Rose, C. F., Verkhratsky, A., & Parpura, V. (2013). Astrocyte glutamine synthetase: Pivotal in health and disease. Biochemical Society Transactions, 41(6), 1518-1524.
Final publication available at : https://doi.org/10.1042/BST20130237

Astrocyte glutamine synthetase: pivotal in health and disease

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List of abbreviations:

Disclosures: The authors have no conflicts to disclose

Key Words: astrocytes, glutamine synthetase, glutamate, glutamine, GABA, ammonia

Astrocytes as homeostatic cells in the central nervous system

Out of four main classes of neuroglial cells of the central nervous system (astroglia, oligodendroglia, NG2 glia and microglia) astrocytes are arguably the most diverse. Indeed, astroglial cells include a huge variety of phenotypes with very different morphology and physiological properties. The largest and most characterised types of astroglia are represented by the *protoplasmic astrocytes* of the grey matter and *fibrous astrocytes* of the white matter. Morphological appearance of these cells as well as their physiology differ substantially between various brain regions. Another main group of astroglial cells covers the *radial glia*, which are bipolar cells with a small cell body and main processes, one of which forms endfeet on the ventricular wall and the other at the pial surface. Radial glia are the main glial type of the embryonic brain and generally absent from the mature brain with the exception of the retina (*Müller glia*) and cerebellum (*Bergmann glia*). Astrocytes are further classified into the *velate astrocytes* of the cerebellum, the *interlaminar and polarised astrocytes* of the primate cortex, *tanycytes* (found in the periventricular organs, the hypophysis and the raphe part of the spinal cord), *pituicytes* in the neuro-hypophysis, and *perivascular* and *marginal* astrocytes. Astroglia also include several types of cells that line the ventricles or the subretinal space, namely *ependymocytes*, *choroid plexus cells* and *retinal pigment epithelial cells* (see [1,2] for review and references).

All these highly diverse cells are united by their function which lies in maintaining the homeostasis of the nervous system [1]. To cater for this, astrocytes perform many different functions, which embrace every known house-keeping and homeostatic task in the CNS (for detailed reviews see [3–12]). Astroglial cells are fundamental for brain development being, for example, chief promoters and supporters of synaptogenesis, synaptic maturation and maintenance [13,14]. In the adult brain these are astrocytes which act as stem cells in the neurogenic niches [15]. Astrocytes define overall brain architecture by forming glial barriers at the pia mater and between the brain parenchyma and vasculature; they divide the grey matter into neuro-vascular units and contribute to regulation of local blood flow; they are primary elements of brain ion homeostasis, they control extracellular pH, they provide neurones with metabolic substrates and release scavengers of reactive oxygen species (for detailed reviews see [3–12]). Astrocytes are also critical for numerous systemic homeostatic functions such as central chemoception [16–18] and regulation of sleep [19]. In the present essay we shall concentrate on a single homeostatic function of astroglia, the function which relates to their ability to maintain turnover of two principal neurotransmitters in the brain, glutamate and GABA, this function being accomplished by glutamate, GABA and glutamine transporters, and by astroglial specific enzyme, the glutamine synthetase (GS).

Astrocytes in glutamatergic neurotransmission: glutamate uptake and release

Fundamentally astrocytes contribute to the homeostasis and regulation of extracellular level of three key neurotransmitters in the CNS, represented by glutamate, GABA and adenosine [8,20,21].

Astroglial role is multifaceted; (i) it includes an uptake of these neurotransmitters from the extracellular cleft, which defines the time course of neurotransmission and (at least in case of glutamate) preventing toxicity; (ii) astrocytes are also capable or releasing neurotransmitters through vesicular and non-vesicular pathways and (iii) astrocytes catabolise neurotransmitters into intermediates, which are then sent back to neurones to be transformed into active molecules, thus maintaining synaptic transmission.

Astroglial uptake of glutamate

Glutamic acid, or glutamate, is an amino acid, which in the CNS, acts as the main excitatory neurotransmitter released from presynaptic terminals through vesicular exocytotic mechanism. To make this mechanism function properly both extra- and intracellular glutamate dynamic should be tightly controlled. Glutamate should be removed from the synaptic cleft and at the same time the synaptic cleft should be guarded from extrasynaptic glutamate or glutamate spillover from neighbouring synapses. Simultaneously the glutamate inside the presynaptic terminal should be rapidly replenished. It is important to note that neurones are incapable of *de novo* synthesis of glutamate; the latter is synthesized in astrocytes [22](see below). Astrocytes also provide a principal pathway for glutamate uptake: about 80% of all glutamate released during synaptic transmission, is taken up by astroglial cells with only about 20 per being accumulated into postsynaptic neurones; presynaptic neurones do not take up glutamate [20,23].

