Glutaminase in microglia: A novel regulator of neuroinflammation

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ABSTRACT

Neuroinflammation is the inflammatory responses that are involved in the pathogenesis of most neurological disorders. Glutaminase (GLS) is the enzyme that catalyzes the hydrolysis of glutamine to produce glutamate. Besides its well-known role in cellular metabolism and excitatory neurotransmission, GLS has recently been increasingly noticed to be up-regulated in activated microglia under pathological conditions. Furthermore, GLS overexpression induces microglial activation, extracellular vesicle secretion, and neuroinflammatory microenvironment formation, which, are compromised by GLS inhibitors in vitro and in vivo. These results indicate that GLS has more complicated implications in brain disease etiology than what are previously known. In this review, we introduce GLS isoforms, expression patterns in the body and the brain, and expression/activities regulation. Next, we discuss the metabolic and neurotransmission functions of GLS. Afterwards, we summarize recent findings of GLS-mediated microglial activation and pro-inflammatory extracellular vesicle secretion, which, in turns, induces neuroinflammation. Lastly, we provide a comprehensive discussion for the involvement of microglial GLS in the pathogenesis of various neurological disorders, indicating microglial GLS as a promising target to treat these diseases.

1. Introduction

Neuroinflammation is the inflammatory response occurring in nervous tissue. Emerging evidence has implicated neuroinflammation as a crucial mediator in the pathogenesis of most central nervous system (CNS) disorders. Major neurodegenerative diseases, such as Alzheimer’s disease (AD) (Akiyama et al., 2000) and Parkinson’s disease (PD) (Tiwari and Pal, 2017), feature neuroinflammation in their etiologies. Besides, acute brain disease or injury like stroke (Gao et al., 2019) or traumatic brain injury (TBI) (Simon et al., 2017) also feature neuroinflammation in their progressions and prognosis.

Neuroinflammation is usually characterized by a series of major
phenomena: first, activation of resident glial cells; second, release of pro-inflammatory cytokines and chemokines; and third, recruitment and infiltration of peripheral cells into the brain given damaged blood brain barrier (BBB). Among them, the activation of microglia, the first line of immune defense, plays a core role in driving neuroinflammation in various CNS diseases (Heneka et al., 2015; Kelly et al., 2020). In AD, once microglia are activated into a pro-inflammatory phenotype, they secrete pro-inflammatory extracellular vesicles (EVs) enriched with neurotoxins like Aβ, Tau, and pro-inflammatory cytokines like IL-1β, which accelerate neuronal death and the progression of AD (Asai et al., 2015; Gao et al., 2019). Microglial activation has been implicated in other neurodegenerative diseases like PD (Tiwari and Pal, 2017).

Fig. 1. Human GLS genes and mRNA transcripts. Schematic illustration of human glutaminase (GLS) genes and mRNA transcripts. (A) Human GLS genes and mRNA transcripts kidney-type glutaminase (KGA) and glutaminase C (GAC). (B) Human GLS2 gene and its mRNA transcripts glutaminase B (GAB) and liver-type glutaminase (LGA). Introns and exons are shown as solid light blue lines and numbered green boxes, respectively. The promoter regions are shown on the 5'-end of each gene. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
GLS has two isoforms in mammals, GLS1 and GLS2 (Fig. 1, Table 1) (Aledo et al., 2001). The genes that encode GLS1 and GLS2 are located on different chromosomes, both of which can be spliced to produce multiple variants. The human GLS1 gene is located on chromosome 2 with a total of 19 exons (Porter et al., 2002). GLS1 precursors are alternatively spliced to produce kidney-type glutaminase (KGA) and glutaminase C (GAC) (Elgadi et al., 1999). KGA mRNA contains exons 1–14 and 16–19, whereas the alternatively spliced transcript GAC uses only the first 15 exons (Elgadi et al., 1999; Porter et al., 2002). Thus, KGA and GAC have the same amino acid sequence for core enzyme action but different C-terminals (Erdmann et al., 2006). The molecular weight of KGA is around 64 KD while the molecular weight of GAC is around 58 KD. The human GLS2 gene is about 18 kb in size, located on chromosome 12, and has 18 exons. GLS2 also has two splicing variants, including glutaminase B (GAB) that contains the full length of GLS2 gene, and liver-type glutaminase (LGA) that only has exons 2–18 (Martin-Rufian et al., 2012). The molecular weight of LGA is around 65 KD while that of GAB is around 77 KD.

### 2. GLS: isoforms, expression patterns, and expression/activities regulation

#### 2.1. Two isoforms of GLS in mammals

GLS has two isoforms in mammals, GLS1 and GLS2. The molecular weight of KGA is around 64 KD while that of GAC is around 58 KD. The human GLS2 gene is about 18 kb in size, located on chromosome 12, and has 18 exons. GLS2 also has two splicing variants, including glutaminase B (GAB) that contains the full length of GLS2 gene, and liver-type glutaminase (LGA) that only has exons 2–18 (Martin-Rufian et al., 2012). The molecular weight of LGA is around 65 KD while that of GAB is around 77 KD.

### 2.2. GLS expression patterns in mammals

#### 2.2.1. The tissue distribution of GLS

GLS1 and GLS2 are expressed in a tissue-specific manner (Fig. 2, Table 1). The GLS1 isoforms are widely distributed in most non-liver tissues (Aledo et al., 2000; Curthoys and Watford, 1995; Smith and Watford, 1990). GLS2 is also expressed in extrahepatic tissues such as pancreas, brain, and immune cells, though at much lower levels than GLS1 (Aledo et al., 2001; Cardona et al., 2015; Gomez-Fabre et al., 2009).

#### 2.2.2. The brain distribution of GLS

Early studies suggested that KGA is the only GLS present in the brain (Haser et al., 1985). Since 2000, Northern analysis and immunocytochemistry revealed that GLS1 (KGA) and GLS2 (GAB and/or LGA) were simultaneously expressed and co-located in mammalian brain, especially in the areas that are enriched with Glu transmission, such as cerebral cortex, hippocampus, striatum, and cerebellum (Aledo et al., 2000; Gomez-Fabre et al., 2000; Olalla et al., 2002b). GLS1 isoforms (KGA + GAC) showed the largest abundance in rodent brains, accounting for more than 90% of the total GLS transcripts, and occupied a dominant position in the brain of mammals (Cardona et al., 2015; Martin-Rufian et al., 2012; Olalla et al., 2002b). For GLS2, the expression of LGA is slightly more abundant than that of GAB in mouse brain, but on the contrary in rat brain, the mRNA level of GAB is four times higher than that of LGA (Martin-Rufian et al., 2012). It may be due to the selective transcriptional regulatory mechanisms that are recruited in distinct tissues and mammalian species.

Being a central enzyme of neurotransmitter Glu recycling, GLS was thought to be exclusively expressed in neurons (Laake et al., 1995; Marquez et al., 2009). This premise has been confirmed by various studies. In 1989, Kaneko et al. reported that neuronal cell bodies exhibit intense phosphate-activated GLS-immunoreactivity in multiple brain regions including paracochlear glial substance, posterior ventral cochlear nucleus, the granule cell layer of the cerebellar cortex (Kaneko et al., 1989). With the development of imaging technologies (e.g. laser scanning confocal microscopy, electron microscopic immunocytochemistry), the abundant expression of GLS in neuronal cells in monkey and human brain was also observed (Cardona et al., 2015; Olalla et al., 2002a).

Importantly, the expression of GLS in glial cells has also been found. For example, immunohistochemical studies have shown expression and activity of GLS protein in rat brain astrocytes, a major type of glia that reciprocally influence synaptic transmission and connectivity (Aoki et al., 1991; Wurdig and Kugler, 1991). Similarly, immunohistochemical assays on postmortem human brain tissue demonstrate strong signals of GLS1 in astrocytes from hippocampus and cerebral cortex from the temporal lobe (Cardona et al., 2015). Although the expression levels of GLS2 are lower than GLS1, GLS2 is also observed in astrocytes. Besides, GLS1 expression is also detected in microglia, another type of glial cells that act as the main immune defender and neuroinflammation mediator.
in the CNS. In the cortical and hippocampal regions of mouse brain, GLS1 immunoreactivities can be observed in Iba1\(^+\) or CD11b\(^+\) microglial cells (Gao et al., 2020, 2019; Zhu et al., 2019). However, there is in lack of information for the presence of GLS2 in microglia, which needs to be verified.

