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CLONING AND EXPRESSION OF A HUMAN GLUTAMATE TRANSPORTER[1]

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A complementary DNA clone was isolated from human brain that encodes a glutamate transporter. Injection of RNA transcribed from this cDNA into *Xenopus* oocytes resulted in expression of a transport activity with a high affinity for glutamate (Km = 78.4μM) and a dependency on external Na^+. The cDNA sequence predicts a protein of 542 amino acids that is highly homologous to the congeneric proteins from rat and mouse brains. RNA blotting analyses revealed the expression both in the brain and in peripheral tissues.

Glutamate is the major neurotransmitter in the brain. It is involved in complex physiological processes like learning and the establishment of memory (1, 2). On the other hand, the elevation of extracellular glutamate concentrations causes neuronal cell death, such as, in the case of ischemic states and neurodegenerative diseases. The glutamate transporters rapidly remove glutamate from the synaptic clefts and prevent the elevation of extracellular glutamate concentrations. Since neurodegenerative diseases, such as, amyotrophic lateral sclerosis (3) and Alzheimer's disease (4), show a reduction of glutamate transporter, it is believed that the glutamate transporter may play an important role in the onset and progress of neurodegeneration (5).

Recently, three subtypes of cDNA encoding the glutamate transporter, rat GLAST (6) (rat (7) and mouse GluT-1(8)), rat GLT-1 (9) and rabbit EAAC1 (10), have been cloned, sequenced and expressed. For further analyses on the relation between the glutamate transporters and neurodegenerative process, we have cloned and characterized a human glutamate transporter (hGluT-1).

[1]The nucleotide sequence data reported in this paper will appear in GSDB, DDBJ, EMBL and NCBI nucleotide sequence databases will the following accession number D26443.

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Materials and Methods

Cloning of cDNA of the human glutamate transporter

Clones ($3x10^5$) derived from the human brain cDNA library (11) (gift from Dr. S. Nakanishi) in λ-ZAPII (Stratagene, La Jolla, CA) were screened under low stringency with a 1504 bp *Pst* I fragment of rat glutamate transporter (rGluT-1) (7) as described (12). Bluescript plasmids pBSSKII(-) were rescued from plaque-purified positives by in vivo excision as described by the manufacturer (Stratagene). RNAs transcribed by T7 RNA polymerase and T3 RNA polymerase from the rescued plasmids were injected into *Xenopus* oocytes. The cDNAs coding glutamate transporters were identified by measurement of the glutamate uptake activity of oocytes as described previously (7). The plasmid phGluT-1, one of the identified clones, was sequenced by the dideoxy chain termination method using Sequenase 2.0 (U.S. Biochemical Corp.) with [^{35}S]dCTP and AutoRead Sequencing Kit in A.L.F. DNA sequencer (Pharmacia LKB).

Expression of the phGluT-1 in oocytes

The synthetic RNA was obtained by transcribing the phGluT-1 with T3 RNA polymerase in the presence of a capping nucleotide. Oocytes were surgically removed from frogs and injected with 40 nl water containing 4-6ng synthetic RNA. After two or three days, they were assayed for glutamate transport. Uptake experiments were initiated by transferring single injected or noninjected oocytes to 50 ml of ND96 (96mM NaCl, 2mM KCl,1mM $MgCl_2$ and 1.8mM $CaCl_2$, Buffered to pH 7.5 with 5mM Hepes) containing [^{3}H]glutamate (50nM, 54.1Ci/mmol; New England Nuclear). After a 60 minute incubation period, the oocytes were rapidly washed and solubilized in 1% SDS. Thereafter, their radioactivity was measured by liquid scintillation counting. At least five oocytes were used for each experimental point. The data were then expressed as the average uptake per hour.

RNA blotting analyses

The human tissue RNA blot was obtained from Clontech Laboratories and the hybridization condition was described by the manufacturer. The 569bp *Eco* RI- *Bgl* II fragment was excised from phGluT-1 and used as a probe.

