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Glutamate transporters from brain

A novel neurotransmitter transporter family

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The removal of neurotransmitters by their transporters in presynaptic nerve terminals and glial cells plays an important role in the termination of synaptic transmission. Many neurotransmitter transporters, which are sodium- and chloride-coupled, have been cloned and shown to constitute a large superfamily. Glutamate is the major excitatory neurotransmitter in the central nervous system. If not efficiently removed, it causes death of neuronal cells. Its transporter couples the flow of glutamate to that of sodium and potassium. Recently three different but related glutamate transporters have been cloned, which have no significant homology to the members of the superfamily.

Neurotransmitter; L-Glutamate; Transporter; Nervous system; Neuronal death

1. INTRODUCTION

The overall process of synaptic transmission is terminated by high-affinity sodium-dependent transport of the neurotransmitters from the synaptic cleft [1,2]. This includes the neurotransmitters γ-aminobutyric acid (GABA), L-glutamate, glycine, dopamine, serotonin and norepinephrine. Another termination mechanism is observed with cholinergic transmission. After dissociation from its receptor, acetylcholine is hydrolysed into choline and acetate. The choline moiety is then recovered by a sodium-dependent transport. As the concentration of the transmitters in the nerve terminals is much higher than in the cleft, typically by 4 orders of magnitude, energy input is required. The transporters which are located in the plasma membranes of nerve endings and glial cells achieve this by coupling the flow of neurotransmitters to that of sodium. The (Na^+,K^+)-ATPase generates an inwardly-directed electrochemical sodium gradient which is utilized by the transporters to drive 'uphill' transport of the neurotransmitters (reviewed in [3–5]).

These transport systems have been investigated in detail by using plasma membranes obtained upon osmotic shock of synaptosomes. It appears that these transporters are coupled not only to sodium, but also to additional ions like potassium or chloride.

These transporters are of considerable medical interest. Since they function to regulate neurotransmitter activity by removing it from the synaptic cleft, specific transporter inhibitors can be potentially used as novel drugs for neurological disease. For instance, attenuation of GABA removal will prolong the effect of this inhibitory transporter, thereby potentiating its action. Thus, inhibitors of GABA transport could represent a novel class of anti-epileptic drugs. Well-known inhibitors which interfere with the functioning of biogenic amine transporters include anti-depressant drugs and stimulants such as amphetamines and cocaine. The neurotransmitter glutamate, at excessive local concentrations, causes cell death, by activating NMDA receptors and subsequent calcium entry. The transmitter has been implicated in neuronal destruction in ischaemia, epilepsy, stroke, amyotropic lateral sclerosis and Huntington's disease. Neuronal and glial glutamate transporters may have a critical role in preventing glutamate from acting as an exitotoxin [6,7].

In the last few years major advances in the cloning of these neurotransmitter transporters have been made. After the GABA transporter was purified [8], the ensuing protein sequence information was used to clone it [9]. Subsequently the expression cloning of a norepinephrine transporter [10] provided evidence that these two are the first members of a novel superfamily of neurotransmitter transporters. This led, using polymerase chain reaction (PCR) and other technologies relying on sequence conservation, to the isolation of a growing list of neurotransmitter transporters (reviewed in [11–13]). This list includes various subtypes of GABA transporters as well as those for all the above-mentioned neurotransmitters, except for glutamate. All of the members of this superfamily are dependent on so-

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dium and chloride and by analogy with the GABA transporter [14] are likely to cotransport their transmitter with both sodium and chloride. Interestingly sodium-dependent glutamate transport is not chloride dependent, but rather is countertransported with potassium [15,16]. In the last few months three distinct but highly related glutamate transporters have been cloned [17–19]. These transporters represent a distinct family of transporters.

