS-7 cells stably expressing GLT1a and GLT1b proteins were subjected to SDS-PAGE and immunoblotted with anti-cGLT1a and anti-cGLT1b antibodies. Anti-cGLT1a antibody (*cGLT1a*) recognized bands in GLT1a transfected COS-7 cells as well as in neuronal cultures and rat brain tissue, but not in GLT1b transfected COS-7 cells. Anti-cGLT1b antibody (*cGLT1b*) recognized bands in a GLT1b stable transfectant COS-7 cell line, in neuronal cultures and in rat brain membranes, but not in GLT1a-transfected cells.

sequence as the published GLT1 clones, consistent with the earlier finding that the N-terminal variant is liver specific. Comparison of the coding region of GLT1b with the genomic sequence determined for human GLT1 (Meyer et al., 1997) reveals that the GLT1b sequence lacks exon 10, and the coding region terminates in the intron after exon 9 (Fig. 1*C*).

Using the sequence information from the partial GLT1b clone, we produced a polyclonal rabbit antibody (Fig. 2). An antibody was also raised against the N-terminal region. In addition, an antibody against the C-terminal region of GLT1a was kindly provided by Dr. Jeff Rothstein. When used to immunoblot rat brain membranes, these three antibodies each produced a band at ~66 kDa as well as at 130 kDa (Fig. 2*A,B*). It is likely that the higher mass band represents a dimer of the lower molecular mass form of the protein. The specificity of these antibodies was tested

by inclusion of peptide against which the antibody was directed, and in each case the peptide completely blocked the appearance of the bands at 66 and 130 kDa (Fig. 2*A*, *pep*). We found that the 11 amino acid peptide based on the unique C-terminal sequence of GLT1b was also able to completely block labeling of the blot by the anti-cGLT1b antibody. As a further test of the specificity of the antibodies, COS-7 cells were transfected with GLT1a and GLT1b cDNA in the pCDNA3 expression vector (Invitrogen), and lysates from GLT1a-expressing cells (Fig. 2*B*, *GLT1a*), GLT1b-expressing cells (Fig. 2*B*, *GLT1b*), neuronal cultures (Fig. 2*B*, *neurons*), and brain were tested for immunoreactivity with anti-cGLT1a and anti-cGLT1b antibodies (Fig. 2*B*, *cGLT1a*, *cGLT1b*). Anti-cGLT1a antibody reacted only with COS-7 cells transfected with GLT1a, neuronal cultures, and rat brain. Anti-cGLT1b antibody reacted only with COS-7 cells transfected with cGLT1b, neuronal cultures, and rat brain. These studies establish the specificity of the anti-cGLT1b antibody and confirm the specificity of the anti-cGLT1a antibody that has been previously demonstrated (Rothstein et al., 1994). These studies also demonstrate the expression of both forms of GLT1 in neuronal cultures.

These cultures contain very few astrocytes, and so the expression observed in the immunoblots was unlikely to be attributable solely to expression in astrocytes. To directly address this question, we used these three antibodies to study the localization of GLT1 in the neuronal cultures. GLT1a and GLT1b were clearly expressed on processes of neurons in these cultures, demonstrated by the immunocytochemical pattern of staining with anti-cGLT1a and anti-cGLT1b as well as by anti-nGLT1 antibodies (Fig. 3). All three of these antibodies produced a punctate staining pattern (Fig. 3*A,C,E*) that could be completely blocked by inclusion of the peptide against which the antibody was directed (Fig. 3*B,D,F*).

The studies so far revealed that the variant form GLT1b was significantly expressed in neurons in culture. The next question we addressed was whether the variant form GLT1b had similar functional properties to GLT1a. COS-7 cells transfected with plasmids expressing GLT1a or GLT1b reliably induced a large increase in sodium dependent uptake activity. The K_m values for GLT1a and GLT1b were similar, at $28 \pm 3 \mu\text{M}$ for GLT1a (two experiments) and $32 \pm 3 \mu\text{M}$ for GLT1b (two experiments). Vector alone produced very little sodium dependent transport activity. In two experiments, the transport activity seen with vector alone was 0.6% of that obtained in COS-7 cells expressing GLT1a or GLT1b.

