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Regulation of Astrocyte Glutamine Synthetase in Epilepsy

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Abstract

Astrocytes play a crucial role in regulating and maintaining the extracellular chemical milieu of the central nervous system under physiological conditions. Moreover, proliferation of phenotypically altered astrocytes (a.k.a. reactive astrogliosis) has been associated with many neurologic and psychiatric disorders, including mesial temporal lobe epilepsy (MTLE). Glutamine synthetase (GS), which is found in astrocytes, is the only enzyme known to date that is capable of converting glutamate and ammonia to glutamine in the mammalian brain. This reaction is important, because a continuous supply of glutamine is necessary for the synthesis of glutamate and GABA in neurons. The known stoichiometry of glutamate transport across the astrocyte plasma membrane also suggests that rapid metabolism of intracellular glutamate via GS is a prerequisite for efficient glutamate clearance from the extracellular space. Several studies have indicated that the activity of GS in astrocytes is diminished in several brain disorders, including MTLE. It has been hypothesized that the loss of GS activity in MTLE leads to increased extracellular glutamate concentrations and epileptic seizures. Understanding the mechanisms by which GS is regulated may lead to novel therapeutic approaches to MTLE, which is frequently refractory to antiepileptic drugs. This review discusses several known mechanisms by which GS expression and function are influenced, from transcriptional control to enzyme modification.

Keywords

Gliosis; glutamate; hippocampal sclerosis; limbic system; metabolism; seizures

1. INTRODUCTION

Astrocytes, the most abundant cell type in the brain, were long regarded as elements of mere structural support, with minor contributions to brain physiology. However, this view has dramatically changed, and astrocytes are now considered pivotal for a multitude of brain functions, such as axonal growth (Katz et al., 1983; Lemke, 2001), energy metabolism (Hertz, 2011; Magistretti and Pellerin, 1999; Pellerin et al., 2002), neurotransmitter

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homeostasis (Danbolt, 2001; Schousboe et al., 2013), water/electrolyte balance (Benfenati et al., 2007; Orkand et al., 1966; Verkhratsky and Steinhauser, 2000), and the immune response (Boddeke et al., 2013). Moreover, astrocytes may be critically involved in the pathophysiology of neurological and psychiatric disorders such as epilepsy (Binder and Steinhauser, 2006; Boison, 2008, 2013; Coulter and Eid, 2012; Haydon and Carmignoto, 2006; Steinhauser and Boison, 2012; Tian et al., 2005), stroke (Nedergaard and Dirnagl, 2005), Alzheimer's disease (Games et al., 1995; Robinson, 2000), multiple sclerosis (Sharma et al., 2010), schizophrenia (Rajkowska et al., 2002; Takano et al., 2010), and suicide/depression (Hasler et al., 2007; Sanacora et al., 2012).

The importance of astrocytes for brain function is underscored by several key features of these cells. Firstly, astrocytes are highly branched cells with cytoplasmic processes surrounding and interacting with critical functional-anatomical domains such as the perivascular and perisynaptic regions (Peters et al., 1991). Astrocyte processes are also juxtaposed to the soma, dendrites and axons of neurons as well as to the plasma membranes of microglial cells, oligodendrocytes, and other astrocytes (Peters et al., 1991). Thus, astrocytes can potentially influence and be influenced by a large number of cells, synapses, and vascular structures. Secondly, astrocytes harbor virtually all of the constitutive metabolic enzymes, as well as several specialized enzymes, most notably glutamine synthetase (GS) (Martinez-Hernandez et al., 1977) and pyruvate carboxylase (Shank et al., 1985). These cells are therefore crucial for the metabolism and homeostasis of glucose, ammonia and glutamate in the brain. Thirdly, the astrocyte plasma membrane is richly endowed with receptors for neurotransmitters (Bowman and Kimelberg, 1984; Kettenmann et al., 1984), growth factors, cytokines, and chemokines (Boddeke et al., 2013). The astrocyte plasma membrane also contains transporters for numerous molecules, such as potassium (Orkand et al., 1966; Verkhratsky and Steinhauser, 2000), water (Nagelhus et al., 1998), glutamate (Danbolt, 2001), glutamine (Chaudhry et al., 2002), glucose (Morgello et al., 1995; Yu and Ding, 1998), ketone bodies and lactate (Bergersen, 2007; Broer et al., 1997; Debernardi et al., 2003). Thus, astrocytes recognize a variety of extracellular stimuli (e.g., neurotransmitters, cytokines, growth factors) and are able to respond to these and other signals by uptake or release of ions, water, amino acids and energy substrates. Finally, astrocytes can change (transform) their phenotype and proliferate in response to certain (usually damaging) conditions. Proliferation of such transformed ("reactive") astrocytes in brain tissue is called reactive astrogliosis (Robel et al., 2011).

Reactive astrogliosis is present in a variety of pathological conditions, including intracranial infections (Garden, 2013), hypoxia/ischemia (Nedergaard and Dirnagl, 2005), Alzheimer's disease (Games et al., 1995), and epilepsy (Cohen-Gadol et al., 2004; Gloor, 1991). Reactive astrogliosis was historically thought to be a secondary "scar tissue" response to loss of neurons; however, an increasing number of studies now suggest that reactive astrocytes are critically involved in the causation of several brain disorders. One such disorder is mesial temporal lobe epilepsy (MTLE), which is among the most common types of medication refractory epilepsies (Hauser et al., 1993). This review discusses the involvement of reactive astrocytes in the pathophysiology of MTLE. The main emphasis will be on the lack of GS in these cells (Eid et al., 2004b; van der Hel et al., 2005), and the mechanisms by which the expression and activity of this enzyme are regulated under normal and pathological conditions. An understanding of these regulatory mechanisms is important, as such information is likely to facilitate the development of novel and more efficacious therapies for MTLE.

