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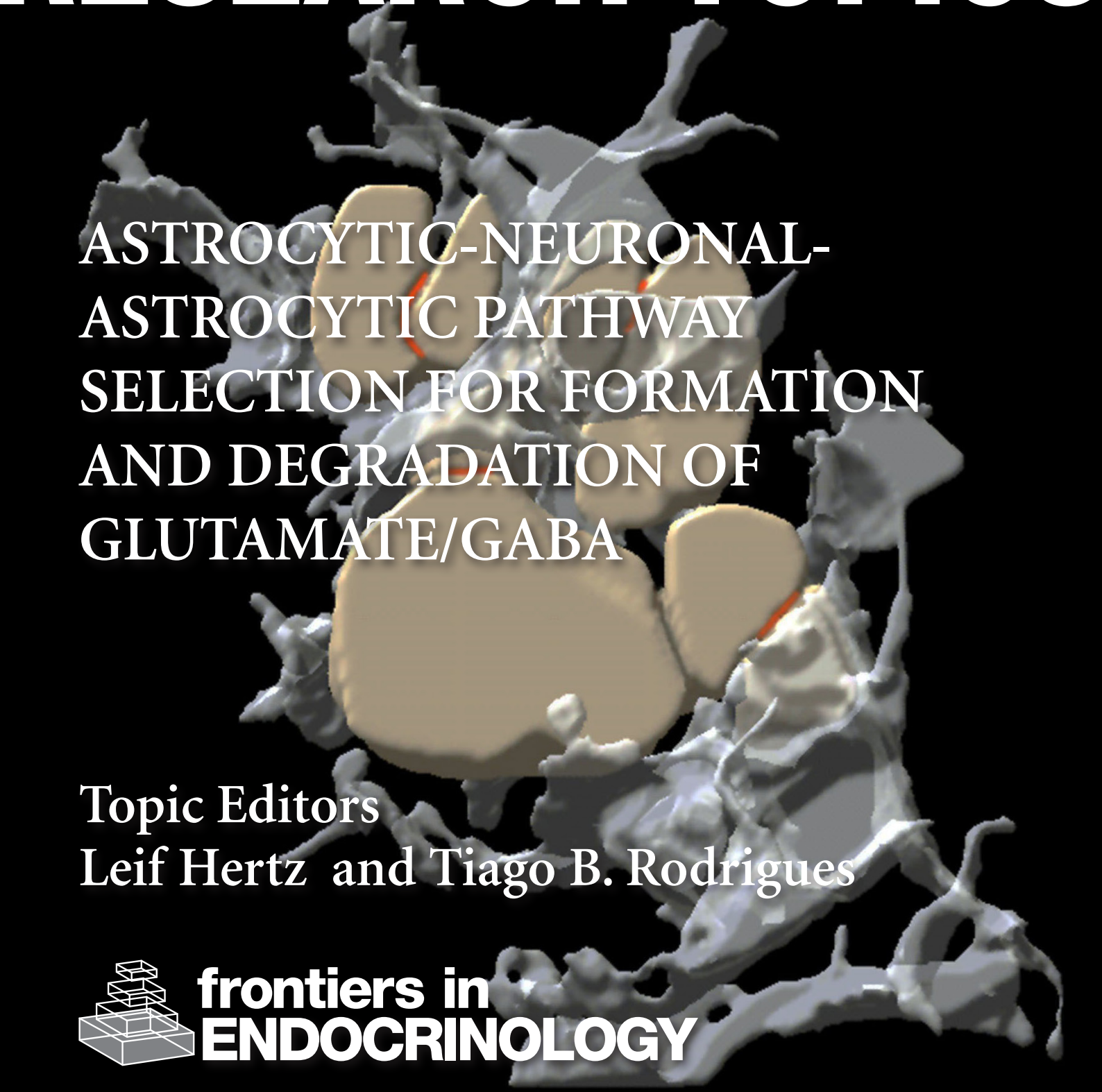

# ASTROCYTIC-NEURONAL-ASTROCYTIC PATHWAY SELECTION FOR FORMATION AND DEGRADATION OF GLUTAMATE/GABA

Topic Editors
Leif Hertz and Tiago B. Rodrigues

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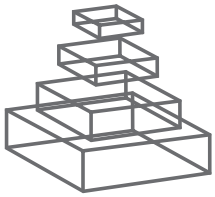

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**ISSN** 1664-8714
**ISBN** 978-2-88919-243-4
**DOI** 10.3389/978-2-88919-243-4


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# ASTROCYTIC-NEURONAL-ASTROCYTIC PATHWAY SELECTION FOR FORMATION AND DEGRADATION OF GLUTAMATE/GABA

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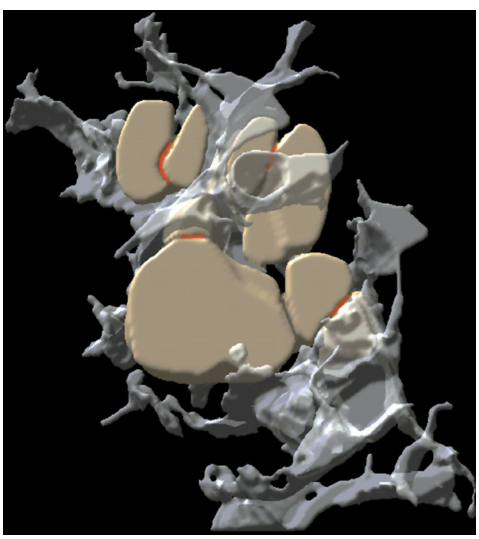

Illustration of close association between neurons (beige, with synaptic clefts in orange) and the astrocytic Bergmann glia (light gray). Slightly modified from Grosche J, Kettenmann H, Reichenbach A. "Bergmann glial cells form distinct morphological structures to interact with cerebellar neurons." (2002) *J Neurosci Res* 68:138-149. Courtesy of Andreas Reichenbach.

Endocrinological research early recognized the importance of intercellular interactions and realized the importance of glutamatergic and GABAergic signaling. In turn this signalling depends on elaborate interactions between astrocytes and neurons, without which neurons would be unable to produce, reuse and metabolize transmitter glutamate and GABA. Details of these subjects are described in this Research Topic by key investigators in this field. It focuses on the intricate and extremely swift pathway producing these amino acid transmitters from glucose in brain but also discusses difficulties in determining expression of some of the necessary genes in astrocytes and related processes in pancreatic islets. However, it does not discuss how closely associated astrocytes and neurons are anatomically, enabling these interactions. This is elegantly shown in this cover image, kindly provided by Professor Andreas Reichenbach (University of Leipzig, Germany).

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frontiers in
ENDOCRINOLOGY

EDITORIAL
published: 03 April 2014
doi: 10.3389/fendo.2014.00042

# Astrocytic–neuronal–astrocytic pathway selection for formation and degradation of glutamate/GABA

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**Keywords: aminoacid transporters, appetite regulation, astrocyte–oligodendrocyte interaction, astrocytic gene expression, brain ammonia, brain aminoacids, brain metabolism, pancreatic islets**

Endocrinological research early recognized the importance of intercellular interactions, initially in processes involved in lactation, pubertal maturation, and regulation of the female ovarian cycle and later in appetite regulation. The importance of glutamatergic and GABAergic signaling during all of these events is now realized. Reference (1) describes existing knowledge of the role of amino acid neurotransmitters in the mechanism of neuronal activation during appetite regulation and associated neuronal–astrocytic metabolic coupling mechanisms. Different responses in these mechanisms are apparently originated in different feeding paradigms associated with appetite stimulation (1).

Formation of transmitters glutamate and GABA requires profound interactions between neurons and astrocytes, as does resupply of released transmitters. Both of these amino acid transmitters are formed in brain from glucose in astrocytes (2, 3), but not in neurons, which lack the enzyme pyruvate carboxylase (PC). The most recent progress in measurement of brain glucose transport and metabolism *in vivo* and its importance for understanding of the glial role in glutamatergic and GABAergic neurons are reviewed in Ref. (4), which also thoroughly describes different approaches to establish mathematical models of brain metabolism and apply them to obtain quantitative metabolic rates (4).

**Figure 1** shows that both PC and pyruvate dehydrogenase are needed to form a new molecule of the tricarboxylic acid (TCA) cycle constituent citrate, from which glutamate is generated via α-ketoglutarate. An important, debated question is whether this process is catalyzed by glutamate dehydrogenase (GDH), as generally assumed, or by aspartate aminotransferase (3, 5), suggested by a large stimulation of glutamate/glutamine formation in astrocytes in the presence of aspartate (5). The latter concept is consistent with extremely high cytosolic and mitochondrial aspartate aminotransferase activity, allowing rapid nitrogen exchange between glutamate and aspartate (6).

Glutamate is converted to glutamine by glutamine synthetase (GS) and transferred to neurons. In glutamatergic neurons, glutamine is converted to glutamate within the mitochondrial membrane, enters the mitochondrial matrix, and is returned to the cytoplasm in a process requiring the malate–aspartate cycle operation (2, 3). GABA formation is slightly more complex, since part of the glutamate – from which GABA is formed by decarboxylation – is treated similarly, but another major part is first partly metabolized via the TCA cycle (2). Maximal glutamate synthesis rates in rats/mice are not achieved until postnatal day 30 (3), associated with huge increases in energy demand and production, and probably with functional gains. After neuronal glutamate or GABA release, some transmitter, mainly GABA, is reaccumulated into neurons but most glutamate is returned to astrocytes. Here a part is oxidized, requiring similar *de novo* synthesis to maintain mass balance, and the remainder is returned to neurons for reuse. Both processes are probably identical in brain (2, 3) and in retina (7), where Müller cells are the major glial cells. They express PC (8) and may synthesize glutamate/GABA like brain astrocytes. By removing most extracellular glutamate in the inner retina and contributing to glutamate clearance around photoreceptor terminals, they contribute to shape (and terminate) synaptic activity (7). Reactive Müller cells are neuroprotective, but may also contribute to neuronal degeneration by reversal of glial glutamate transporters. Dysregulation of retinal glutamate homeostasis is important in many retinopathies. A hormonally induced increase in Müller cell GS protects against neuronal injury, whereas GS inhibition increases cell death (7, 9). The possibility that oxidation of astrocytically generated glutamate represents a major part of astrocyte energy metabolism (3) might contribute to this.