Glutamate uptake by astroglial cells is mediated through plasmalemmal glutamate transporters of the solute carrier 1 (SLC1) family [24] represented by 5 main types of Excitatory Amino Acid Transporters 1 - 5 (EAAT1/SLC1A3, EAAT2/SLC1A2, EAAT3/SLC1A1, EAAT4/SLC1A6 and EAAT5/SLC1A7), of which astrocytes specifically express EAAT-1 and EAAT-2 transporters; these in rodents are known as glutamate/aspartate transporter (GLAST) and glutamate transporter-1 (GLT-1). Transport of glutamate by EAATs is powered by transmembrane gradient of Na^+ ions; translocation of single molecule of glutamate (which is a monovalent anion at physiological pH) is linked to an influx of three Na^+ ions, one H^+ ion and efflux of one K^+ ion, all these movements going downhill along with respective concentration gradients. The glutamate transport cycle, therefore, is associated with net cation influx which defines the electrogeneity of glutamate transporters that could be measured as an inward current. Uptake of physiologically relevant concentrations of glutamate causes substantial sodium influx, which may increase intracellular Na^+ concentration by tens of mM [25,26].

Astroglial release of glutamate

Astrocytes can release glutamate by several different mechanisms: (i) reversal of uptake by plasma membrane glutamate transporters [27], (ii) opening of anion channels, be that be volume-regulated anion channels [28] or Ca^{2+}-activated anion channels [29], (iii) Ca^{2+}-dependent exocytosis [30], (iv) glutamate exchange via the cystine-glutamate antiporter [31], (v) release through ionotropic purinergic receptors [32] and (vi) functional unpaired connexons, “hemichannels”, on

the cell surface [33] [for review see [34]]. These mechanisms can be further classified into the release through plasmalemmal molecular entities, channels and transporters, and by exocytosis, the latter characterized by the formation of an aqueous channel, the fusion pore, upon the merger of vesicular and plasma membranes. All of the mechanisms depend on glutamate concentration in various cellular compartments and the extracellular space. Conceptually, its anionic concentration gradient between the cytosol and the extracellular space directly dictates the release via plasmalemmal channels/transporters. However, due to coupling transport of other ions, EAATs have their reversal potential at ~ +50 mV [25,35], which means that under normal physiological conditions EAATs could not revert to release glutamate [36]. In respect to exocytosis, cytosolic glutamate is sequestered into secretory vesicles via vesicular glutamate transports (VGLUTs) before it gets released through the fusion pore.

The cytoplasmic glutamate concentrations in astrocytes are maintained different than those in neurons [for review see [20]]. The glutamate concentration in the cytosol of synaptic terminals reaches 10-15 mM [37]. This permits VGLUTs (K_m ~1-2 mM) to operate at nearly their maximal rates to concentrate glutamate into synaptic vesicles. Consequently, intravesicular glutamate concentration reaches 60 mM [38], which generates estimated ~1.1 mM glutamate concentration in the synaptic cleft at time of release [39]. Owing to the powerful action of mainly astroglial EAATs, glutamate gets quickly lowered down, so that the resting glutamate concentration in the extracellular space of the CNS is maintained at ~25 nM [40]. Presumably due to the presence of GS, the cytosolic glutamate concentration in astrocytes is lower than neuronal at estimated 0.1-5 mM [37]. This is sufficient to allow for the operation of VGLUTs to concentrate glutamate inside astrocytic vesicles, which intravesicular concentration is estimated at about ~ 20 mM [41]. Consequently, vesicular glutamate release from astrocytes creates localized extracellular glutamate accumulations of 1-100 μM [42].