2.2.3. Subcellular distribution of GLS in the brain

In addition, studies that aim to identify the subcellular localization of different GLS isoforms have also yielded interesting results. The subcellular distribution of GLS1 and GLS2 in neurons was firstly determined. GLS1 locates in neuronal perikarya and GLS2 displays a distinct neuronal nuclear localization (Olalla et al., 2002a). After that, GLS1 has also been observed in astroglial mitochondria from hippocampus and cerebral cortex from the temporal lobe (Cardona et al., 2015). In contrast to the mitochondrial localization of GLS1, GLS2 essentially concentrated in astroglial nuclei, displaying a similar sub-cellular localization pattern in astrocytes to neurons (Cardona et al., 2015). Furthermore, it is important to note that GLS can also present in the cytoplasm of brain cells, suggested by subcellular fractionation and immunocytochemistry studies (Aoki et al., 1991; Marquez et al., 2009). The distinct subcellular distribution may confer differential kinetic behavior to GLS isoforms.

2.3. Biological modulation of GLS expression and activity

Expression of both GLS1 and GLS2 are regulated at transcription, post-transcription, translation, and post-translation levels. Here we review the modulators of GLS transcription, translation, and stability.

2.3.1. Biological modulation of GLS expression

Transcription factors generally bind directly to gene promoter region to activate or repress transcription, or indirectly regulate gene transcription via interacting with other regulatory elements. Our group demonstrated that signal transducer and activator of transcription (STAT) family member STAT1 binds to GLS1 promotor and up-regulates GLS1 expression in monocyte-derived macrophages (Zhao et al., 2012). Similarly, STAT3 also targets GLS1 promotor in astrocytes, therefore enhancing GLS1 expression (Milewski et al., 2019). In tumor cells, the transcription of GLS1 can be induced by hypoxia inducible factor (HIF) family members (Perez-Escuredo et al., 2016; Stegen et al., 2016). HIFs stimulate GLS1 expression and activity likely through recruiting c-Myc and targeting GLS1 promoter to up-regulate GLS1 expression (Perez-Escuredo et al., 2016). Lukey et al. reported that the oncogenic transcription factor c-Jun directly binds to the GLS1 promoter region and induces gene expression in human breast cancer cells, suggesting GLS1-sensitive therapy for certain groups of breast cancer cells (Lukey et al., 2016b). Since c-Jun and HIF signaling pathways are enhanced in
activated microglia (Koh et al., 2015), c-Jun- and HIFs-dependent transcription regulation may also participate in GLS1 hyperexpression in microglia. Besides, GLS2 expression is regulated at transcription levels. For example, p53, which is activated in response to DNA damage or oxidative stress, associates with GLS2 promoter to induce GLS2 expression (Suzuki et al., 2010). Other than p53, GLS2 expression in tumor cells can also be elevated by transcription factors GATA3 (Lukey et al., 2019), n-Myc (Xiao et al., 2015), p63 (Giacobbe et al., 2013), and p73 (Vellutini et al., 2013), suggesting a complicated regulatory network of GLS2 expression. However, except for p53, the rest transcription factors that regulate GLS2 are not reported to be activated in microglia displaying pro-inflammatory feature (Aloi et al., 2015). Thus, the expression and the regulatory mechanisms of GLS2 in microglia remain an open question.

Progressive deletion analysis, mutation analysis, and electrophoretic mobility-shift assays revealed that two CAAT recognition elements near the transcription-initiation site play a crucial role for optimized human GLS2 promoter activity, TATA-like boxes enhance GLS2 basal transcription, and hepatic nuclear factor 1 (HNF-1) motif is a significant distal element for transcriptional regulation of the human GLS2 gene (Perez-Gomez et al., 2003).

GLS expression is also regulated at post transcriptional levels by microRNAs (miRNAs). For instance, miR-23a/b target KGA mRNA and inhibit its translation (Gao et al., 2009a). Thus, miR-23 is involved in regulating the expression ratio of two GLS1 sub-isoforms, KGA and GAC, which influences glutamine metabolism in cancer cells (Masamba et al., 2016). Rathore et al. reported that the p65 subunit of NF-κB could directly bind to the miR-23a promoter and inhibit miR-23a expression in leukemic cells, thus resulting in higher GLS1 expression levels (Rathore et al., 2012). Similarly, oncogenic factor c-Myc indirectly up-regulates GLS1 expression via suppressing miR-23a/b in P493 Burkitt's lymphoma and PC3 prostate cancer cell lines (Gao et al., 2009a; Lukey et al., 2016a). Other miRNAs, such as miR-1 (Zhang et al., 2018), miR-137 (Li et al., 2018), miR-200 (Liu et al., 2017; Lv et al., 2019), and miR-203 (Chang et al., 2017) are also demonstrated to target GLS1 transcripts and inhibit its translation in tumor cells. Although miRNA databases (e.g. targetscan.org) predict that these miRNAs may target KGA only, whether they target both GLS1 sub-isoforms or single ones specifically in microglia requires to be validated through experiments. GLS2 expression can also be regulated by miRNAs such as miR-103 and miR-16. miR-103 directly binds to the 3′UTR of GLS2, suppressing the expression levels of the latter in gastric cancer (Niu et al., 2019). Since miR-103 is a miRNA that is enriched in neural cells, it may explain the low expression levels of GLS2 in the brain (Wang et al., 2014). miR-16, a recently identified miRNAs with anti-inflammatory effect, also targets 3′UTR of GLS2 and inhibits GLS2 expression (Li et al., 2015; Yamada et al., 2020). The down-regulation of miR-16 in activated microglia may contribute to GLS2 expression elevation, which needs further examination.

Furthermore, there are multiple pathways that may indirectly regulate GLS expression. For example, ErbB2 signaling pathway, another oncogenic signaling pathway that is regulated by NF-κB, stimulates GLS1 expression in breast cancer cells with undefined mechanisms (Qie et al., 2014). Cabrera-Pastor et al. reported that increased activation of Tumor Necrosis Factor α (TNFα) receptor induced GLS expression likely through inducing the nuclear translocation of the p50 subunit of NF-κB (Cabrera-Pastor et al., 2018), suggesting that TNFα serves as a GLS expression regulator at different levels.

2.3.2. Biological modulation of GLS activity

Interestingly, GLS isoforms exhibit kinetic differences (Table 1) (Curthoys and Watford, 1995; Marquez et al., 2010). The main ones are the dependence of the activator inorganic phosphate (Pi) (higher in GLS1 than that in GLS2), the relative affinity for the substrate Glu (higher in GLS1 than that in GLS2), and the unique Glu inhibitory effect of GLS1 (Marquez et al., 2010).

GLS is activated through phosphorylation. In their recent study, Han et al. identified Ser314 as a critical phosphorylation site for GAC activation via NF-κB-PKCε axis in lung tumor tissue, and that Ser314 phosphorylation with the resultant GAC activity burst is essential for tumorigenic transformation (Han et al., 2018). Back in 2012, Thangavelu et al. reported that KGA activity is stimulated through an EGF-induced Raf-1/Mek2/Erk signaling pathway (Thangavelu et al., 2012). This group obtained the crystal structure of KGA and found that Ser314 located in the active site of activity core domain and that Ser314 together with other residues formed a loop region (Glu312-Pro329) in the crystal structure of KGA (Thangavelu et al., 2012). Importantly, this loop is near the active site of GLS core. In the ligand-free structure of the enzyme, the loop region forms a closed conformation of the active site through hydrophobic interactions made between Phe318 and Tyr466 and the hydrogen-bonding between the side chain of the Asn319 and the backbone of Asn335 (Thangavelu et al., 2012). Given that Tyr466 and Asn335 in the active site are involved in binding to ligand L-Gln and in catalysis, Han et al. speculated that phosphorylation of GAC on Ser314 might bring increased negative charge at the Glu312-Pro329 loop and subsequently increase the flexibility of the active site to allow L-Gln to enter the open conformation of the active site to be later catalyzed, while dephosphorylation of GAC led the loop back to its closed conformation (Han et al., 2018). Due to that KGA and GAC share the same enzymatic core domain (Thangavelu et al., 2012), the proposed Ser314 phosphorylation-mediated activity regulation should also apply to that of KGA.

