

MINIREVIEW

High Affinity Glutamate Transporters: Regulation of Expression and Activity

GEORGI GEGELASHVILI and ARNE SCHOUSBOE

PharmaBiotec Research Center, Department of Biological Sciences, Royal Danish School of Pharmacy, DK-2100 Copenhagen, Denmark

Accepted March 31, 1997

SUMMARY

L-Glutamic acid is a major excitatory neurotransmitter in the mammalian central nervous system. The termination of the glutamatergic transmission and the clearance of the excessive, neurotoxic concentrations of glutamate is ensured by a high affinity glutamate uptake system. Four homologous types of Na/K-dependent high affinity glutamate transporters, glutamate/aspartate transporter, glutamate transporter 1, excitatory amino acid carrier 1, and excitatory amino acid transporter 4, have recently been cloned and were assigned to a separate gene family, together with two neutral amino acid carriers, alanine/serine/cysteine transporter 1/serine/alanine/threonine transporter and adipocyte amino acid transporter. The genomic organization of these transporters is still under investigation. Very little is known about the nature of the factors and molecular mechanisms that regulate developmental, regional, and cell type-specific expression of the glutamate transporters and

their aberrant functioning in neurodegenerative diseases (e.g., amyotrophic lateral sclerosis and Alzheimer's disease). Some experimental conditions (e.g., ischemia, corticostriatal lesions, hyperosmolarity, culturing conditions) and several naturally occurring and synthetic compounds (e.g., glutamate receptor agonists, dopamine, α_1 - and β -adrenergic agonists, cAMP, phorbol esters, arachidonic acid, nitric oxide, oxygen free radicals, amyloid β -peptide, tumor necrosis factor- α , glucocorticosteroids, unidentified neuronal factors) affect the molecular expression and activity of glutamate transporters. Further elucidation of the molecular events that link epigenetic signals with transcriptional and post-transcriptional mechanisms (e.g., alternative splicing, translation and post-translational modifications) is crucial for the development of selective pharmacological tools and strategies interfering with the expression of the individual glutamate transporters.

L-Glutamic acid is an important nutritional substance involved in several classical biochemical pathways, including ammonia detoxification and gluconeogenesis. However, in the nervous system, L-glutamate plays an even more important role as a chemical transmitter of excitatory signals, a specific function that is being intensely investigated in current neuroscience. Neurotransmitter glutamate is synthesized and stored in specialized glutamatergic neurons and released upon different stimuli into the synaptic cleft. An array of different classes of glutamate receptors situated on the pre- and postsynaptic membranes, and also on astroglia, further transduces integrated signals using increased ion fluxes and second messenger pathways. The termination of

the glutamatergic transmission occurs via clearance of glutamate by a special uptake mechanism, which uses the Na^+ , K^+ electrochemical gradient as a driving force. It consists predominantly of the neuronal and astroglial sodium-dependent high affinity glutamate transporter proteins. The mechanism is capable of translocating the neurotransmitter against its several thousand-fold concentration gradient and thus keeps the resting glutamate concentration in the synaptic cleft below the level ($<1 \mu\text{M}$) that activates glutamate receptors (1–3). However, mounting evidence suggests that the high affinity glutamate transport system, besides maintaining the high signal-to-noise ratio, may play a more active and direct role in glutamatergic signaling mechanisms.

Glutamatergic transmission is believed to be involved in several important brain functions, such as learning and memory (4). However, at elevated extracellular concentra-

This work was supported by the Lundbeck Foundation, the Danish Biotechnology Program, and a guest professorship to G.G. from the Royal Danish School of Pharmacy.

ABBREVIATIONS: ALS, amyotrophic lateral sclerosis; EAAC1, excitatory amino acid carrier; GLAST, glutamate/aspartate transporter; GLT1, glutamate transporter 1; EAAT4, excitatory amino acid transporter 4; ASCT1, alanine/serine/cysteine transporter 1; SATT, serine/alanine/threonine transporter; AAAT, adipocyte amino acid transporter; NMDA, *N*-methyl-D-aspartic acid; PKC, protein kinase C; PKA, protein kinase A; dBcAMP, N^6 , 2'-O-dibutyryl adenosine-3',5'-cyclic monophosphate; AA, arachidonic acid.

tions, glutamate may act as a powerful neurotoxin capable of inducing severe excitotoxic damage of target neurons (5), a mechanism implicated in several neurodegenerative diseases (e.g., ALS, Huntington's disease, and probably Alzheimer's disease) and brain insults (e.g., ischemia, hypoxia, hypoglycemia, status epilepticus) (6–8). The effective removal/uptake of excessive glutamate thus seems to be a crucial rescue mechanism, and failure or loss of the glutamate transport system may aggravate neurotoxic damage. For example, if glutamate uptake is blocked, as little as 1 μM exogenous glutamate is sufficient to induce excitotoxic death in cortical neurons (9). Furthermore, under conditions of energy failure (ischemia, hypoglycemia), when the electrochemical gradient is dramatically reduced or disrupted, glutamate transporters may function in a reversed mode (i.e., carry glutamate from cytoplasm to the exterior) and thus become major contributors to the excitotoxic levels of glutamate (3). The reduced expression of one of the transporter types after global transient ischemia seems to be another significant mechanism for the elevation of the extracellular glutamate concentration (10). A loss of the same glutamate transporter (GLT1, see below) has recently been documented as a major cause of the neurodegenerative processes in sporadic forms of ALS (11). The *in vivo* knockout of the astroglial glutamate transporters resulted in elevated extracellular glutamate levels, excitotoxic neurodegeneration, and progressive paralysis, whereas loss of the neuronal transporter led to epileptic seizures (12). On the other hand, an excessive uptake of glutamate may result in glutamatergic hypofunction, one of the mechanisms suspected to be involved in the development of schizophrenia and other psychoses (13).