2. THE SUBSTRATES OF THE L-GLUTAMATE TRANSPORTER

The mechanism of sodium-dependent L-glutamate transport has been studied initially using tracer flux studies employing radioactive glutamate. These studies indicated that the process is electrogenic, with the positive charge moving in the direction of the glutamate [15,20]. This observation suggested that it is possible to monitor L-glutamate transport electrically using the whole-cell patch-clamp technique [21]. This latter technique has the advantage that the membrane potential can be controlled throughout the transport experiments. It is reassuring that the results obtained with both techniques are in harmony with each other (reviewed in [5] and [22]), and they are summarized here briefly. In addition to L-glutamate, D- and L-aspartate are transportable substrates with affinities in the lower micromolar range. The system is stereospecific with regard to glutamate, the D-isomer being a poor substrate. Glutamate uptake is driven by an inwardly directed sodium ion gradient and at the same time potassium is moving outward. The potassium movement is not due to a passive movement in response to the charge carried by the transporter. Rather it is an integral part of the translocation cycle catalyzed by the transporter. Its role is further described below (section 4). Very recently evidence has been presented that another ionic species is countertransported (in addition to potassium), namely hydroxyl ions [23].

3. STOICHIOMETRY

The first-order dependence of the carrier current on internal potassium [24], together with the well-known first order dependence on external L-glutamate and sigmoid dependence on external sodium, suggest a stoichiometry of 3 Na^+:1 K^+:1 glutamate$^-$ [15,24]. This stoichiometry suggests that one positive charge is moving inward per glutamate anion entering the cell. If an hydroxyl anion is countertransported as well [23], the stoichiometry could be 2 Na^+:1 K^+:1 glutamate:1 OH^-, and transport would still be electrogenic. A stoichiometry of 2 Na^+:1 glutamate is also favored by direct experimental evidence obtained by kinetic [25] and thermodynamic methods [26].

4. BINDING ORDER OF THE SUBSTRATES: THE ROLE OF POTASSIUM

Just as influx of glutamate is coupled to efflux of potassium, efflux of glutamate can be induced by increasing external potassium [27]. This phenomenon is also believed to underlie brain damage during anoxia/ischemia. Under such pathological conditions the external potassium concentration rises and this causes release of glutamate by reversal of the glutamate transporter. If these increased extracellular glutamate levels persist, the neurons die probably because of excessive calcium influx through NMDA receptor channels. Radiotracer studies showed that there is another, potassium-independent, way to cause the efflux of radioactive L-glutamate, namely by addition of unlabelled external L-glutamate (exchange [16]). This suggests that the glutamate translocation step is distinct from that of potassium. In other words it consists of two stages: (1) translocation of sodium and L-glutamate, and (2) reorientation of the binding sites upon binding and translocation of potassium [16]. The binding order of the substrates was probed by comparing the ion-dependence of net flux with that of exchange (or counterflow) [16,28]. According to the model (Fig. 1), during influx (steps 1–8) the two or three sodium ions (see section 3) bind first followed by one glutamate anion. This results in the formation of the translocation complex. Upon translocation of the two substrates to the inside, glutamate is released first followed by sodium (although a random order on the inside cannot be rigorously excluded). Subsequently potassium binds on the inside. Upon translocation of the potassium-loaded transporter to the outside, the potassium is released and a new influx cycle can commence [28]. Efflux of glutamate operates by a reversal of these steps (going clockwise). Hydroxyl anions could be translocated concomitant with glutamate (steps 2–4) or with potassium (steps 6–8). In addition it appears that internal potassium is required for the optimal functioning of the transporter, probably by an allosteric mechanism [28].

5. REGULATION OF TRANSPORTER ACTIVITY

Termination of glutamergic transmission is a key process which is likely to be subject to regulation. Thus far our knowledge on this is fragmentary. It has been shown that arachidonic acid can inhibit glutamate transport [29,30]. Furthermore, short-term exposure of glial but not of neuronal cells to phorbol esters stimulates glutamate transport about 2 fold [31]. This effect is blocked by protein kinase C inhibitors, suggesting that it is a consequence of phosphorylation of the transporter. At this time we can only speculate on a sequence of events which makes physiological sense. The cells containing the transporter must somehow sense that extracellular glutamate is too high, e.g. by the

metabotropic receptor. At the fine glial processes which are located in close proximity to the synapse, glutamate could activate its metabotropic receptor leading to generation of diacylglycerol, which in turn can activate protein kinase C. This causes increased transporter activity and thus more efficient removal of the transmitter.