Results and Discussion

Thirty one clones with strong hybridization signals were isolated during screening of the human brain cDNA library with the *Pst* I fragment of prGluT-1 (7). Three of the 31 clones showed the glutamate transporter activity in the oocyte assay system. The phGluT-1, one of three clones, was sequenced to identify an open reading frame that codes for 542 amino acids with a relative molecular weight of 59,569 (Fig. 1). The first ATG present in the cDNA was assigned as the initiation codon on the basis that it was 6 base pairs downstream from a single in-frame stop codon. The 3'-untranslated region of clone phGluT-1 has two classical polyadenylation signals and ends in a poly(A) tail. Hydrophobicity analysis of putative protein revealed 6 potential hydrophobic segments suitable for the formation of transmembrane α-helical domains. It contains three potential glycosylation sites at positions Asn-35, Asn-206 and Asn-216. The amino acid sequence of the human GluT-1 consists of one less amino acid than the rat (7) and mouse GluT-1 (8), and is 96.9% and 96.7% identical to the rat (7) and mouse GluT-1(8), respectively. The human GluT-1 has 41.8% and 40.4% sequence identity with the human neutral amino acid transporters, ASCT1 (13) and SATT (14), respectively. On the other hand, it has no significant homology with Na^+/Cl^-dependent neurotransmitter transporters of GABA, L-glycine, L-proline, dopamine, serotonin and noradrenaline (15).

Xenopus oocytes injected with hGluT-1 synthetic RNA accumulated up to 25 times as

