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Structure, expression, and functional analysis of a Na^+-dependent glutamate/aspartate transporter from rat brain

(glutamate transport/*Xenopus laevis* oocytes)

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ABSTRACT **Transport systems specific for L-glutamate and L-aspartate play an important role in the termination of neurotransmitter signals at excitatory synapses. We describe here the structure and function of a 66-kDa glycoprotein that was purified from rat brain and identified as an L-glutamate/L-aspartate transporter (GLAST). A GLAST-specific cDNA clone was isolated from a rat brain cDNA library. The cDNA insert encodes a polypeptide with 543 amino acid residues (59,697 Da). The amino acid sequence of GLAST suggests a distinctive structure and membrane topology, with some conserved motifs also present in prokaryotic glutamate transporters. The transporter function has been verified by amino acid uptake studies in the *Xenopus laevis* oocyte system. GLAST is specific for L-glutamate and L-aspartate, shows strict dependence on Na^+ ions, and is inhibited by DL-*threo*-3-hydroxyaspartate. *In situ* hybridization reveals a strikingly high density of GLAST mRNA in the Purkinje cell layer of cerebellum, presumably in the Bergmann glia cells, and a less dense distribution throughout the cerebrum. These data suggest that GLAST may be involved in the regulation of neurotransmitter concentration in central nervous system.**

In the mammalian central nervous system L-glutamate is the main transmitter for most excitatory neurons, which are involved in complex physiological processes, such as learning and memory (1). The excitatory signal is generally removed by reuptake of the amino acids into presynaptic terminals and surrounding glia cells by high-affinity transport systems, which appear to play an important role in the regulation of synaptic transmission (2). Conventional biochemical approaches have resulted in the partial purification of these proteins (3–5). To date, three distinct systems have been described based on different ion requirements: a Na^+-dependent system (3–6), a chloride-dependent system (7, 8), and a Na^+- and Cl^--independent system that is stimulated by Ca^{2+} (9, 10).

Here we report the isolation of a eukaryotic glutamate/aspartate transporter (GLAST) from rat brain and its characterization at the cDNA† and protein level. The deduced primary structure shows appreciable similarity to bacterial glutamate and dicarboxylate transporters. Expression of GLAST in *Xenopus* oocytes demonstrates that it is a high-affinity, Na^+-dependent L-glutamate/L-aspartate transporter. GLAST mRNA is exclusively expressed in brain; it is primarily localized in the cerebellar Purkinje cell layer and is less dense throughout the cerebrum.

MATERIALS AND METHODS

Cloning and Sequence Analysis of a cDNA Encoding GLAST. A rat brain cDNA library in λgt10 (4×10^6 independent recombinants) was constructed from poly(A)$^+$ RNA by using oligo(dT)-primed cDNA synthesis (Pharmacia). Plaques (9×10^5) were screened with the ^{32}P-labeled oligonucleotide AAA/GAAA/GCCITAT/CCAA/GT/CTIATIGC derived from the peptide sequence EMKKPYQLIAQDN. A 3-kilobase (kb) clone was isolated, subcloned into the *Eco*RI site of pGEM3Z (Promega), and sequenced by using the dideoxy chain-termination method (11) applying the T7 sequencing kit (Pharmacia).

***In Vitro* Transcription of GLAST cDNA.** The 3-kb insert of our cDNA clone was excised with *Eco*RI; the ends were filled in by Klenow enzyme and ligated into *Sma* I-digested pSP64-poly(A) vector (Promega). The template DNA was linearized by digestion with *Eco*RI and transcribed into GLAST complementary RNA (cRNA) by using SP6 RNA polymerase (BRL) applying standard methods (12).

***In Vitro* Translation and Immunoprecipitation of GLAST.** One microgram of GLAST cRNA was translated by using rabbit reticulocyte lysate (amino acid depleted; Amersham) in the presence of 30 μCi (1 Ci = 37 GBq) of [^{35}S]methionine according to the supplier's instructions. Immunoprecipitation was performed using C-GLAST-GST, an affinity-purified antibody raised against a recombinant fusion protein consisting of the 49 C-terminal amino acids of GLAST and glutathione *S*-transferase (pGEX-1N vector; Amrad, Victoria, Australia).