2. GS IS DEFICIENT IN ASTROCYTES IN MTLE

MTLE is characterized by recurrent complex partial seizures that originate from a network of brain areas, including the hippocampus, entorhinal cortex, amygdala, lateral temporal cortex, medial thalamus, and inferior frontal lobe (Spencer, 2002). Some of these areas are characterized by pathological changes, most notably patterned loss of neurons (Gloor, 1991; Sommer, 1880), reorganization of synapses with aberrant expression of glutamate receptors (de Lanerolle et al., 1998; Eid et al., 2002), altered properties of microvessels (Eid et al., 2004a; Eid et al., 2005; Heinemann et al., 2012; Lauritzen et al., 2011; Lauritzen et al., 2012a; Liwnicz et al., 1990), and marked proliferation of reactive astrocytes (Cohen-Gadol et al., 2004; Gloor, 1991). The reactive astrocytes in MTLE are characterized by changes in their expression of inwardly rectifying potassium channels (Kir4.1) (Bordey and Sontheimer, 1998; Heuser et al., 2012), water channels (Amiry-Moghaddam et al., 2003; Binder et al., 2012; Eid et al., 2005), monocarboxylate transporters (MCTs) (Lauritzen et al., 2011; Lauritzen et al., 2012a; Lauritzen et al., 2012b), GS (Eid et al., 2004b; van der Hel et al., 2005), and possibly also glutamate transporters (Bjornsen et al., 2007; Tessler et al., 1999). Several additional changes in the phenotype and function of reactive astrocytes in MTLE have been previously described (reviewed in (Boison, 2013)). Based on these findings it has been suggested that reactive astrocytes in the temporal lobe in MTLE contribute to the triggering of seizures via impaired buffering of extracellular potassium (Binder et al., 2006; Bordey and Sontheimer, 1998; Heuser et al., 2012), decreased uptake and metabolism of extracellular glutamate (Cavus et al., 2005; During and Spencer, 1993; Eid et al., 2004b; Petroff et al., 2004; Petroff et al., 2002), release of glutamate from astrocytes (Bezzi et al., 1998; Bezzi et al., 2001; Perez et al., 2012; Santello et al., 2011; Tian et al., 2005), or a combination of the three.

Reactive astrocytes are likely to perturb the glutamate homeostasis in MTLE. Glutamate is a major excitatory and potentially neurotoxic transmitter in the brain, and elevated concentrations of extracellular glutamate and glutamate analogues in the brain can lead to hyperexcitability, seizures and neuronal death (Ben-Ari, 1985; Nadler et al., 1978; Olney et al., 1972). Thus, rapid removal of glutamate from the synaptic cleft and extracellular space is important for termination and fine tuning of excitatory synaptic responses, for prevention of seizures, and for protection of neurons from excitotoxic injury and death. Astrocytes play a crucial role in removal of synaptic and extracellular glutamate because of three key features of these cells (Fig. 1). Firstly, cytoplasmic processes of astrocytes are juxtaposed to excitatory synapses, forming what is often referred to as the tripartite synapse (Halassa et al., 2007; Perea et al., 2009; Peters et al., 1991). Secondly, astrocyte processes take up synaptic glutamate with high efficiency because of the abundance of high-affinity excitatory amino acid transporters on the plasma membrane of the processes (Danbolt, 2001). Thirdly, GS, which is preferentially localized to the cytoplasm of astrocytes (Martinez-Hernandez et al., 1977), converts the glutamate taken up by astrocytes to glutamine according to the following synthetic reaction:

$$\mathrm{Glutamate}+\mathrm{NH_3}+\mathrm{ATP} \rightarrow \mathrm{Gulutamine}+\mathrm{ADP}+\mathrm{P_i}$$

The known stoichiometry of glutamate transport across the astrocyte plasma membrane suggests that rapid metabolism of intracellular glutamate is a prerequisite for efficient glutamate clearance from the extracellular space (Otis and Jahr, 1998).

In vivo brain microdialysis studies of patients with MTLE have shown that the interictal extracellular glutamate concentration is chronically increased in the epileptogenic and astrogliotic hippocampus vs. the contralateral (nonepileptogenic) and nonastrogliotic

hippocampus (Cavus et al., 2005; Cavus et al., 2008). As expected, the concentration of extracellular glutamate increases during a seizure; however, it increases more and is cleared more slowly in the epileptogenic than in the nonepileptogenic hippocampus (During and Spencer, 1993).

A deficiency in GS in reactive astrocytes may explain the extracellular glutamate excess in MTLE. Patients with medication-refractory MTLE exhibit markedly reduced concentration and functional activity of GS in reactive astrocytes in the epileptogenic hippocampus [Fig. 2, (Eid et al., 2004b; van der Hel et al., 2005)]. We have hypothesized that the loss of GS in MTLE slows the metabolism of glutamate to glutamine in astrocytes and leads to accumulation of intracellular (astrocytic) and extracellular glutamate (Eid et al., 2004b). We have further postulated that the perturbed glutamate homeostasis caused by the GS deficiency contributes to the triggering of seizures in MTLE. Thus far, in vivo experiments have supported the hypothesis that GS inhibition in the hippocampus leads to accumulation of glutamate in astrocytes (Perez et al., 2012) and to recurrent seizures that resemble those of human MTLE (Eid et al., 2008; Wang et al., 2009). Moreover, humans with mutations in the GS gene also exhibit epileptic seizures (Haberle et al., 2005; Haberle et al., 2006). Hence, deficiency of GS in astrocytes appears to be critically implicated in the causation of seizure disorders, particularly MTLE. Two important questions therefore arise: “What causes the loss of GS in MTLE? And can restoration of the enzyme in astrocytes represent a novel and highly efficacious therapy for this disorder?” The remainder of this discussion will review how GS is regulated in astrocytes under normal and pathological conditions.

3. REGULATION OF GS IN ASTROCYTES

3.1 General concepts

GS is widely distributed among eukaryotes and prokaryotes, and two major isoforms of the enzyme are known to exist. GSI is present exclusively in prokaryotes, while GSII (or GS, as used here) is found in eukaryotes and prokaryotes (Meister, 1985). The active enzyme in humans comprises two ring-like pentamers that form an active decamer that uses manganese (Mn^{++}) as the cofactor (Krajewski et al., 2008). The mammalian enzyme is preferentially localized to hepatocytes surrounding the terminal hepatic veins (Gebhardt and Mecke, 1983), in skeletal muscle cells, and in most glial fibrillary acidic protein (GFAP)-positive astrocytes in the brain (Blondet et al., 1995; Norenberg, 1979; Riepe and Norenberg, 1978).

Human GS is encoded by the glutamate-ammonia ligase (GLUL) gene, which is localized to chromosome 1q31. A GS pseudogene (GLULP) is present on chromosome 9p13, and three GS-like genes are localized on 5q33 (GLULL1), on 11p15 (GLULL2), and on 11q24 (GLULL3) (Wang et al., 1996). The GLUL gene, which is likely the main source of GS in mammals, has three regulatory regions that control the transcription of mRNA: (1) the far 2.5-kB upstream region, which contains the canonical TATA, CCAATT and GC boxes, as well as sites for binding transcription factor Sp1 (Fahrner et al., 1993; Lie-Venema et al., 1998); (2) the first intron, which is of particular importance for mRNA expression in Hep G2 hepatoma cells; and (3) the 3’ untranslated region, which is thought to influence localization, stability, and translocation of mRNA (Lie-Venema et al., 1998). Thus, GS may be subject to regulation by a variety of mechanisms targeting the GLUL gene and its mRNA, the expression and stability of the protein, and the kinetic properties of the enzyme. Some of these mechanisms will be discussed below (see also Fig. 3 and Table 1 for an overview).