Synthesis of pyruvate from glucose involves one oxidative reaction, leading to cytosolic formation of NADH from $NAD^+$. For regeneration of $NAD^+$, reducing equivalents must be transferred to mitochondria. In brain, this is generally supposed to occur via the malate–aspartate shuttle. Immunohistochemical expression of aralar, an essential constituent of this shuttle, is low in astrocytes (5), but determination of mRNA for its gene in freshly obtained astrocytes and neurons shows equal expression in each cell type (3, 10). A study of the ability of different techniques to demonstrate gene expression in astrocytes showed that a multitude of astrocytic genes, including aralar, seem almost impossible to demonstrate by immunohistochemistry/*in situ* hybridization. Unfortunately, astrocytic gene expression is also occasionally missed by newer microarray studies. Another study evaluated data for GS expression (11), a key enzyme in glutamate/GABA synthesis (**Figure 1**) first shown immunohistochemically in Ref. (12). Anti-GS was concluded to be the most general astrocytic marker, covering all

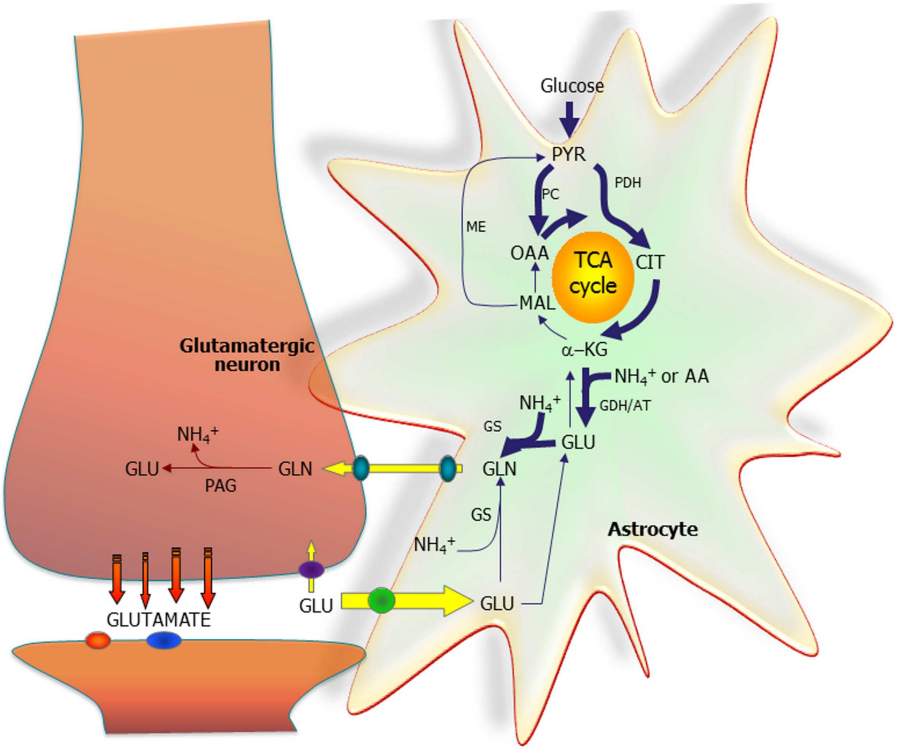


**FIGURE 1 | The astrocytic part of the synapse provides net synthesis of glutamine (GLN), via the concerted action of pyruvate carboxylase (PC) and pyruvate dehydrogenase (PDH), generating oxaloacetate (OAA) and acetyl-CoA, the combination of which leads to synthesis of citrate (CIT)**. This subsequently leads to a net synthesis of α-ketoglutarate (α-KG) allowing synthesis of glutamate (GLU), catalyzed by either glutamate dehydrogenase (GDH) or an amino acid aminotransferase (AA). GLU is used for synthesis of GLN catalyzed by glutamine synthetase (GS). GLN is transferred to the glutamatergic neuron to be used for synthesis of GLU catalyzed by phosphate-activated glutaminase (PAG). Released GLU is taken up into the astrocyte and transformed into GLN completing the GLU–GLN cycle. Alternatively, the GLU taken up may be oxidatively metabolized, which subsequently requires *de novo* synthesis of GLN via the anaplerotic processes indicated in bold arrows. Reproduced from Ref. (2), which together with other contributions discusses metabolic interactions in detail, even in the brain *in vivo*. However, the Figure also shows that $NH_4^+$ is required in astrocytes and released in neurons, and mechanisms transferring $NH_4^+/NH_3$ between the two cell types are discussed in other articles. So are the transporters releasing glutamine from astrocytes and accumulating it in neurons and the powerful transporters accumulating glutamate in astrocytes, as well as associations between glutamate uptake and metabolism. AT, aminotransferase; MAL, malate; ME, malic enzyme; PYR, pyruvate. Figure from Schousboe et al. (2).

astrocytic subtypes, and labeling astrocytic cells but no other cell types *in situ*, in culture or in tumors (11). In spite of several reports to the contrary, anti-GS does not label oligodendrocytes, emphasizing the difficulty of evaluation of cellular localization and the importance of cell-specific features for histological verification. Nevertheless, interactions between oligodendrocytes, astrocytes, and neurons are important for many aspects of brain function (13). It is essential to obtain more information about these basic metabolic interactions, which remain under-studied in spite of the importance of white matter disease. Vesicular release of glutamate occurs in white matter, cells of the oligodendrocytic lineage express glutamate receptors, and oligodendrocytic glutamate toxicity is co-implicated in hypoxic–ischemic, inflammatory, and traumatic brain damage (13). Involvement of astrocytes in white matter disease is also shown in tissue from patients having suffered from multiple sclerosis, through the absence of $\beta_1$-adrenergic receptor, and has potentially wide-ranging consequences (14). Moreover, a normal metabolic response to highly elevated $K^+$ concentrations is absent in cultured astrocytes from the convulsing Jimpy mice (15).

Glutamine exit from astrocytes and entry into neurons are of equal importance to glutamine synthesis for regulation of *de novo* synthesis of glutamate/GABA and for the return of released transmitter via astrocytes to neurons. The system N transporter SN1 resides on perisynaptic astroglial cell membranes and mediates electroneutral and bidirectional glutamine transport (16). Its activity is regulated at many levels, e.g., by extracellular pH, because protons compete with $Na^+$ required for its transport activity. There are consistent observations that SN1 is down-regulated by protein kinase C phosphorylation, probably by internalization (16). Secretion of insulin and glucagon from pancreatic islets resembles other endocrine secretions in their glutamate and GABA dependence, but an even closer resemblance with brain cells is revealed by expression of similar transport processes (17). Islet β- and α-cells contain high levels of glutamate, GABA, and glutamine and their respective vesicular and plasma membrane transporters,

which may play important roles in hormone maturation and secretion. Dependent upon secretion needs, glutamine may enter or leave β-cells via SN1 and be taken up by α-cells by SAT2, one of the SAT isoforms that accumulates glutamine in neurons (17).

Since both glutamate and GABA cycles require ammonia fixation in the astrocytic cytosol, and glutamine deamidation to glutamate in neurons, ammonia shortage occurs in the astrocytic, and ammonia excess in the neuronal cytosol (6, 18). This imbalance requires that excess ammonia in neurons either diffuses via the extracellular space to the astrocyte, probably as $NH_3$, or that it diffuses into mitochondria, becomes fixed to α-ketoglutarate, and forms glutamate, from which ammonia is returned to astrocytes through the aid of amino acid shuttles. Both Ref. (18), an advanced statistical computational model, and Ref. (6), discussing experimental observations, consider the requirement of this process for neuronal GDH to run in its reductive direction as evidence against its occurrence. This and Ref. (19) contradict a previously suggested major role of branched-chain amino acids or alanine shuttles. However, it is suggested that leucine, which enters the brain from the circulation, might supplement glutamine as an astrocytic–neuronal nitrogen carrier (18).

Glutamatergic and GABAergic activity is terminated by cellular uptake (20). The various transporters have different properties and different regulatory mechanisms, and some also act as ion channels. To understand the physiological roles of the individual transporter subtypes, their anatomical distribution must be known. Quantitative information about the expression is essential since functional capacity is determined by the number of transporter molecules. The most important and most abundant transporters for removal of transmitter glutamate in the brain are EAAT2 (GLT-1) and EAAT1 (GLAST), which both catalyze rapid uptake into astrocytes. GAT1 and GAT3 are the major GABA transporters in the brain, with GAT3 being astrocyte-specific.

Inhibition of GDH-mediated glutamate conversion to α-ketoglutarate with any of three inhibitors (epigallocatechin–monogallate, hexachlorophene, and bithionol) impedes glutamate uptake in the brain through cortical membranes expressing GLT-1 (21). This is consistent with this group's previous observations of anatomical and physical linkages between astrocytic glutamate transporters and mitochondria. The inhibitors had no effect in cerebellar membranes, where glutamate is accumulated by GLAST, but they did inhibit GABA uptake, suggesting that the GDH plays a role also in GABA metabolism. GABA enters the TCA cycle via succinate and succinic semialdehyde, but glutamate is required if the succinic semialdehyde formation occurs by transamination (3).

The high rate of glutamate uptake (20) together with the close association between glutamate uptake and metabolism (21) suggests that glutamate must be metabolized at high rates in astrocytes. This is convincingly shown in a review (22), pointing to several studies showing that glutamate uptake in astrocytes is more than high enough to meet the demand for its own energy-consuming uptake, and providing an excellent illustration of the metabolic processes in which ATP is generated. They include those involved in complete oxidation of malate via pyruvate recycling and the cytosolic enzyme malic enzyme (ME) (**Figure 1**). Both Ref. (21) and (22) assume that the initial conversion of glutamate to α-ketoglutarate is mediated by GDH, as always found with isolated cells. However, Balazs found that transaminase-dependent glutamate oxidation accounted for most, but not all, mitochondrial glutamate oxidation (23). Furthermore, in GDH knockout mice most functions remain unchanged (24), except for a reduced glutamate oxidation in cultured, and thus isolated astrocytes. Accordingly, more studies are needed of glutamate/α-ketoglutarate interconversion in intact preparations, a difficult undertaking.

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*Received: 06 February 2014; accepted: 19 March 2014; published online: 03 April 2014.*
*Citation: Hertz L and Rodrigues TB (2014) Astrocytic–neuronal–astrocytic pathway selection for formation and degradation of glutamate/GABA. Front. Endocrinol. **5**:42. doi: 10.3389/fendo.2014.00042*
*This article was submitted to Cellular Endocrinology, a section of the journal Frontiers in Endocrinology.*

frontiers in
ENDOCRINOLOGY

REVIEW ARTICLE
published: 15 August 2013
doi: 10.3389/fendo.2013.00103

# Glutamate and GABA in appetite regulation

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Appetite is regulated by a coordinated interplay between gut, adipose tissue, and brain. A primary site for the regulation of appetite is the hypothalamus where interaction between orexigenic neurons, expressing Neuropeptide Y/Agouti-related protein, and anorexigenic neurons, expressing Pro-opiomelanocortin cocaine/Amphetamine-related transcript, controls energy homeostasis. Within the hypothalamus, several peripheral signals have been shown to modulate the activity of these neurons, including the orexigenic peptide ghrelin and the anorexigenic hormones insulin and leptin. In addition to the accumulated knowledge on neuropeptide signaling, presence and function of amino acid neurotransmitters in key hypothalamic neurons brought a new light into appetite regulation. Therefore, the principal aim of this review will be to describe the current knowledge of the role of amino acid neurotransmitters in the mechanism of neuronal activation during appetite regulation and the associated neuronal-astrocytic metabolic coupling mechanisms. Glutamate and GABA dominate synaptic transmission in the hypothalamus and administration of their receptors agonists into hypothalamic nuclei stimulates feeding. By using $^{13}$C High-Resolution Magic Angle Spinning Nuclear Magnetic Resonance spectroscopy based analysis, the Cerdán group has shown that increased neuronal firing in mice hypothalamus, as triggered by appetite during the feeding-fasting paradigm, may stimulate the use of lactate as neuronal fuel leading to increased astrocytic glucose consumption and glycolysis. Moreover, fasted mice showed increased hypothalamic [2-$^{13}$C]GABA content, which may be explained by the existence of GABAergic neurons in key appetite regulation hypothalamic nuclei. Interestingly, increased [2-$^{13}$C]GABA concentration in the hypothalamus of fasted animals appears to result mainly from reduction in GABA metabolizing pathways, rather than increased GABA synthesis by augmented activity of the glutamate-glutamine-GABA cycle.

