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EXCITATORY AMINO ACID NEUROTRANSMISSION. PATHWAYS FOR METABOLISM, STORAGE AND REUPTAKE OF GLUTAMATE IN BRAIN

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TABLE OF CONTENTS

1. ABSTRACT

In the nervous system, glutamate is an excitatory aminoacid which at higher concentrations has been implicated in a number of disorders. Glutamate is stored in presynaptic vesicles and is released by calcium-dependent exocytosis. After its action on ionotropic receptors (iGluR, related to ionic channels) or metabotropic receptors (mGluR, related to metabolic formation of second messengers), glutamate can be removed from the synaptic cleft through two processes: re-uptake back into pre-synaptic terminals or diffusion out of synaptic cleft for uptake by glial cells. This is achieved by glutamate transporters. In pre-synaptic terminals, glutamate is packed into the specialized secretory vesicles by means of a specific vesicular transporter. The level of glutamate available for neurosecretion is regulated by the vesicular transport activity. In order to achieve a proper concentration of the neurotransmitter in synaptic vesicles, glutamate must be synthesized. Glutamine is obtained in astroglial cells from the glutamate reuptaken, and as it has no neurotransmitter activity, it is the metabolite which regenerates glutamate in neurones (glutamate-glutamine cycle). Moreover, glutamate is also obtained from glucose by an intermediate of TCA cycle. In this paper we want to introduce some aspects of glutamate biosynthesis and release: glutamate receptors, neurotransmitter uptake by the glutamate transporters and neurotransmitter inactivation and new formation by metabolism.

2. INTRODUCTION

Aspartate and glutamate are the simple and common excitatory amino acids, and the inhibitory ones are GABA, glycine and taurine. Of these amino acids, two are intimately associated with CO_2 fixation in the brain, and may be therefore particularly relevant to central ventilation drive, namely, glutamate and GABA. These two amino acids are intimately related to CO_2 fixation in the brain and are derived from the same amino acid, glutamine. Their metabolism is interrelated, and they have opposite and profound effects centrally on ventilatory and cardiovascular functions. Specifically, glutamate may well be the key central neurotransmitter released with stimulation of peripheral chemoreceptors (1,2). Metabolism of glutamate and GABA in the brain are also related to ammonia metabolism. Decrease in brain ammonia is associated with a reduction in α-ketoglutarate and glutamic acid content and an increase in glutamine via the detoxification pathway for NH_3.

The influx of glutamate from plasma across the blood-brain barrier is much lower than the efflux of

glutamate from brain (3-5). Therefore, metabolism of brain glutamate must play an important role in regulating the brain glutamate level. Due to the many different roles associated to glutamate, it is not surprising that its synthesis and metabolism are complex. Thus, administration of the two general precursors, glucose and acetate, in a labeled form indicates that glucose and acetate are synthesizing glutamate via two different citrate cycles in brain. Glucose yields a low specific radioactivity of glutamine via glutamate whereas the opposite is true for acetate. Other metabolites behaving as glucose include glycerol, lactate, pyruvate, α-ketoglutarate and β-hydroxybutyrate; whereas propionate, butyrate, citrate, leucine, GABA, aspartate, proteins and ammonia behave as acetate (6). It seems that glucose is metabolized in neurones (7,8) whereas acetate in astroglial cells (9), where there is a higher glutamine synthase activity (10,11).

In this paper three key aspects of the amino acid neurotransmitter glutamate are reviewed: receptors, transport and metabolism (see figure 1 for a summary).

3. EXCITATORY AMINO ACIDS RECEPTORS

Excitatory aminoacids (aspartate and glutamate) act through two broad classes of receptors: ion channel-linked ionotropic receptors (iGluR) and metabotropic receptors (mGluR), which are coupled with G-proteins inducing intracellular messenger cascades (12).

There are different types of ionotropic and metabotropic glutamate receptors in both neurones and glial cells. Table I shows a summary of the main characteristics of those receptors. Classification of receptors has been possible due to the different affinity to specific agonists and antagonists. A summary of those molecules are given in figure 2.

Glial cells, in particular astrocytes, appear to respond to a great variety of neurotransmitters, including glutamate, hormones and growth factors with activation of metabotropic pathways, which may lead to intracellular pH and/or calcium changes. The distribution of different sets of glutamate receptors in different brain regions may classify neurones and glial cells, and appears to be functionally of some significance, both in normal physiological processes as well as during pathological states. The excitatory neurotransmitter glutamate and its large receptor family is probably the most versatile and complex signaling system in the mammalian brain, and possibly also the most susceptible for pathological disturbances.

3.1. Ionotropic glutamate receptors

Four main subtypes of glutamate-gated channels have been characterized pharmacologically and they have been named according to their preferred agonist, N-methyl-D-aspartate (NMDA), high affinity kainate (KA), alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionate (AMPA), and 2-amino-4-phosphobutyrate (AP4). For each of those agonists, a large diversity of receptors have been described (see table 1).

The most studied receptor is NMDA (13,14), and because of this, glutamate ionotropic receptors are often named as NMDA and non-NMDA. Activation of the ionotropic NMDA and non-NMDA receptors increases transmembrane calcium and sodium fluxes, whereas the metabotropic glutamate receptor activation results in generation of inositol triphosphate and inhibition of adenylate cyclase (15). Nevertheless, metabotropic receptors are also related in phosphorylation of NMDA and non-NMDA receptors.

It seems that glutamate ionotropic receptors, specially NMDA receptors, are related with neurodegenerative diseases, as act when glutamate concentration increases. Thus, a noncompetitive NMDA receptor antagonist (memantine) can be used for the treatment of Alzheimer´s disease (16). It has also been seen that injection of kainate causes selective neuronal degeneration similar to that of Huntington´s disease (17). Evidence from animal models suggests that antagonists of the different glutamate receptors might be beneficial in Parkinson´s disease, Huntington´s chorea and amyotrophic lateral sclerosis but the relevance of these models to the human disease is not clear. However, the identification of numerous receptor subtypes in addition to variabilities of distribution and multiple modulatory sites will provide new solutions to these common neurological disorders.

3.1.1. NMDA receptors

NMDA-type ionotropic receptors have not been demonstrated in glial cells, but only in neurons. Those receptors contain (a) a transmitter binding site, which binds glutamate; (b) a regulatory or coactivator site, which binds glycine, (c) a site within the channel that binds phencyclidine and related compounds, (d) a voltage-dependent Mg^{2+} binding site , and (e) an inhibitory divalent cation site that binds Zn^{2+}. Glycine greatly enhances the actions of NMDA agonists but has no action by itself. Interaction of phencyclidine and related anesthetics to NMDA receptors reproduce most of the symptoms of schizophrenia. A major advance in the understanding of the NMDA receptor was the demonstration by McDonald and Johnston (18) and Flatman *et al.* (19) that NMDA-induced responses are voltage-dependent. The agonist-induced currents are greatest at moderately depolarized potentials (-30 to -20 mV) and are reduced at both more hyperpolarized and depolarized potentials. Consequently, NMDA receptor action is suppressed at the normal resting potential. The work of Nowak *et al.* (20) and Mayer *et al.* (21) demonstrated that the voltage dependency of the NMDA receptor is attributable to extracellular Mg^{2+} ions that block the ion channel only at potentials more negative than -20 or -30 mV.