3.2 Mutations in the GLUL gene

Mutations in the human GLUL gene were first described by Haberle and colleagues (Haberle et al., 2005), who reported two unrelated newborns who had homozygous

mutations in the GLUL gene (R324C and R341C). These mutations were associated with reduced GS activity, severe brain malformations, seizures, multiorgan failure, and early death. A third case of homozygous GLUL mutation was reported later in a 2-year-old infant, who presented with seizures at 13 days of age and developed chronic encephalopathy (Haberle et al., 2011). The considerable morbidity of GLUL mutations is further highlighted by studies of *D. melanogaster* and transgenic mice. Homozygous mutations in the GLUL gene in *D. melanogaster* lead to embryo-lethal female sterility (halted embryonic development with failure of eggs to hatch) (Caggese et al., 1992). Mice with prenatal excisions of the GLUL gene in all cell types die during early embryonic development (He et al., 2007), while mice with selective gene deletions in GFAP-positive astrocytes live until postnatal day 3 (He et al., 2010).

3.3 Hormones and growth factors

Steroid hormones, insulin, and growth factors modulate the expression of GS. The glucocorticoids dexamethasone, hydrocortisone, and cortisol induce transcription of GS mRNA (Hallermayer et al., 1981; Labow et al., 2001), with resultant increases in GS protein and activity (Khelil et al., 1990). The inductive action of corticosteroids on the GLUL gene is partly controlled by a glucocorticoid response element (Patejunas and Young, 1990; Vardimon et al., 1999; Vardimon et al., 1988) and partly by a GS silencer element 5' to the glucocorticoid response element (Avisar et al., 1999). However, the effect of glucocorticoids is not universal, as cortisol fails to induce the astrocyte GLUL gene in certain experimental paradigms such as the rat adrenalectomy model (Tombaugh and Sapolsky, 1990). The sex hormones estradiol and estrogen enhance the transcription of GS mRNA in astrocytes in the hypothalamus and hippocampus (Blutstein et al., 2006) and in C6 glioma cells (Haghighat, 2005), whereas insulin decreases the translation and functional activity of GS (Khelil et al., 1990). Finally, brain-derived neurotrophic factor (BDNF) enhances the expression of GS and the glutamate transporter GLAST in astrocytes (Dai et al., 2012). Because stress (Friis and Lund, 1974), estrous cycle hormones (Pennell, 2009), glucose metabolism (Garriga-Canut et al., 2006), and neuronal reorganization (Ben-Ari, 2008) have been implicated in the pathophysiology of seizures and epilepsy, it is possible that perturbations in these factors influence the expression of GS in MTLE. Whether and exactly how these factors regulate GS in MTLE remain to be established.

3.4 Extracellular interactions

Brain GS is preferentially localized to the cytoplasm of astrocytes (Martinez-Hernandez et al., 1977), and the protein is particularly enriched in astrocyte processes in specific regions of the neuropil. For example, GS is strongly expressed in astrocyte processes surrounding glutamatergic nerve terminals in the hippocampal formation (Derouiche and Frotscher, 1991). GS is also particularly abundant in a laminar pattern in the molecular layer of the dentate gyrus, corresponding to glutamate binding sites (Derouiche and Ohm, 1994). These patterns of GS enrichment likely reflect an increased demand for extracellular glutamate clearance at regions of high excitatory activity.

The intracellular localization of GS in astrocytes is altered in MTLE and also in Alzheimer's disease. While GS is completely absent from large numbers of GFAP-positive astrocytes in the epileptogenic hippocampus in human MTLE, most astrocytes in the subiculum of these patients express GS (Eid et al., 2004b). However, the GS protein is markedly deficient in the most distal astrocyte processes in the subiculum in MTLE when compared with the subiculum in non-epileptic subjects (Eid et al., 2004b). Similarly, in the rat pilocarpine model of MTLE, more GS is found in proximal than in distal astrocyte branches (Papageorgiou et al., 2011). The GS-expressing astrocyte cell bodies are located closer to brain microvessels, whereas the density and volume of GS-immunopositive astrocytes are

not altered (Papageorgiou et al., 2011). GS is also diminished in distal astrocyte processes near perisynaptic regions of the neuropil in patients with Alzheimer's disease, despite a 1.4-fold increase in the density of astrocytes (Robinson, 2001).

Although the mechanisms underlying the spatial enrichment of GS are poorly understood, interactions between the astrocyte and the extracellular milieu are likely to be involved. Firstly, loss of excitatory afferents in vivo reduces the expression of GS in astrocyte processes (Derouiche et al., 1993), and primary astrocytes in culture do not express GS unless cocultured with neurons (Linser and Moscona, 1983). Loss of neurons is often observed in MTLE, and a reduction in astrocyte-neuron interactions may therefore underlie the loss of GS in this disorder. Secondly, the extracellular matrix component hyaluronan decreases GS activity in the retina in a model of glaucoma (Moreno et al., 2005). Hyaluronan and its receptor, CD44, are overexpressed in the astrogliotic hippocampal formation in MTLE (Bausch, 2006; Perosa et al., 2002). Thirdly, loss of the extracellular matrix component aggrecan leads to differentiation of astrocytes with induction of GS, GFAP and the glutamate transporter GLAST (Domowicz et al., 2008). Aggrecan accumulates in the brain in response to early-life seizures and therefore may prevent astrocytes from differentiating into a GS-expressing phenotype (McRae et al., 2010). Finally, studies in peripheral organs have shown that the GLUL gene is induced by beta-catenin, a cytoplasmic protein involved in the Wnt pathway (Audard et al., 2008; Cadoret et al., 2002; Kruithof-de Julio et al., 2005). Translocation of the extracellular adhesion molecule E-cadherin from the plasma membrane to the cytoplasm of pseudopapillary tumor cells of the pancreas leads to increased beta-catenin/Wnt signaling and enhanced expression of the GS protein (Audard et al., 2008). In contrast, Wnt signaling is decreased by the secreted glycoprotein, Dickkopf-1. Increased levels of Dickkopf-1 are present in the astrogliotic hippocampus in patients with MTLE, suggesting that the beta-catenin/Wnt pathway may be implicated in the loss of GS in this disorder (Busceti et al., 2007).

3.5 Redox reactions and beta amyloid

The GS protein is exquisitely sensitive to oxidative and nitrosative stresses. Studies of brain tissue from patients with Alzheimer's disease demonstrate increased markers of oxidative stress in areas of senile plaques and beta amyloid deposits, as evidenced by a decreased W/S (weakly/strongly) ratio of immobilized 2,2,6,6-tetramethyl-4-maleimidopiperdin-oxyl (MAL-6) spin-labeled synaptosomes, and increased phenylhydrazine-reactive protein carbonyl content (Hensley et al., 1995). These indicators of oxidative stress are associated with decreased GS activity (Hensley et al., 1995). Investigations using redox proteomics on temporal lobe specimens from patients with mild cognitive impairment have shown that GS and other proteins are oxidatively modified (Butterfield et al., 2006). Moreover, intracerebral injection of beta amyloid peptide (1–42) in normal rats leads to oxidation of GS (Boyd-Kimball et al., 2005), suggesting that beta amyloid deposits may be implicated in the loss of GS activity in Alzheimer's disease. It is notable that excessive deposition of beta amyloid has been reported in the temporal lobe in patients with MTLE, suggesting that beta amyloid and oxidative stress may be implicated in GS deficiency in this disorder (Mackenzie and Miller, 1994).