**Keywords: GABA, appetite, hypothalamus, NMR spectroscopy, glutamate**

## APPETITE REGULATION: FROM THE PERIPHERY TO THE HYPOTHALAMUS

Appetite is a highly regulated phenomenon, being hunger and satiety crucial factors in controlling food intake. Both food intake and energy expenditure disturbances lead to obesity, a pandemic syndrome frequently associated with the most prevalent and morbid pathologies in developed countries including heart disease, atherosclerosis, diabetes, and cancer (1). Appetite is closely regulated by a coordinated interplay between peripheral and central nervous system pathways. Two major groups of peripheral-derived signals inform the brain about the whole-body energy state: short-term signals produced by the gastrointestinal system and long-term signals produced by adipose tissue (**Figure 1**). There is a vast array of anorexigenic hormones causing loss of appetite secreted from the gut; these include: cholecystokinin (CCK) (2), glucagon-like peptide-1 (GLP-1) (3), peptide YY (PYY) (4), and oxyntomodulin (OXM) (5). Hormones derived from the pancreas, as pancreatic polypeptide (PP) (6), glucagon (7), insulin (8), and amylin (9), also exhibit anorexigenic actions. Finally, adipose tissue-derived anorexigenic signals, such as leptin (10), adiponectin (11), and resistin (12) have been described. On the other hand, gut-derived ghrelin is the only example of a peripheral hormone with orexigenic actions (13, 14), thereby increasing appetite upon its release usually before meals. In spite of intensive research during the last decades, other unidentified peripheral signals playing a role in appetite regulation probably exist. An increased knowledge on peripheral inputs controlling appetite could be relevant for the development of newly successful therapeutical approaches targeting obesity.

Studies employing either discrete lesions in the hypothalamus (15, 16) or surgical transection (17) of neural pathways have shown that central integration of peripheral-derived signals occurs mostly in the hypothalamus. The hypothalamus lies adjacent to three circumventricular organs, which are areas that permit substances to leave the brain without disrupting the blood-brain barrier (BBB), thereby permitting other substances that do not cross the BBB to exert their actions in the brain (18). In the last years, several neurotransmitters involved in hypothalamic appetite regulation have been identified [see, for example reviews (19–22)]. The cornerstone experiment for the identification of a potential neurotransmitter consists on the injection of the respective agent into the hypothalamus or adjacent ventricle of animal models and detection of a rapid increase or decrease in food intake. These experiments allowed not only the identification and characterization of several neurotransmitters involved in hypothalamic appetite regulation, but also to the precise tracking of pathways

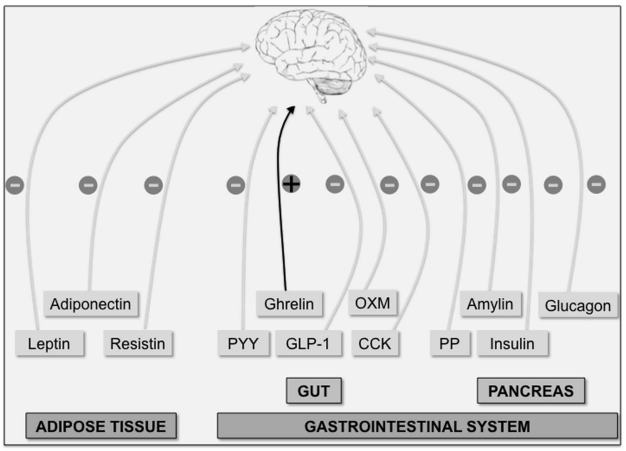


**FIGURE 1 | The brain integrates multiple peripheral signals to control appetite**. Peripheral factors indicative of long-term energy whole-body status are produced by adipose tissue (leptin, adiponectin, and resistin). On the other hand, acute orexigenic (+) ghrelin signal (produced in the gut) and anorexigenic (−) signals such as the gut hormones peptide YY (PYY), oxyntomodulin (OXM), glucagon-like peptide-1 (GLP-1) and cholecystokinin (CCK), and the pancreatic hormones [insulin, glucagon, amylin, and pancreatic polypeptide (PP)] indicate long-term energy status.

containing these signal molecules. Usually, neurotransmitters are classified into peptides, amino acids, and monoamines.

## HYPOTHALAMIC PEPTIDERGIC NEUROTRANSMISSION AND APPETITE REGULATION

In the arcuate nucleus of the hypothalamus, two sets of neuronal populations expressing either orexigenic neuropeptides [Neuropeptide Y (NPY) and Agouti-related peptide (AgRP)], or anorexigenic neuropeptides [Pro-opiomelanocortin (POMC) and Cocaine-amphetamine-related transcript (CART)] co-exist. Neuropeptide Y is synthesized in neurons situated in the far ventromedial aspect of the hypothalamic arcuate nucleus. Within the hypothalamus, NPY-expressing fibers project from the arcuate nucleus to the paraventricular nucleus, where the peptide is released (23). Thus, the administration of NPY to the hypothalamic paraventricular nucleus results in a robust and sustained increase of food intake in rodents (24), eventually leading to obesity when given repeatedly (25). On the other hand, antibody-mediated blockade of NPY action results in decreased food intake in starved animals (26). As neuronal populations expressing NPY are co-localized with AgRP-releasing neurons, the optogenetic (27) or pharmaco-genetic (28) stimulation of AgRP-expressing neurons also drives intense food intake whereas genetic ablation (29, 30) or pharmaco-genetic inhibition (28) decreases food consumption.

Neurons located mainly in the ventrolateral subdivision of the hypothalamic arcuate nucleus contain both the anorexigenic peptide CART and its precursor, POMC. The optogenetic stimulation of POMC-containing neurons reduces food intake (27) whereas genetic ablation of POMC-expressing cells (31, 32) increases appetite and food consumption. The gene encoding POMC gives rise to downstream peptide products, including melanocortins [adrenocorticotropic hormone (ACTH), the α-, β-, and γ-melanocyte-stimulating hormones (MSH) and β-endorphin (33). Release of the α-MSH peptide at the hypothalamic paraventricular nucleus potentially reduces food intake via activation of the melanocortin receptors, MC3R and MC4R. On the contrary, increased food intake and obesity are seen as a result of deletion of MC3R (34) and MC4R (35). In summary, activation of the POMC-expressing neurons in the hypothalamic arcuate nucleus triggers the release of α-MSH, which activates MC4R at the paraventricular nucleus, leading to suppressed food intake and increased energy expenditure. On the other hand, stimulation of AgRP-expressing neurons in the hypothalamic arcuate nucleus releases AgRP peptide, which antagonizes the effect of α-MSH on MC4R thereby increasing food intake.

To date, most effort has been placed on examining direct regulation of hypothalamic NPY/AgRP and POMC/CART-expressing neurons by various circulating factors whereas the role of upstream neural inputs has received comparatively less attention. This is surprising considering that both NPY/AgRP and POMC/CART neurons receive abundant excitatory and inhibitory synaptic input. The two neurotransmitters that account for most of the synaptic activity in the hypothalamus are the amino acids glutamate and γ-aminobutyric acid (GABA).

## HYPOTHALAMIC GLUTAMATERGIC NEUROTRANSMISSION

### NEURONAL-ASTROCYTIC GLUTAMATE METABOLISM

Glutamate is the dominant excitatory neurotransmitter in the central nervous system. In order for a neuron to release glutamate, the neurotransmitter must first be packed at high concentrations into synaptic vesicles, by means of specific vesicular glutamate transporters (VGLUT1, VGLUT2, and VGLUT3) (36). Upon stimulation, glutamate is released into the synaptic cleft to bind and elicit its effects on postsynaptic receptors, whether ionotropic [*N*-methyl-D-aspartate (NMDA), D,L-alpha-amino-3-hydroxy-5-methyl-isoxazole propionic acid (AMPA), kainic acid] or metabotropic receptors (mGluRs), present both in neurons and astrocytes.

Despite of its ubiquitous nature, extracellular glutamate levels are tightly regulated. The release of presynaptic glutamate largely exceeds the amount need for neurotransmission. As high glutamate concentrations could preclude further transmission or become associated with neurotoxicity events unless rapidly cleared, synaptically released glutamate is recycled from the extracellular space by means of excitatory amino acid transporters expressed predominantly on astrocytes (GLT-1 and GLAST). Within astrocytes, recycled glutamate can be metabolized to glutamine via glutamine synthetase or can be assimilated into the tricarboxylic acid (TCA) cycle. Glutamine released from astrocytes is further taken up again by neurons, where the mitochondrial phosphate-specific enzyme, glutaminase, reconverts inert glutamine-to-glutamate for subsequent repackaging into synaptic vesicles: the glutamate-glutamine cycle. Importantly, due to the lack of pyruvate carboxylase in neurons making them incapable of *de novo* synthesis of glutamate from glucose (37), the glutamate-glutamine cycle assures an adequate replenishment of glutamate in the central nervous system (38, 39). However, the glutamate-glutamine cycle faces a drain of compounds by oxidation (40–42), requiring a continuous replenishment of glutamate

and glutamine in astrocytes. *De novo* synthesis of glutamate and glutamine by astrocytes requires an amino group, being that aspartate has been recently suggested as the neuron-born nitrogen donor (43). According to the astrocyte to neuron lactate shuttle hypothesis (ANLSH) (44, 45), energy requirements for astrocyte-mediated glutamate recycling are derived exclusively from the glycolytic glucose metabolism with the concomitant lactate production by astrocytes, the latter becoming the main oxidative fuel for neurons (44, 46, 47).

### GLUTAMATE IN APPETITE REGULATION

The intracerebroventricular injection (48), as well as the lateral hypothalamic injection of glutamate, or its excitatory amino acid agonists, kainic acid, AMPA, and NMDA (49), rapidly elicit an intense food intake in rats. Likewise, intracerebroventricularly injected mGluR5 agonists stimulate feeding in rodents whereas the mGluR5 receptor antagonist (R,S)-2-chloro-5-hydroxyphenylglycine, inhibits food intake (50). Although the above-mentioned studies implicate glutamate signaling in eliciting a stimulation of food intake, until recently the morphological examination of the glutamatergic system was difficult due to the lack of marker molecules specific to glutamatergic neurons. Two highly homologous transmembrane proteins, VGLUT1 and VGLUT2, have been proven to be specific for glutamatergic neurons. On this basis, several studies have identified the presence of a dense plexus of glutamatergic fibers in key hypothalamic areas involved in appetite regulation. For example, elevated expression of mRNA encoding VGLUT2 was found in neurons located in the ventromedial hypothalamus and from the ventrolateral aspect of the arcuate nucleus (51, 52). On the other hand, expression of VGLUT1 is confined to relatively weak labeling in the lateral hypothalamic area (51). Furthermore, by using double-labeling immunohistochemistry, the presence of VGLUT2 immunoreactivity has been shown in appetite-regulating POMC/CART-expressing neurons located in the arcuate nucleus (53, 54), where they receive glutamatergic input from neurons in the ventromedial nucleus of the hypothalamus (55). In addition, Kiss et al. provided evidences for the existence of glutamatergic innervation of NPY-expressing neurons in the rat hypothalamic arcuate nucleus (54).

To evaluate the role of glutamatergic input to NPY/AgRP and POMC/CART-expressing neurons, and more specifically its plasticity as regulated by glutamate NMDA receptors, Liu et al. generated mice lacking NMDA receptors on either AgRP or POMC neurons (56). The authors found that NMDA receptors on AgRP neurons, but not on POMC-expressing neurons, play a critical role in controlling energy balance indicating that fasting-induced activation of AgRP-releasing neurons is associated with markedly increased glutamatergic input (56). Furthermore, through the combination of cell-type-specific electrophysiological, pharmacological, and optogenetic techniques, Yang et al. found that food deprivation elevates excitatory synaptic input. According to these authors, gut-derived ghrelin acts at presynaptic receptors to increase glutamate release and activate NPY/AgRP-expressing neurons through ionotropic glutamate receptors (57).