The emerging understanding of the significant contribution of glutamate carriers to the development of different neurological disorders, as well as understanding of their ability to attenuate the glutamate neurotoxicity, has rapidly turned this transport system into a desirable target for pharmacological intervention. Some aspects of this problem, especially the mechanism of action and kinetic characteristics of glutamate transporters, as well as their distribution, have recently been discussed in several review articles (14–17) and therefore will not be addressed herein. Rather, the present article is an attempt to overview the recent advances in molecular cloning and characterization of the high affinity glutamate

transporters with a special emphasis on the endogenous and pharmacological regulation of their expression and activity.

Molecular characterization of the sodium-dependent high affinity glutamate transporters

A novel gene family of high affinity glutamate transporters. The dramatic increase in information on practically all aspects of high affinity glutamate transport was initiated by the nearly simultaneous cloning of three eukaryotic glutamate carriers: EAAC1 from rabbit intestine (18), GLAST (19), and GLT1 from rat brain (20). The corrected or variant sequences for the same transporters and their homologues in different species were subsequently published (14, 21–38). Recently, a fourth type of high affinity glutamate transporters, human EAAT4 was cloned (39) (Table 1)

Analysis of the deduced amino acid sequence revealed 38–65% identity among the four different transporter types and a very high sequence conservation ($\geq 90\%$ identity) among the species homologues (Table 2). The sequences exhibit no significant resemblance with other mammalian Na^+ - and Cl^- -dependent neurotransmitter transporters or with any other eukaryotic protein sequence deposited in genetic data bases, but they display 25–32% homology with some bacterial dicarboxylic acid carriers (16). Thus, these glutamate carriers, together with the recently cloned human and murine neutral amino acid transporters ASCT/SATT (40–42) and AAAT (43), which have 31–44% identity with the eukaryotic glutamate transporters (Table 1), could be considered a separate gene family.

Chromosomal localization of the glutamate transporter genes. Each of the four types of eukaryotic glutamate transporters is encoded by a distinct gene. However, the production of variant forms due to the differential usage of certain exons cannot be excluded. Very little is known about the genomic structures of these carriers (Table 1). The human GLAST gene stretches over 85 kilobases and contains 10 exons (38). The eight coding exons of the human ASCT1 gene are distributed over 40 kilobases (42) (for more details on the gene promoter region, see Endogenous and pharmacological regulation of the expression of high affinity glutamate transporters). Chromosomal loci for several members of the gene family have recently been established (Table 1), and hypotheses concerning the linkage of these sites with the pathology-

TABLE 1

The gene family of Na^+ -dependent high-affinity glutamate/aspartate and neutral amino acid transporters: basic molecular characteristics of the cloned members.

Transporter	Species	Polypeptide, size (deduced number of amino acids)	mRNA, size (kilobases)	Chromosome/gene loci	Exons
GLAST	Human	542 (26, 27, 38)	4.2 (27, 38)	5p13 (30, 44); 5p11–p12 (38)	10 (38)
	Bovine	542 (33)	3.9 (22)	ND	ND
	Rat	543 (19, 22)	3.9 (22); 4.5 (19); 4.7 (96)	ND	ND
	Mouse	543 (21)	4.6 and 3.0 (21)	15 (30)	ND
GLT1	Human	574 (27); 565 (26)	10 (27, 47)	11p13–p12 (36)	ND
	Rat	573 (14, 20); 453 (23)	11 (20)	ND	ND
	Mouse	572 (34); 573 (29); 574 (35)	10 (29); 12 (34)	2 (29)	ND
	Human	524 (28); 525 (31)	3.5 (28); 3.8 (27, 48); 3.8 and 2.4 (47)	9p24 (32)	ND
EAAC1	Rabbit	524 (18)	3.5 and 2.4 (18)	ND	ND
	Rat	523 (37)	4.4 and 2.8 (48)	ND	ND
	Mouse	523 (34)	4.3 and 2.7 (34)	ND	ND
	Human	567 (39)	2.4 (39)	ND	ND
EAAT 4	Human	567 (39)	2.4 (39)	ND	ND
ASCT/SATT	Human	524 (40); 529 (41)	4.8, 3.5, 2.8, and 2.2 (40)	2 p13–p15 (42)	8 (42)
AAAT	Mouse	553 (43)	2.7 (43)	ND	ND

Amino acid sequence identities (%) among the eukaryotic Na-dependent high affinity glutamate/aspartate and neutral amino acid transporters

	GLAST	GLT1	EAAC1	EAAT4	ASCT/SATT	AAAT
GLAST	96 ^a	46–52	51–56	61–65	40–44	39–42
GLT1		92.5 ^a	49.5–54	38–41	37–41	38–42
EAAC1			90 ^a	41–48	32–39	31–37
EAAT4				100	42	41
ASCT/SATT					99–100	56
AAAT						100

associated loci have been forwarded. Thus, the *Eaat2* locus of the mouse GLT1 gene on chromosome 2 seems to be in the vicinity of several sites (*fi*, *anx*, *Aw2*, *El2*) associated with disorders in the nervous system (e.g., malformation of some cerebellar structures, hyperactivity, tremor, anorexia, alcohol withdrawal-associated neuroexcitability, epilepsy) (29). The human GLT1 gene is assigned to bands p12-p13 of chromosome 11 and thus may be excised in the aniridia-Wilms' tumor-associated deletion that occurs in the 11p13 region (36). The human GLAST gene, mapped to 5p11-p12 (38) or the 5p13 region (44), is situated in the short arm of chromosome 5, which encloses segments deleted in *cri du chat* syndrome (characterized by mental retardation) and is also linked to a predisposition to schizophrenia (44). Linkage of the human EAAC1 gene, mapped to chromosome 9 band q24, with dicarboxylic aciduria and some neurological disorders has also been proposed (32).