2.4. Chemical regulation of GLS activity

Besides the biological regulation of GLS, multiple small molecules such as DON and BPTES have been identified to modulate GLS activities in direct or indirect manners (Table 2) (DeLaBarre et al., 2011; Song et al., 2018).

The structure of DON is similar to Gln, therefore DON successfully inhibits GLS1 and GLS2 through binding to its enzyme active site (Lemberg et al., 2018; Thangavelu et al., 2014). Animal studies showed that DON prevented the cognitive decline of HIV-infected mice through simultaneously normalizing HIV-induced increases of Glu in cerebrospinal fluid and GLS activity in microglia-enriched brain CD11b+ cells, implicating DON as a potential treatment of HAND (Nedelcovych et al., 2017). Afterwards, prodrugs of DON such as JHU-083 were synthesized to overcome peripheral toxicity of DON. JHU-083 exerts similar therapeutic effects in reversing cognitive impairment of HAND mouse and ameliorating social behavior deficits induced by CSDS (Nedelcovych et al., 2019; Zhu et al., 2019). However, due to the lack of selectivity and relatively weak potency, DON and its prodrugs may not be considered as first-class as therapeutic drugs for specific inhibition of GLS. This remains to be further investigated.

Compound 968 is an allosteric inhibitor of GLS1 that may bind between the N and C termini of two GAC monomers at the monomer-monomer interface and inhibit the formation of active GAC dimer (Wang et al., 2010). Currently, the anti-tumor effects of compound 968 are widely investigated, but its anti-inflammatory function in the CNS remains unclarified.

BPTES is another effective non-competitive allosteric GLS1 inhibitor (but not GLS2) with high affinity for free enzymes and enzyme-substrate complexes (Chen et al., 2016; DeLaBarre et al., 2011; Shukla et al., 2012). BPTES binds to the gating loop of GLS1, which affects substrate binding and the formation of active tetrameric GLS1, causing a conformational change that deactivates GLS1. Our previous studies demonstrated that BPTES significantly represses microglial activation and pro-inflammatory EV release in vitro (Gao et al., 2019). Since BPTES’s structure is not similar to Gln or glutamic acid, BPTES does not affect other enzymes that interact with Gln or glutamic acid. However, its medium efficacy (IC_{50} ~ 3μM) and low solubility limit its clinical application (Song et al., 2018). Similar to compound 968, BPTES exhibits high selectivity for GLS1 over GLS2 and thus specifically targets...
| Compound   | Chemical structure | IC_{50} (μM) | Inhibitory mechanism                      | Solubility                  | Specificity to GLS | Selectivity to GLS1/2 | Reference                  |
|------------|--------------------|--------------|-------------------------------------------|-----------------------------|--------------------|-------------------------|----------------------------|
| DON        | ![DON](image1)     | 1000         | GLS active site competitive binding       | 50 mM in water              | No                 | GLS1 & GLS2             | Thangavelu et al., 2014   |
| JHU-083    | ![JHU-083](image2) | N/A          | GLS active site competitive binding       | 62 mg/mL in water           | No                 | GLS1 & GLS2             | Zhu et al., 2019          |
| 968        | ![968](image3)     | 2.5          | GAC dimer formation inhibition            | 50 mM in water              | Yes                | GLS1                    | Wang et al., 2010         |
| BPTES      | ![BPTES](image4)   | 3            | GLS tetramer formation inhibition         | Insoluble in water          | Yes                | GLS1                    | DeLaBarre et al., 2011    |
| CB-839     | ![CB-839](image5)  | 0.030        | GLS tetramer formation inhibition         | Insoluble in water          | Yes                | GLS1                    | Gross et al., 2014        |
| Physapubescin | ![Physapubescin](image6) | 8.60       | Glutamate binding site occupation        | N/A                         | Yes                | GLS1                    | Cheng et al., 2017        |
| Caudatan A | ![Caudatan A](image7) | 36.66       | GLS active site competitive binding       | N/A                         | No                 | GLS1                    | Sun et al., 2018          |
| THDP17     | ![THDP17](image8)  | 3.9          | N/A                                       | N/A                         | N/A                | GLS1                    | Díaz-Herrero et al., 2014 |
| CBX        | ![CBX](image9)     | N/A          | N/A                                       | 100 mg/mL in water          | No                 | N/A                     | Yawata et al., 2008       |
| Ebselen    | ![Ebselen](image10) | 0.008-0.1    | Mixed non-competitive inhibition          | Insoluble in water          | No                 | GLS1 & GLS2             | Thomas et al., 2013       |
| Chelerythrine chloride | ![Chelerythrine chloride](image11) | 0.03-0.3    | Competitive inhibition                    | 11.33 mM in DMSO            | No                 | GLS1 & GLS2             | Thomas et al., 2013       |

(continued on next page)
GLS1-elevated cells. To solve the problem of BPTES’s poor solubility, a series of its analogs were synthesized (Shukla et al., 2012). Among them, CB-839 is the main candidate for a potent, selective, and orally bioavailable inhibitor of both splice variants of GLS1 (Gross et al., 2014). Currently, more than 10 clinical trials are either ongoing or completed that apply CB-839 as an anti-tumor drug in the United States (clinicaltrials.gov). Our recent finding further indicates that CB-839 can be an effective anti-inflammatory drug in attenuating neuroinflammation and brain injury in ischemic mice (Gao et al., 2020).

Except for the commonly used compounds, there are other GLS inhibitors identified. For example, phycoerythrin, a natural alcoholate purified from physalis, is a KGA inhibitor with comparable inhibitory effect with BPTES but less cytotoxicity (Cheng et al., 2017). The cyclic structure overlaps with the binding site of Gln, and the molecule is large enough to completely occupy the cavity of the substrate and product of GLS, thus prevents the substrate from entering. Kinetic studies indicate high affinity of phycoerythrin for KGA and show dual inhibitory (competitive and non-competitive) effects of phycoerythrin on KGA activity. Caudatan A, a natural product isolated from the roots of native Ohvia caudate, has the same inhibitory activity against KGA to BPTES and better solubility (Sun et al., 2018). This compound exerts enzyme inhibition through competitive binding to the KGA active site in aid of the cyclization of isopentyl and bridge ring in caudatan A. In addition, THDP17, a thiourea derivative, shows partial non-competitive inhibition of GLS1 and insignificant cytotoxicity in vitro, and significant inhibition on the intestinal GLS activity (46 ± 3.4%) in vivo (Díaz-Herrero et al., 2014). Moreover, CBX, a gap junction inhibitor, also effectively suppresses excess Gln production in activated microglia and subsequent neurotoxicity, although whether CBX directly targets GLS and exhibits GLS isoform selectivity or not remains unknown (Yawata et al., 2008). The screening of the Library of Pharmacologically Active Compounds (LOPAC1280) for GLS activity further identified ebselein, chelerythrine chloride, and apomorphine hydrochloride as bona fide GLS inhibitors (Thomas et al., 2013). Ebselein and chelerythrine chloride exhibit high inhibitory effects on GLS1 and 5 to 10-fold less activity against GLS2. In contrast, apomorphine hydrochloride inhibits both GLS1 and GLS2 with similar activity. Although non-selective, these chemicals display 10 to 100-fold increased inhibition efficiency than DON and BPTES.

Taken together, tremendous progress has been made in identifying allosteric GLS inhibitors, particularly those derived from BPTES. Emerging evidence has implicated promising anti-inflammatory effects of these inhibitors in the CNS, which are discussed in following sections. However, these molecules still have concerns such as toxicity, non-specificity, low solubility, poor BBB penetration capacity, and other concerns in their clinical application. With more comprehensive screening and modifications, better GLS inhibitors with excellent therapeutic utility in neurological disorders can be developed to alleviate GLS-induced neuroinflammation and neurotoxicity.