In the CNS, glutamate is synthesized *de novo* within astrocytes as a by-product of the tricarboxylic acid (TCA) cycle [43]. Here, as glucose breaks down to pyruvate in the cytosol, pyruvate enters mitochondria and the TCA cycle via pyruvate carboxylase, a ligase that catalyzes the carboxylation of pyruvate to form oxaloacetate (Fig. 1). In turn, glutamate is produced from α-ketoglutarate, a downstream TCA intermediate, by transamination of aspartate *via* mitochondrial aspartate amino transferase. The synthesized glutamate once in the cytosol can then be converted to glutamine by GS, or transported into vesicles via VGLUTs, especially isoform 3 (VGLUT3) [44] (Fig 2). The role of GS and cytosolic glutamate concentrations for exocytotic glutamate release from astrocytes has been experimentally demonstrated [44]. To increase the cytosolic glutamate concentration in astrocytes GS activity was blocked with L-methionine sulfoximine, which led to an augmented exocytotic glutamate release in response to mechanical stimulation [44], while, importantly, Ca^{2+} dynamics were unaffected by this GS blocker. Of note, the mechanical stimulation almost exclusively (97%, based on the pan-VGLUT blocker Rose Bengal) recruits the exocytotic/vesicular release of glutamate [45], but not glutamate release through, e.g., Ca^{2+}-activated anion channels [46]. Therefore, the impairment of GS activity implicates that the increase of cytosolic glutamate concentration provided more

glutamate for VGLUTs to transport across the vesicular membrane into the vesicular lumen, thus increasing the amount of glutamate in vesicles available for release. Further modulation of cytosolic glutamate concentration and its release could also originate from changes that might occur at the level of pyruvate carboxylase. Hence, astrocytes from a Huntington's disease mouse model showed an augmented glutamate release as *de novo* glutamate synthesis was increased due to an upsurge in the expression of pyruvate carboxylase, while the conversion of glutamate to glutamine was unchanged, as no changes in the expression level of GS were observed [47]. Hypothetically, additional modulation of cytosolic glutamate concentration could sprout from the metabolic pathway between pyruvate carboxylase and GS. This could involve various mitochondrial enzymes, such as the previously mentioned aspartate amino transferase. Similarly, SLCs redistributing reactants/products across the inner mitochondrial membrane could also be involved, perhaps mitochondrial glutamate carrier 1 (SLC family 25 member 22) and mitochondrial aspartate glutamate carrier (SLC family 25 member 13, also called citrin).

Astroglial glutamine-glutamate and glutamine-GABA shuttle

Maintenance of glutamatergic transmission requires constant replenishment of glutamate, which in turn relies on astroglial glutamate uptake, glutamate conversion into glutamine and transport of the latter into neuronal presynaptic terminals. This coordinated system of fluxes of glutamate and glutamine in generally known as *glutamate–glutamine shuttle* (Fig. 1). This is an energy dependent mechanism which requires hydrolysis of one molecule of ATP for a single conversion of glutamate to glutamine. Transport of glutamine from astrocytes and into neurones is mediated by amino-acid transporters, which have a specific distribution between neurones and astrocytes [48]. Astrocytes are in possession of the system N transporters (represented by Na^+/H^+ dependent sodium coupled neutral amino acid transporters SN1/SNAT3/SLC38A3 and SN2/SNAT5/SLC38A5 [49,50] which mediate glutamine efflux. Importantly these transporters can be relatively easy switched to the reverse mode after, for example, increase in cytosolic Na+ concentration. Neurones express another system for glutamine transport, mediated by the system A glutamine transporters [51] (the sodium coupled neutral amino acid transporters ATA1/SNAT1/SLC38A1 and ATA2/SNAT2/SLC38A2) which act as influx transporters mediating glutamine accumulation into the neuronal compartment [52]. There are some exceptions however, for example GABAergic neurones express N-transporter SNAT7 [53].

In presynaptic terminals glutamine hydrolysed to glutamate; incidentally this conversion catalysed by phosphate-activated glutaminase, does not require energy. The newly synthesized glutamate is subsequently concentrated in synaptic vesicles bearing VGLUT transporters and thus releasable pool of glutamate is maintained. The supply of glutamate by astrocytes is coordinated with neuronal activity - increase in external concentration of glutamate increases release of glutamine [54], this being mediated through glutamate transporters [55] possibly through an increase in intracellular Na+ concentration.

Astroglia and GABA-ergic transmission

Astroglial glutamine is also critical for inhibitory transmission in the CNS mediated by GABA. Presynaptic terminals synthetise GABA from glutamate that arrives through glutamate-glutamine shuttle; inhibition of the later rapidly inhibits GABAergic transmission [56]; this mechanism is often also referred to as *GABA-glutamine shuttle* or *cycle* (Fig. 2). The cytosolic concentration of GABA in astroglial cells can be quite high, approaching ~2.5 mM [57]. Of note, astrocytes also express GABA transporters GAT-1 and GAT-3, which can be readily reversed upon moderate (about 7 mM) increases in the cytosolic Na+, thus making astrocytes the source of GABA [58].