Hydropathy plots of the amino acid sequences revealed several well conserved segments believed to form transmembrane α -helices. However, the exact number of the membrane-spanning domains, 10 as proposed by Kanai and He-

The serine-rich motif is a highly conserved segment represented with two to four consecutive serine residues in EAAC1 [four residues, 331–334 (18)], GLAST [four residues, 368–371 (19)], EAAT4 [four residues, 388–391 (39)], AAAT [four residues, 363–366 (43)], SATT [three residues, 341–343 (40)], and GLT1 [two residues, 362–363 (34)]. Interestingly, there is another serine triplet in the SATT sequence (residues 199–201), although it is less conserved among the glutamate transporters. The serine clusters may represent substrate-binding sites by analogy with some receptors. However, depending on the model accepted, the serine cluster is either situated within one of the extracellular loops (18) or appears in the cytoplasmic compartment (20).

Several consensus sequences for phosphorylation by protein kinase C and protein kinase A were identified in all cloned glutamate transporters. Regardless of the model proposed, there is one highly conserved site for phosphorylation by PKC in the first intracellular loop present in all transporters. So far, this is the only site shown to be phosphorylated and, thus, to affect the efficacy of the glutamate uptake by the carrier (Ser113 in GLT1) (45). Furthermore, the GLT1

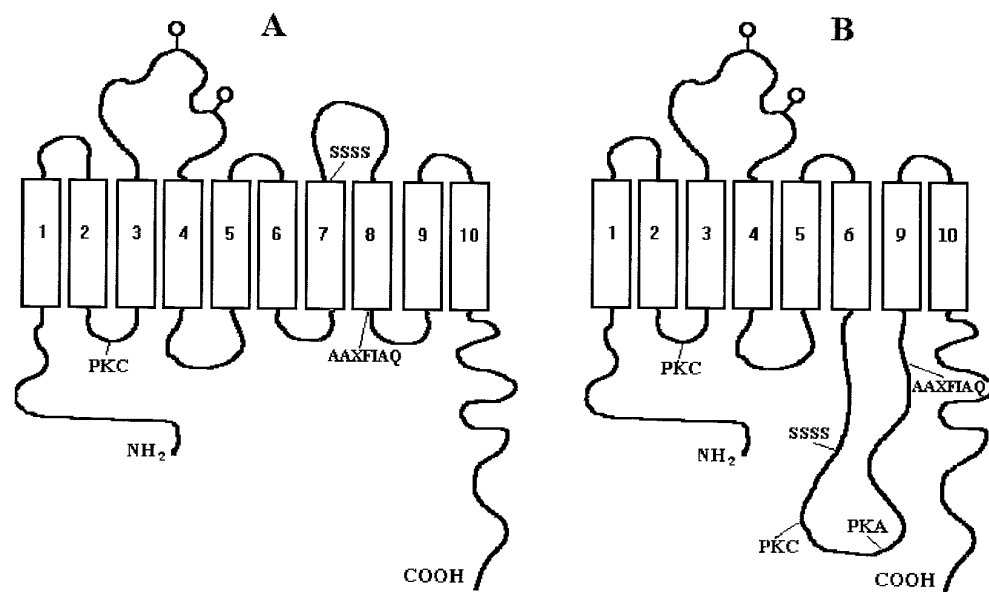


Fig. 1. Topological models for the cloned glutamate transporters proposed by Kanai and Hediger (18) (A) and Pines *et al.* (20) (B). *Rectangles*, predicted transmembrane domains; *upper loops*, extracellular domains. Amino and carboxyl termini and lower loops are located in the cytoplasmic compartment. Only the conserved sites for phosphorylation by cAMP-dependent PKA and PKC, as well as the serine rich motif (SSSS) and the AAXFIAQ motif are indicated. *Hinges*, putative *N*-glycosylation sites in the second extracellular loop. Modified from Kanai *et al.* (16).

and GLAST sequences enclose one additional PKC phosphorylation site in the third intracellular loop, and EAAC1 possesses one analogous site in the second intracellular loop and two in the last cytoplasmic domain represented by the carboxyl-terminal portion of the molecule. The model that suggests eight membrane-spanning domains (20) reveals two additional PKC- and PKA-specific phosphorylation sites located in the third intracellular loop, which are present in all glutamate transporters (Fig. 1). It is noteworthy that some species homologues contain extra, evolutionarily less conserved phosphorylation sites, and several consensus sequences that favor the phosphorylation by different kinases can be found in the putative extracellular domains. The functional significance or even *in situ* occurrence of the ectophosphorylation mechanisms is, however, still debatable.