In the last decade, astrocytes were reported to participate in several neuroendocrine processes although only recently their importance in the control of appetite and energy homeostasis has been established. Astrocytes express receptors for numerous neuropeptides, neurotransmitters, and growth factors, produce neuroactive substances, and express key enzymes necessary for sensing and processing nutritional signals. For example, the anorexigenic hormone leptin is known to affect astrocyte morphology and synaptic protein levels in the hypothalamus (58). Thereby, the observed diet-induced increase in leptin receptor levels in hypothalamic astrocytes is proposed to participate in the onset of obesity. More recently, Fuente-Martín et al. have shown that leptin directly modulates glutamate uptake in astrocytes in a time-dependent manner, stimulating a rapid increase that is downregulated with chronic exposure (59). The initial rapid increase in astrocyte's glutamate captation indicates that leptin could reduce the stimulatory effects of glutamate at nearby synapses, thereby reducing appetite. In addition, when excess glutamate is released to the synaptic cleft, it is eventually recaptured by surrounding astrocytes, together with sodium ions, through the astrocytic glutamate cotransporter, GLAST. As a result, the intracellular sodium ions incorporated have to be extruded to the extracellular space, through the electrogenic $Na^+/K^+$ATPase and $Na^+K^+2Cl^-$ cotransporter, resulting in the intracellular incorporation of potassium ions. Increased intracellular potassium ions concentrations trigger an osmotically driven, aquaporin 4 (AQP4)-mediated, water transport culminating with astrocytic swelling (60). By using diffusion weighting imaging, Lizarbe et al. have recently shown significant increases in the slow diffusion parameters, consistent with astrocyte swelling response, in the hypothalamus of fasted relative to satiated animals (61, 62). On these grounds, we may hypothesize that, whereas an initial leptin-driven glutamate uptake in astrocytes shows anorexigenic potential (by diverting glutamate from neurons and thereby reducing glutamatergic neurotransmission), an excessive glutamate uptake by astrocytes, as occurs under orexigenic fasting conditions, causes astrocyte's swelling and eventual response by amino release to the synaptic cleft (63) (augmenting glutamatergic neurotransmission associated with appetite enhancement).

## HYPOTHALAMIC GABAERGIC NEUROTRANSMISSION

### NEURONAL-ASTROCYTIC GABAERGIC METABOLISM

γ-Aminobutyric acid (GABA) is the main inhibitory neurotransmitter in the central nervous system. The regulation of GABA itself is achieved by several specialized molecular mechanisms mediating transport, sequestration, synthesis, and GABA degradation. GABAergic neurons express both mature isoforms of glutamate decarboxylase, GAD65 and GAD67, to convert the excitatory amino acid glutamate into GABA (64). Moreover, glutamine can be used as an alternative source of GABA. As described in the earlier section, the amino acid glutamine has long been known as the immediate precursor for glutamate. There is increasing evidence for a similar role of this glutamate-glutamine cycle in GABA synthesis [see review (65)]. GABA clearance from the synaptic cleft is mediated by specific, high-affinity, sodium- and chloride-dependent transporters, GAT1, GAT2, and GAT3 and the vesicular GABA transporter (VGAT) (66). After release, GABA elicits a biphasic response via activation of two classes of membrane receptors; either ionotropic ($GABA_A$) or metabotropic ($GABA_B$) receptors. Finally, it is estimated that more than 90% of all GABA

in the mammalian central nervous system is degraded by transamination of GABA and α-ketoglutarate to succinic semialdehyde and glutamate in the mitochondria of astrocytes and neurons (67).

### GABA IN APPETITE REGULATION

A stimulatory role for GABA in the regulation of hypothalamic controlled feeding behavior has been evidenced in the last years. The intracerebroventricular administration of the $GABA_A$ receptor agonist, muscimol, stimulates feeding in satiated pigs, a response blockable by the specific $GABA_A$ receptor antagonist, bicuculline (68). Also, systemic and intracerebroventricular administration of the $GABA_B$ receptor agonist, baclofen, causes an increase in food intake in satiated pigs (69). Moreover, increased food intake obtained after administration of baclofen can be abolished by pretreatment with the $GABA_B$ receptor antagonist, phaclofen (69). In agreement, several evidences indicate that neurons in the hypothalamic arcuate nucleus express to a large extent the GABA transporter, VGAT (70, 71) as well as the GABA synthesizing enzymes, GAD65 and GAD67 (70). Using immunohistochemistry, GAD65/GAD67 and GABA immunoreactivities have been demonstrated in the majority of NPY/AgRP neurons located in the hypothalamic arcuate nucleus (70, 71). On the other hand, in spite of GAD65/GAD67 mRNA presence has been demonstrated in approximately one-third of POMC-expressing neurons (72), VGAT was not detected in hypothalamic POMC cell bodies (53) suggesting the absence of POMC GABA-releasing neurons.

To further understand the role of GABAergic neurons in appetite regulation, Tong et al. have shown that while both NPY and AgRP stimulate food intake when infused into the brain, the weight loss seen when AgRP-expressing cells are destroyed is recapitulated by targeted deletion of their ability to release GABA, rather than NPY or AgRP (73). Furthermore, the severe anorectic phenotype induced by the diphtheria-induced acute ablation of AgRP-expressing neurons in adult mice can be rescued with chronic infusion of a benzodiazepine, known to enhance GABA effect at the level of $GABA_A$ receptor (74). These evidences indicate that the synaptic release of GABA by AgRP-expressing neurons in the hypothalamic arcuate nucleus is required for normal regulation of energy balance. Wu et al. further explored the role of the GABAergic outputs of AgRP-expressing neurons. These authors found that in adult mice lacking AgRP-expressing neurons, pharmacological stimulation of $GABA_A$ receptors in the parabrachial nucleus, by means of local injection of bretazenil (a partial $GABA_A$ receptor agonist) is sufficient to maintain feeding. Wu and colleagues further corroborate these findings by examining the effects of either infusing a GABA antagonist directly into the parabrachial nucleus or selectively ablating AgRP inputs to this area. Both experiments induced a progressive decrease in food intake in mice, indicating that GABAergic inputs from arcuate nucleus AgRP-expressing neurons to the parabrachial nucleus are required to maintain a critical level of appetite stimulus (75). These observations clearly represent a potential shift away from early explanations of energy metabolism regulation, where GABA was thought to facilitate the feeding effect of NPY at target sites in the paraventricular nucleus by blocking opposing POMC transmission (76–78).

## GLUTAMATE AND GABA ACTIONS ON NEURONAL-ASTROCYTIC METABOLIC COUPLING MECHANISM UNDERLYING HYPOTHALAMIC APPETITE REGULATION

To date, glutamate and GABA actions on neuronal-astrocytic metabolic coupling mechanism underlying hypothalamic appetite regulation have been largely unexplored mainly due to the absence of appropriate *in vivo* methodological approaches. Earlier, a variety of *in vivo* Magnetic Resonance Imaging (MRI) and Magnetic Resonance Spectroscopy (MRS) methods have been shown to provide comprehensive information on cerebral activation and the underlying metabolic coupling mechanisms operating between neurons and astrocytes. However, the relatively large voxel size used in the acquisition of *in vivo* $^{13}C$ Magnetic Resonance spectra precludes its use for studying the relatively reduced appetite controlling hypothalamic area of small rodents. Alternatively, High-Resolution $^{13}C$ Nuclear Magnetic Resonance (NMR) spectroscopy investigations of the cerebral metabolism of tracers such as [1-$^{13}C$]glucose or [2-$^{13}C$]acetate contributed comprehensive information on the operation of the neuronal and astrocyte TCA cycles and the transcellular exchanges of glutamate–glutamine or GABA between neurons and astrocytes of the whole brain [see for example (79–82)].

Nevertheless, the relatively large amounts of cerebral tissue needed to prepare brain extracts for high-resolution $^{13}C$ NMR spectroscopy constitutes an important limitation. To overcome the above-mentioned limitations, High-Resolution Magic Angle Spinning (HR-MAS) NMR spectroscopy, a technique yielding high quality spectra from very small tissue biopsies (5–10 mg, a size comparable to the size of the mice brain hypothalamus) was suggested to improve the spatial resolution and to investigate directly hypothalamic metabolism. Whereas $^{1}H$ HR-MAS NMR has been used for metabolic profiling of normal and diseased tissues (83), $^{13}C$ HR-MAS NMR spectroscopy offers the additional advantage of providing information on the operation of the metabolic pathways.

Recently, Violante et al. used $^{13}C$ HR-MAS NMR spectroscopy analysis of mice hypothalamic biopsies, after [1-$^{13}C$]glucose injection, to better understand the mechanisms underlying neurotransmission events and the associated neuronal-astrocytic metabolic coupling mechanisms underlying hypothalamic appetite regulation (84). Following [1-$^{13}C$]glucose injection, glycolytic and TCA cycle intermediates are labeled distinctively, providing information on the relative contribution of the corresponding metabolic pathways. Initially [1-$^{13}C$]glucose is metabolized to [3-$^{13}C$]pyruvate via glycolysis. Labeled pyruvate is then reduced to [3-$^{13}C$]lactate by lactate dehydrogenase, or alternatively enters the TCA cycle, producing [4-$^{13}C$]glutamate and [4-$^{13}C$]glutamine. Moreover [4-$^{13}C$]glutamate can be converted to [2-$^{13}C$]GABA. On this basis, the authors have shown that appetite stimulation, during the feeding-fasting paradigm, increases significantly the $^{13}C$ incorporation in lactate carbons (84). Augmented lactate labeling most probably indicates a relatively increased glycolytic activity. Therefore, increased neuronal firing in the hypothalamus triggered by fasting may stimulate the use of lactate as neuronal fuel leading to increased astrocytic glucose consumption

and glycolysis. Moreover, fasted mice show increased hypothalamic [2-$^{13}$C]GABA content (84) most probably attributable to the existence of GABAergic neurons in key appetite regulation hypothalamic nuclei (70). Increased [2-$^{13}$C]GABA concentrations may be derived either from increased net synthesis or reduced net degradation. Potential increases in GABA synthesis involve an augmented activity of the glutamate-glutamine-GABA cycle, since glutamate and glutamine are considered the main precursors of GABA. Interestingly, despite the increase in the [2-$^{13}$C]GABA enrichment no increase was detected in [4-$^{13}$C]glutamate or [4-$^{13}$C]glutamine. Thus the increased [2-$^{13}$C]GABA concentration in the hypothalamus of fasted animals appears to result mainly from reduction in GABA metabolizing pathways, rather than increased GABA synthesis by augmented activity of the glutamate-glutamine-GABA cycle.

Using a similar methodology, we have recently studied the neuronal-astrocytic metabolic coupling mechanism underlying hypothalamic appetite stimulation in hyperphagic leptin-deficient *ob/ob* mice. After a meal, leptin is released from adipose tissue to bind to the hypothalamic leptin receptor inducing an anorexigenic response consisting of a reduction in food intake and an increase in energy expenditure. On the contrary, in fasting periods, decreased plasma levels of leptin promote increased food intake and diminished energy consumption (85). Disruptions in the leptin signaling systems are often associated with hyperphagia and consequently obesity. In the leptin-deficient *ob/ob* mice, hypothalamic leptin signaling is drastically reduced and hyperphagia develops leading to obesity. We have showed that leptin deficiency in *ob/ob* mice resulted in significantly increased $^{13}$C incorporation from [1-$^{13}$C]glucose in glutamate and glutamine carbons of hypothalamic biopsies suggesting that leptin-dependent hypothalamic activation, contrary to fasting-induced appetite stimulation, involves mainly increases in neuronal oxidation and glutamatergic neurotransmission together with elevated glutamate-glutamine cycling (86). **Figure 2** provides an illustration on the use of $^{13}$C HR-MAS NMR spectroscopy to investigate appetite regulation in small hypothalamic areas during cerebral activation by different feeding activation paradigms. Unlike sensorial or motor paradigms [see for example (87–90)], where only glutamatergic or GABAergic terminals are involved in a simple activation/inhibition mechanisms, both glutamatergic and GABAergic stimulations on different neuronal populations may eventually lead to the dominant orexigenic or anorexigenic response, depending of their relative contributions. Most probably, the observed presence of both glutamatergic and GABAergic neurotransmission in association with different feeding activation paradigms may reflect the existence of complex feedback loops on the neuroendocrine regulation underlying appetite regulation. These feedback loops are crucial homeostatic mechanisms for the hypothalamic neuroendocrine regulation involving the operation of both peripheral signals and intrahypothalamic neurotransmitters.