Injection of GLAST cRNA into *Xenopus* Oocytes and Analysis of the Expressed Amino Acid Transport. Oocytes were isolated from *Xenopus laevis* as described (13), separated by gentle agitation in collagenase type II (2 mg/ml; Sigma) for 1 h, and washed extensively in Barth's modified saline (BS). Integer oocytes (stages V and VI) were selected for injection of 20–50 nl of GLAST cRNA (0.4 mg/ml) or water. The oocytes were maintained in BS at 21°C. To uncover the translation product, five oocytes were incubated in 30 μl of BS containing [^{35}S]methionine (1 mCi/ml) for 20 h. The oocytes were lysed in 80 μl of 20 mM Tris·HCl pH 7.6/0.1 M NaCl/1% Triton X-100/1 mM phenylmethylsulfonyl fluoride. Twenty microliters of 5% deoxycholate/2.5% Nonidet P40/0.5% SDS was added, and immunoprecipitation was performed by using the C-GLAST-GST antibody.

Amino acid transport into the oocytes was routinely assayed 20 h after injection. Individual oocytes were incubated for 15 min at 21°C in 100 μl of BS containing 0.005, 0.02, 0.05, 0.1, 0.2, 0.5, or 1 mM L-[^{14}C]glutamate and L-[^{14}C]aspartate (Amersham). The specific activity was 45 mCi/mmol for the 0.005–0.2 mM amino acid solutions and 9 mCi/mmol for the 0.5 and 1 mM amino acid solutions. Each oocyte was washed five times in 1 ml of BS, homogenized in 100 μl of 2% SDS, and assayed in 10 ml of Bray's solution. In the experiments depicted in Fig. 3*C*, two parameters were changed: the

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Abbreviations: cRNA, complementary RNA; GLAST, glutamate/aspartate transporter.
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†The sequence reported in this paper has been deposited in the GenBank data base (accession no. X63744).

concentration of the cRNA used for the injection was 0.1 mg/ml and uptake was assayed 48 h after injection.

Northern Blot Analysis. Three micrograms of poly $(A)^+$ RNA (14) from the cerebrum, cerebellum, liver, kidney, heart, and skeletal muscle of 18-day-old rats was electrophoresed on a 1% agarose gel containing formaldehyde, transferred to a nylon membrane (NEN), and probed with a ^{32}P-labeled, randomly primed fragment comprising nucleotides 1178–1781 of our cDNA clone (12).

***In Situ* Hybridization.** ^{35}S-labeled RNA probes were prepared by *in vitro* transcription of the 3-kb GLAST cDNA in pGEM3Z by using SP6 (BRL) or T7 RNA polymerase (Boehringer Mannheim) and [^{35}S]UTP. GLAST antisense RNA (or a sense probe as control) was hybridized to 6- to 8-μm cryosections of adult rat brains previously fixed in 4% paraformaldehyde and processed for autoradiography as described (15).

RESULTS

Cloning and Structural Determination of GLAST. During the isolation of the UDPgalactose:ceramide galactosyltransferase from rat brain (S.S. and W.S., unpublished data), we copurified a hydrophobic glycoprotein with a molecular mass of 66 kDa, which, after treatment with endoglycosidase F, yielded a polypeptide of 60 kDa. The purified protein was subjected to limited proteolysis, and the amino acid sequences of four peptides isolated by high-resolution PAGE (16, 17) were determined by Edman degradation. A degenerate oligonucleotide probe corresponding to one of the peptide sequences (Fig. 1) was synthesized and used to screen a rat brain cDNA library. A 3-kb clone was isolated and sequenced. Fig. 1 shows the nucleotide and deduced amino acid sequence of GLAST. The predicted sequence of the polypeptide consists of 543 amino acid residues with a calculated molecular mass of 59,697 Da.