3. GLS: Classic enzyme for neurotransmitter production

Glu has very limited ability to cross the BBB and therefore must be transported by Glu transporters located at BBB or be synthesized by local precursors in neurons (Smith, 2000). GLS is highly expressed in neurons, and GLS1 (NCBI Gene ID 2744) is the main Glu metabolic enzyme in the brain rather than GLS2 (NCBI Gene ID 27165). Previous research demonstrated that inhibition of GLS depletes the stores of Glu (Conti and Minelli, 1994). The importance of GLS in glutamatergic synaptic function has been further confirmed by the fact that GLS1 knockout mice died shortly after birth due to impaired functioning of Glutamatergic neural networks (Bak et al., 2006; Masson et al., 2006). As neurons are unable to synthesize either the neurotransmitter Glu or GABA from glucose, Glu homeostasis is maintained by the Glu/Gln cycle that involves neuron–astrocyte cooperation (Ward and Bradford, 1979). Briefly, Glu is converted to Gln by GLS1 and the newly-produced Gln is packaged into synaptic vesicles in presynaptic neurons. The Glu-filled vesicles are recruited and docked to specialized release sites that are dense electron regions termed the active zones at presynaptic neurons (Chua et al., 2010). Intracellular calcium influxes trigger the release of Glu from presynaptic nerve terminals into synaptic cleft via exocytosis (Boyken et al., 2013). Afterwards, Glu diffuses across the synaptic cleft and binds to Glu receptors located on postsynaptic neurons. The binding of Glu with its receptors alters membrane potential of the postsynaptic neurons and triggers downstream signal transduction cascades. Glu that resides in the synaptic cleft is removed rapidly via four major ways. First, Glu is taken by postsynaptic neurons for neural signal transmission (Colombo and Francoilni, 2019). Second, Glu is endocytosed back to pre-synapse for refilling synaptic vesicles (Sudhof, 2004). Third, some Glu is taken by both pre- and post-synaptic neurons via excitatory amino acid transporters (EAATs) such as EAAT3 and EAAT4 located on neurons (Maragakis and Rothstein, 2001). Fourth, a substantial amount of extra Glu in synaptic cleft is transported to neighboring astrocytes by Glu transporters (EAAT1 and EAAT2) located on astrocytes (Maragakis and Rothstein, 2001). As the resting membrane potential of astrocytes is usually lower than that of neurons, extracellular Glu is favorably efficiently taken by astrocytes through a sodium-dependent mechanism via EAAT1 and EAAT2 (Gegelskivii and Schousboe, 1996). In astrocytes, where the enzyme glutamine synthetase (GS) is expressing at high levels, the majority of Glu is converted back to Gln (Rose et al., 2013). The newly-synthesized Gln is released extracellularly and transported back to neurons by Glu transporters like system N transporters (Chaudhry et al., 2002).

Gln-derived Glu also serves as the precursor of GABA in inhibitory synapses (Nanga et al., 2014). GLS1 and GLS2 isoforms have been found in GABAergic neurons, indicating that GLS is also important for inhibitory neurotransmitter GABA biosynthesis (Cardona et al., 2015). Recent studies presented immunocytochemical evidence of the localization of KGA in GABAergic neurons in cat visual cortex and thalamus (Fisher, 2007; Van der Gucht et al., 2003), demonstrating that Gln is a metabolic precursor of GABA synthesis. Interestingly, co-expression of KGA and Glu decarboxylase (GAD) was also found in rat hippocampal and basal forebrain neurons. GABA is produced from Glu by GAD. Two GAD subtypes in human brain, GAD65 and GAD67, are encoded by different genes (GAD2 and GADI, respectively) on different chromosomes (chromosome 10 and 2, respectively) (Bu et al., 1992). GAD65 is a membrane-anchored protein (585 amino acids) and is mainly

### Table 2 (continued)

| Compound             | Chemical structure | IC50 (µM) | Inhibitory mechanism     | Solubility | Specificity to GLS | Selectivity to GLS1/2 | Reference         |
|----------------------|--------------------|-----------|--------------------------|------------|--------------------|------------------------|-------------------|
| Apomorphine hydrochloride | ![Chemical Structure](image) | 0.3–0.4   | Competitive inhibition   | 12.9 mg/ml in DMSO | No                  | GLS1 & GLS2              | Thomas et al., 2013 |

Diaz-Herrero et al., 2019; Cardona et al., 2015
strictly controlled to ensure dynamic balance of Glu. Therefore, the two main neurotransmitters
1982; Schousboe et al., 1979). The presence of GLS1 in astrocytes and
getically favorable, the production process of Glu and GABA must be
in the CNS, Glu and GABA, can be synthesized by two isozymes encoded
plasmic (594 amino acids) and is responsible for cytoplasmic GABA
responsible for vesicular GABA production. GAD67 is mainly cyto-
larly relevant in periods of massive synaptic activity. In addition, the
exogenous Gln-derived Glu matches the strong energy and biosynthesis needs
stimulation, microglia are activated into a pro-inflammatory phenotype
enlarged Glu and Gln synthesis in cytosol, which, provides an
additional nitrogen source for the Glu/Gln cycle through GS.
Interestingly, GLS2 has a different role other than that of GLS1 in
in cellular metabolism. Hu et al. reported that up-regulated GLS2 expres-
sion increased Glu and α-KG production, which in turn enhanced cellular
mitochondrial respiration and ATP production (Hu et al., 2010). On the
other hand, given that Glu is the precursor of reduced glutathione
(GSH), one most important scavenger of reactive oxygen species (ROS),
up-regulation of GLS2 also increases the level of GSH and decreases ROS
levels to enhance the cell’s ability to resist oxidative stress and protect
cells from oxidative stress-induced apoptosis (Suzuki et al., 2010). In
contrast to the elevated expression and activity of GLS1 in transformed
tumors and that GLS1 activity is positively correlated with tumor ma-
ignancy, GLS2 is usually repressed in tumors, including brain tumors
such as glioblastoma and anaplastic astrocytoma, which suggests an
inhibitory effect of GLS2 on tumor transformation and proliferation
(Kim et al., 2017; Zhang et al., 2016). These results suggest that GLS2
and GLS1 are up-regulated to produce diametrically opposite effects on
tumors, possibly due to their different kinetics, immunology, and mo-
lecular characteristics (Curthos and Watford, 1995; Marquez et al.,
2010). In addition, GLS2 may also play an anti-tumor role through the
interaction of its C-terminal PDZ domain with other proteins containing
the same domain. Regarding the completely different roles of GLS1 and
GLS2, further research is needed to deal with it.

5. Microglial GLS: Key factor for microglial activation

Recently, GLS in microglia has been increasingly noticed due to its
abnormal elevation under pathological conditions. The most well-
recognized pathogenic role of GLS is Glu-mediated excitotoxicity, which is firstly reported in 1957 (Fig. 3B) (Lucas and Newhouse, 1957).
GLS dysregulation in glia causes overproduction of Glu, and excessive
extracellular Glu induces neuronal dysfunction and degeneration (Erd-
mann et al., 2006; Lau and Tymianski, 2010). Interestingly, our recent
findings revealed that GLS plays an important role in microglial acti-
vation and neuroinflammation, suggesting GLS participates in the
pathogenesis of neurological diseases in a much more complex manner
than what has been thought before (Gao et al., 2020, 2019). Upon
stimulation, microglia are activated into a pro-inflammatory phenotype
to release cytokines, chemokines, neurotoxic factors, and EVs, resulting
in neuroinflammation.

5.1. GLS misexpression in activated microglia

In 2004, we reported abnormal elevation of the levels of GLS in HIV-
1-infected macrophages, enhancing extracellular glutamate production
and contributing to potential neurotoxicity (Zhao et al., 2004). This is
the first evidence implying the misexpression of GLS in activated
microglia. In 2006, Takeuchi et al. reported that the pro-inflammatory
cytokine TNFα treatment directly promotes excess Glu release and
causes excitotoxic neuronal death via up-regulating the expression of
Glu in primary mouse microglia (Erdmann et al., 2006; Takeuchi et al.,
2006). Similarly, our group reported increased GLS1 expression in HIV-
1-infected human microglia, implying that the GLS1 up-regulation in
activated microglia is a highly conversed biological process in various
pathological conditions in mammals (Huang et al., 2011a). This premise
is confirmed by multiple investigations in vitro and in vivo. In an in vitro
model of sporadic ALS, soluble iron accumulation in BV2 cells activated
TNFα expression, induced GAC up-regulation, and further contributed to
a large amount of Glu release into extracellular space (Niida-Kawaguchi
et al., 2019). Moreover, Chen et al. found that in Japanese encephalitis
(JEV)-infected microglia, TNFα contributed to GLS up-regulation and increased Glu release. The excessive Glu combined with reduced Glu absorption leads to excitatory cell death (Chen et al.,
2012). In addition, we also demonstrated significant elevation of GLS
expression levels in LPS-stimulated primary microglia and AD mouse
brain ones (Gao et al., 2019). These results all demonstrate that
microglial activation leads to GLS overexpression.