Further evidence supports the involvement of nitrosative/oxidative stress in the GS deficiency in MTLE. Rats treated with kainic acid to induce seizures exhibit increased nitric oxide synthetase activity, increased formation of nitric oxide, and reduced activity of GS in the brain (Swamy et al., 2011). Nitric oxide inhibits the activity of GS by covalent modifications (nitrosylation or nitration) of the enzyme (Kosenko et al., 2003). Induction of seizures in rats using pentylenetetrazole also results in nitration of brain GS, with subsequent reduction in enzyme activity (Bidmon et al., 2008). The reduction in GS activity in this model is *not* associated with a concurrent decrease in GS protein concentration

(Bidmon et al., 2008). Thus, measurements of GS protein by immunological methods (such as Western blots, ELISA, and immunohistochemistry) should be supplemented with measurements of GS enzyme activity, when indicated. Finally, administration of ferric ions (Fe^{+++}) to the brains of rats causes seizures and oxidative damage (Ueda and Willmore, 2000). Ferric ions also inhibit GS and leads to reactive astrogliosis; thus, it is possible that deposition of iron in the brain may contribute to the loss of GS in MTLE (Fernandes et al., 2011; Fernandez et al., 2011). Ferric deposits can occur in pathological conditions associated with brain tissue hemorrhage, such as hypoxia/ischemia, tumors, arteriovenous malformations, and head trauma. These conditions are often linked to development of epilepsy.

3.6 Inflammation and the blood-brain barrier

Neuroinflammation, which is present in epilepsy, can alter the activity and expression of GS. The epileptic brain in patients with MTLE and in animal models is characterized by excessive infiltration of microglial cells and upregulation of interleukin-1 beta (IL-1 beta), tumor necrosis factor alpha (TNF-alpha), IL-6, and NF-kappaB (Vezzani and Granata, 2005). Notably, IL-1 beta triggers seizures when administered to the brains of laboratory animals (Vezzani et al., 1999; Vezzani et al., 2000), and TNF-alpha induces glutamate release from astrocytes in vitro (Santello et al., 2011). Coadministration of IL-1 beta TNF-alpha to human fetal brain cell cultures generates nitric oxide and inhibits GS (Chao et al., 1995). IL-1 beta and TNF-alpha also suppress the induction of GS by dexamethasone in primary mouse astrocytes (Huang and O'Banion, 1998). Finally, treatment of primary astrocyte cultures with lipopolysaccharide increases IL1-beta and downregulates GFAP and GS (Letournel-Boulland et al., 1994). It has been hypothesized that the inhibitory action of cytokines on GS is mediated by an increase in transcription factor c-Jun (Raivich, 2008), which suppresses the expression of GS (Vardimon et al., 1999; Vardimon et al., 2006). Thus, neuroinflammation not only lowers the threshold for seizures, but also reduces the activity of GS, thereby providing a possible causative link between inflammation and excessive glutamatergic neurotransmission.

It is well-known that disruptions of the blood-brain barrier (BBB) can lead to neuroinflammation, and vice versa, that neuroinflammation can result in opening of the BBB (Ransohoff and Brown, 2012; Ransohoff et al., 2003). Several tissue components control the integrity of the BBB, including endothelial cells, vascular basement membrane, pericytes, and perivascular astroglial endfeet. All of these components are altered in patients with MTLE as evidenced by changes in erythropoietin receptors (Eid et al., 2004a), MCTs (Lauritzen et al., 2011), and tight junctions (Rigau et al., 2007) on brain endothelial cells. Furthermore, the BBB in MTLE is characterized by thickening of the basement membrane (Liwnicz et al., 1990); degeneration of pericytes (Liwnicz et al., 1990); and perturbed expression of proteins on perivascular astroglial endfeet, including potassium transporter Kir4.1 (Heuser et al., 2012), dystrophin (Eid et al., 2005), aquaporin 4 (Eid et al., 2005), and MCTs (Lauritzen et al., 2012a). Alterations in the BBB can result in diapedesis of leukocytes and extravasation of plasma components into the brain tissue, with neuroinflammation and seizures as possible consequences (Ballabh et al., 2004; Ransohoff et al., 2003; Salazar et al., 1985; Seiffert et al., 2004). Thus, astrocytes may be affected not only by local tissue inflammation, but also by peripheral blood components leaking into the brain, such as albumin, immunoglobulins, complement, matrix metalloproteinases, and coagulation factors. Serum components, particularly thrombin, have been shown to dedifferentiate astrocytes, resulting in loss of GS (Nelson and Siman, 1990); thus, leakage of thrombin (or possibly prothrombin) across a damaged BBB may be involved in the downregulation of GS in MTLE.

3.7 Metabolites, cofactors, metal ions, and pH

Because GS is intimately involved with regulation of neurotransmitter cycling (in particular, glutamate-glutamine and glutamate-GABA-glutamine cycling) (Chowdhury et al., 2007; Jain et al., 2011; Sibson et al., 2001), it is not surprising that several metabolites associated with neurotransmitter cycling, including the substrates and products of the synthetic reaction catalyzed by GS, play key roles in regulating this enzyme. To better appreciate how the catalytic activity of and flux of substrates through GS can be regulated, it is important to briefly discuss the mechanistic outcome of the kinetic investigations of the purified brain GS (Cooper et al., 1983; Meister, 1985).

The overall mechanism of the GS reaction is complex, and in addition to the synthetic reaction already alluded to above, GS also catalyzes eight other reactions or partial reactions (Cooper et al., 1983; Meister, 1985). However, a detailed discussion of the eight reactions is beyond the scope of this review. It is sufficient to emphasize that a key mechanism of the GS reaction appears to be the formation of an acyl phosphate intermediate followed by a nucleophilic displacement of Pi by ammonia, leading to glutamine formation (Cooper et al., 1983; Meister, 1985). Consistent with this mechanistic construct is the observation that L-methionine-SR-sulfoximine (MSO), an analog of the tetrahedral transition state intermediate, is phosphorylated to MSO-phosphate, which binds tightly to the GS active site and renders GS inactive. Glutamate provides some protection against MSO inactivation, but ammonia does not. Nevertheless, in the presence of ammonia, glutamate provides protection of GS against MSO inactivation (Cooper et al., 1983; Meister, 1985). Because of their implications in the regulation of this enzyme, other catalytic characteristics of GS can be highlighted as follows (Cooper et al., 1983; Meister, 1985): (i) GS possesses separate binding sites for glutamate, ATP, and ammonia, but the substrates do not bind to these sites covalently (Cooper et al., 1983; Meister, 1985). (ii) Ammonia binding to GS requires prior glutamate binding (Cooper et al., 1983; Meister, 1985). Consequently, astrocytic glutamate is important in the role of GS in ammonia detoxification in the brain. (iii) MSO binds to both the glutamate and ammonia binding sites (Cooper et al., 1983; Meister, 1985). Consequently, the convulsant effect of elevated brain MSO can be explained, in part, by elevation of excitatory glutamate and ammonia, neither of which can be detoxified by the action of GS. (iv) Formation of an acyl phosphate intermediate is the key. The slow formation of 2-pyrrolidone-5-carboxylate in the presence of glutamate, ATP, and divalent metal ions (in the absence of ammonia) suggests that GS stabilizes the acyl phosphate intermediate (Cooper et al., 1983; Meister, 1985). Consequently, together with the K_m values of its substrates (see below), these kinetic characteristics of GS can help explain how GS can be regulated by its substrates and the inhibitor MSO. On the other hand, L-aspartate, another excitatory neurotransmitter, is neither a substrate nor an effective inhibitor of GS (Cooper et al., 1983).