A computer-aided search (June 1991) of available data bases revealed significant sequence similarity of GLAST to the glutamate transporters of *Escherichia coli* (18), *Bacillus stearothermophilus*, and *Bacillus caldotenax* and to the dicarboxylate transporters of *Rhizobium meliloti* and *Rhizobium leguminosarum* (19). The overall amino acid sequence identities range from 26% for the dicarboxylate transporter from *R. meliloti* to 32% for the glutamate/proton transporter from *B. stearothermophilus*, which suggests a common transmembrane organization within the family of glutamate transporting proteins. Analysis of the hydrophobicity (20) and hydrophobic moment (21) of the amino acid sequences leads to the following consensus structural model.

The absence of a cleavable signal sequence suggests a cytosolic localization of the N terminus. Six helical segments at positions 48–68, 91–111, 123–145, 238–260, 281–302, and 319–340, all located in the N-terminal half of the protein, are considered as membrane spanning. An arrangement of six short hydrophobic segments of seven to nine residues each, located in the C-terminal region of the protein, is highly conserved throughout the family of glutamate transporters. The region connecting the helical transmembrane segments IV and V exhibits properties of an amphipathic α-helix, a feature also present in the prokaryotic glutamate transporters.

Two putative sites for N-glycosylation are present on the presumed extracytosolic side of the GLAST protein at posi-