With the help of several pioneer studies, the mechanisms that lead to
microglial GLS misexpression have been unveiled. In 2012, our group
observed that interferon α (IFNα) treatment or HIV-1 infection signifi-
cantly elevates the activity of STAT1 in monocyte-derived macrophages
(Zhao et al., 2012). STAT1 directly binds to the promoter of GLS1,
enhancing the transcription of the latter (Zhao et al., 2012). Our ob-
servations indicate the inflammatory cytokine-induced GLS1 up-regula-
tion for the first time. Following our study, Milewski et al. found that the inflammatory cytokine TNFα induces an elevation of
STAT3 activity via enhancing the phosphorylation of the latter. The
activated STAT3 may then binds to GLS1 promoter to induce GLS1 expres-
sion in astrocytes, under the stimulation of TNFα (Milewski et al.,
2019). Another transcription factor, NF-κB, also positively regulates GLS
expression in CD11b+ myeloid-derived cells, providing more potential
axis in the positive regulation of GLS1 transcription by inflammatory
cytokines (O’Driscoll et al., 2013). Furthermore, GLS1 promoter has
predicted binding sites for T-box, GATA, SP, KLF families that are or
may be dysregulated in activated microglia, implying the importance
of inflammatory transcription factors in GLS expression (Li et al., 2017;
Nath et al., 2004).

Besides, the elevated GLS expression in activated microglia may also be
due to post-transcriptional regulation. Our previous research has
demonstrated that only GAC, but not KGA, is significantly elevated in
activated microglia in vitro and in brain tissues isolated from early-stage
AD mouse and post-mortem HAND patients (Gao et al., 2019; Huang
et al., 2011a). The differential expression of KGA and GAC can be
explained in two ways. First, alternative splicing machinery can be
activated in stimulated microglia under pathological conditions, which
interferes with the normal splicing process of GLS1 to produce more
GAC mRNAs (Gonzalez-Pena et al., 2016). Second, KGA and GAC
possess unique C-terminals (Marquez et al., 2010). The shortened 3’ UTR
increases the stability of GAC mRNA and protects it from inhibitory post-
transcriptional regulation, such as miR-23 binding (Aledo et al., 2009;
de la Rosa et al., 2009; Elgadi et al., 1999; Gao et al., 2009b).

Thus, multiple studies have demonstrated the mechanisms of
microglial activation-mediated GLS1 misexpression, however, we are
still far away from fully understanding the pathways that regulate GLS1
Fig. 3. Physiological and pathological functions of microglial GLS1. (A) GLS1, locating within the mitochondrial matrix in loose association with the inner membrane, is the enzyme that for glutamine (Gln) metabolism. In physiological conditions, Gln, transported into the mitochondrial matrix by mitochondrial Gln transporter, is deamidated into glutamate (Glu) by GLS1 and oxidatively deaminated into α-ketoglutarate (α-KG) by Glu dehydrogenase (GDH). α-KG enters the tricarboxylic acid (TCA) cycle to provide materials and energy for the cell. Succinate, the product of α-KG, contributes to the expression of HIF family. Malate generates pyruvate and nicotinamide adenine dinucleotide phosphate reduced (NADPH) after exiting the TCA cycle. Oxaloacetate (OAA) produces aspartate that is involved in nucleotide synthesis. OAA is converted into citrate that supports synthesis of acetyl-CoA, lipids, NAPDH, and reactive oxygen species (ROS). Besides, the activity of TCA cycle can also be regulated by glucose-derived acetyl-CoA. (B) Under pathological conditions, extracellular cytokines, such as IFNα and TNFα, can raise the activities of SRAT1/3 signaling pathways. Abnormal GLS expression is caused by the direct binding of STAT1/3 on the promoter regions of GLS genome. GLS1 up-regulation causes overproduction of glutamate (Glu) in microglia, increasing extracellular Glu levels, which, in turn, leads to excitotoxicity and neuronal degeneration. The excessive Glu in microglia may also elevate TCA cycle activity. The metabolic changes induce the accumulation of HIF and reactive oxygen species (ROS), activating MAPK-NF-κB and HIF-1α signaling pathways. In consequence, the expression and secretion of pro-inflammatory factors including TNFα and IL-1β are promoted, resulting in microglial activation. The accumulation of TNFα and other cytokines form a positive feedback loop to further enhance the expression of GLS1, accelerating microglial activation and immune response. The dysregulation of TCA cycle also generates higher levels of fatty acids and ATP, providing materials and energy for the biogenesis of EVs. Thus, activated microglia release more EVs enriched with pro-inflammatory factors and contribute to the formation of pro-inflammatory microenvironment in the CNS. In summary, GLS1 acts as a core in the initiation and progression of neuroinflammation and neuronal damage by inducing excitotoxicity, activating microglia, and promoting pro-inflammatory EV release.
expression in microglia. With more comprehensive investigations, the regulatory network of microglial GLS expression may be completely drawn in a near future.

5.2. GLS and microglial activation

The misexpression of GLS1 in activated microglia implies a role of GLS in regulating the phenotype transition of microglia. Through applying perturbation-of-function assay, the exact effects of microglial GLS1 are unmasked. We recently observed that the overexpression of GLS, especially GAC, activates microglia, ascertained by the up-regulation of expression in microglia. With more comprehensive investigations, the network between GLS1 overexpression and microglial activation.

In view of the essential roles of GLS in microglial activation, the underlying mechanisms have emerged as an important research topic. The first and the most intuitionistic one is that microglia may be activated by the excessive Glu. Interestingly, we previously demonstrated that microglia do not express any ionotropic or metabotropic Glu receptors except for mGluR2/3, and excessive extracellular Glu cannot directly induce microglial activation, which, does not accord with the premise. However, this traditional view has undergone recent revision as evidence has accumulated that several Glu receptors are expressed on microglia and help mediate the neuronal-microglial bidirectional communication. For example, scientists reported that microglia express NMDA receptors and the stimulation of these receptors triggered microglial activation and caused neuronal damage that signals back to activate microglia in vitro (Kaindl et al., 2012; Raghunatha et al., 2020). Similarly, other groups reported that the stimulation of mGluR2/3 (Taylor et al., 2005) and mGluR5 (Spampinato et al., 2018) triggered microglial activation and a pro-inflammation shift. These reports indicate that the transformation of microglia from a normal to a pro-inflammatory phenotype, which further intensifies the abnormal accumulation of Glu in microglia. Vice versa, GLS1 up-regulation exerts additional pro-inflammatory cytokine production and microglial activation.

Apart from the activation of Glu receptor, the competitive binding of anti-inflammatory miRNAs by GLS transcripts becomes a possible mechanism that mediates microglial activation. For example, databases for miRNA target prediction (e.g. targetscan, miRanda, etc.) showed that GLS1 has conserved binding site for miR-125-5p. Microglial miR-125 attenuates polarization of pro-inflammatory phenotype by targeting TNFα/IRF4 and iNOS in GLS gain-of-function group (Gao et al., 2020, 2019). GLS1 overexpression also induces excess release of pro-inflammatory cytokines (Gao et al., 2019). Furthermore, the treatment of GLS inhibitor BPTES compromises the activation of primary microglia stimulated by lipopolysaccharide (LPS) or TNFα (Gao et al., 2019). The administration of GLS1 inhibitor CB-839 and JHU-083 attenuates neuroinflammation in the mouse models of neurological disorders, confirming the part that GLS1 has played in microglial activation (Gao et al., 2020; Zhu et al., 2019). Therefore, mounting reports have implicated GLS as a regulator of microglial activation, although it remains vague whether GLS serves as an inducer or downstream effector of microglial activation.

Overall, although we are still in urgent need to excavate the roles of GLS in microglia, results above indicate a bidirectional regulatory network between GLS1 overexpression and microglial activation. Microglia, activated by extrinsic cues such as TNFα and IFNγ stimulation, exhibit elevated expression of GLS1 due to the enhanced activities of STATs and NF-κB. The activated microglia express higher levels of pro-inflammatory cytokines, which further intensifies the abnormal accumulation of Glu in microglia. Vice versa, GLS1 up-regulation exacerbates microglial activation via excess production and release of Glu, altered cellular metabolism, and the up-regulation of NADPH oxidase that produces increased levels of ROS. Therefore, a positive feedback loop is formed that finally results in uncontrolled activation of microglia, exaggerating neuroinflammation.