The second extracellular loop (mentioned in both models) contains two evolutionarily conserved NXS/T consensus sequences for N-linked glycosylation, shared by all members of the glutamate transporter gene family. EAAT4 possesses a third N-glycosylation site in the same domain (39). Rat and mouse EAAC1 sequences also possess one additional glycosylation site in the second extracellular loop, whereas the human and rabbit EAAC1 sequences lack this particular site but have an extra NXS/T consensus triplet in the first extracellular domain (37).

The functional significance of the evolutionarily preserved sites and domains has yet to be deciphered, although they likely underlie the common functional principles among all members of the glutamate transporter gene family. However, very little is known about the structural basis of the probable functional diversity of these transporter molecules, crucial information urgently needed for the design of transporter type-specific pharmacological tools (e.g., inhibitors/activators). Studies using site-directed mutagenesis and high resolution crystallographic analysis of the tertiary structure may shed more light on this issue.

Endogenous and pharmacological regulation of the expression of high affinity glutamate transporters

Regulation of transcription, mRNA processing, and translation. Information on the elements regulating the glutamate transporter gene transcription is limited. Recently, it has been established that the human GLAST (EAAT1) gene is organized in 10 exons, but the promoter region has not been characterized (38). However, in a previous study concerning the neutral amino acid transporter ASCT1, which belongs to the same family of high affinity amino acid transporters, the exon/intron arrangement and functional 5' end elements of the corresponding gene were revealed (42). The promoter region seemed to be devoid of the well defined *cis* elements (e.g., TATA and CAAT boxes) but contained at least five consensus sequences for the Sp1 transcription factor and two potential recognition sites for the *Egr* gene family of transcription factors (*krox24*, *krox20*, *Egr3*, *NGFI-C*), which are known to be involved in cell differentiation processes. Moreover, the ASCT1 gene promoter region exhibits the pattern of GC-rich repeats typical for the most early growth response genes (e.g., *Egr* family, *jun* D). Taking into account the considerable phylogenetic relationship among the three glutamate transporters and ASCT1/SATT (e.g., 39–44% amino acid sequence identity, similar hydropathy profiles and presumed *trans*-membrane organi-

zation, conservation of the crucial function-related motifs, several matching exon splice sites in GLAST and ASCT1) (38), it is likely that similar general design (e.g., exon/intron structure) and regulation may be characteristic of other high affinity glutamate transporter genes as well. This suggestion may primarily apply to EAAC1, which, like ASCT1, exhibits a wide tissue distribution and is probably regulated by the similar "housekeeping" gene-promoter types. In addition, recent data hint at the likely presence of the tonicity-responsive element within the EAAC1 gene promoter (46).

Very little is known about the factors that regulate the primary processing and the further fate of the glutamate transporter mRNAs. The above-postulated intron/exon structure for the glutamate transporter genes suggests differential excision/splicing of certain exons and, thus, the generation of variant mRNA species and their translation products. Several mRNA size classes encoding EAAC1 and GLAST (mGluT) have been detected (18, 21, 34, 47, 48; Table 1). Although this heterogeneity was primarily attributed to the differential polyadenylation, the occurrence of mRNA species with diverse coding capacity cannot be excluded. Moreover, in one study, the existence of additional GLT1 subspecies was shown (23). The occurrence of several closely related molecular forms within each glutamate transporter prototype may further contribute to the refined tuning of the glutamate uptake. Several studies have demonstrated that the steady state levels of functional glutamate transporters do not necessarily correlate with the levels of corresponding mRNAs (Table 3). For example, GLT1 mRNA was detected in some hippocampal neurons, whereas no corresponding protein could be found in the same cells (49). In ALS patients, dramatic loss of GLT1 was not matched by down-regulation of the corresponding mRNA (11). In amino acid-deprived NBL-1 cell cultures, the dramatic rise in EAAC1 content and activity was accompanied by significant decrease in the EAAC1 mRNA levels, followed by the rapid up-regulation and the restoration of the basal steady state level of this transcript (50). In contrast, transient ischemia caused nearly synchronous down-regulation of GLT1 and corresponding mRNA in rat hippocampus (10). Glutamate and kainate dramatically up-regulated the expression of GLAST protein in cultured astrocytes, whereas no apparent changes in the corresponding mRNA levels could be detected (51). In contrast, dBcAMP seemed capable of up-regulating both GLAST and its corresponding mRNA. Moreover, in dBcAMP-treated cultures, the glutamate receptor agonist *t*ACPD stimulated a further increase in GLAST mRNA levels but did not alter the corresponding protein levels (51). These results suggest that the metabolic turnover rates of glutamate transporters (i.e., translation versus degradation rates) and turnover rates of the corresponding mRNAs (transcription versus degradation) do not necessarily change in concert and are probably regulated differentially depending on cell phenotype, environmental cues, and signaling pathways used. For example, factors that specifically increase the GLT1 mRNA lability seem to be up-regulated in neural cells that express proliferative/invasive phenotype (e.g., in brain tumors) (48). Differential polyadenylation as well as the specific regulatory sequences that reside in the 3'-untranslated regions of the glutamate transporter mRNAs may control the degradation rates of these transcripts. It is noteworthy that the remarkable size of the GLT1 mRNA (10–11 kilobases) (20) is pri-

TABLE 3

Effect of different substances and experimental conditions on the expression and activity of the Na⁺-dependent high-affinity glutamate transporters