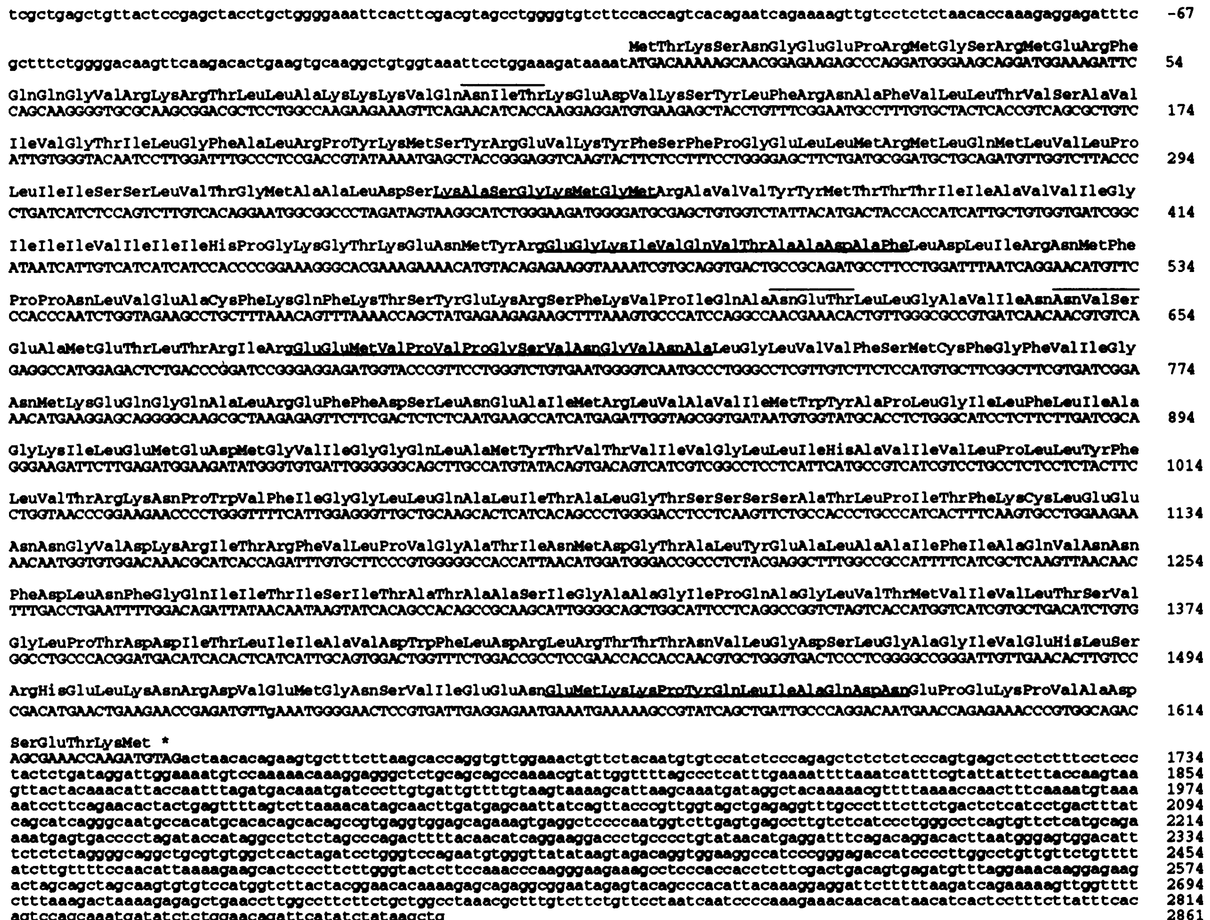

FIG. 1. Nucleotide sequence of GLAST cDNA and deduced primary structure. Position 1 refers to the first nucleotide and amino acid residue of the predicted GLAST coding region. N-Glycosylation site consensus motifs are overlined. Amino acid sequences determined by Edman degradation of purified peptide fragments released by V8 or Lys-C proteolytic cleavage of the purified GLAST protein are underlined.

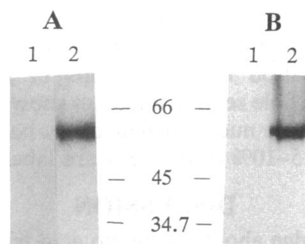

FIG. 2. *In vitro* translation/*in vivo* expression of GLAST. (*A*) *In vitro*-transcribed GLAST cRNA (lane 2) or water as a control (lane 1) was used for *in vitro* translation followed by immunoprecipitation of GLAST, SDS/PAGE analysis, and autoradiography. (*B*) Immunoprecipitation of [^{35}S]methionine-labeled GLAST synthesized by *Xenopus* oocytes injected with GLAST cRNA (lane 2) or water as a control (lane 1). Molecular sizes (in kDa) are indicated.

tions Asn-206 and Asn-216. They are localized in the extended connecting loop between transmembrane segments III and IV, a region not present in the prokaryotic proteins. One additional N-glycosylation motif is located at the N terminus (Asn-26). Possible phosphorylation sites for protein kinase C are found between helix II and III (Ser-116) and the C terminal of helix VI (Thr-341 and Thr-372). A phosphorylation consensus motif for cAMP-dependent protein kinase is present in the N terminus (Thr-26) and the C terminus (Thr-387).