6. Microglial GLS: Important controller of pro-inflammatory EV release

Emerging studies have implied CNS microenvironment as the fundamental matrix in mediating various intercellular signals including the inflammatory ones (Fig. 3B) (Gordon et al., 1998; Mack and Wolburg, 2013). We and others have reported that GLS participates in the formation of pro-inflammatory microenvironment via interactive regulation with EVs (Gao et al., 2020, 2019; Huang et al., 2011a; Wang et al., 2017b).
6.1. GLS and EV release regulation

EVs are heterogeneous nanoscale membranous vesicles that are secreted by almost all types of CNS cells (Xia et al., 2019). Due to diverse biogenesis pathway, EVs are divided into exosomes (30–100 nm), microvesicles (50–1000 nm), and apoptotic bodies (500–2000 nm) (Xia et al., 2019). Exosomes are originated from the endosomal system via the inward budding of multivesicular bodies (MVBS) (Baierova et al., 2012; Tamai et al., 2010; Trajkovic et al., 2008). Microvesicles are assembled with small membrane domains and released by directly outward budding of plasma membrane (Mause and Weber, 2010; Raposo and Stoorvogel, 2013). Apoptotic bodies are produced via shedding from dying cells (Hauer et al., 2017). EVs are abundantly present and have been considered as an important part of the microenvironment in the CNS. They transfer their cargos (miRNAs, proteins, and lipids, etc.) among cells horizontally to mediate intercellular communication. Under virus infection and LPS stimulation, microglia release more EVs that are loaded with pro-inflammatory molecules (Wu et al., 2018). Interestingly, an increase of EV release is found in GLS1-overexpressing Hela cells (Wu et al., 2018) and GLS increase is found positively correlated with the increase of EV release and the enrichment of cytokines (e.g., TNFsα) in EVs from rat ischemia brain (Gao et al., 2020). Importantly, GLS inhibitors BPTES and CB-839 significantly decreased EVs release from HIV-1-infected macrophages and immune-activated microglia, implying the contribution of GLS in EVs release and content loading. Another study of our team confirmed this premise as we found that GLS1 overexpression enhanced microglial EVs secretion and TNFsα packaging (Gao et al., 2019). These pro-inflammatory EVs derived from GAC-overexpressed microglia induce TNFsα and iNOS expression in resting microglia, facilitating the spread of inflammatory signals (Gao et al., 2019).

Interestingly, GLS is released into the extracellular space via EVs from HIV-1-infected macrophages and immune-activated microglia (Wu et al., 2015). Extracellular GLS further promotes Glu production and causes neurotoxicity (Wu et al., 2015). The virus infection- or immune activation-induced neurotoxicity can be blocked by GW4869, an inhibitor of EV release, implying that the neurotoxicity of HIV-1-infected macrophages and immune-activated microglia is dependent on GLS-containing EVs (Wu et al., 2015). Further research suggests that GLS may regulate EV biogenesis from HIV-1-infected macrophages and immune-activated microglia via its key downstream products α-KG and ceramide as addition of α-KG or ceramide rescued EV release during BPTES treatment (Wu et al., 2018). Besides, GLS may boost ROS generation, therefore influence EV secretion. We found that TNFsα treatment increases EV levels, protein levels of GLS (only GAC isoform), and the Glu production in astrocytes, exaggerating ROS production. The elevation of EV release can be blocked by GLS inhibitors, the antioxidant NAC, or genetic knockout of GLS, which indicates that GLS-ROS axis is involved in EV release from astrocytes (Wang et al., 2017a).

Given these results, we are interested to find out how GLS’s key downstream products α-KG and ceramide contribute to EV biogenesis in glial cells. We speculate that the excessive generation of these products by GLS interferes with the normal TCA cycle activity. It is likely that the enhanced TCA cycle activities under GLS overexpression generate more metabolites including ATPs and ceramide, providing energy and material support for EV release. Current studies show that extracellular ATP activates its receptor P2X7 and triggers the release of GAPDH and other robust vehicle-mediated unconventional proteins such as calpain, signaling-related proteins. These proteins enhance acid sphingomyelinase activity, resulting in rapid exosome release and membrane shedding (Takeouchi et al., 2015; Valimaki et al., 2016). Ceramide induces the coalescence of small microdomains into larger domains and triggers domain-induced budding of EV vesicles into multivesicular endosomes (Trajkovic et al., 2008). Thus, TCA cycle dysregulation may be a possible mechanism for GLS-induced EV release, which requires confirmation in future studies.

6.2. GLS and EV function regulation

It is worth-noting that the overexpression of GLS in microglia also alters the contents of EVs, other than modifying the release of EVs (Gao et al., 2019). Our miRNA array analysis suggests that pro-inflammatory miRNAs (such as miR-130, miR-145a, miR-23b, and miR-146a, etc.) levels are up-regulated and anti-inflammatory miRNAs (such as miR-124 and let-7b) levels are down-regulated in EVs derived from GLS overexpressed microglia. Changes in the contents of EVs affect their function. The EVs derived from GAC-overexpressed microglia promote inflammatory molecules (such as TNFsα and iNOS) expression and release in resting/quiescent microglia probably via three ways. First, our group has found that brain cells under inflammatory stimulations release EVs highly enriched with TNFsα as well as other pro-inflammatory molecules (Gao et al., 2020). After being internalized, these EVs release pro-inflammatory molecules into recipient cells, leading to the direct elevation of these molecules intracellularly. Second, we have observed elevated levels of GLS in EVs derived from activated microglia (Wu et al., 2015). EV-mediated transferring of GLS from activated microglia to resting ones promotes the expression of pro-inflammatory molecules in the latter. Third, EVs derived from microglia with GLS overexpression contain less anti-inflammatory miRNAs like miR-124, compared to controls (Gao et al., 2019). miR-124 is widely associated with the suppression of multiple pro-inflammatory pathways including TLR4 signaling and MEC2-STAT3 axis (Periyasamy et al., 2018a, 2018b). The lack of miR-124 in microglial EVs removes the existing roadblocks for the expression of pro-inflammatory molecules in physiological conditions, accelerating the spreading of neuroinflammation.

The detailed mechanisms of GLS overexpression-induced pro-inflammatory molecule loading into EVs remain indistinct to date. Pro-inflammatory cytokines and miRNAs were enriched in EVs derived from GLS-overexpressed microglia, which may be passively packaged into EVs since these molecules are expected to have high expression levels in the cytosol of activated microglia. On the other hand, although there is a lack of direct evidence currently, we cannot exclude the potential existence of GLS-mediated active exosomal cargo sorting. Squadrito et al. showed that in macrophages and endothelial cells, endogenous RNAs bind to miRNAs and modulate the sorting of the latter into EVs by controlling free miRNA levels in cytosol (Squadrito et al., 2014). It is interesting to examine whether the same mechanism is recruited in exosomal miRNA sorting under GLS overexpression conditions.

7. Microglial GLS: Pathological contributors of neurological diseases

Many studies have demonstrated significant up-regulation of microglial GLS under pathological conditions that include AD, HAND, multiple sclerosis (MS), ALS, Rett syndrome (RTT), and other neurological diseases (Gao et al., 2019; Jin et al., 2015; Tian et al., 2008; Ye et al., 2013). It is suggested that microglial activation-induced neural injury can be mediated by GLS. In this section, we discuss recent findings of microglial GLS as the pathogenic mediator in aforementioned diseases (Table 3).