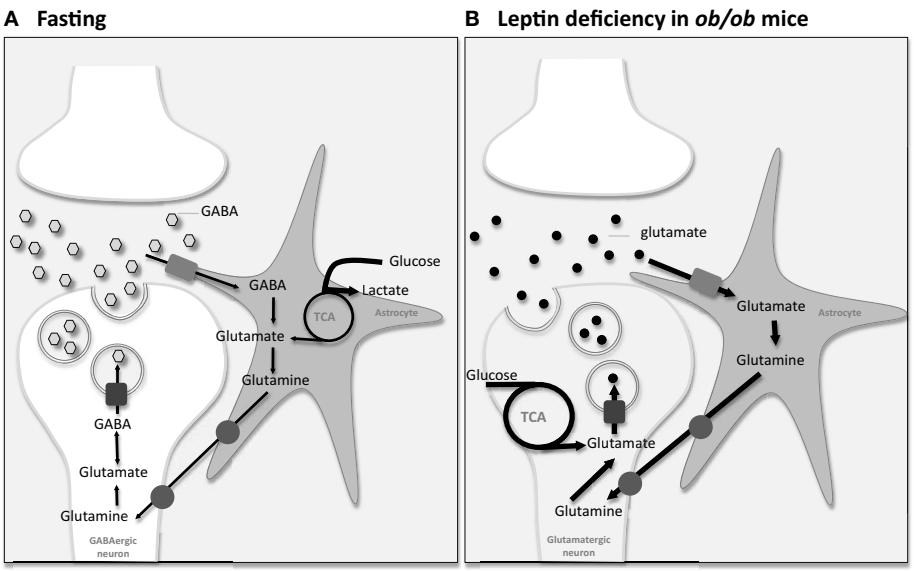


**FIGURE 2 | Information on the integrated neuronal-astrocytic metabolic coupling mechanisms underlying appetite regulation can be investigated using [1-$^{13}$C]glucose injection followed by analysis by $^{13}$C High-Resolution Magic Angle Spinning (HR-MAS) Nuclear Magnetic Resonance (NMR) spectroscopy analysis of mice hypothalamus biopsies**. **(A)** Fasting-induced changes: Violante et al. showed that increased neuronal firing in the hypothalamus triggered by fasting may stimulate the use of lactate as neuronal fuel leading to increased astrocytic glucose consumption and glycolysis (dark bold arrows). Moreover, fasted mice showed increased hypothalamic [2-$^{13}$C]GABA content most probably attributable to the existence of GABAergic neurons in the hypothalamus. Despite elevated [2-$^{13}$C]GABA, no increase was detected in the main precursors of GABA, glutamate, and glutamine, suggesting a reduction in GABA metabolizing pathways rather than increased GABA synthesis by augmented activity of the glutamate-glutamine-GABA cycle (84). **(B)** Leptin-deficiency-induced changes: we have shown that leptin deficiency in hyperphagic *ob/ob* mice resulted in significantly increased $^{13}$C incorporation from [1-$^{13}$C]glucose in glutamate and glutamine carbons of hypothalamic biopsies suggesting that leptin-dependent appetite activation involves mainly increases on neuronal oxidation and glutamatergic neurotransmission together with elevated glutamate-glutamine cycling (dark bold arrows) (86).

## CONCLUDING REMARKS

Although to date most effort has been placed on examining direct regulation of hypothalamic appetite by neuropeptide-expressing neurons, it is evident that hypothalamic neurons further release and respond to excitatory and inhibitory amino acid neurotransmitters, as glutamate and GABA. Neuropeptides and amino acids neurotransmitters may both function as independent transmitters, or alternatively, neuropeptides may work by modulating the actions of glutamate and GABA and vice-versa. Herein, current knowledge on neuronal-astrocytic interactions underlying glutamate- and GABA-dependent hypothalamic appetite stimulation was reviewed. Apparently, different feeding paradigms associated with appetite stimulation account for different responses in neuronal-astrocytic metabolic coupling mechanisms. Whereas the fasting state is associated both with the use of lactate as neuronal fuel leading to increased astrocytic glucose consumption and with augmented hypothalamic GABAergic neurotransmission, elevated neuronal oxidation, and glutamatergic neurotransmission with increased glutamate-glutamine cycling are hallmarks of leptin deficiency in hyperphagic rodents. Further information on the neuronal-astrocytic metabolic pathways underlying appetite stimulation during different feeding paradigms, which can be achieved by the combined use of $^{13}$C HR-MAS NMR spectroscopy and metabolic tracers, may be a valuable tool for finding novel anti-obesity central-based therapeutical targets.

## ACKNOWLEDGMENTS

TCD holds a post-doctoral fellowship from the Fundação para a Ciência e Tecnologia, Portugal (SFRH/BPD/46197/2008).

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**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

*Received: 31 May 2013; accepted: 01 August 2013; published online: 15 August 2013.*
*Citation: Delgado TC (2013) Glutamate and GABA in appetite regulation. Front. Endocrinol. **4**:103. doi: 10.3389/fendo.2013.00103*
*This article was submitted to Frontiers in Cellular Endocrinology, a specialty of Frontiers in Endocrinology.*

frontiers in
ENDOCRINOLOGY

REVIEW ARTICLE
published: 15 August 2013
doi: 10.3389/fendo.2013.00102

# Astrocytic control of biosynthesis and turnover of the neurotransmitters glutamate and GABA

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Glutamate and GABA are the quantitatively major neurotransmitters in the brain mediating excitatory and inhibitory signaling, respectively. These amino acids are metabolically interrelated and at the same time they are tightly coupled to the intermediary metabolism including energy homeostasis. Astrocytes play a pivotal role in the maintenance of the neurotransmitter pools of glutamate and GABA since only these cells express pyruvate carboxylase, the enzyme required for *de novo* synthesis of the two amino acids. Such *de novo* synthesis is obligatory to compensate for catabolism of glutamate and GABA related to oxidative metabolism when the amino acids are used as energy substrates. This, in turn, is influenced by the extent to which the cycling of the amino acids between neurons and astrocytes may occur. This cycling is brought about by the glutamate/GABA – glutamine cycle the operation of which involves the enzymes glutamine synthetase (GS) and phosphate-activated glutaminase together with the plasma membrane transporters for glutamate, GABA, and glutamine. The distribution of these proteins between neurons and astrocytes determines the efficacy of the cycle and it is of particular importance that GS is exclusively expressed in astrocytes. It should be kept in mind that the operation of the cycle is associated with movement of ammonia nitrogen between the two cell types and different mechanisms which can mediate this have been proposed. This review is intended to delineate the above mentioned processes and to discuss quantitatively their relative importance in the homeostatic mechanisms responsible for the maintenance of optimal conditions for the respective neurotransmission processes to operate.

**Keywords: glutamate, GABA, astrocyte, neurotransmitter, homeostasis, energy**

## INTRODUCTION

### HISTORICAL PERSPECTIVE OF AMINO ACID NEUROTRANSMISSION: GLUTAMATE AND GABA

The general concept in biochemistry that glutamate is a key metabolite linking amino acid and glucose metabolism via the tricarboxylic acid (TCA) cycle was extended when Eugene Roberts discovered GABA in the brain. They showed that it was synthesized from glutamate by decarboxylation of the C-1 carboxylic group thereby adding an important functional property to glutamate (1). Further studies of glutamate and GABA metabolism led to the biochemical term "the GABA shunt" (2) which interconnects the TCA cycle with glutamate and GABA metabolism (**Figure 1**). While the basic biochemical metabolic pathways (see below) involving glutamate and GABA metabolism were worked out from 1950 and onward it was not until the end of this decade that pioneering electrophysiological studies of spinal cord neurons led to the understanding that glutamate and GABA act as excitatory and inhibitory substances (3, 4). Due to the fact that these amino acids were present in much higher concentrations than the classical neurotransmitters acetylcholine and the monoamines it took several years until it was generally accepted that these two amino acids are not just metabolites but they constitute the quantitatively most important neurotransmitters in the central nervous system [see reviews by Ref. (5) and (6)].

## CONTENT

### BIOSYNTHETIC PATHWAYS FOR TRANSMITTER GLUTAMATE AND GABA

#### *Glutamate*

It is commonly accepted that the enzyme catalyzing the hydrolysis of glutamine to glutamate, glutaminase is the functionally most important enzyme for the biosynthesis of glutamate [see Ref. (7)] and since this enzyme is activated by phosphate it is routinely referred to as phosphate-activated glutaminase (PAG). This enzyme was first described by Krebs (8) in an extensive study of glutamine degradation and synthesis in different tissues including the brain. The expression of PAG is higher in neurons than in astrocytes (9, 10) but PAG is not specifically associated with glutamatergic neurons as GABA synthesis in GABAergic neurons is dependent upon glutamate derived from glutamine taken up from surrounding astrocytes (see below).

There are actually alternative enzymatic reactions which are able to produce glutamate from the TCA cycle intermediate α-ketoglutarate. These are glutamate dehydrogenase (GDH) and the aminotransferases. Among the latter aspartate aminotransferase (AAT), alanine-aminotransferase (ALAT), and the branched chain aminotransferase (BCAAT) are the most likely candidates to be involved in glutamate biosynthesis [for reactions and references, see Ref. (11)]. While the aminotransferase reactions would

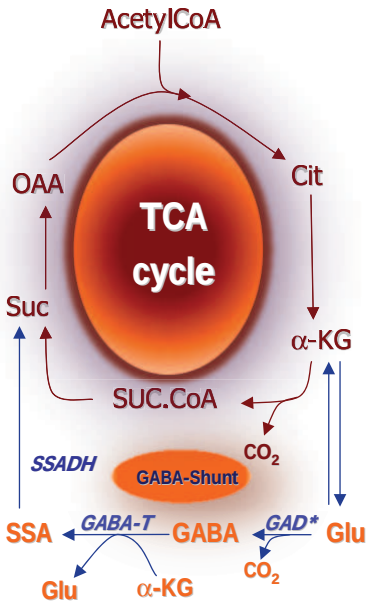


**FIGURE 1 | Schematic representation of the TCA cycle and the GABA shunt which provides an alternative pathway for conversion of αKG to succinate**. (*) Please note that glutamate decarboxylase is localized in the cytosol whereas all other enzymes depicted are mitochondrial. αKG, α-ketoglutarate; SSADH, succinate-semialdehyde; GABA-T, GABA-transaminase; GABA, γ-aminobutyrate; GAD, glutamate decarboxylase; Glu, glutamate.