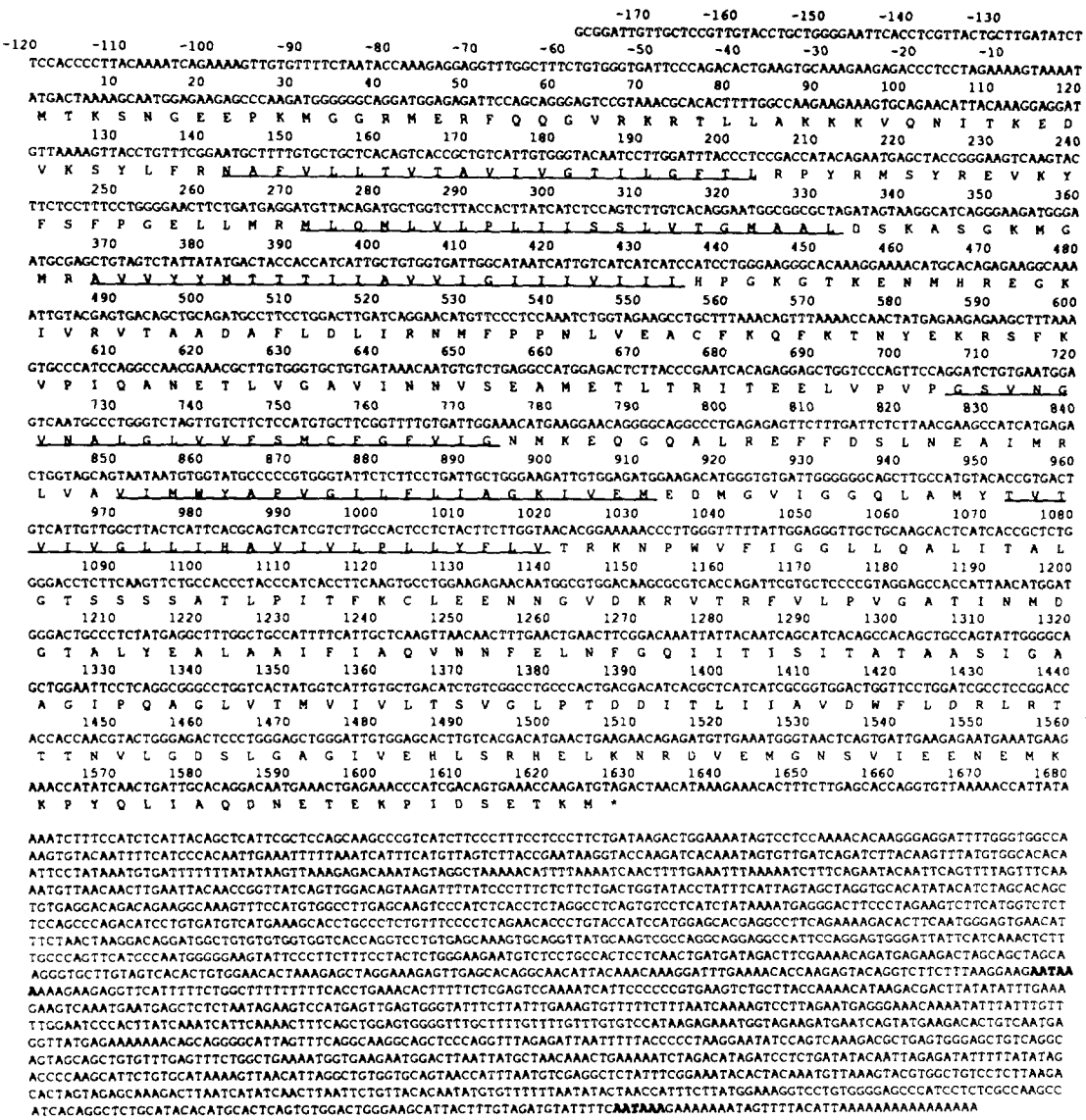

Fig.1.Nucleotide and deduced amino acid sequences of the human GluT-1.
Six putative membrane-spanning domains are underlined. * shows the stop codon. Two polyadenylation sequences (AATAAA) are indicated by boldface.

much glutamate as uninjected oocytes (Fig.2A). The uptake was absolutely dependent on extracellular Na^+, but was not on extracellular Cl^-. Fig.2B shows the rate of [^{3}H]glutamate uptake into *Xenopus* oocytes injected with a synthetic RNA of phGluT-1 at various glutamate concentrations. Nonlinear regression analysis of the data indicates a Michaelis constant (Km) of 78.4μM and a maximum velocity of uptake (Vmax) of 64.6pmol per oocyte per hour (mean of 2 experiments).

The hGluT-1-mediated transport was also examined for the stereospecificity and the pharmacological properties (Table 1). The uptake of [^{3}H]glutamate was effectively

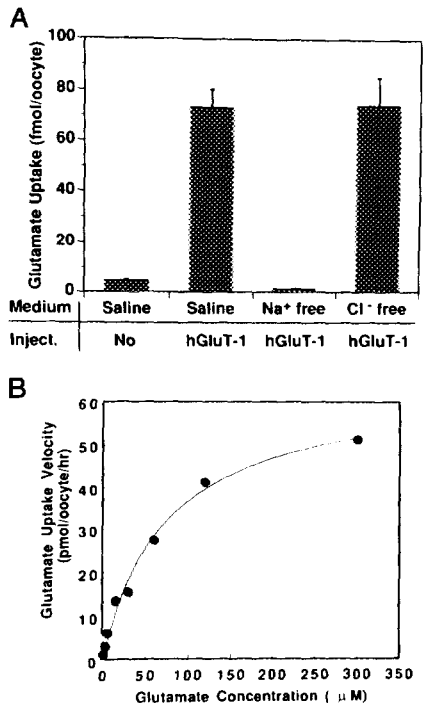

Fig.2.Na^+, Cl^- and concentration dependence of L-glutamate uptake into *Xenopus* oocytes injected with hGluT-1 mRNA.

(A) Effects of Na^+ and Cl^- on glutamate uptake by hGluT-1 mRNA-injected *Xenopus* oocytes.
Oocytes were assayed in either ND96 (Saline) or a similar solution in which Na^+ was replaced by equimolar choline (Na^+free) or which Cl^- was replaced by gluconate (Cl^- free). The glutamate concentration was 50nM in all assays. Each value is the mean ± SEM of results from at least five oocytes.
(B) Effects of glutamate concentrations on glutamate uptake.
Uptake velocity of 50nM [^{3}H]glutamate was determined in the presence of increasing concentration of unlabeled L-glutamate during a 60 minute incubation. The theoretical curve for saturation analysis of [^{3}H]glutamate was drawn by nonlinear least squares analysis as described previously (8).