6. RECONSTITUTION AND PURIFICATION

A sodium- and potassium-coupled L-glutamate transporter from rat brain has been purified to near homogeneity using reconstitution of transport as an assay [32]. The properties of the pure transporter are fully preserved. They include ion dependence, electrogenicity, affinity, substrate specificity, and stereospecificity. SDS-PAGE revealed one main band with an apparent molecular mass of around 80 kDa and a few minor bands. Later the apparent molecular mass of the main band was corrected to 73 kDa [33]. Comparison of polypeptide composition with L-glutamate transport activity throughout the fractionation procedure reveals that only the 73 kDa band can be correlated with activity. Polyclonal antibodies against the 73 kDa band were able to selectively immunoprecipitate the 73 kDa polypeptide as well as most of the L-glutamate transport activity, as assayed upon reconstitution, from crude detergent extracts of rat brain membranes [33]. Thus, the 73 kDa polypeptide represents the L-glutamate transporter, a conclusion reinforced by its cloning [18].

Using the antibodies raised against the glutamate transporter, the immunocytochemical localization of the transporter was studied at the light and electron microscopic level in the rat central nervous system. In all regions examined (including cerebral cortex, caudatoputamen, corpus callosum, hippocampus, cerebellum, spinal cord) it was found to be located in glial cells rather than in neurons. In particular, fine astrocytic processes were strongly stained. Putative glutamatergic axon terminals appeared nonimmunoreactive [33]. The uptake of glutamate by such terminals (for which there is strong previous evidence) therefore may be due to a subtype of glutamate transporter different from the glial transporter. Using a monoclonal antibody raised against this transporter, a similar glial localization of the transporter was found [34].

7. MOLECULAR CLONING AND PREDICTED STRUCTURE OF THE TRANSPORTERS

Transporters for many neurotransmitters were cloned on the assumption that they are related to the GABA [9] and norepinephrine [10] transporters (reviewed in [11–13]). This approach was unsuccessful for the glutamate transporter. Very recently three different glutamate transporters were cloned using different approaches: GLAST [17], GLT-1 [18] and EAAC 1 [19]. The former two appear to be of glial [17,33], the latter

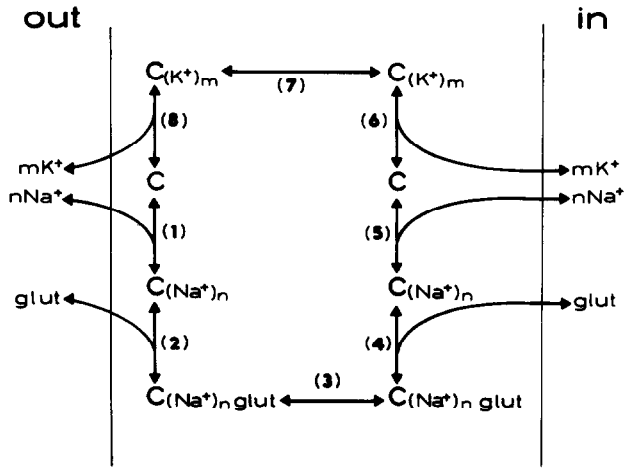

Fig. 1. A model for the mechanism of L-glutamate translocation. The details are described in the text. As indicated, $n = 3$ or 2, $m = 1$. Not indicated is the possible countertransport of one hydroxyl ion per glutamate. Reprinted with permission from Biochemistry 29 (1990) 11209–11214.

