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Short Communication

Rat and Human Glutamate Transporter GLAST1 Stable Heterologous Expression, Biochemical and Functional Characterization

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Human embryonic kidney cell lines (HEK293) which express heterologously and permanently the human and rat GLAST1 (high affinity, Na^+-dependent, CNS-specific L-glutamate transporter) have been established by the transfer of the two minigenes under the control of the cytomegalovirus promoter by electroporation. The transfected HEK_h GLAST1 (human) and HEK_r GLAST1 (rat) cell lines strongly express the glutamate uptake system which exhibits all biochemical and electrophysiological properties determined so far in the transiently expressing *Xenopus* oocyte system except the K^+ dependence. These cell lines are a valuable tool for further biochemical, physiological, and pharmacological studies on this uptake system of the most important excitatory neurotransmitter.

Key words: Electrogenic properties / Excitatory neurotransmitter / Heterologous expression of Glast-1 / High affinity uptake system.

L-Glutamate is the major neurotransmitter at excitatory synapses in vertebrate CNS besides being a key intermediate in the metabolism of any cell type of CNS. It is involved in very divergent excitatory neurotransmission, even in hippocampal long term potentiations, potentially the basis of learning and memory (Monaghan *et al.*, 1989).

The regulation of the activity of the glutamate uptake system is of great importance for the plasticity of excitatory synaptic processes not only with regard to transmission efficiency of different synapses such as long term potentiation (LTP) of glutamatergic synapses in hippocampus (Bliss and Collingridge, 1993) but also with regard to excitotoxicity of persisting high concentrations of glutamate in the synapse (Choi *et al.*, 1987; Beal, 1992a, b). The excitotoxic mechanism is regarded as a causal process in several neurodegenerative diseases. The glial glutamate transporter system apparently plays a key role in hyp- or anoxic degeneration of neurons following apoplexia (Nicholls and Attwell, 1990). The L-glutamate uptake system maintains the steep neurotransmitter gradient, extracellularly 2–5 μM but intracellularly 1–10 mM (Erecinska and Silver, 1990) and clears the synaptic cleft in microseconds from the excitatory concentration between 0.1 and 1 mM during excitation down to 1 μM in the resting state.

Synaptic L-glu concentration must be under precise, rapid and efficient control for a precise transmission particularly in view of the neurotoxicity of L-glu at prolonged interaction with the postsynaptic membrane. 0.1 mM L-glu over a period of five minutes leads to the death of neurons (Choi *et al.*, 1987; Nicholls and Attwell, 1990; Beal, 1992a, b).

The Na^+-dependent, high-affinity glutamate transporter system sustains this steep concentration gradient. This has been demonstrated in glutamate uptake studies in brain slices, homogenates, and synaptosomes (Logan and Snyder, 1972; Shank and Champbell, 1984; Robinson *et al.*, 1993), astrocytes (Hertz *et al.*, 1978; Flott and Seifert, 1991), glia cells (Hertz *et al.*, 1978; Flott and Seifert, 1991) and neurons (Dreier *et al.*, 1982). L-glu uptake systems integrated into the plasma membrane of nerve and glia cells bordering the synaptic cleft achieve the rapid fine tuning of the synaptic glutamate concentration.

We have recently described the first member of the Na^+-dependent, high affinity L-glutamate transporter family of mammalian CNS GLAST1 (Storck *et al.*, 1992). Like other neurotransmitter transporters GLAST1 is also a polytopic integral plasma membrane protein of 543 aa residues (59697 kDa) but has only six well-defined transmembrane helical domains instead of 12 h in transporters of the other neurotransmitters but a rather undetermined C-terminus. The lack of sufficient sequence homologies prohibited the discovery of the L-glutamate transporter family by homology screening which was very successful in the isolation of GABA (Guastella *et al.*, 1990), noradrenaline (Pacholczyk *et al.*, 1991), dopamine and serotonin transporters (Amara and Kuhar, 1993), glycine (Smith *et al.*, 1992), and L-proline (Fremeau *et al.*, 1992). Northern blot analysis of mRNA of cerebrum, cerebellum, liver, kidney, heart, and skeletal muscle of rats proved the brain-specific expression as a 4.5 kb mRNA and *in situ* hybridization with GLAST1-specific antisense RNA its expression in Bergmann glia cells of cerebellum hippocampus and throughout the brain.