Ammonia detoxification

In addition to its importance in neurotransmission, GS also plays a significant role in the assimilation of ammonia by the brain. Ammonia, a metabolite mainly produced within the gastrointestinal system (through protein degradation and amino acid deamination), is primarily regulated by the urea cycle; exclusively found in the liver. Ammonia-rich venous blood from the gastrointestinal tract first passes through the liver maintaining the circulating concentration of ammonia between 35-50 µM. Ammonia, composed of gas (NH_3) and ion (NH_4^+) components, can easily cross all plasma membranes through diffusion, channels and transporters [59]. In the setting of liver disease, blood ammonia levels can increase as high as 1mM leading to toxic concentrations in the brain and the onset of hepatic encephalopathy, a neuropsychiatric disorder involving cognitive alterations and motor impairments. Hyperammonemia also arises in infants with inborn errors of urea cycle enzymes causing seizures and coma. Surviving children have a high incidence of mental retardation and cerebral palsy. Toxic levels of ammonia trigger changes in both pH and membrane potential and have a profound effect on metabolism [59]. The brain is particularly susceptible to increased concentrations of ammonia and heavily relies on GS in astrocytes to prevent toxicity and neurological dysfunction. It has been demonstrated that an increase in brain ammonia elicits an increase in GS activity [60] however Cooper et al. [61] showed that under normal physiological conditions, GS activity in the brain operates at near maximal capacity, preventing any further induction. In either case, the observation of elevated concentrations of ammonia in liver disease reveals that the capacity of GS to detoxify ammonia in the brain is limited and therefore cannot compensate to maintain ammonia at physiological levels. Moreover, GS activity in brain has been shown to be inhibited in liver disease [62] since an increase in cerebral nitric oxide stimulates peroxynitrite-mediated nitration of tyrosine residues of GS resulting in inactivation [63]. In turn, a decrease in capacity to clear ammonia in the brain detrimentally leads to sustained elevation in brain ammonia and, thus, persisting neurological dysfunction [64].

GS mutations in brain

First described by Häberle et al., [65], homozygous mutations in the GS gene (GLUL), as observed in two newborns lead to a reduction in GS activity and, consequently, severe brain malformations, seizures, multiorgan failure and early death. Surviving infants develop chronic

encephalopathy during the neonatal period. Furthermore, mice with prenatal excisions of the GS gene die during early embryonic development [66]. Consequently, in addition to causing alterations in glutamate/glutamine homeostasis, deficiency in GS consequently impairs the detoxification of ammonia; concentrations of ammonia have been demonstrated to rise between 100-200 μM [67]. These levels of ammonia are comparable to those found in liver disease associated with hepatic encephalopathy [68].

CNS disorders and alterations in glutamine synthetase

As GS is an essential part of a complex astrocyte-neuron signaling process, changes in expression and activity of GS will lead to neurological dysfunction. A number of different brain pathologies are associated with alterations in GS expression/activity. Patients diagnosed with medial lobe temporal lobe epilepsy exhibit markedly reduced GS expression and activity in the hippocampus despite astroglial proliferation/reactive astrocytes [69,70].
However, an upregulation of GS has been demonstrated in the hippocampal dentate gyrus during seizure acquisition in the amygdala kindling model of epilepsy [71] with, however, no change in GS activity found in frontal cortex of epilepsy patients [72]. Overall, it appears that alterations in GS activity/expression are region selective depending on the kind of epilepsy. Similarly, a decreased in GS expression was found in the striatum of rats with Parkinsonian tremor [73]. Postmortem brain tissue collected from Alzheimer's patients have revealed a reduction in GS activity [74,75]. Furthermore, it has been shown that the amount of GS protein in Alzheimer's brains is inversely correlated with the number of beta-amyloid plaques [76] and that a decrease in GS is associated with the presence of amyloid beta deposits, as demonstrated in a mouse model of Alzheimer's disease [77]. Moreover, in the brains of patients with schizophrenia, an increase and decrease in GS mRNA has been found in specific regions as well [78]. Furthermore, there are several lines of evidence that support retinal GS is implicated in diabetic retinopathy. Down regulation of GS has been demonstrated to occur during the early stages of diabetes[79–81] . The susceptibility of certain brain structures damage (i.e CA1 area of the hippocampus) to hypoxic damage is associated with the disappearance of GS; this observation, however, has not been demonstrated in brain regions less susceptible to hypoxia [82]. Brain GS mRNA expression has also been found to be decreased in patients with depression [83–85]. In addition, increased expression of GS through ischemic postconditioning has been shown to be neuroprotective in ischemic rats [86]. Overall, changes in GS expression/activity are associated with neurodegenerative diseases and mental disorders (Fig. 3).