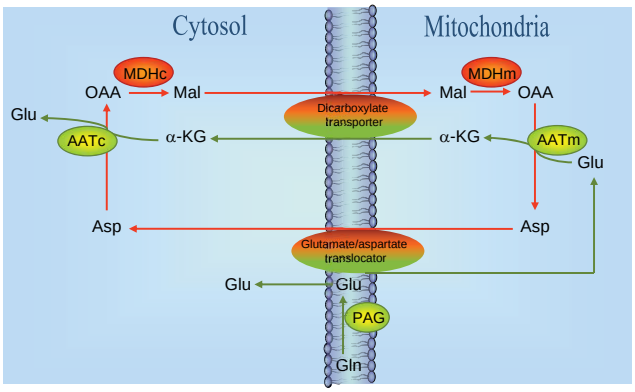


**FIGURE 2 | Schematic drawing of the reactions involved in the biosynthesis of neurotransmitter glutamate**. Reactions involve the elements of the malate-aspartate-shuttle (43), i.e., the glutamate/aspartate translocator and the dicarboxylate transporter as indicated. Notice that a significant fraction (about 50%) of glutamate derived from the reaction catalyzed by phosphate-activated glutaminase (PAG) is directly entering mitochondria presumably via the glutamate/aspartate translocater. It has also been suggested that PAG is catalytically active in the mitochondrial matrix (23, 24) although this is controversial [see text and Ref. (22)]. MDHc, cytosolic malate dehydrogenase; MDHm, mitochondrial malate dehydrogenase; AATc, cytosolic aspartate aminotransferase; AATm, mitochondrial aspartate aminotransferase; OAA, oxaloacetate; Mal, malate; Glu, glutamate; Asp, aspartate; GLN, glutamine; α-KG, α-ketoglutarate.

normally be in thermodynamic equilibrium the GDH reaction in the brain is unlikely to be at thermodynamic equilibrium due to low levels of ammonia and NADH/NADPH (12). The $K_m$ for ammonia in the GDH-catalyzed reaction is in the millimolar range, i.e., well above the level of ammonia found in the brain; however, in a mitochondrial microenvironment close to the PAG reaction in glutamatergic neurons, the level of ammonia might be high enough for the reaction to proceed in the direction of reductive amination as initially suggested by Waagepetersen et al. (13) and discussed by Bak et al. (14). In the liver, however, where the substrate conditions are different the GDH reaction may well be at thermodynamic equilibrium (15). As pointed out by Cooper (12) the GDH-catalyzed reaction is often coupled to the aminotransferases, which in the brain would result in ammonia being produced from the amino group in the amino acids since the GDH reaction would preferentially catalyze oxidative deamination of glutamate. This aspect will be discussed in more detail below.

Based on experiments performed in cultured glutamatergic cerebellar granule cells using phenylsuccinate to inhibit the ketodicarboxylate carrier in the mitochondrial membrane (16) a model was proposed according to which (**Figure 2**) neurotransmitter glutamate is generated by the concerted action of glutaminase and the malate-aspartate-shuttle (MAS). According to this model glutamate formed by the action of PAG located in the mitochondrial membrane (17) gets access to the mitochondrial matrix where it is transaminated by AAT to form α-ketoglutarate which is translocated to the cytoplasm where it undergoes a second transamination via AAT to produce glutamate. That this mechanism is indeed working is supported by the demonstration that while oxidation of the carbon skeleton of glutamate in the TCA cycle is dependent on activity of GDH (18), the formation of glutamate from α-ketoglutarate is catalyzed by an aminotransferase which is most likely mitochondrial AAT (19). This glutamate is subsequently used for vesicular release (16). The translocation processes require the operation of the aspartate-malate shuttle (see **Figure 2**). In keeping with this the release of transmitter glutamate could be inhibited not only by phenylsuccinate that inhibits the dicarboxylate carrier (20) but also by aminooxyacetic acid that inhibits AAT (20). It has been argued that glutamate generated in the PAG catalyzed hydrolysis of glutamine is released from the mitochondrial membrane into the cytosol and not into the matrix [see **Figure 2**; (17, 21, 22)] but experiments using [$^{13}$C]glutamine and histamine to inhibit glutamine transport into the mitochondria have provided evidence that glutamate generated in the PAG catalyzed reaction does indeed get access to the mitochondrial matrix (23, 24).

### *GABA*

While no single enzyme can be used as a specific marker for glutamatergic neurons (see above), the demonstration by Saito et al. (25) using an antibody which specifically recognizes the GABA synthesizing enzyme glutamate decarboxylase (GAD) that GAD is associated selectively with GABAergic neurons has led to the notion that GAD is a selective marker for GABAergic neurons. The subsequent finding of GAD-like immunoreactivity in certain glutamatergic neurons in the hippocampus (26, 27) may, however,

indicate a more complex distribution of GAD the functional implications of which may not be easy to understand. It should be noted in this context that GABA not only acts as transmitter but also functions as a neurotrophic agent [see Ref. (28), and references herein]. Cloning studies have revealed that isoenzymes of GAD exist since two cytosolic forms of GAD with molecular weights of 65 and 67 kDa, respectively have been described [see Ref. (29) for review]. The general notion is that the intracellular distribution of the two isozymes is heterogeneous, since $GAD_{65}$ is selectively associated with GABAergic nerve endings and therefore plays an important regulatory role in synthesis of neurotransmitter GABA (30). This is compatible with the observation that $GAD_{65}$ knock-out ($GAD_{65}{}^{-/-}$) animals are susceptible to induced seizures (31, 32). $GAD_{67}$ is found throughout the cytosol of these neurons and therefore may serve as the biosynthetic enzyme for the GABA pool associated with metabolism [i.e., consistent with a housekeeping function; see Ref. (33)]. It may be noted that $GAD_{67}$ knock-outs ($GAD_{67}{}^{-/-}$) are lethal while heterozygotes are viable and have no seizure sensitivity contrary to the $GAD_{65}$ knock-outs (31, 34).

As illustrated in **Figure 3** the biosynthesis of neurotransmitter GABA may be somewhat more complex than originally thought of as studies of GABA synthesis from [$^{13}$C]glutamine have revealed that the synthesis involves mitochondrial TCA cycle metabolism (35, 36). As pointed out above hydrolysis of glutamine by PAG leads to generation of glutamate in the mitochondrial matrix and this glutamate needs to be transaminated to form α-ketoglutarate in order for the carbon skeleton to leave the mitochondria (see **Figure 2**). Since GAD is a cytosolic enzyme (37) the decarboxylation of glutamate to form GABA can take place only in the cytosol. As a result of the fact that at least some of the glutamate generated in the PAG reaction may be released directly into the cytosol (17) GABA can be formed either directly from glutamate in the cytosol or from glutamate originating from α-ketoglutarate produced in the TCA cycle from oxidatively deaminated glutamate involving GDH. These two pathways seem to be almost equally important for biosynthesis of transmitter GABA (35, 36). The functional significance of the mitochondrial involvement in GABA biosynthesis is not well understood but it may provide additional possibilities for regulatory mechanisms to operate.

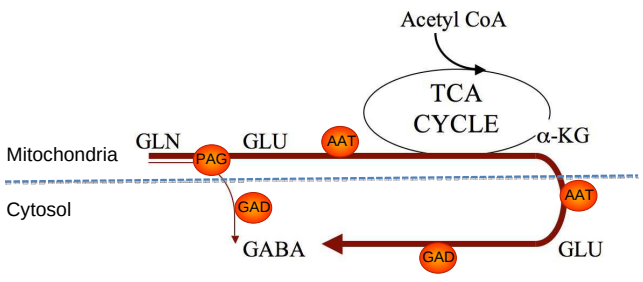


**FIGURE 3 | An illustration of two pathways for GABA synthesis from glutamine**. The bold arrow illustrates the predominant pathway where the carbon skeleton of glutamine is metabolized via the TCA cycle prior to synthesis of GABA. The thin arrow illustrates the direct synthesis of GABA from glutamine without involvement of the TCA cycle. Note that AAT is present in both the cytosol and the mitochondrial matrix. αKG, α-ketoglutarate; PAG, phosphate-activated glutaminase; ATT, aspartate aminotransferase; GAD, glutamate decarboxylase.

## METABOLIC PATHWAYS FOR DEGRADATION OF GLUTAMATE AND GABA

While the primary event responsible for inactivation of the neurotransmitter action of glutamate and GABA is related to the high-affinity transporters for these amino acids, the subsequent intracellular metabolic pathways for degradation of glutamate and GABA are of functional significance [Ref. (38) and references herein]. A discussion of the transporters will follow below.

The enzymes of interest are glutamine synthetase (GS), GDH, and aminotransferases (or transaminases) in relation to glutamate. In case of GABA the pertinent enzymes are GABA-transaminase (GABA-T) and succinic semialdehyde dehydrogenase (SSADH). GS is a cytosolic enzyme while GDH, SSADH, and GABA-T are located in the mitochondria [Ref. (34) and references herein]. Except for GS which is strictly astroglial (39) these enzymes are expressed both in neurons and astrocytes. Transgenic or knock out animals of these enzymes (GS, GDH, and SSADH) have provided evidence that they are of significant, functional importance for the maintenance of homeostatic mechanisms related to the cellular contents as well as to the neurotransmitter function. Thus, GDH knock outs exhibit impaired astrocytic glutamate oxidation and increased glutamine synthesis pointing to a more prominent cycling of glutamate which may be compatible with the observation that synaptic glutamatergic function remained intact (40). On the other hand, GS homozygote knock outs are lethal and heterozygotes exhibit increased susceptibility to seizures (41). $SSADH^{-/-}$ animals exhibit altered neurotransmitter GABA metabolism and increased seizure activity which may result in lethal status epilepticus [see Ref. (34)].

## ROLE OF ASTROCYTES PROVIDING GLUTAMINE, THE PRECURSOR FOR GLUTAMATE AND GABA

Glutamine serves a very important role in glutamatergic and GABAergic neurotransmission, as it constitutes the only precursor for the biosynthesis of these two amino acid neurotransmitters (see above). Due to the fact that GS as stated above is exclusively expressed in astrocytes these cells play a unique role in supplying the precursor for the transmitter in both glutamatergic and GABAergic neurons [see Ref. (38) and references therein]. This requires a symbiotic relationship between the neuronal and astrocytic compartments since an exchange of glutamine and the transmitter amino acids is necessary. This is illustrated in **Figure 4** which is a schematic representation of the glutamate/GABA-glutamine cycle operating between the nerve endings of glutamatergic or GABAergic neurons and surrounding astrocytes. This cycle was originally thought to operate in a stoichiometric fashion maintaining the carbon skeleton of the transmitter amino acids intact (42). However, the demonstration of a significant oxidative metabolism of both glutamate and GABA via the TCA cycle [see Ref. (43)] makes it impossible for this cycle to operate in a stoichiometric way as at least part of the glutamate and GABA skeleton will be lost as carbon dioxide. It is therefore imperative that such a loss is compensated for by *de novo* synthesis of the carbon skeleton of glutamate and subsequently glutamine. As ultimately the precursor