inhibited by L-glutamate, L-aspartate, and D-aspartate at 100μM (92%, 98% and 94% inhibition, respectively), but not by D-glutamate at 100μM (7% inhibition). L-Homocysteate (L-HC), an excitatory transmitter candidate, is a substrate of low-affinity but not of high-affinity glutamate transport (16). Consistent with this, L-HC (100μM) weakly inhibited the glutamate uptake (16% inhibition). Two glutamate uptake inhibitors, DL-threo-β-hydroxyaspartate (THA) and L-cysteine sulfinate (L-CS), that were not selective in different brain regions (17, 18), inhibited the glutamate uptake (90% and 91% inhibition at 100μM, respectively). Two region specific inhibitors of glutamate uptake, dihydrokainate in the rat forebrain and L-α-aminoadipate in the rat cerebellum (17, 18,19), weakly inhibited the glutamate uptake (85% and 62% inhibition at 3mM, respectively). 4-Acetamide-4'-isothiocyano-2,2'-disulfonic acid stilbene (SITS), a selective inhibitor of glial glutamate transporter (20), weakly inhibited the glutamate uptake (50% inhibition at 1mM). The pharmacological profile of hGluT-1 is similar to

Table 1.Pharmacological characterization of [^{3}H]glutamate uptake into *Xenopus* oocytes injected with hGluT-1 mRNA

Compound	Concentration (mM)	Displacement (%)
L-Glutamate	0.1	92±2
D-Glutamate	0.1	7±1
L-Aspartate	0.1	98±1
D-Aspartate	0.1	94±1
L-Homocysteate	0.1	16±6
DL-Threo-β-hydroxyaspartate	0.1	90±2
L-Cysteine sulfinate	0.1	91±3
Dihydrokainate	3	85±1
L-α-Aminoadipate	3	62±4
SITS	1	49±5

Glutamate transport assays (50nM[^{3}H]glutamate) were conducted as described in Materials and Methods for 60 minutes, with or without the indicated agents. Data show percentage displacements of specific [^{3}H]glutamate uptake. Values are presented as mean ± standard errors of the means and represent the average of at least five oocytes.

the rat (7,21) and mouse GluT-1(8), and different from the previously characterized glutamate transporters in the rat brain (17,18,19).

The expression of hGluT-1 mRNA was analyzed by RNA blotting of the poly(A)$^+$RNA from the various human tissues (Fig.3). The strongest hybridization signal of 4.0

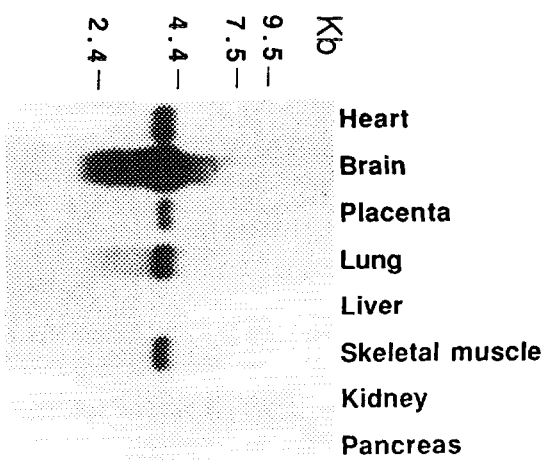

Fig.3.Tissue distribution of mRNA for the glutamate transporter, hGluT-1. Northern hybridization was performed as described in Materials and Methods.

kilobases was detectable in the brain. An intermediate signal was detectable in the heart, lung, skeletal muscle and placenta, and a faint signal was detected in the liver and kidney. No signal was detectable in the pancreas. No signal with other size was detectable in all tissues examined.

The human glutamate transporter, hGluT-1, was cloned and characterized. The hGluT-1 shows a unique pharmacological profile and is the novel glutamate transporter as is the rat GluT-1. The analysis of the hGluT-1 will develop new pharmacological and molecular probes for studying neural cell death after ischemia or of neurodegenetive diseases, such as, Alzheimer's disease or amyotrophic lateral sclerosis.

Acknowledgments

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