The glutamate transport function of the putative transporter (GLAST1) was analyzed by expression in *Xenopus* oocytes and L-[^{14}C]-glutamate uptake into oocytes after GLAST1-specific cRNA injection into oocytes. Its Na^+ dependence, the K_m value and influence of the D,L-threo-3-hydroxyaspartate, an inhibitor of the Na^+-dependent high

affinity glutamate uptake, were determined by the voltage-clamp technique (Klöckner *et al.*, 1993; Klöckner *et al.*, 1994). In view of the broad scope of the biochemistry and pharmacology of the GLAST1 uptake system which in comparative studies shows the strongest expression of the three L-glu transporters (GLAST1, GLT1, EAAC transporter) we established heterologous, stable GLAST1 (rat and human) expressing cell line.

For the stable heterologous expression of GLAST1 in HEK293 cells the blunt-ended 3 kb rat and 3.2 kb human GLAST1 cDNAs were cloned into the BsstI site of the eukaryotic expression vector pRc CMV which carries the neoresistance (gene) as selection marker. GLAST1 expression takes place under the strong CMV promoter. The XbaI-linearized vector was used for DNA transfer by electroporation into HEK293 cells. High G418 concentrations (400 μg/ml) were used for clone selection. The 5′ end of the rat and human GLAST1 cDNA insert had the optimal Kozak sequence (Kozak, 1984) which was generated by PCR as described before (Storck *et al.*, 1992). The transfected HEK cell clones showed normal growth.

For the biochemical characterization of HEK GLAST1 cell clones total RNA of HEK cell clones resistant to G418 was isolated (Chomczynski and Sacchi, 1987), size fractionated in formaldehyde-agarose (1.2%) gels, transferred to a GeneScreen Plus membrane and hybridized to a ^{32}P-random-labelled 600 bp EcoRI/PstI fragment under standard conditions. A 4.5 kb GLAST1-specific RNA was detected in RNA of the pooled cells of single HEK293 clones. The degree of expression of GLAST1 in the HEK clones was estimated by comparing the intensities of the Northern blot hybridization signals of the 4.5 kb GLAST1 signal and the 1.4 kb GAPDH signal. Expression of GLAST1 was regarded to be stable after 50 passages.

The homogeneity of the cell clones expressing GLAST1 was established by assaying cytosol of single HEK cells for GLAST1-specific RNA by reversed transcription and subsequent PCR. Cytosol of single cells was sampled under the conditions of the single electrode voltage clamp technique under IR-DIC video microscopy (Stuart *et al.*, 1993). The nested oligonucleotide primer method was applied with two outer primers yielding a 430 bp fragment and in the re-PCR two inner oligonucleotide primers yielding a 384 bp fragment.

The RT-PCR reaction mix was divided into two aliquots, one to probe for GLAST1 and the other for GAPDH with the respective primers.

We proved the GLAST1 expression in HEK cell clones on the protein level in a radio-labelling experiment: semiconfluent HEK cell layers were incubated in methionine-free medium with [^{35}S]methionine for 10 hours. Cells were harvested and lysed. Labelled GLAST1 was immunoprecipitated with GLAST1-specific antibodies similar to the immunoprecipitation of GLAST1 expressed in *Xenopus laevis* oocytes (Storck *et al.*, 1992). The dissociated immunocomplex was size-fractionated by SDS PAGE and analyzed by autoradiography. Two bands of 64 and 70 kDa were radioactively labelled (not shown). HEK cells expressing GLAST1 were grown on cover slips, permeabilized with methanol and reacted with GLAST1-specific antibodies and the second fluorescein-labelled antibody for fluorescence microscopy. Figure 1 visualizes the strong labelling of the plasma membrane of GLAST1-positive clones as compared with the wild type. Besides the plasma membrane the cytosolic structures (Golgi system) close to the nucleus also lighten up. We have recently applied the effective separation of the 64 and 70 kDa isoform of GLAST1 by lectin affinity chromatography. The 64 kDa isoform differs from the 70 kDa isoform by the degree and complexity of their glycosylation, the 64 kDa GLAST1 being a high mannose fucosylated glycoprotein binds specifically to Lentil lectin and the mature 70 kDa isoform is specifically bound by wheat germ lectin which recognizes terminal galactose and sialic acid residues transferred by sugar transferases of the medial and trans-Golgi system during the passage to the plasma membrane. By differential affinity chromatography we separated the two forms of GLAST1 from 2×10^8 HEK_{GLAST1} cells (clone 38). The two transporter proteins were characterized by GLAST1 peptide-specific antibodies.