7.1. Microglial GLS and AD

Microglia-mediated neuroinflammation is a key pathological feature of AD as the depletion of microglia significantly halts disease-related molecule accumulation (Asai et al., 2015). To date, growing evidence has indicated important roles of microglial GLS and Glu in the pathogenesis of AD. Studies over the past two decades report decreased N-acetylaspartyl Glu levels in patient brain, however, Hollinger et al. reported opposing findings (Hollinger et al., 2019), suggesting more complex underlying pathological mechanisms than previously known.
Table 3
The pathological roles of microglial GLS in neurological disorders.

| Neurological disorders                  | GLS expression patterns | GLS effects                          | Reference                     |
|-----------------------------------------|-------------------------|--------------------------------------|-------------------------------|
| Alzheimer’s disease (AD)                | GLS1: Up-regulated in microglia | Activating microglia neurotoxicity | Gao et al., 2019              |
| HIV-1-associated neurocognitive disorders (HAND) | GAC: Up-regulated in the brain and microglia | Inducing Glu neurotoxicity | Zhao et al., 2004; Tian et al., 2008; Erdmann et al., 2009; Huang et al., 2011; Ye et al., 2013; Nedelcovych et al., 2019 |
| Multiple sclerosis (MS)                 | GLS: Up-regulated in the brain and microglia | Inducing Glu neurotoxicity | Werner et al., 2001; Shijie et al., 2009; Kono et al., 2019 |
| Amyotrophic lateral sclerosis (ALS)     | GAC: Up-regulated in the brain and microglia | Inducing neuronal damage | Van Den Bosch et al., 2006; Yuan et al., 2017; Niida-Kawaguchi et al., 2019 |
| Rett syndrome (RTT)                     | GLS1: Up-regulated in microglia | Enhancing abnormal Glu release | Marazawa and Jin, 2010; Jin et al., 2015; Khoury et al., 2020 |
| Depression                              | GLS: Up-regulated in microglia | Activating microglia, Inducing neuronal damage | Dowlath et al., 2010; Zhu et al., 2019 |
| Acute brain disease/injury              | GLS1: Up-regulated in microglia | Activating microglia, Inducing neuronal damage | Chamoun et al., 2010; Gao et al., 2020 |
| Chronic hyperammonemia/hepatic encephalopathy | GLS: Up-regulated in cerebellar microglia and astrocytes | Increasing Glu and GABA release, inducing motor incoordination | Cabrera-Pastor et al., 2018 |

Our study has revealed that in AD mice brain tissue and LPS-activated cultured primary microglia, GAC isoform is specifically up-regulated (Gao et al., 2019). Overexpression of GAC in cultured primary microglia induces its pro-inflammatory phenotype, indicating microglial GLS as a key inducer of neuroinflammation in AD (Gao et al., 2019). The treatment of GLS inhibitors BPTES and JHU-083 significantly suppresses the transition of pro-inflammatory microglial phenotype in vitro and ameliorates the cognitive performance of AD mice, respectively, indicating a great association of GLS with AD pathogenesis (Gao et al., 2019; Hollinger et al., 2020).

Recent studies have implied the involvement of microglial EAs in AD pathogenesis (Trotta et al., 2018). The inhibition of EEA release has yielded promising effects on attenuating AD phenotype in animal model (Asai et al., 2015). As we discussed above, GLS1 is a key regulator of microglial EV release and function (Gao et al., 2019; Wu et al., 2018). The intraperitoneal administration of CB-839 also leads to a significant reduction of EV numbers in AD mouse brains, suggesting GLS inhibition may obtain therapeutic effects via suppressing EV release (unpublished data). In short, microglial GLS dysregulation promotes microglial activation, pro-inflammatory EV release, and the neuroinflammatory microenvironment formation, contributing to the pathogenesis of AD (Gao et al., 2019).

7.2. Microglial GLS and HAND

GLS-mediated neurotoxicity is also reported to participate in the pathogenesis of HAND. Mononuclear phagocyte (such as macrophages and microglia) immunity plays a significant role in HAND. Our previous works have described that HIV-1-infected macrophages generate excessive Glu through mitochondrial GLS1, which causes Glu receptor NMDA over-stimulation, caspase signaling activation, and neuronal apoptosis. The production of Glu can be inhibited by GLS-specific siRNA or inhibitor (Erdmann, 2007; Erdmann et al., 2007; Tian et al., 2008; Zhao et al., 2004). After HIV-1 infection, the expression and subcellular localization of GLS isoforms in macrophages also changed. The GLS1 isoform GAC is specifically up-regulated, which is consistent with the GAC level in frontal cortical microglia derived from the HIV-1-infected patients (Huang et al., 2011a). The GAC isoform is released from monocytes into cytosol and the extracellular space (Wu et al., 2015). However, no changes in the KGA isoform are observed (Erdmann et al., 2009). What’s more, similar results are also detected in HIV-infected primary microglia (Huang et al., 2011a). Another in vivo experiment shows that the expression of GLS1 in neuron is increased in a murine model of HIV-1 encephalitis, indicating the neurotoxic effects of GLS during HIV-1-infection-induced inflammation (Ye et al., 2013).

Other studies have shown that both interferon and HIV-1 infection activate phosphorylation of STAT1 which increases GLS1 expression and Glu production, driving neurotoxicity (Pais et al., 2008; Zhao et al., 2012). Moreover, during HIV-1 infection, oxidative stress also enhances neuroinflammation by causing mitochondrial GLS1 release (Tian et al., 2008). Importantly, both our works and others’ have suggested that GLS1 may serve as a potential therapeutic target of HAND. Our group have shown that siRNA-based GAC knockdown reversed neuronal toxicity in HAND (Huang et al., 2011b) and Nedelcovych et al. have demonstrated pharmacological inhibition of GLS1 by DON and JHU083 reversed cognitive impairment and microglial activation in the EcoHIV-infected mouse model of HAND (Nedelcovych et al., 2019).

7.3. Microglial GLS and MS

MS is an inflammatory demyelinating disease of the CNS. Mounting reports have demonstrated that macrophages and microglia near the center lesion express higher level of GLS in white matter of MS patients (Werner et al., 2001). In experimental autoimmune encephalomyelitis (EAE) mouse model, the immunostaining of spinal cord cross-section also shows GLS overexpression in inflammatory lesions. The GLS-catalyzed production of Glu causes excitotoxicity which is involved in axonal damage (Werner et al., 2000). These results indicate that GLS-mediated Glu excitotoxicity may contribute to the pathogenesis of MS. The blockade of Glu release by hemichannel inhibitor CBX or GLS1 inhibitor DON can be a promising treatment for MS (Shijie et al., 2009). GLS inhibitor DON significantly reduces Glu release, prevents neuron death, and promotes functional recovery in EAE mice in a dose-dependent manner (Shijie et al., 2009). In addition, another GLS inhibitor BPTES also displays a protective effect for MS in an EAE mouse model, suggesting that inhibition of glutaminolysis represents a potential novel treatment strategy for patients with systemic lupus erythematosus (SLE) and Th17-related autoimmune diseases (Kono et al., 2019).
7.4. Microglial GLS and ALS

In ALS, abnormally accumulated-Glu-induced excitotoxicity is a key cause of neuronal death (Van Den Bosch et al., 2006). In the sporadic amyotrophic lateral sclerosis model, the expression of GAC is up-regulated significantly, which results in the release of a large amount of Glu (Niida-Kawaguchi et al., 2019). In a TNFα-stimulated in vitro ALS model, the over-active status of GAC can be repressed by DON, leading to less Glu production. Similarly, GLS1 inhibitor ebsonel has shown promise as an effective treatment for ALS for its strong Glu-reducing and anti-oxidant properties (Yuan et al., 2017). However, the decrease of phosphate-activated GLS activity is observed in G93A-tTA cell line, a motor neuronal model of familial ALS, which favors the decrease of Glu synthesis (D’Alessandro et al., 2011). Thus, the roles of GLS in ALS pathogenesis remain controversial and more detailed studies for the involvement of GLS in neuroinflammation and neurodegeneration in ALS are badly needed.

7.5. Microglial GLS and RTT

RTT is a neurodevelopmental disorder that primarily affects young girls, characterized by deceleration of brain growth, loss of motor and vocalization skills, cognitive capability deficiency, and autistic features, etc. (Chabrou and Zoghbi, 2007). In the methyl-Cpg-binding protein 2 (MeCP2) mutation-dependent Rett syndrome model, microglia release high level of Glu (about five times the level of the wild type control) which causes toxicity to dendrites and synapses (Maezawa and Jin, 2010). The excessive Glu production is mediated by GLS, ascertained by that the elevation of GLS expression levels was found in MeCP2-deficient microglia and the blockage of abnormal Glu generation by the treatment of GLS inhibitors DON, JHU29, and D-JHU29 (Khoury et al., 2020). Besides, hyperammonemia has been a known feature of RTT since Rett’s report in 1977 (Percy, 2016). Increased ammonia, apart from Glu, is highly likely to contribute to RTT pathology in MeCP2-deficient mice. Follow-up studies identified that the up-regulation of GLS in RTT is highly likely due to the MeCP2 deficiency-induced pro-inflammatory signaling cascade, which includes SNAT1 expression, ROS accumulation, and NF-κB activation (Jin et al., 2015). Thus, GLS may act as a core element in regulating neuroinflammation and neurotoxicity, the two wings of RTT pathogenesis, by controlling Glu generation.