Functional Expression of GLAST. To verify that our cloned cDNA encodes the purified GLAST polypeptide, cRNA was prepared by *in vitro* transcription of GLAST cDNA and used for *in vitro* translation in the presence of [^{35}S]methionine. Immunoprecipitation using the affinity-purified C-GLAST-GST antibody raised against a recombinant GLAST glutathione *S*-transferase fusion protein revealed a single band at 60 kDa in SDS/PAGE (Fig. 2*A*, lane 2), in good agreement with the molecular mass of the deglycosylated form. To determine the amino acid specificity and ion dependence of GLAST, the protein was first synthesized *in vivo* by injecting GLAST cRNA into *Xenopus* oocytes. Subsequently, oocytes were incubated in medium supplemented with [^{35}S]methionine, lysed, and subjected to immunoprecipitation with the C-GLAST-GST antibody. A labeled 60-kDa protein was immunoprecipitated from the oocyte homogenate (Fig. 2*B*, lane 2), which comigrated in SDS/PAGE with the *in vitro*-synthesized polypeptide (Fig. 2*A*, lane 2) as well as the deglycosylated, native GLAST protein isolated from rat brain. Injection of water instead of GLAST cRNA yielded no labeled product (Fig. 2*B*, lane 1). To test whether GLAST is indeed capable of transporting L-glutamate and L-aspartate, amino acid uptake by oocytes injected with GLAST cRNA was assayed by incubation in medium containing either ^{14}C-labeled L-glutamate or L-aspartate followed by the measurement of radioactive material taken up by the oocytes. Twenty hours after GLAST cRNA injection, the oocytes accumulated ≈30 times more L-glutamate and 15–20 times more L-aspartate than the water-injected controls. Amino acid uptake showed saturation kinetics (Fig. 3*A*) with V_{max} = 600 ± 200 pmol per oocyte per h, K_m = 77 ± 27 μM for L-glutamate and V_{max} = 290 ± 130 pmol per oocyte per h, K_m = 65 ± 30 μM for L-aspartate as determined by a Lineweaver–Burk plot.