GS expression in other brain cells?

In addition to GS being particularly localized in astrocytes, GS expression has been measured in other cells of the brain. During pathophysiological conditions, such as in patients with Alzheimer's disease, GS was found to be expressed in neurons; a result that was not replicated in post-mortem tissue from control brains. Furthermore, GS has also been demonstrated to be expressed in microglia of simian immunodeficiency virus(SIV)-infected macaques [87]. However,

the role of GS, as well as the cause or effect of GS expression in other cells of the brain, remains unclear [88].

Conclusion

Proper astrocyte function is imperative for glutamatergic/GABA physiology. Alterations in GS, an exclusive enzyme found in astrocytes, have been shown to be associated with a number of neurological disorders. In addition to GS playing a vital role in glutamate homeostasis, it is also bears the brunt of ammonia detoxification in the brain. The precise neurotoxic effects following alterations in GS, whether due to glutamate or ammonia neurotoxicity, deserves to be further investigated.

Acknowledgements. Authors' research was supported by Alzheimer's Research Trust (UK) Programme Grant (ART/PG2004A/1) to A.V. and by National Science Foundation (CBET 0943343) grant to V.P.

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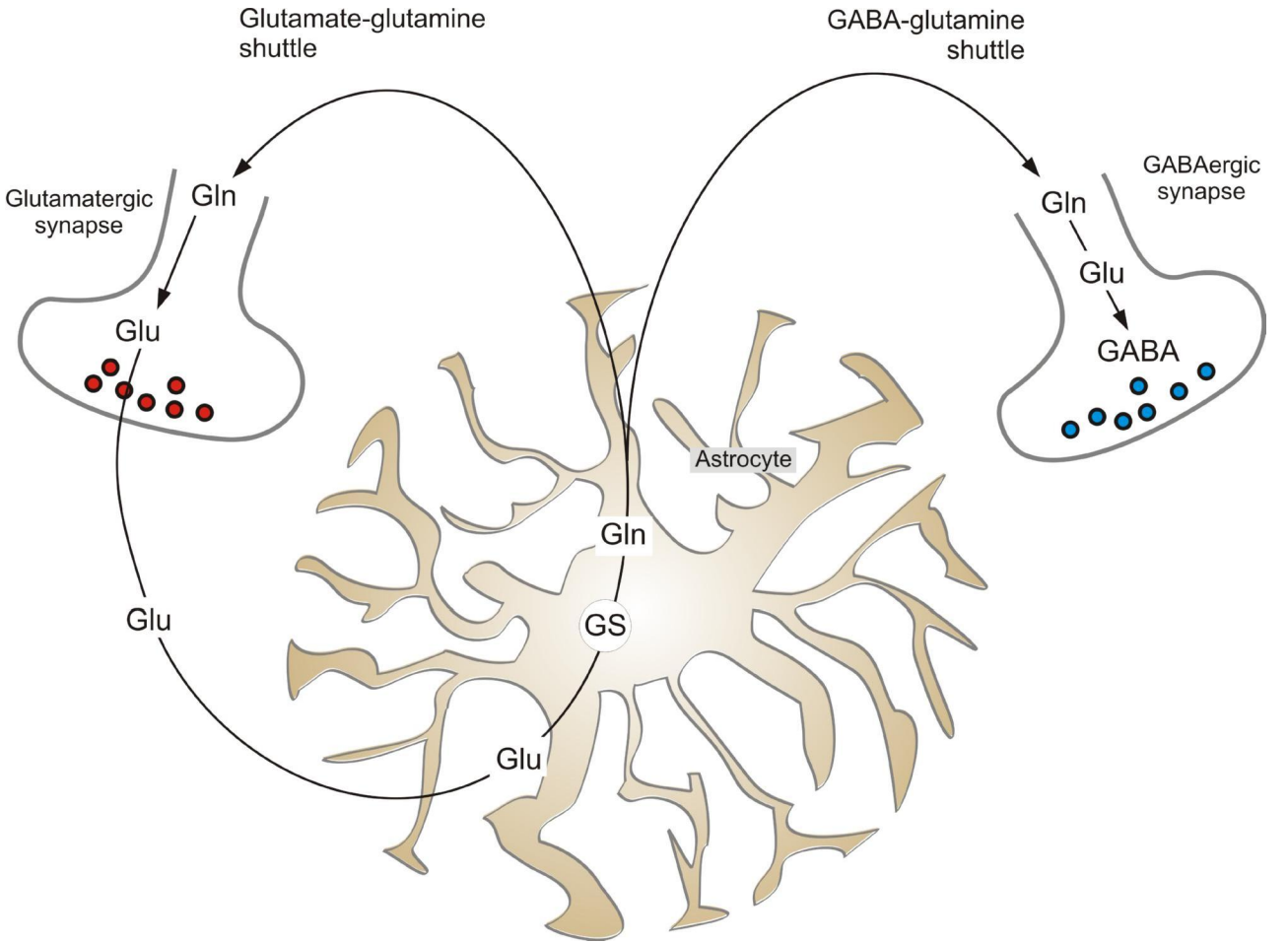
Figure legends