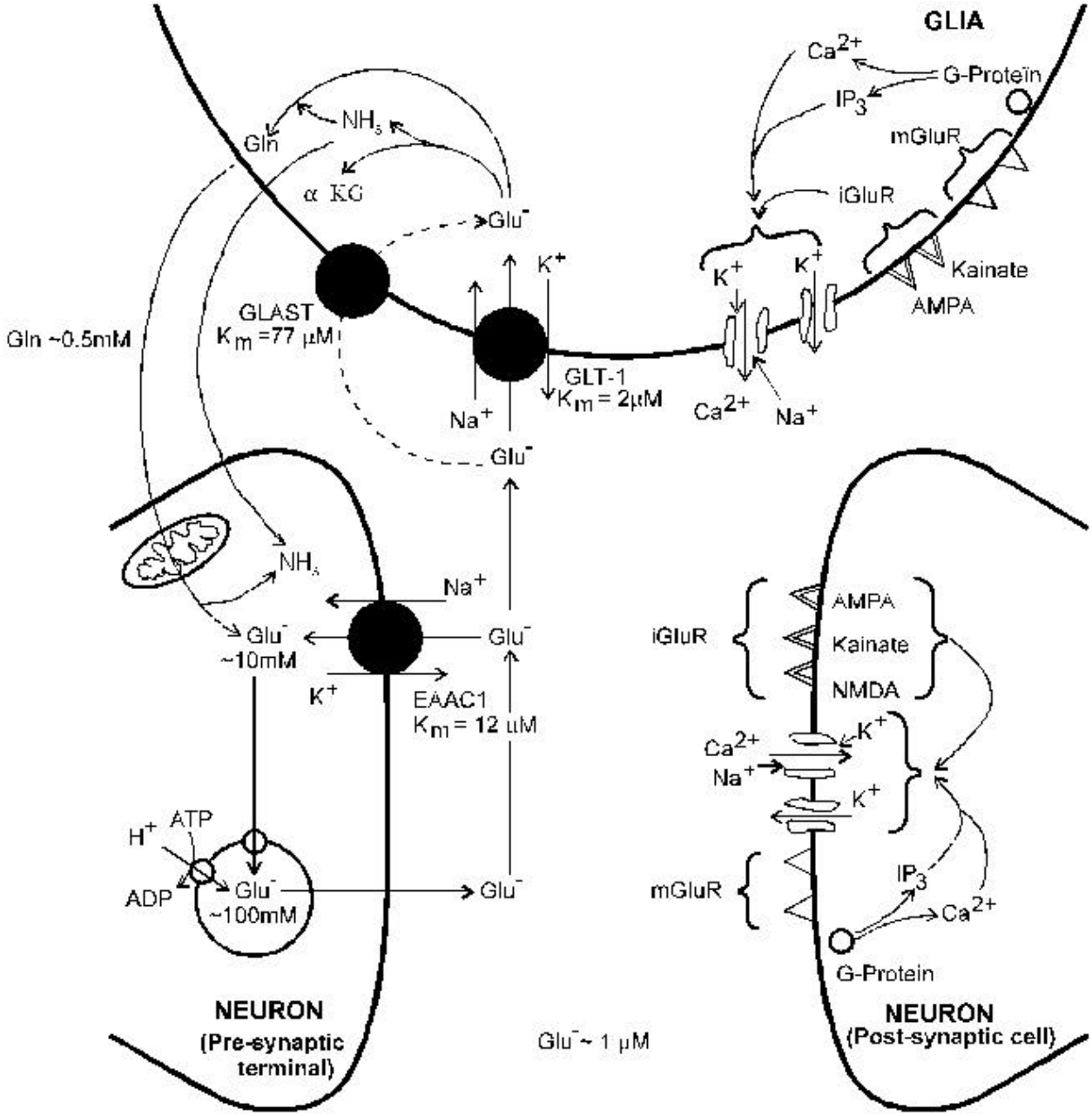
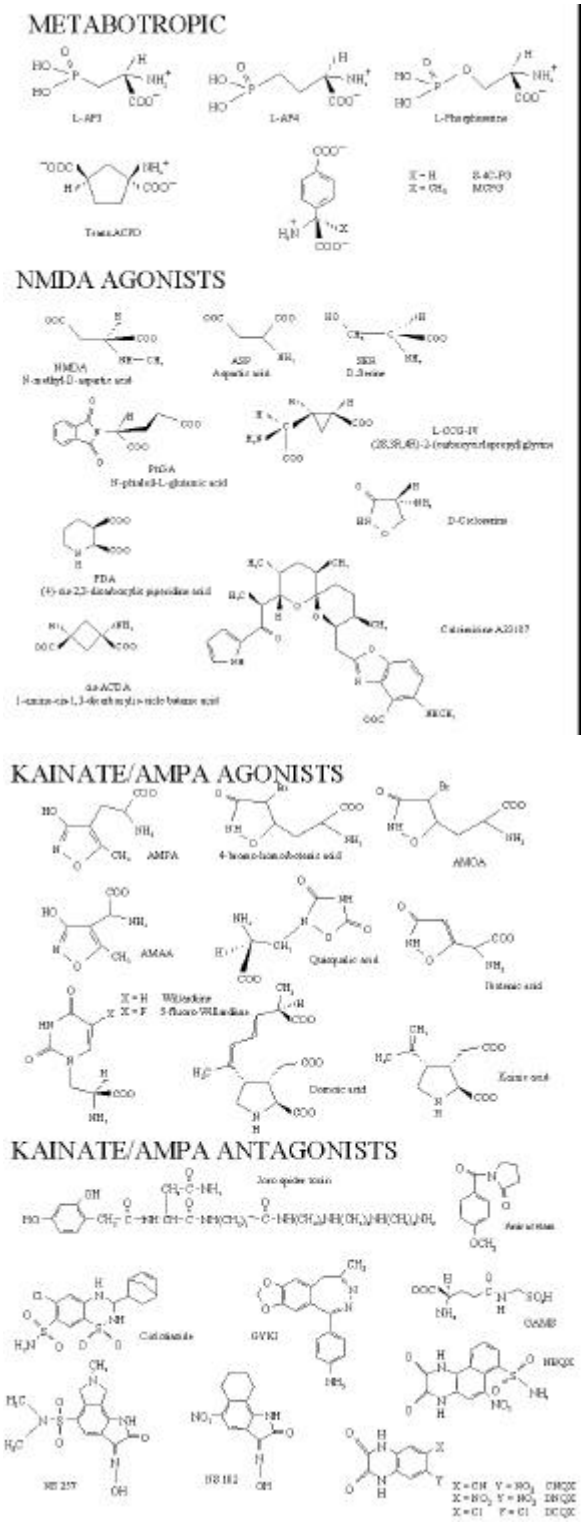


Figure 1. Glutamate mobilization at glutamatergic synapses. Glutamate stored in synaptic vesicles is released by fusion of those vesicles to the presynaptic membrane. Stimulation of ionotropic glutamate receptors (iGluR) by glutamate opens associated ion channels and induces an excitatory postsynaptic potential. Metabotropic glutamate receptors (mGluR) are associated with G proteins and, depending on the subtype they increase IP or affect cAMP concentration. Glutamate is removed from the synaptic cleft through two processes: re-uptake back into pre-synaptic terminals and diffusion out of synaptic cleft to be re-uptaken by glial cells. The rapid re-uptake of glutamate is believed to be mediated by a Na^+ and K^+ depending high-affinity glutamate transporter (EAAC1, with K_m for glutamate of 12μM). The low glutamate concentration is re-uptaked in glial cells by a higher-affinity transporter (GLT-1, with K_m of 2 μM). The low affinity glial cells transporter (GLAST, with K_m of 77 μM) is able to transport aspartate and glutamate and it is a mechanism of protection in order that glutamate concentration doesn't increase too much in brain, because of its "excitotoxic" effect. Glutamate in glial cells is thought to be metabolized to glutamine (by glutamine synthetase) and to α-ketoglutarate (by glutamate dehydrogenase or glutamate oxaloacetate transaminase). Those metabolites can be used as precursors of glutamate synthesis in pre-synaptic neurones. Glutamate is packed again into synaptic vesicles by a Na^+ independent transport driven by the internal positive membrane potential generated by the vesicular ATP-dependent H^+ transport.