Uptake of L-alanine, L-leucine, L-glutamine, L-arginine, L-methionine, and L-malate by GLAST-expressing oocytes ranged between 0.8 and 1.2 of the control values (data not shown).

To investigate the possibility that GLAST is a member of one of the three known L-glutamate/L-aspartate transport systems in mammalian brain, we measured amino acid transport by GLAST in the presence or absence of Na^+. The uptake of L-glutamate and L-aspartate by GLAST cRNA-injected oocytes was absolutely Na^+ dependent (Fig. 3*B*). When NaCl was replaced in the incubation medium by Tris·HCl, L-glutamate and L-aspartate uptake in GLAST cRNA-injected oocytes was reduced to background levels.

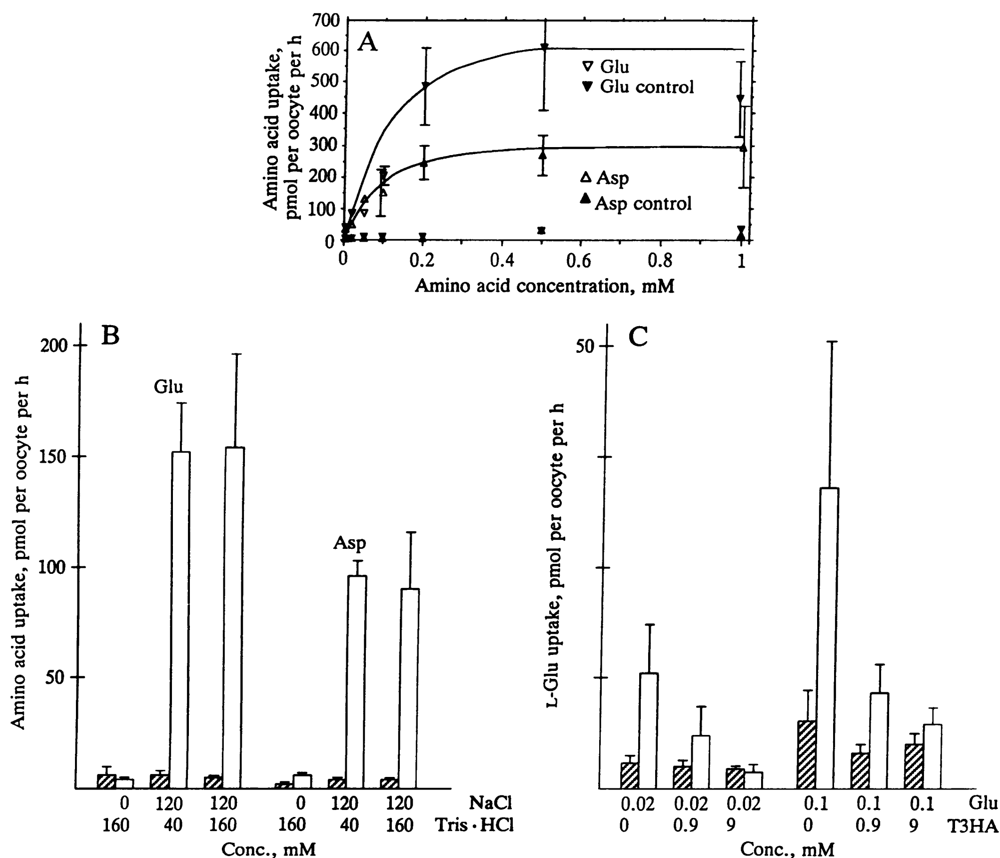

FIG. 3. Functional analysis of GLAST in *Xenopus* oocytes. (*A*) Saturation kinetics of L-glutamate (Glu) and L-aspartate (Asp) uptake by oocytes injected with GLAST cRNA (open triangles) or water (filled triangles). Each point is the mean ± SD (n = 3). (*B*) Na^+ dependence of GLAST-mediated L-glutamate/L-aspartate transport. Transport of L-glutamate and L-aspartate (at 0.2 mM) into *Xenopus* oocytes injected with GLAST cRNA (open bars) or water (hatched bars) was measured in the presence or absence of Na^+. The incubation medium contained 0.3 mM $Ca(NO_3)_2$, 0.41 mM $CaCl_2$, 0.82 mM $MgSO_4$, and various concentrations of NaCl and Tris·HCl (pH 7.6) as indicated. Values are the mean ± SD (n = 3). (*C*) Inhibition of L-glutamate uptake by DL-*threo*-3-hydroxyaspartate (T3HA). Symbols are as in *B*. The Na^+ concentration in BS is 88 mM (13). Values are the mean ± SD (n = 3).

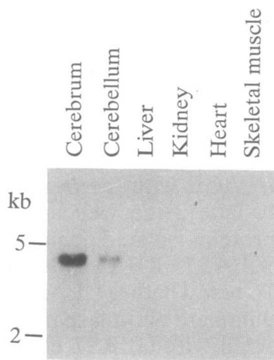

FIG. 4. Northern blot analysis of GLAST expression in rat tissues. The positions of 2- and 5-kb markers are indicated. The position of GLAST mRNA is at 4.5 kb.

To ensure the high Tris concentration was not inhibitory in our assay, we also incubated oocytes in the presence of Na^+ and Tris at a high concentration. As seen in Fig. 3B, amino acid uptake was not affected by the Tris concentration, demonstrating that transporter function is indeed Na^+ dependent.

For further characterization, uptake experiments were performed in the presence of DL-*threo*-3-hydroxyaspartate, the strongest known inhibitor of Na^+-dependent glutamate transport assayed in brain slices (22) and astrocytes (2) (Fig. 3C). At 0.1 mM glutamate, an inhibitor concentration of 9 mM resulted in a 90% reduction of glutamate uptake in the oocyte system.

Localization of GLAST Expression. The distribution of GLAST mRNA in different rat tissues was examined by Northern blot analysis (Fig. 4). The result demonstrates that GLAST is specifically expressed in brain. *In situ* hybridization of frozen rat brain sections using an *in vitro*-transcribed GLAST antisense RNA probe reveals a prominent expression of GLAST in the cerebellar cortex and a more even distribution in the cerebrum as documented by a dark-field image (Fig. 5A). Bright-field microscopy at higher magnification illustrates the localization of GLAST message in the cerebellum in more detail (Fig. 5B). Here the hybridization signal is restricted to the Purkinje cell layer. Control experiments using the respective sense RNA revealed no significant accumulation of silver grains (Fig. 5C). In the cerebrum, higher magnification reveals clumps of grains scattered throughout the whole section (data not shown). These signals coincide with single nuclei visualized by hematoxylin/eosin staining. About 5–10% of the cells are labeled by grains.