Figure 1: Astrocytes maintain glutamatergic and GABA-ergic transmissions through glutamate-glutamine and GABA-glutamine shuttle.
After entering the astrocyte, glutamate is converted into glutamine, which is then transported back to the presynaptic terminals, where it is converted into glutamate in excitatory and into GABA in inhibitory synapses; these neurotransmitters subsequently are accumulated into synaptic vesicles.

Figure 2: Regulation of glutamate in exocytotic glutamate release from astrocytes.
Glucose is broken down to pyruvate in the cytosol. In the mitochondrion (mito) pyruvate entry through the tricarboxylic acid cycle via pyruvate carboxylase (PC) leads to production of oxaloacetate (OAA) and a downstream intermediate, α-ketoglutarate (α-KG). In turn, glutamate (Glut) is synthesized in astrocytes *de novo* from α-KG by transamination of aspartate *via* mitochondrial aspartate amino transferase (AAT). The synthesized glutamate once in the cytosol can then be converted to glutamine (Gln) by glutamine synthetase (GS), or transported into vesicles via vesicular glutamate transporters, especially isoform 3 (VGLUT3). Drawing is not to scale.

Figure 3: Alterations in GS and neuropathologies


Glutamate-glutamine
shuttle
GABA-glutamine
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Astrocyte
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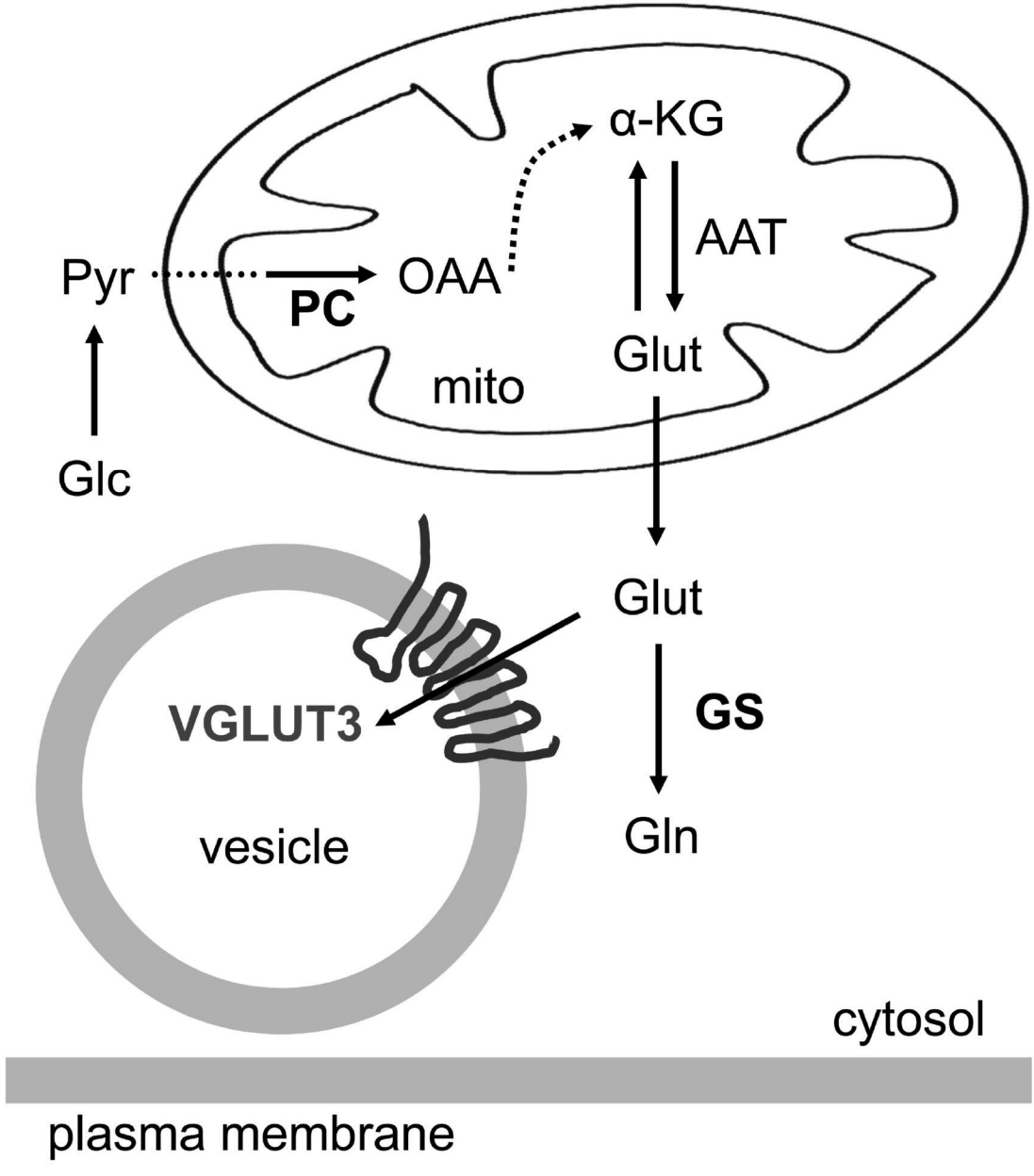
α-KG
AAT
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PC
OAA
Glut
mito
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Glut
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GS
vesicle
Gln
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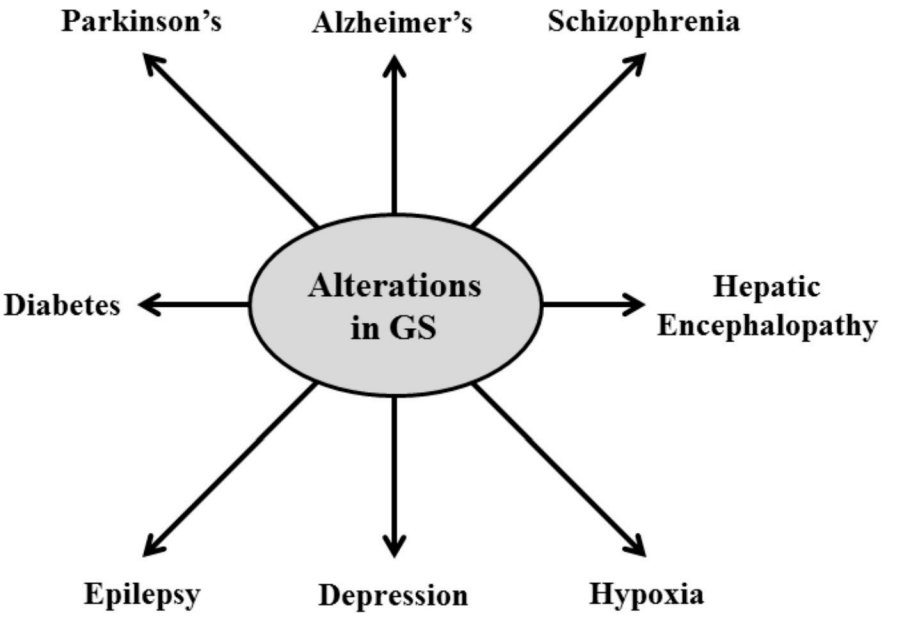
Parkinson's
Alzheimer's
Schizophrenia
Diabetes
Alterations in GS
Hepatic Encephalopathy
Epilepsy
Depression
Hypoxia