7.6. Microglial GLS and depression

Recent observations have implicated the strong association of inflammation with depression, including major depressive disorder, bipolar disorder, and dysthymia (Wium-Andersen et al., 2013). Increased expressions of pro-inflammatory biomarkers have been found among depressed individuals, and pro-inflammatory agents were able to induce depressive symptoms (Dowlati et al., 2010). GLS activity is specifically enhanced in microglia-enriched CD11b+ cells isolated from the prefrontal cortex and hippocampus of chronic social defeat stress (CSDS) mouse, a widely used depression animal model (Zhu et al., 2019). Acute treatment by JHU-083, a prodrug of DON designed to improve CNS penetration (Rais et al., 2016), compromises GLS activity, thus reverses CSDS-induced inflammatory activation of CD11b+ cells (Zhu et al., 2019). JHU-083 also ameliorates deficits in social behavior induced by CSDS, revealing GLS1 as a promising target for treating depression.

7.7. Microglial GLS and neuroinflammation after acute brain disease/injury

GLS dysregulation has been extensively implicated in acute brain disease or injury (Dorsett et al., 2017; Gao et al., 2020). In middle cerebral artery occlusion (MCAO) rat, GLS1 is highly expressed and evidently co-localized with Iba1 (Gao et al., 2020). Cerebral ischemia also increases the loading of pro-inflammatory molecules into EVs (such as TNFα and Cox2) (Gao et al., 2020). The administration of GLS inhibitor CB-839 can significantly reduce pro-inflammatory factor expression and inhibit EV secretion, alleviating neuroinflammation and brain injury (Gao et al., 2020). These evidences show that GLS1 is an important contributor to neuroinflammation in the pathogenesis of cerebral ischemia. Unlike MCAO, the involvement of GLS in TBI remains vague. Brain injury initiates excitotoxic cascade of Glu and other excitory amino acids, giving rise to neuronal death in the surrounding tissue of the original injury site (Dorsett et al., 2017). Clinical studies also showed that cerebral extracellular Glu levels are associated with mortality and functional outcomes for TBI patients: high Glu levels are predictive of poor outcome, whereas a delayed Glu increase leads to the worst prognosis (Chamoun et al., 2010). These results imply that GLS may be involved in the elevation of Glu in TBI, although no quantification data are available to confirm the elevation of GLS expression and activities.

7.8. Microglial GLS and chronic hyperammonemia and hepatic encephalopathy

Chronic hyperammonemia is a major contributor to cognitive and motor dysfunctions in patients with chronic liver diseases (Felipo et al., 2012) and has recently been demonstrated to induce neuroinflammation (Hernandez-Rabaza et al., 2016a; Rodrigo et al., 2010). As we mentioned in previous sections, scientists reported that chronic hyperammonemia induced pro-inflammatory cytokine release and increased TNFα receptor activation in rat cerebellar microglia and astrocytes (Cabrera-Pastor et al., 2018). Chronic hyperammonemia-induced neuroinflammation further led to changes in Glu and GABA receptor expressions (Hernandez-Rabaza et al., 2016b). GLS expression in cerebellar microglia and astrocytes is also elevated upon hyperammonemia-induced neuroinflammation after NF-κB activation through the nuclear translocation of its p50 subunit (Cabrera-Pastor et al., 2018). These results suggest that GLS and Glu signaling is affected by chronic hyperammonemia and that GLS might serve as a potential target for intervention. Furthermore, EV release and their protein cargo were found alternated in hyperammonemia-induced neuroinflammation, and that plasma EVs from hyperammonemic rats carry molecules that sufficiently trigger neuroinflammation in cerebellum and induces motor incoordination in normal rats (Izquierdo-Alarcon et al., 2020).

Apart from discussions above, GLS has been indicated to have a part in other diseases, like diabetes (Watford et al., 1984) and schizophrenia (Gaisler-Salomon et al., 2009). However, as this review focus on the role of microglial GLS, we will not discuss the involvement of GLS in these diseases in detail.

Taken together, microglial GLS has been deemed as a key enzyme that directly participates in microglial activation and the consequent inflammatory response and excitotoxicity. To date, various types of GLS inhibitors have been designed and applied in the pre-clinical studies in aim to treat neurological disorders. Inspiring results have been obtained. However, due to the importance of Glu in transmitting excitatory nerve conduction signals in the CNS, more comprehensive works are urgently needed to precisely modulate GLS-mediated Glu generation without interfering normal brain functions.

8. Conclusions and future directions

To date, great efforts have been made to demonstrate the various isoforms of GLS, together with the differences in enzymatic activity, expression regulation, and pharmaceutical modulation among each isoform. More importantly, abundant functional studies have unveiled the key roles of GLS in neurotransmission, cell metabolism, and microglial activation, a recently discovered function. Our and other groups all suggest that GLS misexpression and dysfunction result in the generation
of excessive Glu, the alteration of inflammatory factor expression, and the disruption of metabolic homeostasis, causing microglial activation. GLS up-regulation also enhances microglial EV release and promotes the package of pro-inflammatory molecules into EVs, which, in turns, leads to the formation of neuroinflammatory microenvironment. The enormous influence of GLS on microglial activation and neuroinflammation indicates GLS as a key pathological contributor in various neurological disorders including neurodegenerative diseases, RTT, acute brain disease/injury, and chronic hyperammonemia and hepatic encephalopathy.

It is also worth-noting that the more involvement of microglial GLS in neurological disorders we observe, the more underlying mechanisms we need to explore. One crucial issue we are facing now is to dissect the relationship and underlying mechanisms of the misexpression of GLS in activated microglia and the GLS-mediated microglial activation. This is “a chicken-and-egg problem” which requires tremendous efforts to fully surmount it. Besides, KGA and GAC exhibit distinct expression patterns in activated microglia in culture and in disease brains, as discussed above. To explore the non-canonical roles of those two isoforms, therefore, becomes an interesting issue (Aledo et al., 2000; de la Rosa et al., 2009). Except for different degrees of stability, whether or not the distinct C terminal confers other properties of the two GLS1 requires more comprehensive studies to explore.

Furthermore, the development of pharmaceutical compounds that modulate GLS in a precise manner is a challenging job. Due to the central role in excitatory neurotransmission (Marquez et al., 2010), GLS activity should be modified with great cautiousness to prevent function impairment of the brain. One approach is to develop novel pharmaceuticals with differential inhibitory effects of GLS activity. Another one is to utilize strategies for targeted delivery such as EV-based delivery. Being natural nano-vesicles that are non-immunogenic and highly bio-stable, EVs may deliver GLS inhibitors to microglia via being equipped with homing molecules to (Alvarez-Erviti et al., 2011), which is under investigation.

Of note, we have also identified GLS as an important regulator of macrophage activation, indicating the involvement of GLS in the inflammatory responses in other organ systems besides the CNS (Huang et al., 2011a; Wu et al., 2015; Zhao et al., 2012). For example, cytokine storm has been identified as a key mechanism for acute respiratory distress syndrome (ARDS), the main cause of death in coronavirus disease 2019 (COVID-19) (Chanappanavar and Perlman, 2017; Huang et al., 2020). Cytokine storm is caused by the uncontrolled release of pro-inflammatory cytokines and chemokines from immune effector cells like macrophages (Bird, 2018). The commonly used therapeutics including corticosteroid and interferons therapies may exacerbate disease due to enhanced plasma viral load or delayed administration (Chanappanavar and Perlman, 2017). For the crucial roles in macrophage activation, GLS may be a promising target for alleviating COVID-19-induced cytokine storms, which is currently under investigation. On the other hand, GLS has a critical role in the formation of tumor micro-environment where macrophages constitute a major immunosuppressive component in almost every step of tumorigenesis and transformation (Mantovani et al., 2017; Noy and Pollard, 2014). Via targeting Glu metabolism by GLS inhibitors, JHU-083 and DON, the transformation (Mantovani et al., 2017; Noy and Pollard, 2014). Via targeting Glu metabolism by GLS inhibitors, JHU-083 and DON, the irreversible neuronal damage.

9. Declarations

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Author contributions

JCZ XX conceived the manuscript. LD XX CL XX YW collected references. LD XX CL XX YW wrote the manuscript. LD XX prepared illustrations.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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