DISCUSSION

Current knowledge about the amino acid transporter, which limits the neurotransmitter action of L-glutamate released by glutamatergic synaptic terminals, has emerged from amino acid uptake studies on rat brain synaptosomes, brain slices, glia cell preparations, and primary cultures (2, 23) as well as glioma and neuroblastoma lines (24). The Na^+-dependent high-affinity uptake system exhibits a high specificity for glutamate and aspartate (25). K_m values ranging from 14 to 220 μM have been reported (2, 26), supporting the idea that this transport system may consist of several components.

We isolated a cDNA clone encoding GLAST, a 66-kDa glycoprotein, which is specifically expressed in brain and exhibits significant homology to procaryotic glutamate transporters. The amino acid transport properties of GLAST were investigated in the *Xenopus* oocyte expression system. The expressed transporter is highly specific for L-glutamate and L-aspartate. Other amino acids including L-alanine, L-leucine, L-glutamine, L-arginine, and L-methionine are not transported in significant amounts. In contrast to the mitochondrial dicarboxylate transporter, GLAST is unable to catalyze the uptake of L-malate, a structural relative of L-aspartate. K_m values were determined to be 77 ± 27 μM for L-glutamate and 65 ± 30 μM for L-aspartate. This coincides with values measured in primary astrocyte cultures (67 μM/77 μM), whereas for neuronal primary cultures, higher affinities (20 μM/32 μM) have been reported (27). Transporter function is strictly dependent on Na^+. Additionally DL-*threo*-3-hydroxyaspartate, known as a strong inhibitor of the Na^+-dependent glutamate uptake (22) and capable of causing neuronal degeneration (28), was demonstrated to be a potent inhibitor of GLAST.