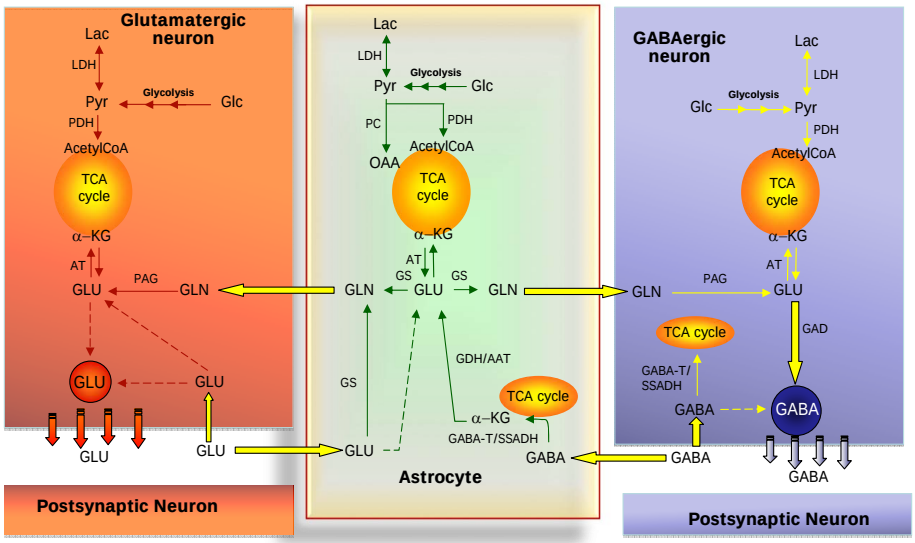


**FIGURE 4 | Schematic cartoon depicting the metabolic interactions between a glutamatergic neuron, a GABAergic neuron, and a nearby astrocyte**. In all three cell types, glucose (Glc) is metabolized to pyruvate via the multi-step process of glycolysis and either reduced to lactate (by lactate dehydrogenase, LDH) or oxidized to acetyl-CoA (by pyruvate dehydrogenase complex, PDH) which will subsequently be oxidized in the TCA cycle. In astrocytes, pyruvate may also undergo carboxylation to form oxaloacetate (OAA), an anaplerotic reaction catalyzed by pyruvate carboxylase (PC). At the glutamatergic synapse, glutamate (GLU) released as neurotransmitter will be taken up by nearby astrocytes and amidated to glutamine (GLN) by glutamine synthetase (GS) and returned to the neuron for re-use as neurotransmitter, the so-called glutamate-glutamine cycle. In neurons, GLU is re-formed from GLN by the mitochondrial enzyme phosphate-activated glutaminase (PAG). A similar cycle exists at the GABAergic synapse; however, the carbon skeleton of GABA enters the TCA cycle as indicated. GABA is transformed to the TCA cycle intermediate succinate via two reactions catalyzed by GABA-transaminase (GABA-T) and succinic semialdehyde dehydrogenase (SSADH). The GABA-T catalyzed reaction produces GLU from α-KG that may then be used as precursor for GLN synthesis and eventual synthesis of GABA in the neuron. This process is known as the GABA shunt since it by-passes two reactions of the (astrocytic) TCA cycle. In GABAergic neurons, GABA is synthesized from GLU by the enzyme glutamate decarboxylase (GAD). Notice that in all three cell types, GLU is in transamination equilibrium (catalyzed by aminotransferases, AT) with α-KG linking TCA cycle metabolism with GLU and GABA homeostasis. Also notice that re-uptake of released GLU and GABA takes place to some extent as well and that the GABA shunt works in GABAergic neurons when GABA is taken up pre-synaptically.

must originate from the TCA cycle in the form of an intermediary which most likely is α-ketoglutarate (**Figure 5**) an anaplerotic mechanism must be operating involving *de novo* synthesis of oxaloacetate. In the brain this is most likely catalyzed by pyruvate carboxylase (PC) which has a higher activity than the other enzymes capable of synthesizing oxaloacetate, i.e., malic enzyme (ME) and phosphoenolpyruvate carboxykinase (44). Interestingly, PC is like GS an astrocytic enzyme (45, 46) and accordingly *de novo* synthesis of the carbon skeleton of glutamate and GABA via glutamine requires astrocytic participation [see Ref. (43, 47)]. It has been estimated that this anaplerotic, *de novo* synthesis of glutamine may account for replenishment of approximately 25–30% of the glutamate transmitter pool [see Ref. (43)]. It would be assumed, that this would be associated with a comparable complete oxidation of glutamate to carbon dioxide, a process requiring pyruvate recycling (**Figure 6**). It may therefore seem somewhat enigmatic that the activity of pyruvate recycling in the brain as well as in cultured brain cells appears relatively modest (43).

## CELLULAR DISTRIBUTION OF GLUTAMINE TRANSPORTERS

As a consequence of the astrocytic localization of *de novo* synthesis of glutamine, a mechanism must exist which can mediate transport of glutamine out of the astrocytes and into glutamatergic and GABAergic neurons, respectively. This mechanism consists of a unique set of glutamine membrane transporters located either on astrocytes or the two sets of neurons. These transporters are referred to as System A transporters in neurons and System N transporters in astrocytes (14, 48). The System A family includes SNAT1, 2, and 4 and System N consists of SNAT3 and 5 (14). Each of these cloned transporters have alternative names making the nomenclature confusing [for a comprehensive review, see Ref. 38)]. These transporters are regulated in complex ways with regard to expression and kinetics underlining the functional importance of the transporters (38).

## ROLE OF GLUTAMATE TRANSPORTERS IN MAINTENANCE OF THE GLUTAMATE NEUROTRANSMITTER POOL, I.E., UPTAKE OF GLUTAMATE IN GLUTAMATERGIC NEURONS VERSUS ASTROCYTES

Detailed studies of the cellular localization of the most abundantly expressed glutamate transporters, GLAST (EAAT-1) and Glt-1 (EAAT-2) have provided compelling evidence that these glutamate transporters are expressed predominately in astrocytes (5, 49, 50). In addition to these astrocytic glutamate transporters, a neuronal counterpart (EAAC-1 or EAAT-3) has been cloned (51) and it has been proposed that if located in the plasma membrane of neurons, this transporter could participate in the maintenance of the pool of transmitter glutamate and GABA (52–54). However, a recent detailed study of the expression of EAAC-1 relative to that

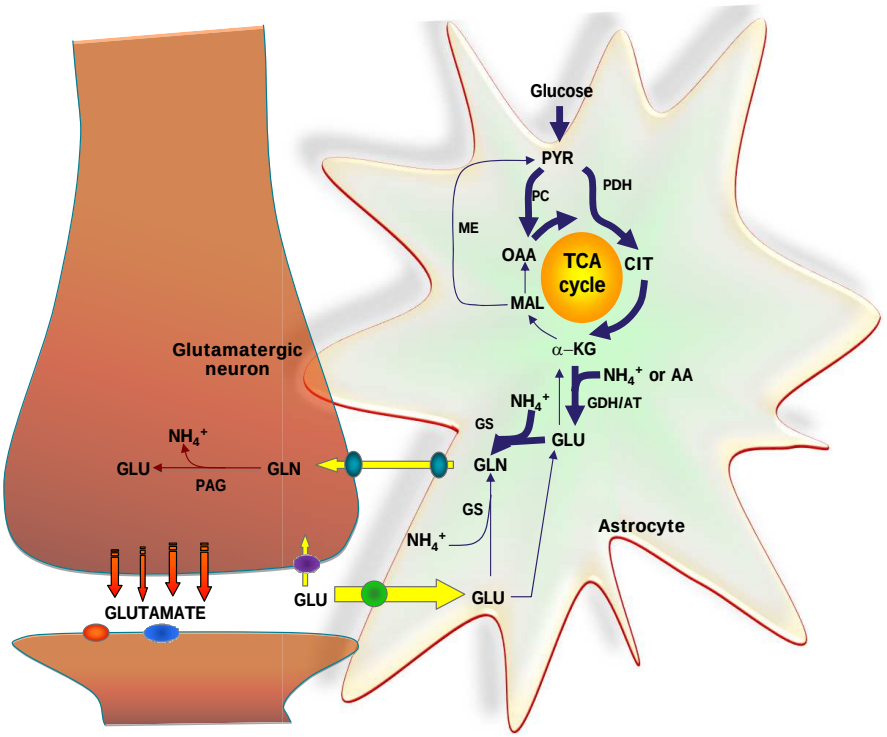


**FIGURE 5 | The astrocytic part of the synapse provides a net synthesis of glutamine (GLN) via the concerted action of pyruvate carboxylase (PC) and pyruvate dehydrogenase (PDH) generating OAA and acetyl-CoA, the combination of which leads to synthesis of CIT**. This subsequently leads to a net synthesis of α-ketoglutarate (α-KG) allowing synthesis of glutamate (GLU) catalyzed by either glutamate dehydrogenase (GDH) or an amino acid aminotransferase (AA). Glutamate is used for synthesis of glutamine (GLN) catalyzed by glutamine synthetase (GS). Glutamine is transferred to the glutamatergic neuron to be used for synthesis of glutamate catalyzed by phosphate-activated glutaminase (PAG). Released glutamate is taken up into the astrocyte and transformed into glutamine completing the glutamate-glutamine cycle. Alternatively the glutamate taken up may be oxidatively metabolized which subsequently requires *de novo* synthesis of glutamine via the anaplerotic processes indicated in bold arrows.

of GLT-1 and GLAST has shown that the expression level of the neuronal glutamate transporter EAAC-1 is very low and hence, it is enigmatic how this transporter can contribute significantly to removal of transmitter glutamate released from glutamatergic neurons thereby via re-uptake into the presynaptic nerve endings replenishing the transmitter pool of glutamate (55). Nevertheless, a functional study in glutamatergic neurons has shown that even in the presence of glutamine in the incubation medium, block of glutamate transport using the specific glutamate transporter inhibitor threo-benzyloxy-aspartate (TBOA) caused a significant decrease in the cellular glutamate content after repetitive release of glutamate from the transmitter pool (56). Additionally, it has been shown that the non-metabolizable glutamate analog D-aspartate is taken up into presynaptic nerve endings (57) as well as into cultured glutamatergic neurons with only a marginal astrocytic contamination (58). Altogether, these studies point to a quantitatively more important role of astrocytic glutamate uptake relative to that associated with neurons in the inactivation of transmitter glutamate. This notion is in keeping with the functional role of the glutamate-glutamine cycle and it fits with the model proposed by Hertz and Schousboe (59) based on analysis of kinetic data for glutamate uptake into cultured neurons and astrocytes.

## ROLE OF GABA TRANSPORTERS FOR MAINTENANCE OF GABA NEUROTRANSMISSION

The concept of termination of GABA mediated neurotransmission via high-affinity transport is based on pioneering studies by Elliott and Van Gelder (60) demonstrating that GABA was accumulated by cerebral cortical slices from the incubation medium against a concentration gradient. The high-affinity nature of the transport mechanism was demonstrated by Iversen and Neal (61) also using brain slices and this study showed GABA uptake in both neurons and astrocytes. Detailed kinetic studies of GABA uptake in cultured neurons and astrocytes [for review, see Ref. (62, 63)] have shown that the neuronal GABA uptake is more efficacious than that in astrocytes as schematically illustrated by Hertz and Schousboe (59). Hence, only 10–20% of synaptically released GABA will be taken up into astrocytic processes whereas the remaining GABA is re-accumulated in the presynaptic nerve endings of the GABAergic neurons where it may be re-utilized as a neurotransmitter after packaging in vesicles (64). This is in contrast to the scenario described above for glutamatergic neurotransmission in which the majority of the released transmitter is taken up into astrocytes which ensheathe the synapse.