For purified sheep brain GS, its pH optimum is in the range of 7.0 to 7.4 (Meister, 1985). However, its pH optimum can vary between 4.8 and 8.5, depending on the divalent cations present (Meister, 1985). At pH 7.2, Mg^{++} is more effective than Co^{++} or Mn^{++} at their respective pH optima (Meister, 1985). Equivalent activities are observed with these two divalent cations. In the presence of Mg^{++}, the apparent K_m values for ATP, L-glutamate, and ammonium ion are, respectively, 2.3, 2.5, and 0.18 mM for sheep brain GS (Meister, 1985). Unlike the liver enzyme, brain GS does not respond to physiological levels of feedback modifiers or end-product metabolites derived from L-glutamine (Wedler and Toms, 1986).

There is evidence that brain GS is a $^{Mn++}$ metalloprotein (Denman and Wedler, 1984; Wedler et al., 1982; Wedler and Ley, 1994; Wedler et al., 1994). Consistent with this conclusion are the observations that, regardless of whether the transferase activities or the biosynthetic activities of sheep brain GS were measured, the apparent K_m for Mn^{++} (~20

μM) was approximately two orders of magnitude lower than the corresponding apparent K_m for Mg(II) (1.7–3.5 mM) (Wedler and Toms, 1986). In conjunction with the differential roles of divalent cations in activating brain GS (Meister, 1985), the finding that brain GS is a Mn metalloprotein, having a high affinity for Mn^{++}, strongly suggests that intracellular levels of Mn^{++} in astrocytes constitute one critical factor in regulating brain GS activity and flux (Wedler et al., 1994), because brain GS is largely astroglial in localization (Martinez-Hernandez et al., 1977). Brain levels of manganese become elevated in several neurodegenerative (e.g., Alzheimer's disease and Parkinson's disease) and metabolic (e.g., hepatic encephalopathy) diseases and in manganese toxicity (Lai et al., 1999; Lai et al., 2000). Of relevance to the regulation of brain GS is the finding that significant levels of the elevated Mn are likely detected in astrocytes, because these cells are known to accumulate Mn avidly (Lai et al., 1999; Lai et al., 2000; Wedler et al., 1994). Consequently, the elevation of astrocytic Mn levels may exert modulatory effects on GS activity and flux and ultimately on glutamate-glutamine and glutamate-GABA-glutamine cycling in those disease states (Chowdhury et al., 2007; Jain et al., 2011; Lai et al., 1999; Lai et al., 2000; Sibson et al., 2001; Wedler et al., 1994). A case in point is hepatic encephalopathy (Lai et al., 1999; Lai et al., 2000) (see below). Furthermore, the mechanistic interrelations between elevated Mn levels, GS regulation, and neurotransmitter cycling may have important pathophysiological implications in seizure disorders.

When their brain levels are elevated, two substrates of GS, namely ammonia and glutamate, are excitotoxic and exert convulsant effects (Cooper and Lai, 1987; Lai and Cooper, 1986, 1991; Lai et al., 1989). Indeed, GS is the only brain enzyme efficient at removing excess brain ammonia (Cooper and Lai, 1987; Cooper et al., 1983). Thus, when GS is functionally compromised, it can no longer convert ammonia into the comparatively harmless glutamine. One result of the functional compromise of GS is that brain ammonia will rise and ultimately become proconvulsant. The pathophysiological mechanisms of hepatic encephalopathy provide some insight into how brain GS can be functionally compromised. Hepatic encephalopathy is associated with increases in brain levels of ammonia and Mn (Cooper and Lai, 1987; Lai and Cooper, 1986, 1991; Lai et al., 1999; Lai et al., 2000; Lai et al., 1989), the increase in Mn being especially notable in the basal ganglia.

In hepatic encephalopathy, astrocytes typically show Alzheimer Type II histopathological changes (Norenberg, 1988). Two neurotoxins (namely, ammonia and Mn) implicated in this disease are likely to be mechanistically responsible for these pathological changes in astrocytes. Ammonia treatment of primary cultures of astrocytes also induces them to swell and exhibit pathological morphologies very similar, if not identical to Alzheimer Type II histopathological changes (Norenberg, 1988). Ammonia and Mn^{++} act synergistically in inhibiting glutamate uptake by astrocytes in culture (Hazell and Norenberg, 1997). Similarly, Mn^{++} treatment of astrocytes in primary culture induces them to swell (Rama Rao et al., 2007) and the effects of ammonia and Mn^{++} on astroglial swelling are at least additive, if not synergistic (Hazell and Norenberg, 1997; Jayakumar et al., 2004; Rama Rao et al., 2007).

Treatment of astrocytes in primary culture with Mn^{++} induces decreased expression of glutamate transporters and GS in these cells (Deng et al., 2012). This effect of Mn^{++} can lower the capacity of Mn-treated astrocytes to detoxify brain ammonia (Deng et al., 2012) by (i) lowering the catalytic capacity of astroglial GS (through decreasing GS expression) and (ii) decreasing the availability of externally supplied glutamate, a key GS substrate (Hazell and Norenberg, 1997). Moreover, in chronic Mn toxicity, GS expression is decreased, and swelling of astrocytes [e.g., resulting from combined treatment with ammonia and Mn^{++}] (Norenberg, 1988; Rama Rao et al., 2007) leads to decreased GS activity, even though astrocytes are accumulating Mn^{++}, which, upon entering the

cytoplasmic space of astrocytes, may bind to GS and thereby activates it (Wedler and Toms, 1986). Taken together, these observations prompted us to conclude that when the brain, and consequently the extracellular, levels of ammonia and Mn^{++} are elevated, as is the case in hepatic encephalopathy (Cooper and Lai, 1987; Hazell and Norenberg, 1997; Jayakumar et al., 2004; Lai and Cooper, 1986; Lai et al., 1999; Lai et al., 2000; Norenberg, 1988; Rama Rao et al., 2007), the astroglial capacity to detoxify ammonia via the action of GS is decreased because of lower availability of glutamate, a substrate of GS, and decreased expression of GS (Deng et al., 2012; Morello et al., 2007). The net result of these pathophysiological mechanisms derived from elevation of extracellular glutamate and ammonia increases the likelihood of seizures.