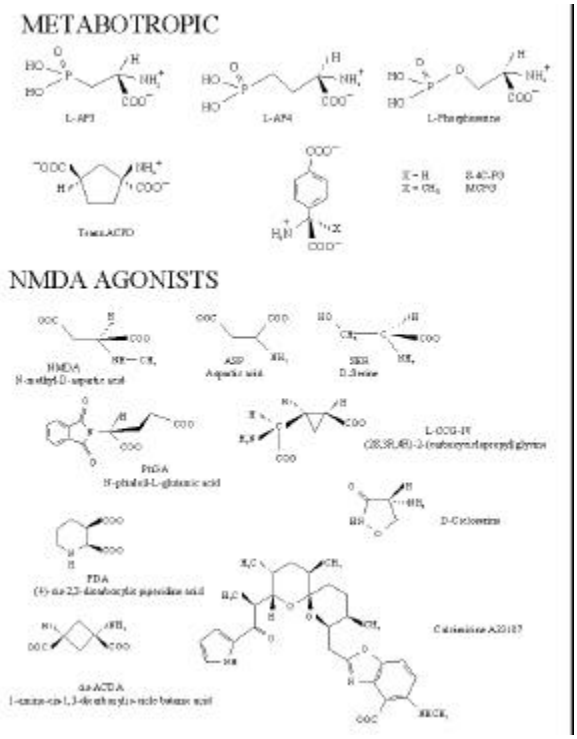


Figure 2. Some of the agonists and antagonists of glutamate receptors. Classification of glutamate receptors is possible due to the effect of some agonists and antagonists. Here it is shown the chemical structures of the most common used.

Table 1. Glutamate receptors

Receptor	Component	Transmembrane Regions	G-Proteins	Transduction systems	Agonists potency
	NMDAR1	4	--	Open	GLU>NMDA
NMDA	NMDAR2A	4		ionic channels	--
	NMDAR2B	4		Na^+, Ca^{++}	--
	NMDAR2C	4			--
	NMDAR2D	4			--
	GluR1	4	--	Open	QA>DOM≈AMPA>GLU>KA
AMPA	GluR2	4		ionic channels	QA>AMPA>GLU>KA
	GluR3	4		Na^+, K^+ (Ca^{++})	QA>AMPA>GLU>KA
	GluR4	4			QA>AMPA>GLU>KA
	GluR5	4	--	Open	DOM>KA>QA≈GLU>AMPA
Kainate	GluR6	4		ionic channels	DOM>KA>QA>GLU>AMPA
	GluR7	4		Na^+, Ca^{++}	DOM>KA>GLU>QA>AMPA
	KA1	4			KA>QA>DOM>GLU>AMPA
	KA2	4			KA>QA>DOM>GLU>AMPA
	mGluR1	7	G_q o G_i/G_o	Activates PLC	QA>IBO≈GLU>tACPD
Metabotropic	mGluR2	7	G_i/G_o	Inhibits AC	GLU≈tACPD>IBO>QA
	mGluR3	7	G_i/G_o	Inhibits AC	GLU≈tACPD>IBO>QA
	mGluR4	7	G_i/G_o	Inhibits AC	AP4>GLU>tACPD (QA inactive)
	mGluR5	7	G_q o G_i/G_o	Activates PLC	QA>GLU≈IBO>tACPD
	mGluR6	7	G_i/G_o	Inhibits AC	--

Glutamate receptors are classified as ionotropic (NMDA, AMPA and kainate) and metabotropic. Depending on the receptor type they show different transmembrane regions and different affinity for the agonists.

In the central nervous system the N-methyl-D-aspartate (NMDA) receptor channel plays an important role in synaptic plasticity and neuronal development. It has an heteromeric configuration consisting of the epsilon (NR2) subunits, which potentiate the channel activity and modulate the functional properties, and zeta 1 (NR1) subunit, which is essential to form functional NMDA receptors channels (22).

3.1.2. Non-NMDA receptors

AMPA and kainate receptors have been regarded as rather impermeable to divalent cations, in particular to Ca^{2+} (23), although in certain neurons Ca^{2+}-permeable kainate receptors were observed (24-26). Pharmacological studies indicate that the AMPA and kainate receptors are responsible for the voltage-independent portion of the synaptic response in many neuronal pathways. Kainate receptors could probably have multiple actions, e.g. the opening of a voltage-independent cation channel as well as a modulatory action, possibly via calcium channels.

The finding that L-AP4 can potently block synaptic transmission in these systems yet is ineffective as an antagonist of the other well characterized excitatory amino acid agonists indicate that L-AP4 acts at receptors others than those identified by NMDA, kainate or AMPA. Early studies suggested that the AP4 receptor could be identified in presynaptic membranes as a sodium-independent, chloride-dependent L-glutamate binding site.

3.2. Metabotropic glutamate receptors

The metabotropic glutamate receptors (mGluR) are both functionally and pharmacologically different from the family of the ionotropic receptors. The mGluR is coupled to a G protein(s) and evokes a variety of functions by mediating intracellular signal transduction (27,28). It also differs from known ionotropic receptors in agonists selectivity (28,29) (See table 1 and figure 2).

Seven subtypes of mGluR are known to exist (12) but their roles in synaptic physiology are not very understood. They are classified upon their second messenger as follows: mGluR1 and mGluR5 are associated to inositol phosphate (IP) and to changes in calcium concentration and belong to the Group I. mGluR2 and mGluR3 belong to Group II and mGluR4, mGluR6-mGluR8 are related to Group III. All receptors from Groups II and III inhibit the cAMP production.

Postsynaptic group I mGlu receptors may modulate both AMPA and NMDA receptor mediated currents, probably via phosphorylation of the respective ion channels. Group II/III receptor-activation produce neuroprotective effects. In cerebellar Purkinje cells, application of the mGluR agonist *trans*-1-aminocyclopentane-1,3-dicarboxylic acid, or the active enantiomer, 1S,3R-ACPD, results in a depolarization associated with an inward current and an elevation of intracellular calcium (30).

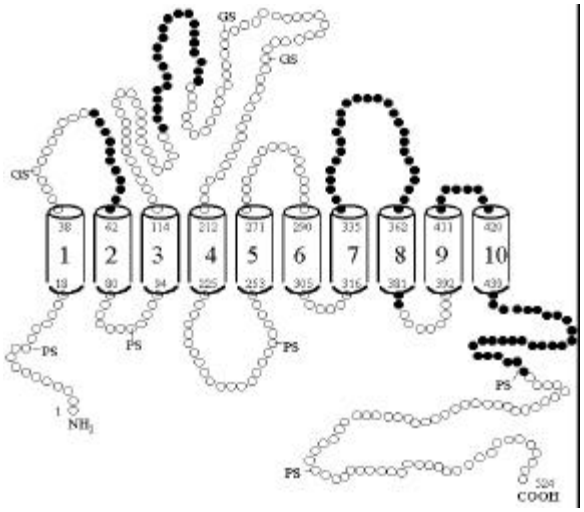


Figure 3. Structural model of EAAC1. EAAC1 transporter has 10 transmembrane sections. It is a glycoprotein with 3 external glycosilation sites (GS) and has 5 internal potentially phosphorylations sites (PS). Domains corresponding to regions of extended sequence identity with GLT-1 and GLAST are shown as filled circles.