of neuronal origin [19]. Indeed, the three transporters are not related to the above superfamily [17–19]. On the other hand, they are very similar to each other (Fig. 2), displaying ~ 50% identity and ~ 60% similarity. They also appear to be related to the proton-coupled glutamate transporter from *E. coli* and other bacteria (**glt-P** [35]) and the dicarboxylate transporter (**dct-A** [36]) of *Rhizobium meliloti*. In these cases the identities are around 25–30%. Thus, they form a distinct family. They contain between 500–600 amino acids. GLT-1, which encodes the glutamate transporter which was purified [18,32,33], has 573 amino acids and a relative molecular mass of 64 kDa, in good agreement with the value of 65 kDa of the purified and deglycosylated transporter [33]. Hydrophaty plots are relatively straightforward at the amino terminal side of the protein and the three different groups have predicted 6 transmembrane α-helices at very similar positions (Fig. 2 and [17–19]). On the other hand, there is much more ambiguity at the carboxyl side where 0 [17], 2 [18] or 4 [19] α-helices have been predicted. However, all three groups note uncertainty in assigning transmembrane α-helices in this part of the protein, taking into account alternative possibilities including membrane spanning β-sheets [17]. It is clear that experimental approaches to delineate their topology are badly needed. The most similar part of glt-P is also included in the alignment. In this stretch, out of 98 amino acids, 36 are identical in all 4 transporters and another 18 are similar. By themselves the three neurotransmitter transporters display a ~ 75% identity and close to 90% similarity in this stretch. Especially striking is the stretch of 7 amino acids **AAI(V)FIAQ** (407–413, GLT-1 numbering). It is tempting to speculate that the only polar residue, glutamine (**Q**), could be involved in glutamate binding. The pres-