GLT1 has a distinctive pharmacology, and the transport associated with cortical synaptosomes (Ferkany and Coyle, 1986; Robinson et al., 1993; Koch et al., 1999) and forebrain neurons in culture (Wang et al., 1998a) has been shown to resemble GLT1 in that it is inhibitable by DHK (Table 1). However, an anomaly has been that although SOS is a relatively weak (K_i value, 1157) inhibitor of the human GLT1 homolog EAAT2 (Arriza et al., 1994), it is of moderate potency in neuronal cultures and in oocytes expressing GLT1 (Wang et al., 1998a) (Table 1). We tested the potency of DHK, SOS, as well as the pan-transporter inhibitor PDC against glutamate transport mediated by GLT1a and GLT1b expressed in COS-7 cells (Fig. 4). In the experiment shown, the K_i values for DHK, SOS, and PDC against GLT1a were 52, 100, and $5.3 \mu\text{M}$, respectively. Against GLT1b these values were 45, 112, and $6.3 \mu\text{M}$, respectively.

Table 1 summarizes the experiments with GLT1a and GLT1b expression in COS-7 cells, and several conclusions can be drawn. First, there was no significant difference ($p > 0.05$) between

Figure 3. Immunocytochemistry of GLT1a and GLT1b expression in cultured forebrain neurons. *A, C, E*, Cultured neurons at 18 d *in vitro* immunostained with anti-cGLT1a (140 ng/ml) (*A*), anti-cGLT1b (3.2 $\mu\text{g/ml}$) (*C*), and anti-nGLT1 (1.5 $\mu\text{g/ml}$) (*E*) antibodies. All GLT1 antibodies detected immunoreactivity on neuronal processes and plasma membranes in a similar punctate pattern, suggesting synaptic localization of GLT1 proteins. Note that immunostaining by anti-cGLT1a and anti-cGLT1b antibodies was not distinguishable. *B, D, F*, Immunoreactivity was completely blocked using an excess of peptide against which each GLT1 antibody was directed.

GLT1a and GLT1b with respect to the K_i values obtained for DHK, SOS, and PDC. Second, the K_i values obtained with COS-7 cells very closely resembled the values obtained in neuronal cultures, with the exception of SOS, for which a value in COS-7 cells in the present study was obtained that was approximately twice the value obtained in neurons in our previous study. Third, the K_i value for DHK was consistent with the value obtained in neuronal cultures, COS-7 cells, and cortical synaptosomes, but was six to seven times higher than we obtained in oocyte expression studies (Wang et al., 1998a). According to these results, glutamate transport in neuronal cultures can be almost completely accounted for by the expression of GLT1a and GLT1b in neurons in these cultures.

Previous studies have shown that in astrocyte cultures, in which neurons were not present, GLT1 mRNA and protein expression were barely detectable (Swanson et al., 1997; Schlag et al., 1998). In addition, these studies showed that GLT1a was markedly upregulated by exposure to neuron conditioned medium or to dibutyryl cAMP. It was of interest to determine whether GLT1b as well as GLT1a were both downregulated in astrocytes without neurons present and whether they would both be coordinately upregulated. In three experiments we confirmed that astrocyte

Table 1. K_i or K_m values (μM) for DHK, L- α -AA, PDC, KA, and SOS interaction with glutamate transport systems

	DHK	L- α -AA	PDC	KA	SOS
GLT1a (rat; in COS-7 cells)	53 $\pm$ 3 ^a	ND	5 $\pm$ 1 ^a	ND	107 $\pm$ 6 ^a
GLT1b (rat; in COS-7 cells)	47 $\pm$ 6 ^a	ND	6 $\pm$ 2 ^a	ND	126 $\pm$ 9 ^a
GLT1 (rat; in oocytes) ^b	8 ^a	>1 mM ^a	ND	ND	312 ^c
Neuronal cultures ^b	65 ^a	>1 mM ^a	5 ^a	103 ^a	56 ^a
Cortical synaptosomes (rat) ^d					
High efficiency site	110 ^a	720 ^a	2.1 ^a	72 ^e	2.6 ^e
Low efficiency site				3500 ^e	55 ^e