Substance/condition	Species/tissue/cell type/system	Transporter	Activity	Protein	mRNA	Reference
dBcAMP	Mouse cortical astrocytes	GLAST	↑↑	↑↑	↑	(51, 52)
Glutamate	Mouse cortical astrocytes	GLAST	↑↑	↑↑	NC	(51)
Kainate	Mouse cortical astrocytes	GLAST	↑↑	↑↑	NC	(51)
	Rat striatal synaptosomes	ND	↑	ND	ND	(65)
dBcAMP + AMPA	Mouse cortical astrocytes	GLAST	NE ^a	↑ ^a	NE ^a	(51)
dBcAMP + tACPD	Mouse cortical astrocytes	GLAST	NE ^a	NE ^a	↑ ^a	(51)
AP3	Rat striatal synaptosomes	ND	↓	ND	ND	(65)
PCP	Rat striatal synaptosomes	ND	↓	ND	ND	(65)
DOPA	Rat striatal homogenates	ND	↓	ND	ND	(66)
Phenylephrine	Rat cortical astrocytes	ND	↑	ND	ND	(67)
Isoproterenol	Rat cortical astrocytes	ND	↓	ND	ND	(67)
AA	Xenopus oocyte	h GLAST	↓	ND	ND	(73)
		h GLT1	↑	ND	ND	(73)
Nitric oxide	Rat synaptosomes	ND	↓	ND	ND	(80)
	Mouse cortical astrocytes	ND	NE	ND	ND	(71)
Free oxygen radicals	Rat and mouse cortical astrocytes	ND	↓	ND	ND	(71, 83)
Amyloid β peptide	Rat cortical astrocytes	ND	↓	ND	ND	(88)
Phorbol ester (TPA)	Rat C6 glioma cells	ND (EAAC1 ?)	↑	ND	ND	(45)
Dexamethasone	Rat hippocampal astrocytes	ND	↓	ND	ND	(94)
Wnt1 protein product	Rat PC12 cells	GLAST	↑	ND	↑	(96)
TNF _α	Human fetal astrocytes	GLAST and EAAC1	↓	NE	NE	(95)
Neuronal factors	Mouse cerebellar astrocytes	ND	↑	ND	ND	(56)
	Rat cortical astrocytes	GLT1 and GLAST	↑	↑	↑	(98)
Horse serum	Mouse cerebellar astrocytes	ND	↑	ND	ND	(57)
Electric stimulation	Rat frontal cortex	ND	↑	ND	ND	(66)
Ischemia	Rat hippocampus	GLT1	ND	↓	↓	(10)
Corticostratial lesions	Rat neostriatal homogenate	GLT1 and GLAST	↓	↓	ND	(61)
Corticostratial and fimbria-fornix lesions	Rat hippocampal and striatal homogenates	GLT1 and GLAST	↓	↓	ND	(62)
Hypertonic stress	Bovine renal NBL-1 cell line	EAAC1	↑	ND	↑	(46)
Amino acid deprivation	Bovine renal NBL-1 cell line	EAAC1	↑	↑	↓↑	(50)

ND, not determined; NE, no effect; TPA, O-Tetradecanoylphorbol-13-acetate; TNF, tumor necrosis factor.

^a Relative to dBcAMP-treated control.

marily due to an unusually large noncoding portion (approximately 8–9 kilobases), which probably encloses a stability-regulating element. However, these suggestions remain speculative, and the precise molecular events involved in the utilization rates of glutamate transporter mRNAs have yet to be explored.

Regulation via post-translational modifications. The two types of post-translational modifications of glutamate transporters so far documented, phosphorylation and glycosylation, may play regulatory roles in the brain. Thus, short term exposure of astrocytes or C6 astrocytoma cells to phorbol esters, activators of protein kinase C, results in significant enhancement of glutamate uptake and in increased phosphorylation of glutamate transporter GLT1 at the conserved serine 113 residue (45). So far, PKA-dependent phosphorylation of glutamate transporters at the conserved consensus site (14) and its possible functional relevance has not been reported. Exposure to dibutyryl cAMP dramatically up-regulates the glutamate uptake activity in cultured astroglia (52), but the effect seems to be mainly the result of increased production of the transporter molecules (51).

N-Glycosylation of GLAST had no impact on the kinetic characteristics of the transporter when expressed in *Xenopus* oocytes (53), but in another recent study, the glycosylation of

EAAC1 seemed to be essential for the expression of transporter activity (46). No signaling mechanisms that regulate the extent of the glycosylation have yet been identified.

Regulation by activity of glutamatergic neurotransmission. How do the changes in the glutamate concentration in extracellular milieu affect the high affinity glutamate transport capacity of neural cells? Several types of metabotropic and ionotropic glutamate receptors are expressed in neurons and astrocytes (54, 55), and their possible involvement in the regulation of glutamate uptake is an obvious issue. More than a decade ago, Schousboe and co-workers (56) reported the up-regulation of the glutamate uptake in astroglial cultures supplemented with conditioned media from neuronal cultures, an observation subsequently confirmed by others (57). The magnitude and specificity of the effect seemed to be dependent on the regional origin of the cultures used. It has been proposed that some unidentified substance(s) secreted by neurons, including glutamate, could enhance the apparatus for glutamate uptake in astrocytes. Recent studies have provided further evidence that glutamate is capable of modulating the quantity of glutamate transporters in neural cells as well as mediating biochemical modifications of the existing transporter molecules. A more than 70% decrease in D-aspartate uptake in striatum 10 days