The L-glutamate transport by GLAST1 in the permanent HEK cell line was characterized by the voltage clamp technique as described for *Xenopus* oocytes expressing GLAST1 (Klöckner *et al.*, 1993; Klöckner *et al.*, 1994).

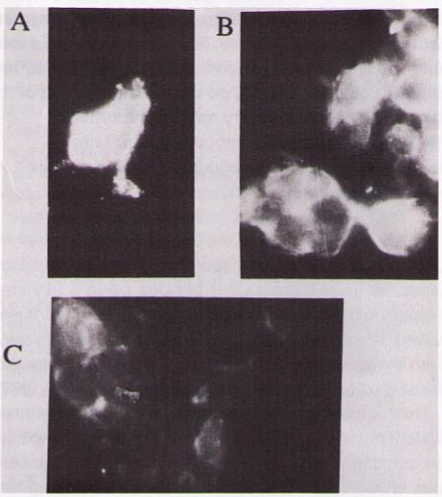

Fig. 1 Immunofluorescence of HEK_{GLAST1}.
Control HEK cells and HEK_{GLAST} cells were grown on cover slips, fixed, permeabilized, preincubated with BSA, then washed and covered with the first GLAST1-specific antibodies for four hours, rinsed thoroughly again with PBS and then reacted with goat anti-rabbit IgG tagged with dichlorofluorescein for one hour. Cover slips were thoroughly washed with PBS and immediately studied under the fluorescence microscope (Zeiss, Oberkochen, Germany). Notice the intense fluorescent cell boundaries and intracellular structures. (A) Single HEK_h GLAST1 cell; (B) cell colony, (C) control, nonimmunserum was used as first antibody.

The GLAST1-mediated L-glutamate uptake by HEK_{GLAST1} cells is L-glutamate and Na^+ ion-dependent. HEK_{GLAST1} cells grown on cover slips were superfused with the bathing solution with different L-glutamate and Na^+ ion concentrations. A membrane potential of −60 mV was applied during all measurements. The depolarizing inward current I_{GLAST1} at 100 µM L-glutamate was set to 1 for normalizing the subsequent current at [L-Glu] of 5, 10, 20, 40 mM with $[Na^+]$ = 140 mM.

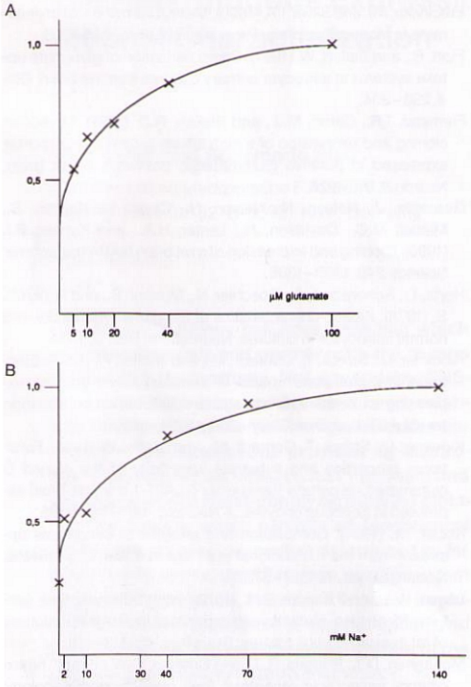

Fig. 2 Dependence of I_{GLAST1} from (A) L-Glutamate at 140 mM $[Na^+]$, and (B) $[Na^+]$ at 40 µM L-Glutamate.
For experimental conditions see Klöckner *et al.*, 1993; Klöckner *et al.*, 1994. Figures at the y-axis are relative I_{GLAST1} values.