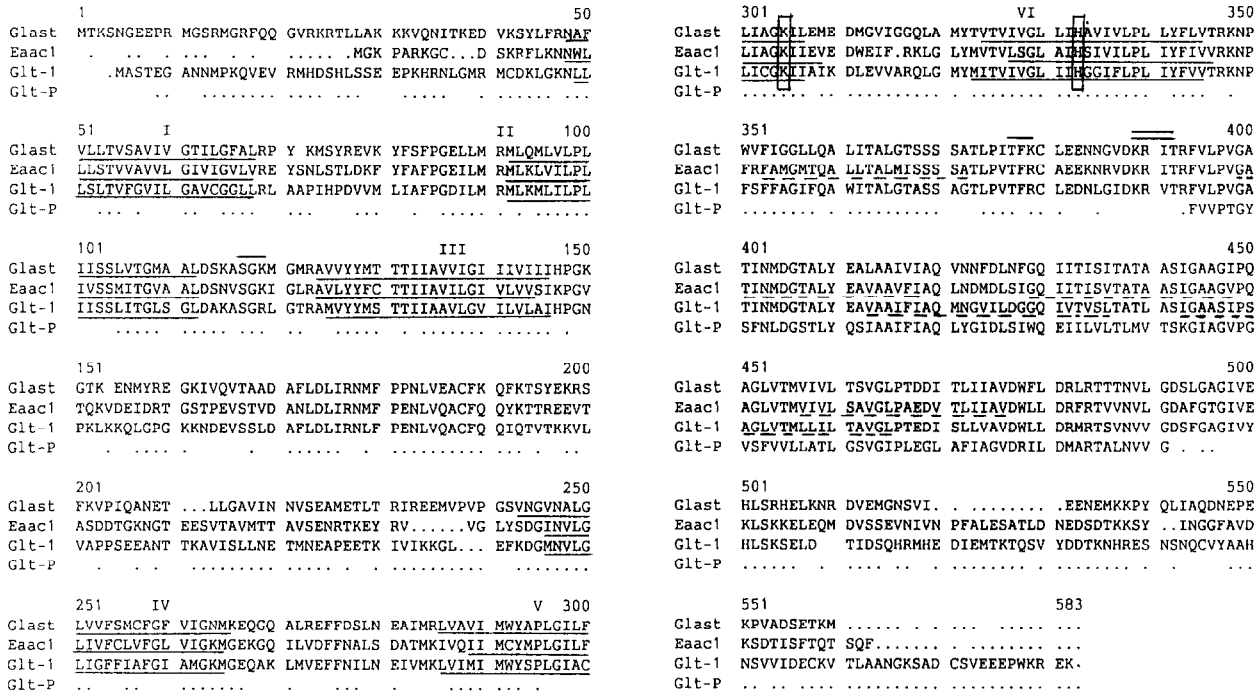

Fig. 2. Alignment of glutamate transporter sequences. Sequences are taken from the following references: **GLAST** [17]; **EAAC 1** [19], **GLT-1** [18] and **glt-P** [35]. Two sequencing errors discovered after the publication of GLT-1 [18] have resulted in a correction of the amino acid sequence, between amino acids 260 and 289, as shown in the figure. Only the part of glt-P which is most homologous to the three neurotransmitter transporters, is displayed. The first six putative transmembrane α-helices, as assigned in the original publications, are underlined by bars. The other assigned helices, which are ambiguous, are underlined by interrupted bars. Gaps are introduced to optimize identical sequence positions. Putative phosphorylation sites, conserved in the three neurotransmitter transporters, are overlined once for protein kinase C sites and twice for the protein kinase A site. The conserved charged amino acids, lysine in helix 5 and histidine in helix 6, are boxed.

ence of charged amino acids, located in the membrane and conserved in the three transporters, e.g. lysine in helix 5 and histidine in helix 6 (Fig. 2), may point to their importance. However, this will have to be clarified by site-directed mutagenesis. One of the proposed models (GLT-1, [18]) is shown here (Fig. 3) to point out some other structural features. These include potential glycosylation sites in the large extracellular loop between helices 3 and 4 and putative protein kinase A and C phosphorylation sites. Two of the putative protein kinase C phosphorylation sites and a potential protein kinase A site are conserved in all three transporters (overlined in Fig. 2). This could hint that one or both of them are used physiologically. On the other hand the observation that phorbol esters activate glutamate transport in glial but not in neuronal cells [31] could argue against this. Again, site-directed mutagenesis studies are likely to clarify this issue.

In addition to the heterogeneity of glutamate transporters revealed by molecular cloning, recent pharmacological studies also provide evidence for this [37–40]. A careful pharmacological characterization of the three cloned glutamate transporters should indicate if there are more of these transporters yet to be cloned. Furthermore, it is likely that the new family will not necessarily be limited to glutamate transporters, but includes others

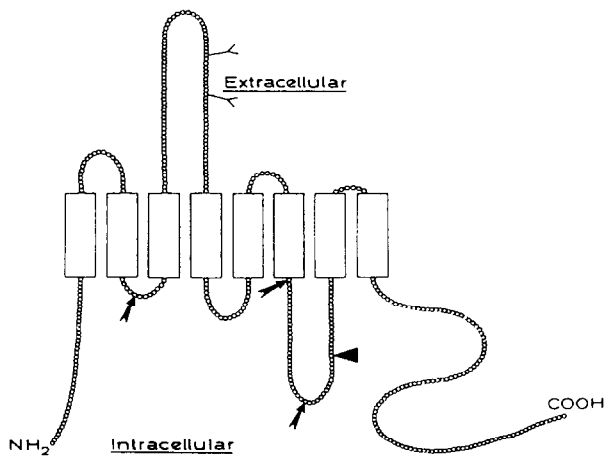

Fig. 3. Schematic representation of the L-glutamate transporter GLT-1 showing its proposed orientation in the plasma membrane. Putative transmembrane segments are shown as rectangles. One putative protein kinase A and three putative protein kinase C phosphorylation sites are indicated by an arrowhead and arrows, respectively. The remaining two potential protein kinase C sites in the putative extracellular loop between helices 3 and 4 are not shown. The two putative glycosylation sites are also located on this loop and are indicated as branched lines. Adapted from Nature 360 (1992) 464–467.

transporting different neurotransmitters or other solutes.

8. CONCLUSIONS

Recent progress in molecular cloning of glutamate transporters has revealed that, contrary to expectations by many, they do not belong to the recently characterized superfamily of neurotransmitter transporters. The members of the latter family are sodium- and chloride-coupled. On the other hand, the family of glutamate transporters are coupled to sodium and potassium, and this should of course be reflected in structural differences. A major challenge is to reveal the structural basis of this difference in ion specificity as well as other features, including substrate binding and regulatory domains. Other issues to be solved are identification of other family members and the topology of the transporters. The powerful combination of biochemical and molecular biological approaches should be able to shed light on many of these issues.

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