^a K_i values.^bWang et al. (1998).^c K_m values.^dGarlin et al. (1995).^eIC₅₀ values.

cultures expressed little GLT1a and also that they expressed little GLT1b. After stimulation with 250 μM dibutyryl cAMP for 14 d we found that the expression of both GLT1a and GLT1b were greatly increased (Fig. 5).

The starting point of this work was to characterize the glutamate transporters present in nearly pure cultures of rat forebrain neurons that contained very few astrocytes. In these cultures we demonstrated expression of GLT1a, as well as a variant form, GLT1b. This variant form has not previously been shown to be significantly expressed in the brain, although a previous study demonstrated by RT-PCR that transcripts for GLT1b were detectable in RNA derived from the mouse brain (Utsunomiya-Tate et al., 1997). To assess the significance of GLT1b as a variant form of GLT1, we next investigated the expression of GLT1b mRNA and protein in the rat brain. For Northern blot analysis, we exploited the fact that the two variant forms possess completely different 3'-UTR sequences [GenBank accession numbers AF451299 (GLT1b) and AY069978 (GLT1a)]. Therefore, probes directed at the 3'-UTR regions should be able to distinguish between the two transcripts. In a developmental survey we found that the expression of both GLT1a and GLT1b increased with age (Fig. 6). By P10, strong signals for GLT1a and GLT1b were present. Interestingly, a 3.3 kb transcript was detectable using the GLT1b probe; at higher levels of loading, a transcript at similar, but different, relative mass (3.5 kb) was detectable using the GLT1a probe (data not shown). The experiment shown is representative of four independent experiments that were performed. To study protein expression, a regional survey by immunoblot analysis was performed (Fig. 7). Heavy expression of GLT1b as well as GLT1a was found in the cortex, hippocampus, striatum, thalamus, and midbrain. The patterns of expression for GLT1b and GLT1a were similar, but nonidentical. The experiment shown is one of three independent experiments that were performed.

These Northern and immunoblot studies demonstrated that GLT1b transcript and protein are significantly expressed in the normal rat brain. The next question we addressed was the cellular localization of GLT1b. The immunoblot and immunocytochemical studies reported in Figures 2 and 3 established that the variant GLT1b protein was expressed in forebrain neurons in tissue culture. The studies reported in Figure 5 demonstrated the expression of GLT1b in astrocytes in culture as well. GLT1a, which has been shown previously to be exclusively an astrocyte transporter in the normal, mature brain, was found together with GLT1b in neurons in culture. Because the expression of GLT1a in

cultured neurons is possibly caused by some aspect of the culture procedure and not representative of its expression *in vivo*, the question was asked then whether *in vivo* GLT1b would also be localized to astrocytes exclusively or whether it would be found to be expressed in neurons *in vivo*.

To answer this question, electron microscopic localization studies were performed in rat brain using pre-embedding immunocytochemistry with detection by SIG (Fig. 8). Figure 8A shows an example of labeled terminals. SIG particles occurred at sites away from and adjacent to the plasma membrane. In contrast, labeling within astrocytes occurred predominantly at the membrane (portion facing asymmetric synapse indicated by an asterisk). In the panel shown in Figure 8B, three PSDs are indicated by open arrows. Astrocytes (LAs) are labeled in several locations. Asterisks indicate labeling adjacent to synapses. Figure 8C again shows a labeled astrocyte (LAs) as well as an unlabeled astrocyte (UAs). A dendritic shaft is labeled LSh and contains three particles. Silver-enhanced gold particles were close to or immediately adjacent to membranes of astrocytes, but were more often located in the cytoplasm, presumably associated with cytoplasmic membranes, in neurons. Preadsorption of the anti-cGLT1b antibody abolished the SIG immunolabeling completely. These results demonstrate that GLT1b protein is detectable in neuronal elements, as well as in astrocytes, in the normal mature brain.