Another metabolic and signaling mechanism known to regulate astroglial GS is pH (Nissim, 1999). Elevation of intracellular pH markedly stimulates astroglial glutamine synthesis (Nissim, 1999). A few mechanisms may account for this pH effect (Nissim, 1999). Potassium ion-induced alkalinization of astrocytes is associated with enhanced glutamate uptake and glutamine synthesis by astrocytes (Nissim, 1999). On the other hand, even though this enhanced glutamate uptake provides more substrate for GS, the increased astroglial accumulation of glutamate may decrease intracellular pH in astrocytes because of transporter-mediated inward H^+ and outward K^+ and OH- transport (Nissim, 1999). Consequently, this acidosis induced by astroglial glutamate accumulation may exert an inhibitory effect on GS (Nissim, 1999). Nevertheless, the quantitative dynamics of the regulation of astroglial glutamine synthesis by elevation of intracellular pH remains incompletely understood and is an important area that merits further investigation.

3.8 Exogenous compounds

Several exogenous compounds can influence the expression and activity of GS. Perhaps the most extensively studied such compound is MSO, which competes for binding with glutamate in the active site of GS (Ronzio and Meister, 1968). MSO is phosphorylated by GS in the presence of ATP and results in an irreversible, noncovalent inhibition of the enzyme (Eisenberg et al., 2000; Ronzio and Meister, 1968). The effects of MSO on brain function were first discovered when animals were fed nitrogen chloride-treated (agenized) zein (a maize protein) and subsequently developed hysteria and seizures. The neuropsychiatric signs were caused by MSO, which developed as a byproduct during the agenization process (Bentley et al., 1949). Later studies showed that administration of MSO to mammals inhibited GS and resulted in slowing of the glutamine-glutamate-GABA metabolic pathway (Liang et al., 2006), with increased glutamate in astrocytes (Perez et al., 2012), and epileptic seizures as some of the consequences (Eid et al., 2008; Folbergrova et al., 1969).

Over forty additional exogenous inhibitors of GS have been identified [see Eisenberg et al. for an extensive review (Eisenberg et al., 2000)]. While most of the inhibitors are synthetic, some are found in nature, e.g. tabtoxinine-beta-lactam produced by *Pseudomonas syringae pv. tabaci* (Langston-Unkefer et al., 1984), alanosine produced by *Streptomyces alanosinicus* (Anandaraj et al., 1980), and a 110-kD protein identified in tomato roots (Gallardo and Canovas, 1992).

Considerably fewer exogenous compounds have been shown to *increase* the activity or expression of GS. The phytoalexin resveratrol enhances glutamate uptake and GS activity in C6 glioma cells (dos Santos et al., 2006). Beta-N-oxalyl-L-alpha,beta-diaminopropionic acid (beta-L-ODAP), which is a toxin derived from the seeds of *Lathyrus sativus*, increases GS activity by 155 percent in astrocytes in the rat cerebral cortex. The effect is dose-dependent and requires protein translation (Miller et al., 1993). Finally, treatment of mice in utero with

2,2',4,4' tetrabromo diphenyl ether (BDE47) results in significant upregulation of GS mRNA in the brain (Haave et al., 2011).

4. CONCLUSIONS

GS, which is found in astrocytes, is a critical enzyme for metabolism of ammonia, glutamate, and GABA in the central nervous system, and perturbations in the homeostasis of these metabolites are likely to profoundly affect brain function. An increasing number of studies have revealed a spectrum of deficiencies and aberrant expression patterns of brain GS in patients with epilepsy, stroke, Alzheimer's disease, multiple sclerosis, schizophrenia, and suicide/depression. While the exact causes of these alterations are not fully understood, several mechanisms are possible, such as: (a) perturbations in glucocorticoids, sex hormones, and growth factors; (b) oxidative and nitrosative stresses; (c) loss or gain of extracellular interactions; (d) neuroinflammation/BBB dysfunction; and (e) perturbations of metabolites, cofactors, metal ions, and pH. Knowledge of the mechanisms by which GS is altered in brain pathology is likely to guide the development of novel therapeutic interventions targeting the expression and activity of this important enzyme.

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Research highlights

- Glutamine synthetase (GS) is critical for metabolism of brain glutamate and ammonia
- Brain GS is deficient in several brain disorders, including epilepsy
- Deficiency in GS may lead to perturbed neurotransmission and epileptic seizures
- Here we discuss the mechanisms that regulate GS in the brain
- Knowledge of such mechanisms may facilitate new therapies