4. EXCITATORY AMINO ACIDS TRANSPORTERS

Uptake of the acidic amino acids from the extracellular enviroment is mediated by sodium-dependent transport systems of high affinity. Five subtypes of high-affinity glutamate transporters have been cloned independently (31-35). Although they have different nomenclature, the actual nomenclature used for human glutamate transporters is EAAT (excitatory amino acid transporter) followed by a number. Those transporters have been related to the previous known transporters from other species by amino acid sequence identity. Here we describe the structure, distribution, functional expression and pharmacology of these transporter subtypes.

4.1. EAAT1 and GLAST1

EAAT1 has a molecular weight of 59.5 kDa and 96% amino acid sequence identity with the rat GLAST1 sequence (33). GLAST is a specific transporter for L-glutamate and L-aspartate and plays an important role in the termination of neurotransmitter signals on excitatory synapses. It is specifically expressed in brain being distributed in glial cells. In cerebellum, GLAST message is specifically expressed in the striatum gangliosum made up primarily by Purkinje and Bergmann glia cells (33).

Storck *et al.* (33) isolated and sequenced a 3 kb clone from a rat brain cDNA library. The predicted sequence of the polypeptide consists on a 543 amino acid residues protein. A tentative model for the rat GLAST protein comprises 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 (see figure 3).

Significant sequence similarity of GLAST to the glutamate transporters of *E. coli* (36), *Bacillus staerothermophilus* and *Bacillus caldotenax* and to the dicarboxylate transporters of *Rhizobium meliloti* and *Rhizobium leguminosarium* (37). The overall aminoacid sequence identities range from 26 to 32%. It shares no sequence similarity with the *E. coli* Na^+-dependent glutamate transporter (38,39) or the cloned mammalian neurotransmitter transporters studies so far (40,41). The transporter function has been verified by aminoacid uptake and electrophysiological studies in the *Xenopus laevis* oocyte system.

The results provide evidence for GLAST-1 carrying out a high affinity, sodium-dependent L-glutamate transport with a proposed stoichiometry of 3 Na^+, 1 L-glutamate$^-$/1K^+. There is no evidence for the cotransport of protons (42). Aminoacid uptake shows saturation kinetics: K_m(L-glu)=77 ±27 mM (33) and other amino acids including L-alanine, L-leucine, L-glutamine, L-arginine and L-methionine are not transported in significant amounts. DL-*threo*-3-hydroxy-aspartate, a strong inhibitor of the Na^+-dependent glutamate uptake (43) and capable of causing neuronal degeneration (44), is a potent inhibitor of GLAST. A decrease in Na^+-dependent L-glutamate transport in patients with Alzheimer's disease has been reported (45). Whether GLAST plays a pivotal or ancillary role in these disorders remains to be elucidated.

4.2. EAAT2 and GLT-1

EAAT2 has a molecular weight of 62.1 kDa and a 95% identity with the correct sequence (46) of rat GLT-1 (32). GLT-1 is expressed in astrocytes and maintains the low extracellular glutamate concentration of approximately 1μM below neurotoxic levels. The L-glutamate transporter cDNA was obtained by immunoscreening of a λ_{Zap} library from rat brain (47) with an antibody raised against the purified glial transporter. The cDNA sequence predicts a protein of 573 amino acids with 8-9 putative transmembrane α-helices (32) (figure 3). It is a glycoprotein with 2 potential N-linked glycosylation sites and 2 phosphorylation sites (48). GLT-1 does not share significant overall homology with any known eukariotic protein, including the Na^+/glucose transporter and the growing superfamily of neurotransmitter transporters but with the protein-coupled L-glutamate transporter glt-p of *E.coli*.

The transport system has three substrates: Na^+, L-glutamate and K^+ (49-51). In an electrogenic process, the transporter takes up Na^+ and L-glutamate and extrudes K^+. Like the others (Na^+-K^+)-coupled L-glutamate transporters (52), GLT-1 is stereospecific, being strongly inhibited by L-glutamate but not by the same concentration of D-glutamate. The apparent K_m in intact cells is 10 μM higher than in membrane preparations (53) and in the purified and reconstituted transporter (54).

Glutamate analogues: L-aspartate, D-aspartate, cysteine sulphinate, *threo*-3-hydroxy-DL-aspartate and L-*trans*-pyrolidine-2,4-dicarboxylate are competitive inhibitors of GLT-1. L-*trans*-pyrolidine-2,4-dicarboxylate inhibits

transport of L-glutamate but does not prevent it from binding to its receptors (55). The GLT-1 system is potently inhibited by dihydrokainate (DHK) and L-α-aminoadipate (L-AAD), while the EAAC1 system (see 2.3.) is inhibited by L-AAD and is essentially DHK insensitive.

The decrease in GLT-1 function cannot be attributed to selective astrocytic degeneration as there is no loss of staining of the astrocytic marker, GFAP. In fact, previous studies have suggested that astrogliosis and not a loss of astrocytes may be present in ALS motor cortex (56). Studies have been carried out to try to identify the reason for the selective loss of GLT-1 in ALS. There is no evidence for a decrease in gene expression as there was no change in the levels of the mRNA coding for the protein in ALS patients (57). Other possible explanations for a loss of GLT-1 could involve translation mechanisms whereby the gene is transcribed into mRNA but defects in the translation could interfere with the synthesis of the protein. Alternatively, the protein may be translated normally but may be much more unstable than normal. Finally, the decrease in activity may be due to posttranslational modifications, possibly induced by an increase in the levels of cellular free radicals.

4.3. EAAT3 and EAAC1

EAAT3 has a molecular weight of 57.2 kDa and 92% identity with the rabbit sequence termed EAAC1 (58). EAAC1 is of both neuronal and epithelial origin. In brain it provides a presynaptic glutamate uptake mechanism to terminate the action of released glutamate at glutamatergic synapses. But it is also expressed in γ-aminobutyric acid (GABA)-ergic cerebellar Purkinje cells, where it provides glutamate as a precursor for GABA synthesis.

To isolate the cDNA encoding rabbit EAAC1, Kanai and Hediger (58) screened a rabbit intestinal cDNA library for their ability to induce [^{14}C] glutamate uptake in *Xenopus* oocytes. They isolated a cDNA which encodes a 524-residue protein. This protein has approximately 10 putative membrane-spanning regions but alternative models with a different number of membrane-spanning regions can be constructed. EAAC1 has a significant homology to the H^+-coupled gltP glutamate transporters of *E. coli*., *B. stearothermophiles* and *B. caldotenax* and to the dctA dicarboxylate transporter of *Rhizobium meliloti.* They have sequence indentities ranging between 27% and 32%. There is neither homology to the Na^+-Cl^--dependent GABA/neurotransmitter transporter family (40,41,59) nor to the *E. coli* Na^+/glutamate transporter gltS (38).

EAAC1-mediated transport is electrogenic and dependent on extracellular Na^+ but not on Cl^-. Studies in salamander retinal glial cells and in oocytes expressing EAAC1 revealed that glutamate transport coupled to the cotransport of two Na^+ and the countertransport of one K^+ and one OH^- (60).

Due to the presence of a large hydrophobic stretch near the C terminus (residues 357 to 444, figure 3), alternative models with a different number of membrane spanning regions are concievable. The N-terminal part of EAAC1 is hydrophilic and lacks a signal peptide. A motif-like cluster of serine residues is present (residues 331-334). Similar clusters have been identified in the ligand-binding sites of receptors for acetylcholine and biogenic amines.

The function and pharmacology of the expressed protein are characteristic of the high-affinity glutamate transporter already identified in neuronal tissues. Membrane currents were measured in Xenopus oocytes injected with EAAC1 cRNA. L-glutamate, L-aspartate and D-aspartate evoked inward currents, almost with the same amplitude, whereas currents induced by D-glutamate and L- or D-homocysteinate were smaller. The glutamate receptor ligands NMDA and kainate did not induce current whereas quisqualate evoked a significant inward current. EAAC1-mediated L-glutamate transport is saturable and the K_m value is 12,2±1,2 μM, indicative of a high-affinity transport (31).