DISCUSSION

In this report we provide evidence that a variant form of GLT1, designated GLT1b, as well as the originally described form, GLT1a, is significantly expressed in forebrain neurons in tissue culture. The expression of these two forms accounts for the dihydrokainate-sensitive glutamate transport displayed by these cultures (Wang et al., 1998a). Others have shown by single-cell PCR that transcripts for GLT1 are present in hippocampal cultures (Brooks-Kayal et al., 1998) and that GLT1a protein is expressed in hippocampal neurons in microcultures (Mennerick et al., 1998). In addition to demonstrating the presence of GLT1b in neurons in culture, the results of Northern blot and immunoblot analysis presented here also establish that GLT1b transcripts and protein are significantly expressed in the rat brain.

Cellular localization of GLT1b

Because neurons can induce the expression of GLT1a in astrocytes (Swanson et al., 1997; Schlag et al., 1998), it was possible that the expression of GLT1b in neurons in our cultures was a peculiarity of tissue culture not representative of its expression

Figure 4. Effect of transport inhibitors on $[^3\text{H}]$ L-glutamate uptake in GLT1a- and GLT1b-transfected COS-7 cells. *A, B*, The effect of DHK on $[^3\text{H}]$ L-glutamate uptake by GLT1a (*A*) and GLT1b (*B*). In this particular experiment the IC_{50} values for GLT1a and GLT1b were 52 and 45 μM , respectively. *C, D*, The effect of SOS on $[^3\text{H}]$ L-glutamate uptake by GLT1a (*C*) and GLT1b (*D*). The IC_{50} values for GLT1a and GLT1b were 100 and 102 μM , respectively. *E, F*, The effect of PDC on $[^3\text{H}]$ L-glutamate uptake by GLT1a (*E*) and GLT1b (*F*). The IC_{50} values for GLT1a and GLT1b were 6.3 and 5.3 μM , respectively. In each case, results are single experiments representative of at least four experiments that were performed (Table 1).

patterns in the brain, in which GLT1 protein has been found to be mainly associated with astrocytes (Rothstein et al., 1994; Chaudhry et al., 1995; Lehre et al., 1995). However, a sensitive *in situ* hybridization study in the brain showed widespread expression of mRNA for GLT1 in neurons (Berger and Hediger, 1998). Our demonstration of GLT1b protein in neurons *in vivo* is consistent with and, in fact, anticipated by this demonstration of GLT1 transcripts in neurons. Whether the GLT1 transcripts that are present represent primarily GLT1a or GLT1b remains a question, however. It should be possible to address this issue using variant specific probes targeting the different 3'-UTRs of the two forms.

A previous study that used several different antibodies directed against different regions of GLT1 shared by the two variant forms failed to detect immunolabeling in neurons (Lehre et al., 1995).

Figure 5. Similar upregulation of GLT1a and GLT1b in astrocytes in response to dibutyryl cAMP. Astrocyte cultures contained undetectable levels of GLT1a or GLT1b (Control). After treatment with 250 μM dibutyryl cAMP (dbcAMP) for 14 d, both GLT1a and GLT1b were significantly upregulated. Membrane protein (5 μg) solubilized in 1% SDS was loaded in each lane.

At the light microscopic level, this can be simply explained by the preponderant astrocyte labeling obscuring the neuronal labeling, as has been noted in an *in situ* hybridization study of GLT1 mRNA (Berger and Hediger, 1998). At the EM level, the failure of antibodies against shared epitopes to detect neuronal labeling cannot be explained in this way. Three considerations are relevant. First, it has been shown that antibodies of proven specificity against different regions of a molecule may produce very different patterns of labeling, for reasons that are unclear (Aoki et al., 1999). Second, fixation techniques are critical, and we found best preservation of antigen using paraformaldehyde and avoiding glutaraldehyde. Third, care needs to be taken to use ultrathin sections close to the surface of the stained tissue section because only at the surface is penetration of reagents optimal. For these specific reasons (varying results with different antibodies, denaturation of antigen by the fixation conditions, and limited penetration of reagents into tissue), the failure of an antibody to detect an antigen cannot be taken as evidence excluding the presence of that antigen. Based on our rigorous demonstrations of the specificity of the anti-GLT1b antibody, the fact that it is clearly expressed in neurons in culture, the presence of immunoreactivity in neurons using this antibody *in vivo*, and the previous demonstration of mRNA for GLT1 in neurons throughout the brain (Berger and Hediger, 1998), we feel confident that GLT1b is, in fact, expressed in neurons.