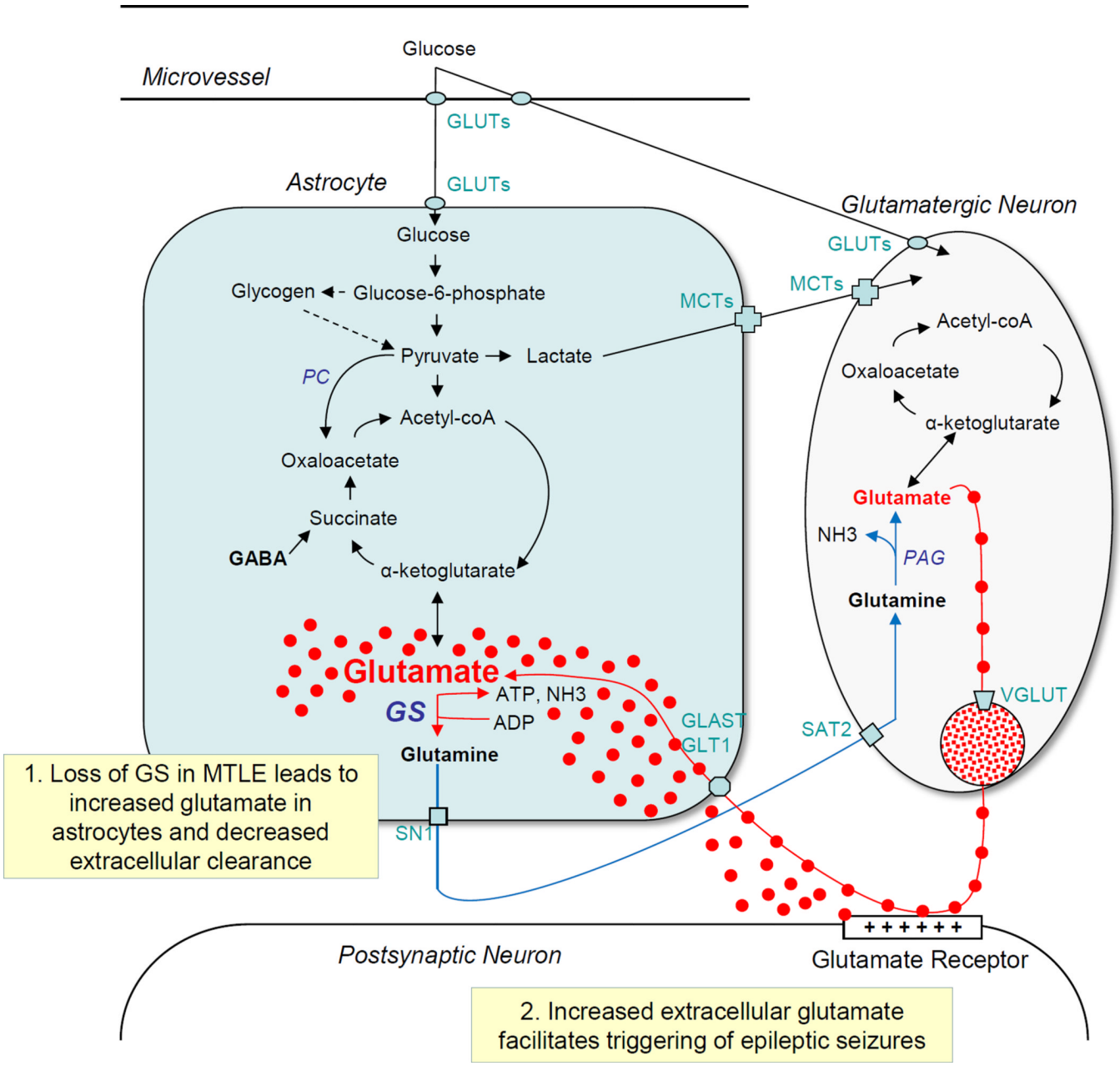

Figure 1.

Simplified diagram of the brain glutamate metabolism illustrating the involvement of astrocytes, neurons, transporter molecules, and key enzymes. Note that extracellular glutamate is taken up by the astrocyte via glutamate transporters (GLAST, GLT1) and converted to glutamine by glutamine synthetase (GS) in the astrocyte cytoplasm. Loss of GS is hypothesized to increase glutamate in astrocytes and impair the clearance of extracellular glutamate (Box 1). Increased extracellular glutamate is likely to facilitate the triggering of epileptic seizures (Box 2). Abbreviations: GLUTs, glucose transporters; PAG, phosphate activated glutaminase; MCTs, monocarboxylate transporters; MTLE, mesial temporal lobe epilepsy; PC, pyruvate carboxylase; SAT2, system A transporter 2; SN1, system N transporter 1; VGLUT, vesicular glutamate transporter.

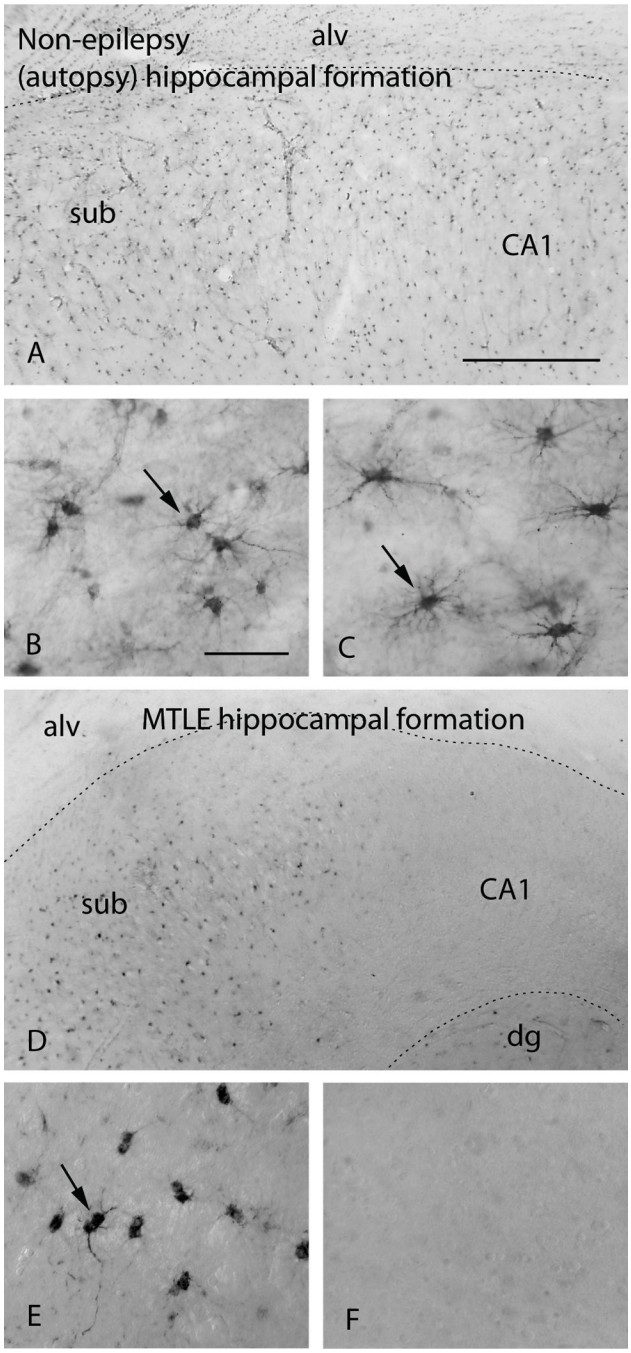

Figure 2.

Immunohistochemistry for GS reveals abundant labeling of astrocytes (arrows) in the subiculum (sub, A, B) and CA1 (A, C) of the hippocampal formation in a human autopsy subject without epilepsy. In contrast, GS is severely deficient in astrocytes in the hippocampal formation in a patient with mesial temporal lobe epilepsy [MTLE, (D–E)]. The deficiency is particularly pronounced in CA1 (D, F). Note that astrocytes in the subiculum of the MTLE hippocampus lack staining for GS in the most distal astrocyte processes (arrow in E) vs. the normal (autopsy) hippocampus (arrow in B). B and C are high power fields of the subiculum and CA1 respectively in A. E and F are high power fields of the subiculum and CA1 respectively in D. Abbreviations: alv, alveus; CA1, Cornu Ammonis subfield 1;

dg, dentate gyrus; MTLE, mesial temporal lobe epilepsy. Scale bars: A=0.5 mm (same magnification as D); B=0.1 mm (same magnification as C, E, F). Reproduced from (Eid et al., 2004b), with permission from The Lancet/Elsevier.