after cortical lesions was observed by Shifman (58). However, it seemed that the application of ganglioside GM1 after the lesions was capable of preserving the D-aspartate transport activity, despite the dramatic loss of glutamatergic synapses. Therefore, it could be assumed that at least the portion of the transporter activity that decreased for several days after the lesions was not of synaptic origin. Thus, the reduction of the uptake capacity might occur in nonlesioned cells (e.g., astroglia) because of the chronic deficit of glutamate or, possibly, other factors that are normally secreted by the glutamatergic neurons. Such a mechanism could also be responsible for the down-regulation of glutamate uptake seen in earlier studies using axotomy of glutamatergic neurons (59, 60). More recently, two groups independently demonstrated that the disruption of cortical glutamatergic pathways led to selective although transient down-regulation of two glial glutamate transporters GLAST and GLT1 but not of the neuronal transporter EAAC1 in rat striatum and hippocampus (61, 62). On the other hand, transient ischemia, a condition resulting in a severalfold, long-lasting elevation of the extracellular glutamate concentration (63), has been shown to cause up-regulation of glutamate/aspartate transport sites (64). These studies suggested that glutamate and/or other factors released from glutamatergic nerve endings may be capable of affecting the levels and kinetic characteristics of glutamate transporters in surrounding cells, most likely via receptor-mediated signaling.

In support of this concept, long term treatment of astroglial cultures with glutamate or kainate seemed to stimulate D-aspartate uptake in a dose-dependent manner, a phenomenon paralleled by significant up-regulation of the GLAST protein content. Moreover, in dBcAMP-treated astrocytes, which acquired the differentiated stellate phenotype characterized with higher glutamate uptake capacity (52), the agonists of two distinct glutamate receptor classes, (R, S)- α -amino-3-hydroxy-5-methylisoxazole-4-propionic acid and *trans*-(1S, 3R)-1-amino-1,3-cyclopentanedicarboxylic acid, induced a further up-regulation of GLAST and of the corresponding mRNA, respectively (51). These results favor the idea that glutamate may serve as a primary signal for the regulation of GLAST expression in astroglial cultures, which are practically devoid of other neuronal factors. This notion, however, does not exclude the possibility of glutamate receptor agonist-stimulated induction and release of astroglial factors, which in turn affect the levels of the glutamate transporter via secondary signaling cascades. Any scenario, however, should consider the glutamate receptor stimulation at the start and the activation of transcriptional and/or post-transcriptional mechanisms culminating in *de novo* synthesis of GLAST. Obviously, it is tempting to suggest that glutamate receptor-mediated up-regulation of glutamate transport in astroglial cultures represents a regulatory feedback loop that occurs in the brain.

A study by Saransaari and co-workers (65) has shed more light on the involvement of glutamate receptors in the fast regulation of the high affinity glutamate transporter activity. Phencyclidine, a noncompetitive antagonist of NMDA receptors, slightly reduced the glutamate uptake rate in crude synaptosomes from mouse cerebral cortex, whereas an antagonist of metabotropic glutamate receptors, (R, S)-2-amino-3-phosphonopropionic acid, exhibited more pronounced inhibition (approximately, 25%). In contrast, kainate, a selective nontransportable inhibitor of glial GLT1/EAAT2 transport-

ers (27), stimulated high affinity glutamate uptake in the crude synaptosomal preparation, which probably contained a substantial portion of glial elements as well. These effects likely involve relatively fast direct modifications of the existing glutamate transporters rather than the changes in their number. Glutamate and other glutamate receptor agonists are indeed capable of mediating the intracellular processes known to interfere with glutamate transporter activity (e.g., production of nitric oxide, arachidonic acid, production of diacylglycerol and subsequent activation of protein kinase C, etc.). However, these events may be regulated by other neurotransmitters as well. Therefore, for the sake of clarity, these aspects of the glutamate transporter regulation are discussed in separate sections.

Biogenic amines and related second messenger pathways. Several biogenic amines used in the nervous system as neurotransmitters affect high affinity glutamate uptake. Dopamine inhibits glutamate carrier activity in rat striatal homogenates and at a lower concentration offsets, probably via D₂ dopamine receptors, the glutamate transporter activation induced by *in vivo* electric stimulation of the frontal cortex (66). Lesions of dopaminergic pathways or pharmacological blockade of the D₂ receptors, on the other hand, do not affect the basal level of glutamate uptake (66). The inhibitory mechanism may involve the D₂ dopamine receptor-mediated decrease in the basal production of cAMP, a second messenger known to stimulate glutamate uptake rates and expression of the glutamate transporter GLAST (51, 52). In cultured rat astrocytes, stimulation of α_1 -adrenergic receptors causes an activation of the glutamate uptake, apparently due to increase in V_{max} , whereas β -receptor agonists have an inhibitory action. Surprisingly, under the same experimental conditions, the uptake of D-aspartate, which is believed to be a substrate for the same transport system, was not affected. No effects of 5-hydroxytryptamine or α_2 agonists on high affinity glutamate uptake have so far been demonstrated (67). These effects of α_1 - and β -adrenergic agonists on glutamate uptake may represent one of the linking pathways in the neurotransmitter "cross-talk" that apparently takes place in the nervous system.