The dependence of I_{GLAST1} and [L-Glu] is plotted in Figure 2A. Values plotted in Figure 2B are normalized to I_{GLAST1} at 140 mM Na^+. Likewise I_{GLAST1} was measured at 40 µM L-glutamate in dependence of increasing $[Na^+]$. The difference in $[Na^+]$ concentration to 140 mM was adjusted with choline chloride. The two curves are described by $I = I_{max}\,[L\text{-}Glu]^n/[L\text{-}Glu] + K_m$, when a K_m of 10–15 µM for L-Glu and a Hill coefficient, n, of 1.3 is assumed, and a K_m for Na^+ = 41 mM with a Hill coefficient of n = 3.3. Therefore the Na^+/glutamate cotransport procedes with a stoichiometry of 3:1 as measured in *Xenopus* oocytes before (Klöckner *et al.*, 1993). On average at −60 mV holding potential 40 µM L-glutamate induced an I_{GLAST1} of 150 pA.

L-glutamate transport can be inhibited by structural analogs of L-glutamate or L-aspartate (Storck *et al.*, 1992).

Table 1 summarizes the apparent K_m values and normalized I_{max} of several substrates and inhibitors.

These examples clearly demonstrate the practicability and usefulness of the HEK_{GLAST} cell lines for pharmacological studies on the L-glutamate uptake system as an *in vitro* system but also for several open biochemical and physiological questions particularly with reference to the regulation of the glutamate uptake system.

The influence of the membrane potential was analyzed by a voltage-current ramp experiment (Figure 3) in which the L-glutamate-induced current I_{GLAST} is continuously registered during the shift from +50 mV to −100 mV. The difference of the currents (I_{GLAST1} in pA) under control conditions (HEPES buffer, upper recording) and with 40 µM L-glutamate (ECS = HEPES + 40 µM L-glutamate) is plotted as a function of voltage. The data suggest that the netto charge translocation over the plasma membrane as-

Table 1 $K_{m(app)}$ and Normalized I_{max} of Substrates and Inhibitors of GLAST1.

Substrate	Apparent K_m (µM)	Normalized I_{max} (mean ± SD)	n
L-glutamate	12 ± 4	1	43
D-glutamate	1500 ± 300	0.90 ± 0.20	5
L-aspartate	6.5 ± 3*	0.76 ± 0.16*	11
D-aspartate	6.1 ± 3*	0.50 ± 0.14*	5
L-cysteinesulfinate	8 ± 4	0.86 ± 0.05*	11
L-cysteate	6.3 ± 3*	0.93 ± 0.11	10
L-homocysteinesulfinate	50 ± 100	0.92 ± 0.10	3
L-homocysteate	1500 ± 250	0.91 ± 0.08	4
D,L-threo-3-hydroxyaspartate	16 ± 5	0.75 ± 0.15*	8
L-α-aminoadipate	1100 ± 400	0.93 ± 0.13	7
β-aminoadipate (1 mM)	–	0	3
D-α-aminoadipate (1 mM)	–	0	3
quisqualate (1 mM)	–	0	3
dihydrokainate (1 mM)	–	0	3
N-p-aminobenzoyl-L-glutamate (1 mM)	–	0	3

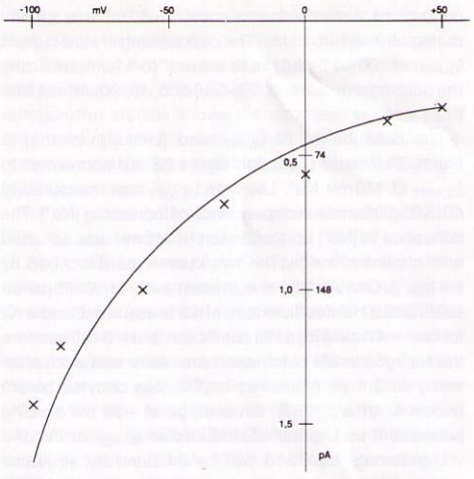

Fig. 3 Influence of Membrane Potential on L-Glutamate Induced Current I_{GLAST1} by a Voltage-Current Ramp between +50 mV and −100 mV.

sociated with the L-glutamate uptake is influenced by the electrical potential of the cell on the inward transport of positive ions (Na^+) together with the neurotransmitter L-glutamate.

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