The abnormal glutamate transport that is associated with certain neurodegenerative diseases and which occurs during ischemia and anoxia could be due to abnormalities in the function of this protein.

4.4. EAAT4

EAAT4 is a human L-aspartate/L-glutamate transporter which is expressed in the cerebellum and at lower levels, in brain stem, cortex and hippocampus. cDNA encoding EAAT4 was obtained from human cerebellar messenger RNA (34). The amino acid sequence exhibits 65%, 41% and 48 % amino-acid identity to the human glutamate transporters EAAT1, 2 and 3 respectively.

EAAT4-mediated transport is electrogenic, dependent on Na^+ and K^+ and it has properties of a ligand-gated chloride channel. Its transport is saturable and the K_m values derived from current measurements are K_m (L-asp)=184±0.46 μM and K_m(L-glu)=3.3±0.4 μM (34). Pharmacological properties of EAAT4 are similar to those previously described to the other glutamate transporters.

Structural analogs of glutamate, L-*trans*-2,4-pyrrolidine dicarboxylic acid (PDC), L-quisqualate and L-α-aminoadipate, elicit currents in oocytes expressing EAAT4. This current is predominantly carried by chloride ions and chloride-dependent glutamate transport activities have been reported in synaptosomes and other brain preparations but they have not demonstrated the cotransport of chloride ions with substrates (61,62). This chloride conductance is not blocked by endogenous oocyte chloride channel blockers. Thereafter, EAAT4 functions as a transporter, reducing the amount of neurotransmitter available for activating postsynaptic receptors and as a glutamate-gated chloride channel, modifying the neuronal excitability by ist capacity for enhancing chloride permeability.

4.5. EAAT5

EAAT5 is a human glutamate transporter whose mRNA is mostly expressed in the retina. Arriza *et al.* (35) isolated the transporter EAAT5 from a salamander retina glutamate transporter cDNA to screen a human retinal λgt10

cDNA library. The gene product is 560 amino acid residues in length. EAAT5 has 46% identity with EAAT1, 43% identity with EAAT4, 37% with EAAT3 and 36% identity with EAAT2.

The amino- and carboxy- terminal sequences of the excitatory amino acid transporters are poorly conserved. In EAAT5 C-terminus there is a sequence motif found in synaptic membrane proteins: E-S or T-X-V-COOH (63). The C-termini interact with several PDZ (a modular protein-binding motif) domains in PSD-95 (postsynaptic density-95kDa protein). The presence of this PDZ domain suggests that the transporter is a component of the signal transduction pathway. Its channel-like properties may indicate a role in retinal physiology different from neurotransmitter clearance.

EAAT5 uptake is sodium- and voltage-dependent and chloride-independent. It has stereospecificity for L-glutamate versus D-glutamate and L-aspartate versus D-aspartate with K_m values for L-glutamate and L-aspartate of 64± 6μM and 13±5μM, respectively. L-*trans*-pyrrolidine-2,4-dicarboxylic acid (tPDC) and *threo*-β-hydroxyaspartate (THA) are potent blockers but neither generated a current with a voltage dependence similar to that of glutamate (35). However, ion substitution experiments show that the currents seen are carried by chloride ions. These properties are similar to those described in retinal neurons suggesting that the transporter can be involved in visual processing (35).

4.6. Synaptic vesicles glutamate transporter

Glutamate is preserved in secretory vesicles at pre-synaptic terminals. A unique group of proteins found on secretory vesicles are the transporters needed for the accumulation of neurotransmitters from cytoplasm into these vesicles. The vesicular glutamate transporter is one between at least four different vesicular transporter types which have been biochemically identified (64,65).

Synaptic vesicles and microvesicles, enclosed in endocrine cells like pinealocytes, possess an active glutamate-specific transporter that is dependent on the extravesicular Cl^- concentration, on an electrochemical proton gradient across the vesicle membrane (66-69) and on the temperature (67).

The dependence of glutamate uptake on ATP-generated proton electrochemical potential was analysed in a highly purified preparation of synaptic vesicles from rat brain (70). The glutamate anion is transported into synaptic vesicle by a Na^+-independent vesicle transport, driven by the internal positive membrane potential generated by the vesicular ATP-dependent H^+ transport (66,67,70). Anyway, it seems that glutamate uptake is solely dependent on $\Delta\psi$, suggesting that protons are not directly involved.

The vesicle carrier has a low substrate affinity (K_m= 1.6 mM) and is highly specific for L-glutamate (5 mM D-glutamate reduces uptake by 30% while 5 mM L-aspartate has no effect) (71). Other glutamate analogues which interact with various glutamate receptor subtypes don't affect transport (71). It has been observed a good correlation between acidification and inhibition of glutamate uptake by glutamate analogues such as 1-aminocyclohexane-*trans*-1,3-dicarboxylic acid (72). Glutamine, aspartate and GABA do not inhibit L-glutamate uptake (67).

Due to the fact that the transporter has not been cloned yet, there is no knowledge about its structure, sites involved in vesicular transporter function nor the sites that determine substrate specificity. Nevertheless, future molecular analysis will help us to understand some events such as cell-specific expression, specific targeting, bioenergetic properties and neuropharmacology.

5. METABOLIC INTERACTIONS BETWEEN NEURONS AND ASTROCYTES

If one tries to envisage the metabolic capabilities required to maintain glutamatergic, aspartatergic, and GABA-ergic transmission (Glu, Asp, and GABA) in a simple cell type residing behind a blood-brain barrier through which there is rapid exchange of CO_2, H_2O, and NH_3 and a rapid net influx of glucose, but no net accumulation of either glutamate, aspartate, GABA, or glutamine (73), one would end up with a cell like that sketched in figure 4. Neurons are able to synthesize both glutamate and GABA from glutamine, and astrocytes form and release glutamine (which has no transmitter activity) after accumulation of either glutamate or GABA.

Extracellular glutamate is, to a larger extent, accumulated into astrocytes both in the intact brain (74) and in cultured cells (75,76), but much of the accumulated glutamate (how much probably depends upon the experimental conditions) is degraded as a metabolic fuel to CO_2 and H_2O in astrocytes and thus not converted to glutamine (75,77). New glutamate and GABA precursor molecules will, therefore, have to be synthesized from glucose. Since carboxylation of pyruvate to oxaloacetate, an intermediate of the TCA cycle, occurs in astrocytes, net synthesis of α-ketoglutarate in the TCA cycle can also take place in these cells.

The rate of glutamate metabolism to CO_2 is high in astrocytes but not in the two neuronal types, especially not in the glutamatergic cerebellar granule cells. The rapid CO_2 formation in astrocytes from glutamate appears to represent mainly a net utilization of glutamate (78), not just an isotope exchange between glutamate and α-ketoglutarate. Utilization of glutamate or glutamine as a metabolic substrate is not restricted to cultured cells but has also been observed in brain slices and dissociated cell preparations (79,80). As long as the glutamate utilized as a metabolic fuel originally is produced from glucose behind the blood-brain barrier, this is not a violation of the well-established fact that the entire adult brain *in vivo* under normal conditions almost exclusively utilizes glucose as its substrate for energy metabolism (81).