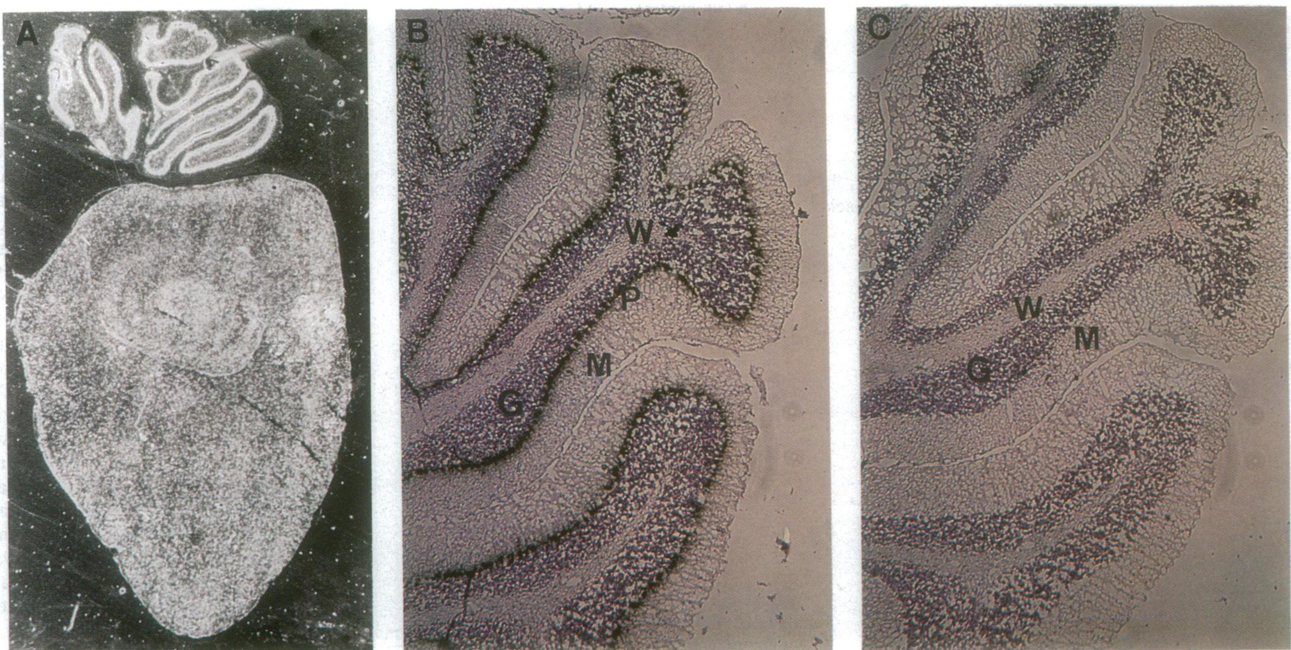

FIG. 5. *In situ* hybridization analysis of GLAST expression in rat brain. Distribution of GLAST message in rat brain examined by *in situ* hybridization of vertical (*A*) and horizontal (*B* and *C*) 6-μm cryosections using GLAST antisense cRNA (*A* and *B*) or corresponding sense cRNA as a control (*C*). (*A*) Dark-field image; hybridization signals appear as white grains. (*B* and *C*) Bright-field image showing cerebellar gyri, counterstained with hematoxylin/eosin. (×130.) G, granular layer; W, white matter; P, Purkinje cell layer; M, molecular layer.

The functional properties of GLAST examined so far are in good agreement with those reported for the Na^+-dependent high-affinity glutamate transporter at excitatory synapses. We therefore propose that GLAST is a neurotransmitter transporter possibly playing a role in the termination of the excitatory signal at synapses using glutamate and/or aspartate.

Localization of GLAST expression in brain was analyzed by *in situ* hybridization. In the cerebrum GLAST mRNA was found to be widely distributed. Hybridization signals coincide with single nuclei scattered throughout all examined regions with only slightly greater expression in the hippocampus, which may be attributed to the higher cell density in this region. In cerebellum, GLAST message is specifically expressed in the stratum gangliosum made up primarily by Purkinje and Bergmann glia cells. Purkinje cells do not use glutamate or aspartate as neurotransmitters, but receive excitatory signals from climbing and parallel fibers. Several types of glutamate receptors have been demonstrated to exist on Purkinje cells (29). Therefore it seems likely that GLAST is expressed in Bergmann glia cells closely associated with the respective synapses.

As we could not detect high levels of GLAST mRNA in the hippocampus and no message in the cerebellar granular layer, structures known to contain glutamatergic synapses at high density, we propose that GLAST is a member of a family of Na^+-dependent glutamate transporters, which are differentially expressed in various regions of the brain.

The rat brain GLAST seems to be evolutionary related to prokaryotic Na^+-independent glutamate-transporting proteins but shares no sequence similarity with the *E. coli* Na^+-dependent glutamate transporter (30, 31) or the cloned mammalian neurotransmitter transporters characterized so far (32–39).

Unlike the 12-transmembrane-helix model proposed for these neurotransmitter transporters, the tentative model presented here for the rat GLAST protein comprises only six α-helical membrane-spanning segments in its N-terminal half with approximately the same spacing as the first six helices of previously reported neurotransmitter transporters. The sequence stretches forming the six putative transmembrane helices are essentially free of charged amino acids with no indication of amphipathic structures. The putative glycosylation sites occupy similar positions as in the other neurotransmitter transporters. A feature uncommon for transporter proteins is the presence of an extended C-terminal region with moderate hydrophobicity. This most strongly conserved part of the protein is made up of at least six relatively short hydrophobic stretches of seven to nine amino acids, each of which is too short to form a transmembrane α-helix. It should be noted, however, that other secondary structures are known to provide the dimensions and conformation to allow spanning of the membrane with fewer amino acids. Assuming a β-sheet as an underlying secondary structure, several regions can be found in the C-terminal half of GLAST that show considerable separation of polar and unpolar amino acid side chains.

The transport systems for excitatory amino acids are not only important for terminating the excitatory signal but also for maintaining the concentration of L-glutamate and L-aspartate below toxic levels. Defective transport of excitatory amino acids has been postulated to be involved in neurodegenerative diseases and epilepsy (1). Finally a decrease in Na^+-dependent L-glutamate transport in patients with Alzheimer disease has been reported (40). Whether GLAST plays a pivotal or ancillary role in these disorders remains to be elucidated.

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