Arachidonic acid and other polyunsaturated fatty acids. AA is a polyunsaturated fatty acid currently recognized as a diffusible signaling molecule capable of modulating several cellular and intercellular processes (4, 68). AA was found to reduce the rates of high affinity glutamate uptake in different brain preparations (69, 70). The inhibitory effect is relatively fast and reversible, but it is not restricted solely to glutamate transporters because some other sodium-dependent transport systems seemed to be inhibited as well (69, 70). *Cis*-polyunsaturated fatty acids but not saturated or *trans*-unsaturated fatty acids, mimic the inhibitory action of AA with a rank of potency that matches the degree of unsaturation. Platelet activating factor, a lipid messenger that may be produced parallel to AA by a common synthetic pathway, has no effect on glutamate uptake within the physiological concentration range (70, 71). Biotransformation of AA via the cyclo- or lipoxygenase pathways is not an essential requirement for the expression of the inhibitory effect. AA inhibits purified and liposome-reconstituted rat glutamate transporter GLT1 directly from the water phase, not via incor-

poration into the phospholipid bilayer (72). The AA-sensitive inhibition of glutamate transport in intact systems (cells, synaptosomes, tissue slices) and proteoliposomes exhibits comparable kinetic characteristics. Therefore, it was concluded that direct interaction of AA with the glutamate transporter accounts for the AA-sensitive inhibition of the high affinity glutamate uptake in cells (72). However, another recent study (73) revealed differential modulation of three human glutamate transporter subtypes by arachidonic acid in the *Xenopus* oocyte expression system. Thus, micromolar levels of AA inhibited EAAT1/GLAST-mediated glutamate uptake via 30% reduction of V_{\max} not accompanied with changes in K_m . In contrast, EAAT2/GLT1-mediated glutamate uptake was enhanced due to a 2-fold increase in the apparent affinity for glutamate, whereas no changes in the maximal velocity were detected. Finally, the efficacy of EAAT3/EAAC1 was increased only slightly (73). If such a regulatory mechanism is operating *in situ*, AA may differentially affect the net glutamate uptake depending on the actual ratio of glutamate transporter subtypes in the vicinity of AA release sites. The significance of this study is the demonstration of the opposite effects of the naturally occurring bioactive substance on the components of the high affinity glutamate transport system. Molecular deciphering of the dual effect of AA may significantly contribute to a search for subtype-, hence region-specific, synthetic modulators of high affinity glutamate transport.

The AA-dependent modulation of glutamate uptake may be of physiological relevance. AA is produced by various stimuli (68), including the activation of NMDA-type glutamate receptors in neurons (8, 74) and metabotropic glutamate receptors in astroglia (75), and thus may mediate the glutamate-dependent regulation of glutamate transporters. It is noteworthy that besides the direct interaction with glutamate transporters, AA may regulate glutamate uptake via the activation of PKC (68), which is capable of modifying the activity of the transporters (45). Thus, induction of AA in pathological conditions [e.g., seizures and ischemia (76)] and synaptic plasticity processes [e.g., long term potentiation (77)] may contribute (via the glutamate inhibition of transporters) to the enhancement of glutamate neurotoxicity and efficacy of glutamatergic synaptic transmission, respectively.

Nitric oxide. Nitric oxide is a gaseous, short-lived second messenger suspected to be involved in several physiological and pathological processes, including glutamate-mediated neurotoxicity and long term potentiation (78, 79). NO was shown to inhibit (approximately 21–23%) high affinity glutamate uptake in rat synaptosomes (80) but not in cultured mouse astrocytes (71). The inhibition of the high affinity glutamate transport by NO in rat brain preparations is primarily mediated by the free radical form of the compound and is dependent neither on the reaction of NO with free oxygen radicals nor on the production of cGMP (80). Since NO is produced in response to the stimulation of glutamate receptors, primarily of NMDA-type receptors (81, 82), this diffusible messenger could be added to a list of potential mediators of the glutamate-sensitive regulation of high affinity glutamate transport. However, it should be emphasized that no inhibition of glutamate uptake has so far been detected *in vitro* after

the *in vivo* applications of the NO-precursors/generators (80). Thus, it has yet to be explored whether NO-mediated inhibition of glutamate uptake occurs in the brain.

Free oxygen radicals. Reactive oxygen radicals generated by H_2O_2 or the xanthine/xanthine oxidase system were shown to inhibit glutamate uptake in rat cortical astrocytes in culture. The maximal effect (approximately 60% inhibition) developed within 10 min and lasted for at least 1 hr (83). In another study (71) using glucose oxidase as a generator of reactive oxygen species in murine astroglial cultures, the inhibitory effect (30–50%) of free radicals was detected within the concentration range typically ensured by activated microglia. Surprisingly, the higher concentrations of reactive oxygen intermediates seemed practically incapable of influencing the glutamate uptake system (71). AA metabolites may be a source of reactive oxygen radicals and, *vice versa*, these radicals may stimulate phospholipase A2 and hence produce AA. However, AA and free oxygen radicals interfere with glutamate transporters in an additive manner, apparently via distinct mechanisms. The mechanism of the oxygen radical-mediated inhibition suggests direct oxidation of the transporter protein SH-groups (83), although inhibition of enzymes known to support the sodium and glutamate gradients (e.g., Na, K-ATPase, glutamine synthetase) cannot be excluded. Production of reactive oxygen species, which is a hallmark of pathological conditions (e.g., ischemia, hypoxia, hypoglycemia) characterized by cell energy failure/depolarization, may be mediated via the excessive stimulation of glutamate receptors (7) and may thus constitute an independent, aberrant circuit in the glutamatergic regulation of high affinity glutamate uptake system.