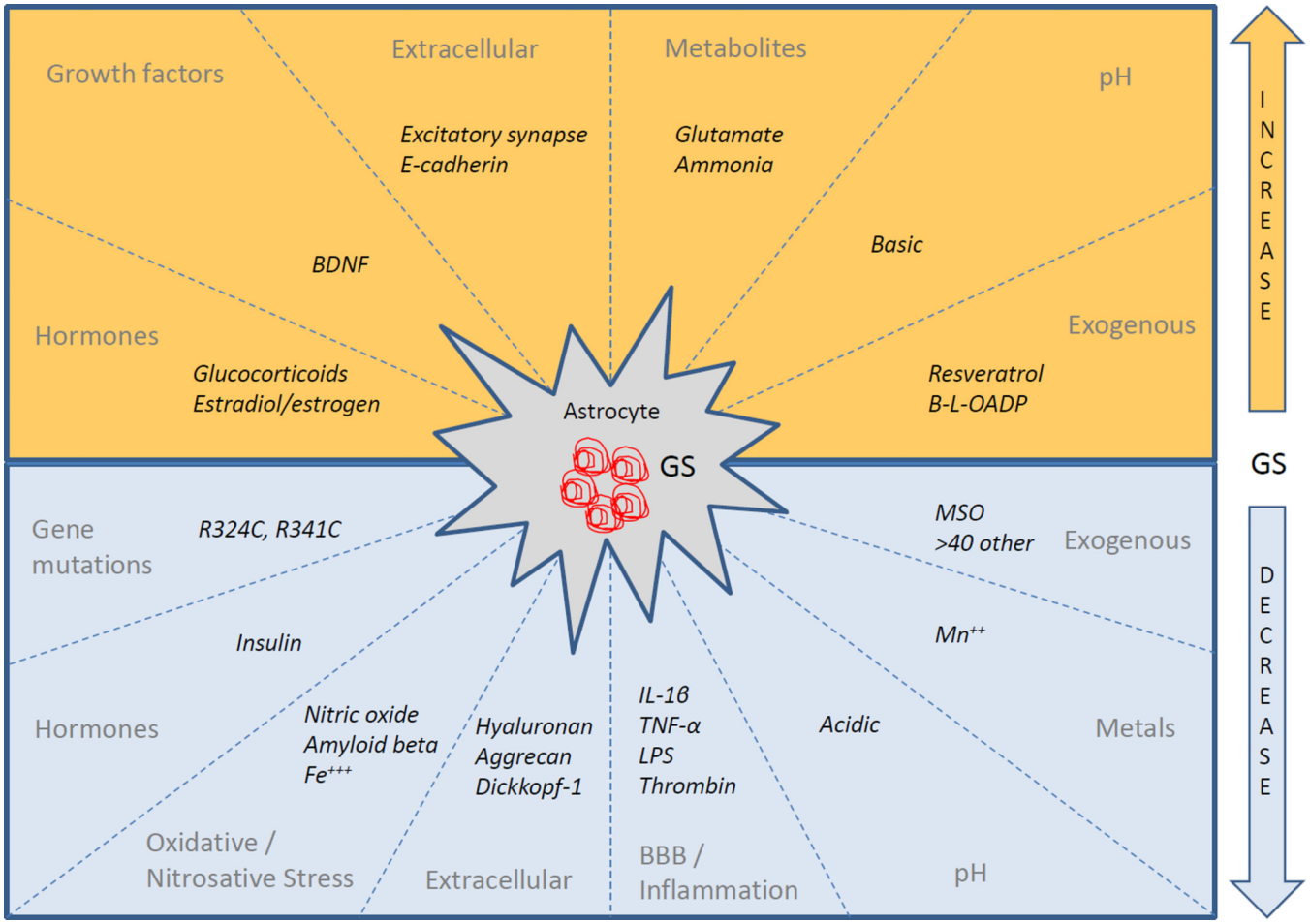

Figure 3.
Summary of factors that increase (top half) or decrease (bottom half) the expression or activity of GS. For further details and explanation of abbreviations, see Table 1

Table 1

Regulation of GS

Factors	Net effect on GS	Mechanism of Action	References
Gene mutations			
R324C, R341C	↓	Disrupts synthesis of GS	(Haberle, et al. 2005)
Hormones			
Glucocorticoids	↑	Induces gene via GRE and GS silencer element 5' to GRE	(Patejunas & Young 1990, Vardimon, et al . 1999, Vardimon, et al. 1988)
Estradiol/estrogen	↑	↑ transcription of mRNA	(Blutstein, et al. 2006)
Insulin	↓	↓ translation and activity of GS	(Khelil, et al. 1990)
Growth factors			
BDNF	↑	Unknown	(Dai, et al. 2012)
Extracellular interactions			
Excitatory synapses	↑	Glutamate or other factor	(Derouiche & Frotscher 1991)
Hyaluronan	↓	↓ activity	(Moreno, et al. 2005)
Aggrecan	↓	↓ differentiation of astrocytes	(Domowicz, et al. 2008)
E-cadherin (pancreas)	↑	↑ Beta-catenin/Wnt-signaling	(Audard, et al. 2008)
Dickkopf-1	↓	↓ Beta-catenin/Wnt-signaling	(Busceti, et al. 2007)
Redox and beta amyloid			
Oxidative stress	↑	GS oxidation → loss of function	(Butterfield, et al. 2006, Hensley, et al. 1995)
Nitrosative stress	↓	↑NOS activity-> GS nitrosylation/nitration -> loss of function	(Bidmon, et al. 2008, Kosenko, et al . 2003, Swamy, et al. 2011)
Fe3+	↓	GS oxidation -> loss of function	(Fernandes, et al. 2011)
Beta amyloid	↓	GS oxidation -> loss of function	(Boyd-Kimball, et al. 2005)
Inflammation/BBB disruption			
IL-1 beta + TNF-alpha	↓	↑NO -> GS nitrosylation/ nitration -> loss of function; ↓, induction by glucocorticoids	(Chao, et al. 1995, Huang & O'Banion 1998)
LPS	↓	↑ IL-1 beta	(Letournel-Boulland, et al. 1994)
Thrombin	↓	↓ differentiation of astrocytes	(Nelson & Siman 1990)
Metabolites, cofactors metal ions and pH			
Glutamate	↑	Unknown	(Suarez, et al. 2002)
Ammonia	↑	Unknown	(Suarez, et al. 2002)
Increased pH	↑	Unknown	(Nissim 1999)
Decreased pH	↓	Unknown	(Nissim 1999)
Mn	↓	Unknown	(Deng, et al. 2012)
Exogenous compounds			
Resveratrol	↑	↑ GS activity	(dos Santos, et al. 2006)
Beta-L-ODAP	↑	↑ GS activity via protein translation	(Miller, et al. 1993)

Factors	Net effect on GS	Mechanism of Action	References
Methionine sulfoximine	↓	Irreversible inhibition of GS	(Ronzio & Meister 1968)
>40 other compounds, e.g. : –Tabtoxinine-beta-lactam –Alanosine	↓	V arious	Reviewed in (Eisenberg, et al. 2000)

Abbreviations: GRE, glucocorticoid response element; NO, nitric oxide; NOS, nitric oxide synthetase; Beta-L-ODAP, beta-N-oxalyl-alpha, beta-diaminopropionic acid; Wnt, wingless/integrated