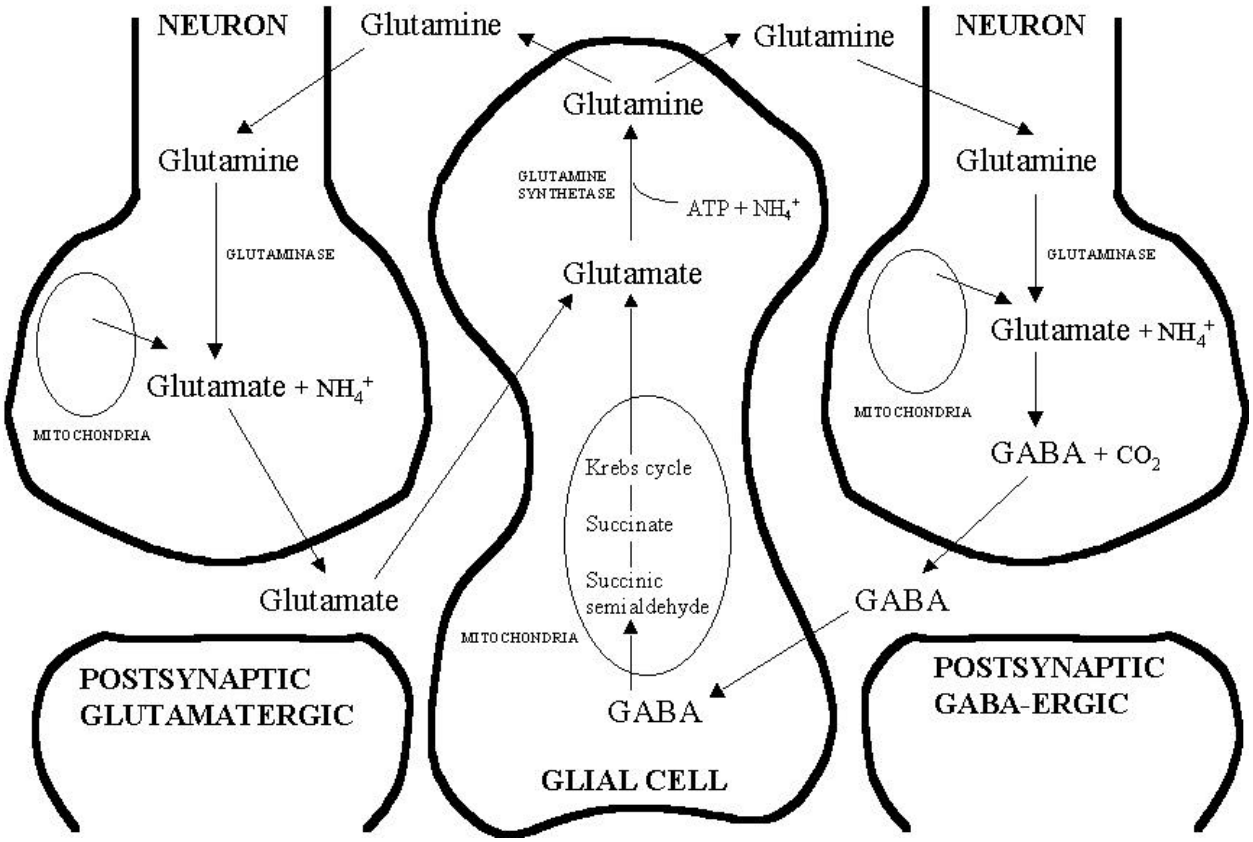


Figure 4. Schematic diagram of glutamate/glutamine and GABA/glutamine cycles. In neurons, glutamate and γ-aminobutyric acid (GABA) are synthesized from glucose or glutamine. Neurotransmitter released in synaptic cleft can then interact with receptor sites. To terminate this effect, transport to astroglial cells is performed with higher affinity than to neuron cells. In astroglial cells there is a higher glutamine synthase activity and glutamate and GABA are metabolized to glutamine, which has no neurotransmitter effects and can be recycled to neurons to form glutamate or GABA.

Exposure to very high potassium concentrations (> 20 mM) causes depolarization and a depolarization-induced increase in free intracellular calcium concentration in synaptosomes (82), neurons, and astrocytes (83) and enhances metabolic interactions between these two cell types by facilitating glutamate (and GABA) release from neurons and CO_2 fixation in astrocytes, which in turn promotes astrocytic formation of transmitter precursors for neurons. During exposure to slightly elevated potassium. In the following section, we describe some properties of the enzymes involved in the glutamic acid metabolism (figure 5).

5.1. Glutamine synthetase (E.C. 6.3.1.2.)

Glutamine synthetase catalyzes several reactions (84), although the main reaction is the following one:

$Glu + NH_3 + ATP <-----> Gln + ADP + P_i$

The enzyme has two important functions: assimilation of ammonia and biosynthesis of glutamine. The enzyme from brain has been studied in rat, ox, sheep, pig and human. It has been isolated from a variety of sources and the proteins vary greatly in their ability to catalyze the reverse reaction. With the mammalian enzyme, however, the forward rate relative to the reverse rate is about 10 to 1 (85).

The purification of the enzyme usually proceeds through four steps consisting on an acetone powder extract, precipitation by acid, hydroxylapatite and DEAE-cellulose column chromatography (86); with a yield of 15-30% and a purification of about 200-fold.

Glutamine synthetase is composed of eight subunits which are identical (44,000-50,000 daltons) showing rather similar amino acid composition (84). The enzyme has a molecular weight of 400,000 daltons, distributed in two tetramers (87). Distribution of glutamine synthetase and glutaminase are uneven in central nervous system. Berl (88) determined the distribution of glutamine synthetase in 16 brain areas of the adult rat. The site of glutamine synthesis, from glutamate amidation via glutamine synthase (GS), is in glial cells (89); and with a cytosolic location (9). Additionally, glia cells contain carbonic