Amyloid β peptide. Several studies have demonstrated that Na-dependent glutamate uptake capacity was decreased in tissues obtained from patients with Alzheimer's disease (84). It is still debatable whether aberrant glutamate metabolism plays a significant role in Alzheimer's disease. However, *in vitro* studies show that in neuron-astrocyte co-cultures, β -amyloid peptide (βA), a major constituent of the insoluble and cytotoxic amyloid plaques, dramatically increases the vulnerability of neurons to excitotoxic damage (85). βA may induce apoptotic death in the neuronal cell line MES 23.5 via Ca^{2+} entry through NMDA-gated channels and subsequent production of NO (86), the latter of which is capable of inhibiting the glutamate transporters (80). A decrease in glutamate transport may also occur via βA -dependent inactivation of glutamine synthetase (87), an astroglial enzyme that converts translocated glutamate into glutamine and thus is thought to support high uptake capacity. Recently, a direct inhibition of astroglial high affinity glutamate transporters by free radicals derived from βA peptide has been demonstrated (88). The effects of amyloid precursor protein or its soluble excitoprotective derivatives on the high affinity glutamate uptake have so far not been investigated.

Factors down-regulating the GLT1 glutamate transporter in ALS. Recently, an aberrant glutamate transport has been found to be associated with ALS, a progressive neurodegenerative disorder characterized by loss of motor neurons in the cerebral cortex, brain stem, and spinal cord. Decreased high affinity glutamate uptake has been observed in synaptosomes from spinal cord and

motor cortex in patients with ALS (89). Moreover, chronic pharmacological inhibition of glutamate transporters in cultured organotypic spinal cord slices caused a weak neurotoxicity resulting in a slow degeneration of motor neurons (90). The defective glutamate uptake in ALS seemed to be due to a dramatic loss (up to 95%) of the GLT1 glutamate transporter but not of the GLAST or EAAC transporters (11).

The molecular mechanisms that regulate the GLT1 expression are yet unknown. The dramatic down-regulation of GLT1 in sporadic ALS cases may be primarily due to the aberrant translational and post-translational mechanisms because no apparent changes in the steady state GLT1 mRNA levels were observed (11). Thus, the block of the translation or the fast internalization/degradation of the persistently damaged translation product, GLT1, have been suspected (11). In fact, it seemed that the transgenic mice that overexpress mutant human Cu/Zn superoxide dismutase, an apparent pathogenic factor in some familial (genetically transmitted) forms of ALS, develop ALS-like symptoms paralleled with an excessive nitration of GLT1 and a marked loss of this transporter (91).

On the other hand, three variant GLT1 cDNAs that differ in length and composition of the 5' untranslated region have recently been cloned, and although neither of these three versions seemed to be ALS-specific, they may possess distinct capacities with regard to mRNA stability and translational efficacy. Moreover, one of the GLT1 cDNA variants contains a canonical consensus sequence for binding NF- κ B, a nuclear transcription factor activated in response to various signals, including cytokines (92).

Some growth factors were shown to attenuate the ALS progression in clinical trials (93). However, several growth factors, cytokines, and hormones so far tested had no apparent effect on the expression of glutamate transporters *in vitro* (91), and only tumor necrosis factor- α and glucocorticoids were capable of inhibiting the glutamate uptake in cultured astrocytes without affecting the transporter protein levels (94, 95). However, horse serum, which compared with fetal calf serum contains higher concentrations of some unidentified differentiation-promoting factors, markedly up-regulates glutamate uptake in cerebellar astrocytes (57). In an early study, yet unknown macromolecular factors released from neurons were strongly suspected to up-regulate glutamate transport in astroglial cultures, although the precise molecular nature of this phenomenon could not be established because of the unavailability of molecular probes for individual glutamate transporters (91). Interestingly, the *Wnt1* oncogene protein product, a para- and autocrine factor secreted by neuronal PC12 cells, is capable of inducing the expression of the glial GLAST transporter activity (96). The virtually total loss of GLT1 in neuron-free primary astroglial cultures, which has been revealed in recent studies (51, 95, 97), and at least partial preservation of this astroglia-specific transporter in the mixed (neuron-astroglia) cultures (98)¹ confirms the neuronal origin of the regulatory factor(s). Thus, the primary cultures of neural cells seem to constitute a prospective model for elucidation of the GLT1

regulation, a crucial issue in deciphering the detrimental loss of this transporter in ALS.

Conclusion. The developmental, regional, and tissue- and cell type-specific patterns of the expression of high affinity glutamate transporters seem to be precisely regulated, although little is known about the nature of the primary environmental cues and consequent events responsible for the molecular fine tuning of the expression of these transporters. Nevertheless, rapidly accumulating data suggest that the expression of glutamate transporters is amenable to regulation by different pharmacological agents and naturally occurring substances, which probably underlie the regulatory effects of some experimental manipulations. Further studies of the molecular events that link environmental signals with transcriptional and post-transcriptional mechanisms (e.g., alternative splicing, translation and post-translational modification) are of prime importance for the development of selective tools to elucidate the regulation and expression of particular glutamate transporters.

Acknowledgements

The secretarial assistance of Ms. Hanne Danø and Mrs. Tea Gobronidze is cordially acknowledged.

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Send reprint requests to: Prof. A. Schousboe, D.Sc., PharmaBiotec Research Center, The Neurobiology Unit, Department of Biological Sciences, The Royal Danish School of Pharmacy, 2 Universitetsparken, DK-2100 Copenhagen, Denmark. E-mail: as@charon.dfh.dk