Despite the evidence for glutamate uptake in excitatory terminals (Iversen and Storm-Mathisen, 1976; Divac et al., 1977; Storm-Mathisen, 1977; Storm-Mathisen and Iversen, 1979; Cuenod et al., 1982; Gundersen et al., 1993; Gundersen et al., 1995), the identification of the responsible transporter

Figure 6. Expression of GLT1a and GLT1b mRNA during development. Total RNA was extracted from the forebrain of postnatal day 1 (P1), day 10 (P10), day 27 (P27), or adult (ad; 200 gm) Sprague Dawley rats. Ten micrograms of total RNA was loaded per lane. DNA probes representing 3'-UTR regions of GLT1a or GLT1b hybridized to RNA at ~11 kb. The GLT1b probe clearly recognized another RNA ~3 kb. At longer exposure times, a 3.5 kb band was seen with the GLT1a probe (data not shown). Expression of both GLT1a and GLT1b progressively increased through development.

has been elusive (Danbolt, 2001). Our finding of GLT1b in axon terminals suggests that GLT1 may be an important pre-synaptic transporter.

Ventura and Harris (1999) found that ~60% of excitatory synapses in the hippocampus had astrocytic processes opposed to them, and of these, astrocytic processes surrounded less than half of the synaptic apposition. Furthermore, astrocytic processes occurred along the path between only ~33% of neighboring synapses, whereas 66% of these paths were exclusively along neuronal membranes. Therefore, the presence of glutamate transporters presynaptically and postsynaptically in neurons is likely to be important in controlling spillover from occurring between excitatory synapses (Asztely et al., 1997; Rusakov and Kullmann, 1998). EAAC1 is expressed in a somatodendritic localization (Rothstein et al., 1994; Velaz-Faircloth et al., 1996; Coco et al., 1997; Shashidharan et al., 1997). An immunogold study in the macaque monkey demonstrated sparse distribution of EAAC1 in the postsynaptic density, at the edge, and in the perisynaptic zone

Figure 7. Regional distribution of GLT1a and GLT1b proteins in rat brain. Immunoblots representing different rat brain regions were probed with anti-cGLT1a or anti-cGLT1b antibodies. Both proteins were detected in all regions. Strongest expression was observed in the cerebral cortex.

Figure 8. Anti-cGLT1b immunoreactivity in neurons and astrocytes of layer 1 of somatosensory cortex, as revealed by electron microscopic immunocytochemistry using SIG label. *A*, SIG label is present in an axon terminal (LT) and in an astrocyte (LAs). Here and in all other panels, SIG particles are indicated by arrows in neuronal elements and by arrowheads in astrocytes, whereas open arrowheads point to postsynaptic densities. SIG labeling is present along the plasma membrane of an astrocyte, including portions juxtaposed to asymmetric junctions (asterisk). *B*, An astrocyte is labeled LAs at two sites adjacent to two different asymmetric synapses (asterisks). *C*, A shaft, possessing a contiguous spine in the same section, is labeled LSh. An astrocyte is labeled LAs, and SIG particles are located adjacent to the plasma membrane. Note that not all astrocytes are labeled (e.g., *C*, UAs). Scale bar, 500 nm.

of asymmetric synapses in the entorhinal cortex (He et al., 2000). The localization of GLT1b to the postsynaptic membranes of a subset of excitatory synapses suggests a possible role for GLT1 as well in the neuronal clearance of synaptically released glutamate. The extent of the expression of GLT1b in presynaptic terminals and postsynaptically in spines and dendrites can only be answered by a quantitative study of the expression of GLT1b in neurons, preferably with serial reconstruction of cellular elements. Such a study is currently in progress, but is beyond the scope of the present report.