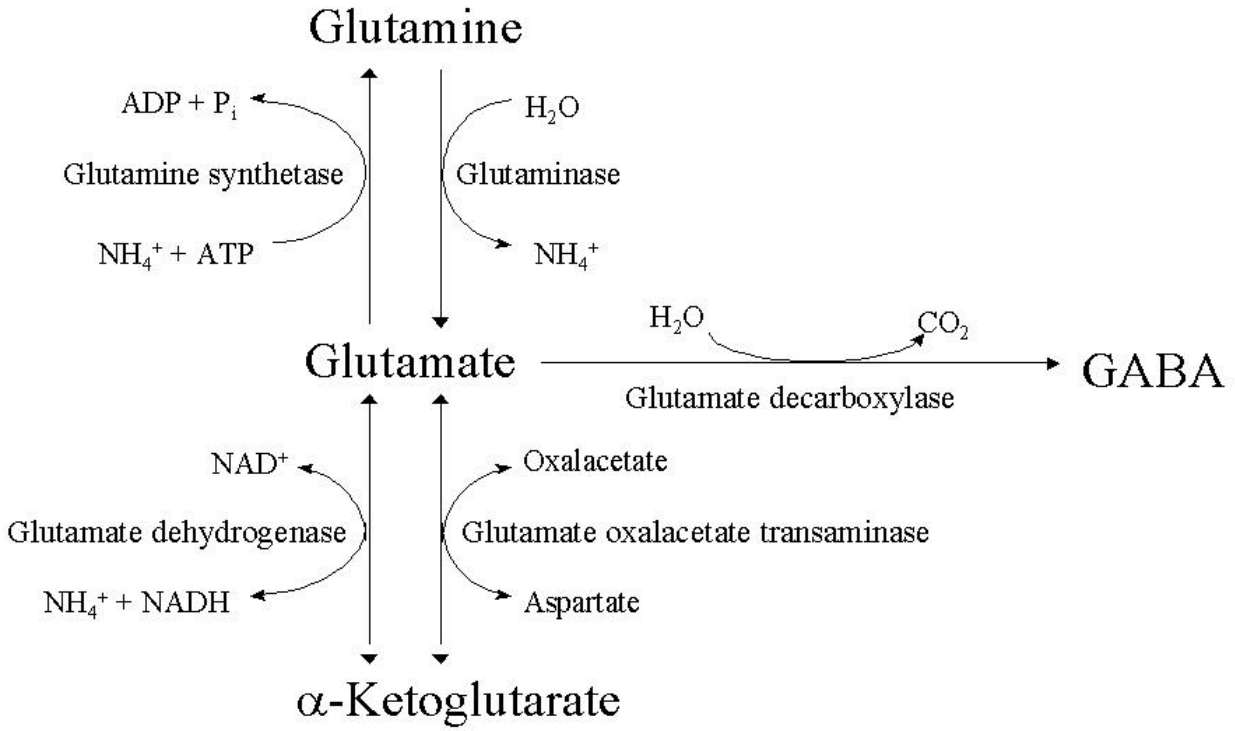


Figure 5. Glutamate metabolism. Glutamate from mammalian brain can be obtained from glutamine (glutaminase) or from α-ketoglutarate (glutamate dehydrogenase and glutamate oxalacetate transaminase). Degradation of glutamate can generate α-ketoglutarate by reversible reactions, GABA (glutamate decarboxylase) or glutamine (glutamine synthetase).

anhydrase, which catalyzes the CO_2 hydration reaction and HCO_3^- formation. Therefore, glia cells could be important in acid-base regulation and related amino acid metabolism in CNS. Glutamine formed by GS can move into adjacent nerve endings where it is utilized either in metabolic pathways unrelated or not directly related to neurotransmitter glutamate and GABA synthesis or in formation of neurotransmitter glutamate and GABA via glutaminase and glutamate decarboxylase, respectively.

The enzyme is irreversibly inhibited by methionine sulfoxamine (MSO) (90). ATP and magnesium are necessary for the binding of glutamate to the enzyme, whereby it becomes activated. Tate *et al.* (84) calculated in rat liver K_m for ATP = 2.3 mM; and Deul *et al.* (86) K_m for ammonia = 0.3 mM. Certain anions, particularly bicarbonate and chloride, activate the enzyme when nonsaturating concentrations of L-glutamate are used. Although liver glutamine synthase is activated by 2-oxoglutarate, brain enzyme is less affected by this compound (84). The mammalian enzymes are inhibited by inorganic phosphate and carbamyl phosphate (91). This effect can be due to the reaction of ATP synthesis from ADP and carbamyl phosphate, catabolized also by glutamine synthetase.

5.2. Glutaminase (E.C. 3.5.1.2.)

The reverse reaction of glutamine synthase is catalyzed by the ubiquitous enzyme glutaminase, which is present in both neurons and astrocytes (92). Nevertheless, glutaminase is predominantly a neuronal enzyme and it has been localized in mitochondria (93,94). Pig brain mitochondria have been shown to contain two major forms of glutaminase, one soluble located in the matrix and one membrane-bound enzyme located in the inner membrane and both activated by phosphate (95).

Soluble glutaminase has been purified from pig brain (96,97) and some other sources (98-100). The soluble enzyme is present as a dimer (101) with a relatively low specific activity and a highly aggregated form of the enzyme with a considerably greater specific activity. By an incubation with phosphate, the soluble enzyme is reversibly and slowly aggregated (101-103). Because of this complicated regulatory behavior, some evidences have been suggested that the membrane-bound and soluble enzyme may have different function in the brain (95).

Kinetic studies of the two enzymes have been performed by Nimmo and Tipton (101) and they show K_m values for glutamine of about 0.8-1.4 mM and 3.4-9.7 mM. One of the products, glutamate, inhibits the enzyme strongly, whereas the other product ammonia has only a slight inhibitory action on the enzyme. Glutamate inhibition is mixed (K_i^{slope}= 1.6 mM and $K_i^{intercept}$= 3.3 mM).

In astrocytes exposed to 1.2 mM valproate, glutaminase activity increased 80% in primary culture by day

2 and remained elevated by day 4; glutamine synthetase activity was decreased 30% (104).

5.3. Glutamate dehydrogenase (E.C. 1.4.1.2.-4.)

Glutamate dehydrogenases catalyze the following reversible reaction:

L-glutamate + H_20 + NAD(P) <-----> α-ketoglutarate + NH_3 + NAD(P)H

although it appears that the reaction velocity is higher when the formation of glutamate is studied.

Three different enzymes are considered depending on the use of NAD/NADH (E.C. 1.4.1.2.), NADP/NADPH (E.C. 1.4.1.4.) or both (E.C. 1.4.1.3.) as coenzymes. Neural glutamate dehydrogenase (E.C. 1.4.1.3.) can use both coenzymes, although it has been shown that NAD is used more effectively than NADP (105). The direction of neural glutamic dehydrogenase (E.C. 1.4.1.3.) activity appears to be regulated in part by the tissue NAD(P)/NAD(P)H concentration ratio (105, 106). In rat brain GDH activity exists in two distinct forms differing in solubility, kinetic parameters, resistence to heat inactivation and allosteric properties (107). These forms have been designed soluble and particulate GDH (107,108).

When the ratio is high, e.g., in the absence of glucose, oxidative deamination of glutamate occurs. In the presence of glucose, when the ratio falls, and α-ketoglutarate is not rate limiting, reductive amination of α-ketoglutarate is favored. Kinetic parameters have been studied for both directions of the reaction (105).

GDH's from various organs of the same species are similar, if not identical. Complete cross-reaction occurs between antibodies induced by bovine liver GDH and extracts of bovine spleen, brain and heart. Most animal GDH are inhibited by GTP and activated by ADP (109). GDH from bovine brain has been partially purified by Grisolia *et al.* (106) and shows a specificity either for NAD or NADP. This enzyme has a molecular weight of 332,000 daltons, as judged from the amino acid sequence of the six identical subunits (110).

As compared with other enzymes, the V_{max} of GDH in synaptic mitochondria from rat brain is 20-40% lower than V_{max} of aspartate aminotransferase, but 4-5fold higher than V_{max} of phosphate dependent glutaminase (111).

5.4. Transaminases

Transaminases play an important role in the aminoacid metabolism, as they are able to catabolize a reversible transference of an amino group from an aminoacid to a ketoacid acceptor. Those enzymes use pyridoxal phosphate as a coenzyme, which will perform the transference of the amino group. Among those transaminases, aspartate aminotransferase or glutamate oxalacetate transaminase (E.C. 2.6.1.1.) catabolizes the reversible transference of amino group of Asp to α-ketoglutarate and generates oxalacetate and glutamate. There have been found two isoenzymes of aspartate aminotransferase in animals: a citoplasmic and a mitochondrial. Both are dimeric proteins of 45,000 daltons and 2,000 daltons subunits (112).

Aspartate amino transferase (AAT) activity seems to be higher than glutamate dehydrogenase (GDH). The ratio AAT/GDH is between 10 and 20 in rat brain (113) or in squid giant nerve (114). Transamination of glutamate by using aspartate amino transferase generates aspartate, which is also a neurotransmitter; whereas glutamate dehydrogenase yields ammonia but not another neurotransmitter. The higher activity of AAT can be therefore due to prevent the toxic action of ammonia.

Another transaminase is alanine aminotransferase (E.C. 2.6.1.2.), an enzyme which transfers amino from alanine to α-ketoglutarate to yield pyruvate and glutamate. This enzyme presents also a cytoplasmic and a mitochondrial isoenzymes.

5.5. Glutamic acid decarboxylase (E.C. 4.1.1.15.)

The enzyme removes the α-carboxyl group of glutamate to produce a γ-carboxyl amino acid called γ-amino butyric acid (GABA). This decarboxylation of glutamate to GABA is not very different from decarboxylation of L-DOPA or tryptophan to dopamine and serotonin. Like those enzymes, glutamic acid decarboxylase requires the cofactor pyridoxal phosphate (vitamin B_6).