Implications of the existence of a variant form of GLT1 for the physiology of glutamate transport

It is well established that the C terminus of membrane proteins may interact with other proteins by way of specialized domains to form macromolecular complexes (Niethammer et al., 1996; Brakeman et al., 1997; Dong et al., 1997; Ziff, 1997; Kim and Haganir, 1999; Sheng and Pak, 1999; Sheng and Sala, 2001). The existence of C-terminal variants of GLT1 suggest that there may be different interacting networks for each form. Specifically, the last four amino acids of GLT1b constitute a PSD-95/DLG/ZO-1 domain-interacting consensus sequence lacking in GLT1a. It will be of great interest to determine whether GLT1b has interacting protein partners and whether these are distinct from the interacting protein partners of GLT1a.

Ultrastructural localization of GLT1b in neurons

A surprising finding of this work is that in neurons the SIG labeling was typically at sites removed from the plasma membrane. One interpretation of this finding is that GLT1b in neurons in the plasma membrane may be masked by membrane-anchoring proteins that interact with the C terminus, competing with the C terminus directed antibody. Another possibility is that the bulk of the GLT1b in neurons is dormant and localized to cytoplasmic membranes until mobilized to the cell surface as a result of exposure to a signal, perhaps related to activity. This would be consistent with studies showing significant translocation of EAAC1 to the plasma membrane as a result of activation of protein kinase C (Dowd and Robinson, 1996; Davis et al., 1998).

Developmental variation

We used 3'-UTR-specific probes to assay separately GLT1a mRNA and GLT1b mRNA across development, and our finding of increasing postnatal expression is in basic agreement with a previous *in situ* hybridization study using probes directed against the coding sequence of GLT1 (Sutherland et al., 1996) and an immunocytochemical study using an anti-cGLT1a antibody (Furuta et al., 1997). The localization of GLT1a at greatest density in astrocyte membranes adjacent to synapses (Lehre and Danbolt, 1998) as well as in dendritic shafts and axon terminals (this study) suggests that the increasing expression of both GLT1a and GLT1b may be related to synaptic enrichment and activity in the postnatal period.

Implications of variant forms of GLT1 in the pathophysiology of glutamate transport

Glutamate efflux via glutamate transporters in neurons is a source of the extracellular glutamate that accumulates in ischemia (Szatkowski et al., 1990; Attwell et al., 1993; Lipton and Rosenberg, 1994). DHK has been shown recently to significantly block the release of glutamate during ischemia (Seki et al., 1999), and this finding might indicate a role for GLT1 expressed in neurons.

Glutamate transport and GLT1 protein expression are depressed in ~50% of patients with sporadic amyotrophic lateral sclerosis. There is evidence suggesting that aberrant RNA processing of GLT1 transcripts underlies these defects (Lin et al., 1998). Because at least one familial neurodegenerative disease, frontotemporal dementia, has been shown to be caused by a mutation resulting in a defect in splicing (Poorkaj et al., 1998; Morris et al., 1999; Varani et al., 1999), the existence of a variant form of GLT1 raises the possibility that a mutation affecting the splicing of this protein might produce deleterious consequences.

In summary, two variant forms of GLT1 are expressed in neurons in culture and account for glutamate transport in these cultures. GLT1b, which we cloned from a neuronal library made from these cultures, is altered in its C terminal, and its message and protein are significantly expressed in the brain. GLT1b is expressed in presynaptic terminals and in dendritic shafts as well as in astrocytes in the normal mature rodent brain. The quantitative significance of this expression has yet to be determined. The existence of variant forms of GLT1 and the expression of GLT1 in neurons is likely to have important implications for the physiology and pathophysiology of glutamate transport and excitatory neurotransmission.

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