The enzyme is highly substrate specific, although Homola and Dekker (115) showed that some glutamate analogs can also be decarboxylated.

6. DYSFUNCTION OF EXCITATORY AMINOACID NEUROTRANSMISSION

It is important to maintain low levels (1-3μM) of extracellular glutamate as excessive receptor stimulation or excessive ammonium generated by the glutamate dehydrogenase can lead to neural injury and/or death ("excitotoxicity").

Glutamate appears to be remarkably potent and rapidly acting neurotoxin. Exposure to 100 μM glutamate for 5 min is enough to destroy large numbers of cultured cortical neurons (116). By the way, glutamate neurotoxicity may be blocked by antagonist compounds and attenuated by antagonists added after glutamate exposure (116).

Neurodegenerative diseases, such as Alzheimer's disease (AD), Huntington's disease (HD), Parkinson's disease (PD) and amyotrophic lateral sclerosis (ALS), particularly affect old people and result in: i) a modification of the individual personality; ii) the need for constant help from relatives; iii) a high economical costs for family and institutions. In Spain alone, over 500.000 people are affected with those diseases with this increasing as the aged population increases. Due to the increased lifespan in

developed societies, neurodegenerative diseases have been increased in the European Union and represent one third of deaths (after cancer and cardiovascular problems). It is though that with Alzheimer's disease alone, at the beginning of next century, 8 million Europeans will be affected.

Many reports have been published on increased levels of glutamate in certain neurodegenerative diseases such as AD, HD, PD and ALS (117-120). Increased extracellular glutamate has also been implicated in the onset of neurodegeneration associated with hypoxic damage (stroke). The release in glutamate following hypoxia has been suggested to be due, at least in part, to a calcium-independent mechanism following the reversal of the neuronal glutamate uptake carrier (121). This increase in extracellular glutamate acts afterwards postsynaptically to increase cellular calcium levels with subsequent cell death.

The mechanisms by which glutamate is increased in neurodegenerative diseases is unknown. The cause of this increased extracellular glutamate has been ascribed to a decrease in the activity of glutamate dehydrogenase (PD) (122) or a decrease in the number of Na^+-glutamate transporters (ALS, HD) (123). As it is a neurotoxic, it is likely that the high glutamate concentration observed in neurodegenerative diseases are the most likely cause of the neurodegeneration.

Marangos *et al*. (124) stated the glutamate hypothesis for early Alzheimer's disease: "Cell death due to neuronal toxicity could result from excessive synthesis or release of glutamate or a glutamate-like substance, faulty glutamate reuptake, decreased glutamate degradation, or decreased inhibition of excitatory neurons. Any of these aberrant processes could, early in the disease, increase local levels of glutamate and so initiate a slow, progressive degeneration and eventual death of neurons".

Several post-mortem studies have compared brain glutamate levels in Alzheimer's disease to those in control subjects. Some investigators (125-127) have found lower glutamate levels in the frontal cortex and the temporal cortex of Alzheimer's patients than in control subjects. Cerebro spinal fluid (CSF) concentration of free glutamate was significantly higher in patients with Alzheimer's disease than in comparison subjects (117). It should be noted that measurements of glutamate in CSF are likely to give better approximation of glutamate concentrations at synapses than are plasma glutamate concentrations.

In Huntington's disease, glutamate and GABA concentrations decrease in striatum and caudate nucleus from brain (118,128). However, no reduction at all was observed in the frontal cortex of patients. A likely possibility is that the low glutamate content of the caudate and the putamen in HD results from chronic failure of the normal reuptake mechanism for glutamate released at synapses, with or without any excessive rate of release of this neurotransmitter. This possibility is supported by the finding of Cross *et al.* (129), who observed large reductions in high-affinity glutamate uptake sites in autopsy specimens of caudate and putamen from HD patients.

If either excessive release or decreased reuptake of glutamate occurred in the striatum in HD, concentrations of glutamate might become high at synapses, with resulting damage to neurons. Some of the excess glutamate accumulate in synaptic clefts in HD would be carried away in the extracellular fluid, thus eventually causing a lowered glutamate content in striatal tissue. This fact is supported by an increased glutamate concentration in CSF of living HD patients as it was observed for Alzheimer's disease patients.

In Parkinson's disease, Schapire *et al.* (130) demonstrated a reduced activity of complex I of the mitochondrial respiratory chain in the region of substantia nigra in brain. Deficiencies on complex II and IV have been also observed in muscle biopsia from PD patients (131). Cedarbaum *et al.* (122) observed a deficiency of GDH but not on pyruvate dehydrogenase complex. Since complex I is the point of entry for reducing equivalents (as NADH) to the respiratory chain, a decrease in complex I activity might result in feedback endproduct inhibition of GDH. Decreased levels of GDH might exert an excitotoxic glutamate effect via NMDA receptors on striatal dopamine nerve terminals (132) and contribute to cellular degeneration in PD.

Amyotrophic lateral sclerosis (ALS) is a disease resulting in degeneration of the motor cortex, the brain-stem and the spinal chord. While there are a number of hypotheses underlying this disease, an increase in glutamatergic neurotransmission has been proposed as a key event in the disease onset and recently, this has been ascribed, at least in part, to a defect in the function of the GLT-1 glutamate transporter which is localized on astrocytes (123).

Recent *in vitro* studies have demonstrated that a blockade of the GLT-1 transporter either by transport inhibitors or by an antisense approach resulted in a slow, selective loss of motor neurons, thus strengthening the case for a critical role for GLT-1 in the etiology of ALS (120,133). Furthermore, this decrease in uptake does not appear to be associated with a decrease in transporter expression levels (57) and there is no evidence of specific protein mutations associated with the disease. Because of the potential role of free radicals in ALS and the upset in function of the superoxide dismutase enzyme in the familial form of ALS, previous studies have suggested that an increase in free radicals may impair the function of GLT-1 (134).

The role of free radicals in the etiology of ALS has been strengthened by the selection of point mutations in the cytosolic Cu/Zn superoxide dismutase (SOD-1) associated with the familial form of ALS (FALS) (135). These changes were not detected in control individuals and do not represent normal allelic variants. SOD-1 catalyses the dismutation of the superoxide radical (O_2^-) to hydrogen peroxide (H_2O_2) and represents the first line of defense against oxygen toxicity. The mechanism responsible for tissue damage associated with reduced SOD activity remains to be defined. Direct toxicity due to the superoxide radical is probably minor in comparison to the generation of

the hydroxyl radical (OH.) which is much more reactive. In addition, the superoxide may interact with endogenously formed nitric oxide to form peroxynitrite which can oxidize methionine residues in proteins and peptides as well as thiols and thioethers (136). It has therefore been suggested that an increase in free radicals in ALS may be, at least in part, responsible for the upset in functioning of the GLT-1 transporter and that the disease may have both a free radical and excitatory amino acid basis.

Glutamate, and particularly the glutamate transporter system, have also been implicated in the ischemic damage associated with anoxia/hypoxia (stroke). For the first few minutes of ischemia, there is a slow acid shift of the cellular pH with a slow rise of extracellular potassium concentration and a subsequent decrease in extracellular sodium and calcium. The rise in potassium depolarizes the cells to around -20mV (anoxic depolarization) and the release of glutamate (137). The mechanism by which glutamate is released in ischemia has been controversial. Some reports suggest that the release is calcium-dependent, suggesting conventional vesicular release, while others claim that the release is calcium-independent, implying a non-exocytotic mechanism such as the reversed operation of the glutamate uptake carrier (138).

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