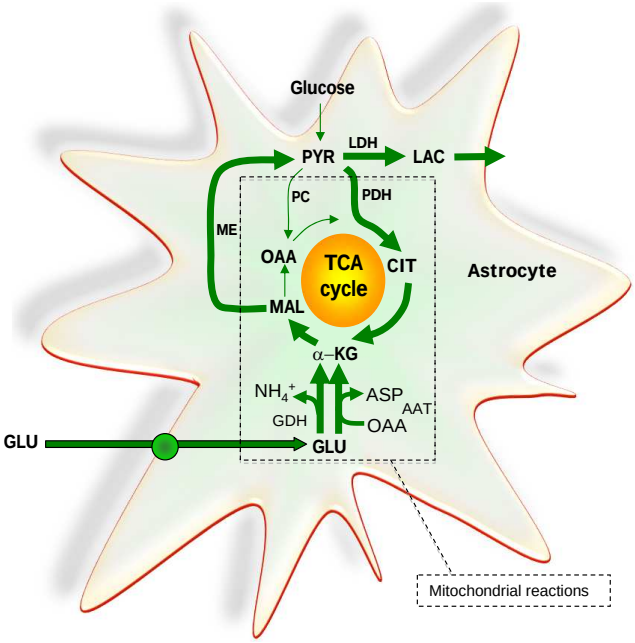


**FIGURE 6 | The extent to which glutamate (GLU) is oxidized in astrocytes seems to increase particularly during higher glutamate concentrations**. A net synthesis of tricarboxylic acid (TCA) cycle intermediates occurs when the initial reaction is catalyzed by glutamate dehydrogenase (GDH) which paves the way for the complete oxidation of the carbon skeleton of glutamate. This requires pyruvate recycling via the concerted action of malic enzyme (ME) and pyruvate dehydrogenase (PDH) converting malate into acetyl CoA producing NAD(P)H. Acetyl CoA is oxidized completely in one turn of the TCA cycle. A partial oxidation of glutamate is acquired when pyruvate (PYR) is reduced to lactate (LAC) instead of being oxidized to acetyl CoA. The redox state of the cell is likely important in the regulation of the destiny of the glutamate molecule. As an alternative to the activity of GDH, aspartate aminotransferase (AAT) facilitates the formation of α-ketoglutarate (α-KG) from glutamate at the expense of oxaloacetate (OAA); thus no net synthesis of TCA cycle intermediates is obtained. In contrast to the complete oxidation initiated by the activity of GDH, AAT enables the truncated TCA cycle which refers to the net synthesis of aspartate from glutamate, a pathway shown to accelerate during hypoglycemic conditions. All enzymes except ME and LDH are located in the mitochondria. PC, pyruvate carboxylase; CIT, citrate.

The high-affinity GABA transporters have been cloned and four distinct transport proteins have been described three of which are specific for GABA and one is able to transport both GABA and the osmolyte betaine [for references, see Ref. (38)]. The nomenclature of these transporters is unfortunately somewhat confusing but it is recommended to utilize the HUGO nomenclature, i.e., GAT1, GAT2, and GAT3 for the GABA specific transporters and BGT1 for the betaine-GABA transporter (38, 65, 66). Among these transporters, GAT1 is the most prevalent being expressed both in GABAergic neurons and in astrocytes. GAT2 is expressed neonatally in the brain but its expression decreases as a function of postnatal development. GAT3 may be considered primarily astrocytic while BGT1 is only sparsely expressed in the brain [for references, see Ref. (38)]. While pharmacological studies have pointed to an interesting function of BGT1 and possibly GAT3 for the control of the availability of GABA at extrasynaptic GABA receptors (67) the low expression level of BGT1 has questioned the quantitative significance of this transporter for GABA homeostasis (68).

The fact that aberrations in GABA neurotransmission are associated with a number of neurological diseases including epilepsy [see Ref. (34) for references] has prompted detailed pharmacological studies of GABA transporters to be performed [for references, see Ref. (66)]. This has resulted in development of the clinically active antiepileptic drug tiagabine which is a specific inhibitor of GAT1 (34) and more recently such studies have been focusing on development of inhibitors acting preferentially on non-GAT1 transporters (69). This has led to synthesis of a GABA analog which preferentially inhibits BGT1 and which exhibits anticonvulsant activity (70). This is an indication that astrocytic GABA transport may represent an interesting pharmacological target as proposed previously (71, 72).

## AMMONIA HOMEOSTASIS RELATED TO THE GLUTAMATE-GLUTAMINE CYCLE AND SHUTTLING OF NITROGEN BETWEEN NEURONS AND ASTROCYTES

As seen in **Figure 4** the glutamate-glutamine cycle leads to production of ammonia in glutamatergic neurons by the conversion of glutamine to glutamate catalyzed by PAG. At the same time ammonia is needed in the astrocytes for the synthesis of glutamine from glutamate catalyzed by GS. This obviously requires a mechanism by which the ammonium ion can be transported out of the neurons and into the surrounding astrocytes. While ammonia ($NH_3$) may be able to diffuse through the plasma membrane it is less likely that the charged ammonium ($NH_4^+$) ion can penetrate the lipid double layer of these membranes and at physiological pH essentially all ammonia is in the ionized form of ammonium. It has therefore been proposed that an amino acid based shuttle mechanism may be operating between the neuronal and the astrocytic compartments [see Ref. (14)]. The operation of one of the models is based on the use of alanine as the nitrogen-carrier (13, 73) while the other analogous model has proposed that branched chain amino acids (BCAAs) and their congeneric keto-acids play this role (74). Regardless of the model there is a requirement for a reductive amination of α-ketoglutarate to glutamate catalyzed by GDH to take place with a simultaneous transamination of this glutamate to either alanine of a BCAA to occur [see Ref. (11) for details]. As the thermodynamic equilibrium of the GDH-catalyzed reaction and the high $K_m$ value for [$NH_4^+$ of GDH counteract such amination (75)] it has been proposed that the intra-mitochondrial milieu possibly coupled to a metabolomic complex between PAG, GDH, and the transaminases in question, i.e., ALAT and BCAA-aminotransferase may facilitate the reactions (11). Actually, a metabolomic coupling between GDH and ALAT as well as BCAA-aminotransferase has been experimentally demonstrated (76, 77).

A recent review paper by Rothman et al. (78) has provided a careful analysis of the functional capability of the above mentioned shuttles to support the flux of nitrogen from neurons to astrocytes needed to balance the rate of *de novo* synthesis of glutamine to compensate for oxidative metabolism of glutamate in the astrocytic compartment. While the rate of ammonia fixation by the GDH reaction and the activity of the transaminases involved in the

proposed shuttles may be sufficient to match the rate of glutamine synthesis, the modeling studies show that the actual fluxes of either the alanine/lactate or the BCAA/branched chain keto-acid shuttles are insufficient to account for the need of ammonia nitrogen to account for glutamine synthesis. Hence, the exact mechanisms involved in ammonia nitrogen transfer between the neuronal and astrocytic compartments remain to be fully elucidated.

## ENERGY SUBSTRATES FOR THE OPERATION OF GLUTAMATE/GABA CYCLING. GLYCOGEN, GLUCOSE, LACTATE, OR GLUTAMATE

### *Energy cost related to cycling*

Since the transporters for glutamate and GABA are dependent on an intact trans-membrane sodium ion gradient maintained by the operation of the $Na^+$-$K^+$-ATPase it is clear that transport of the amino acids is strongly dependent on chemical energy supplied by ATP hydrolysis [see Ref. (43)]. Additionally, in case glutamate transported into astrocytes is metabolized to glutamine one molecule of ATP is used per glutamate in the GS catalyzed reaction. As will be discussed below, glutamate may alternatively function as an energy producing molecule in case it is oxidatively metabolized via the GDH-catalyzed reaction and subsequent metabolism of the carbon skeleton to $CO_2$ in the TCA cycle. However, since glucose is the most important energy fuel for the brain under normal physiological conditions (43), the following sections shall deal with glucose and its associated molecules glycogen and lactate as energy sources. Glutamate as an energy source will be discussed separately.

### *Glycogen as energy source*

The brain has a small but apparently functionally significant store of glycogen which is selectively located in the astrocytes [Ref. (79) and references herein]. It can be used during an energy crisis, i.e., reduced blood supply and/or aglycemia but recent studies have provided evidence that glycogen is a highly dynamic molecule which via the glycogen-shunt (**Figure 7**) channels a significant part of the glucose carbon entering the brain into glycolysis (79, 80). Studies utilizing drugs selectively inhibiting glycogen phosphorylase, the key enzyme in glycogenolysis have convincingly demonstrated that a continuous functional activity of the glycogen-shunt is a prerequisite for the maintenance of optimal glutamatergic activity and for functional and behavioral activities associated with such neurotransmission including memory formation and consolidation [for references, see Ref. (79) and (81)]. This obviously places the astrocytes in a key functional role since as mentioned above glycogen is exclusively found in astrocytes.

### *Glucose versus lactate as energy source*

Whether or not glucose is metabolized through the glycogen-shunt its carbon skeleton enters the glycolytic metabolic pathway as glucose-6-phosphate and through a series of reactions referred to as the glycolytic pathway, the glucose molecule consisting of six carbon atoms is split into two molecules of pyruvate, i.e., two C-3 units (43). This results in a net production of two molecules of ATP if the glycogen-shunt is not operating since the hexokinase step consumes an ATP while three molecules of ATP are gained when the glycogen-shunt is in operation since the hexokinase step is circumvented. Therefore from a purely energetic efficiency point of view there is a dramatic advantage of the operation of the glycogen-shunt increasing the energy production of the glycolytic pathway by 50%. The two moles of pyruvate formed in glycolysis from glucose can either be converted to two moles of lactate or they can be oxidized to $CO_2$ in the pyruvate dehydrogenase (PDH)/TCA cycle reactions. In the latter case the $NADH + H^+$ produced in the oxidative processes of the glycolytic pathway are available for oxidative phosphorylation in the mitochondrial respiratory chain while in the former case the reduced co-enzyme is oxidized in the lactate dehydrogenase (LDH) catalyzed conversion of pyruvate to lactate [see Ref. (43) for further details]. It must be emphasized that in case pyruvate is to enter the PDH/TCA cycle pathway a mechanism capable of transferring reduced co-enzyme equivalents from the cytoplasm to the mitochondria and vice versa must exist or else the cytoplasmic content of $NAD^+$ will be depleted. The shuttling mechanism for transfer of reduced co-enzyme equivalents across the mitochondrial membrane is of fundamental importance and the most important mechanism for this in the brain is the MAS discussed above in relation to the biosynthetic pathway for transmitter glutamate (43). While there is consensus about the presence of the MAS in neurons its expression level in astrocytes has been questioned (82). It should be noted, however, that a transcriptomic analysis of acutely isolated astrocytes has provided evidence that this shuttle is present in astrocytes (9, 83) which is in keeping with the high expression of all enzymes necessary for oxidation of glucose and the actual

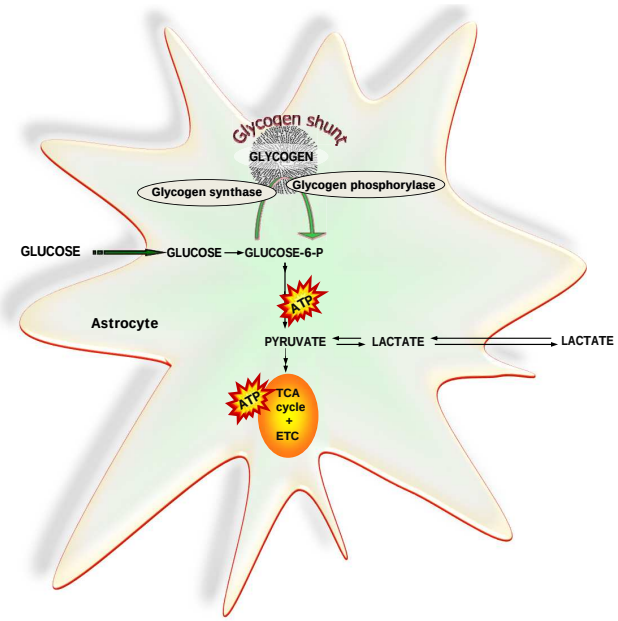


**FIGURE 7 | A simplified schematic representation of glucose metabolism via glycolysis or via the "glycogen-shunt" illustrating how glucose units may be metabolized *via* incorporation into and subsequent hydrolysis from the branched glycogen molecule preceding metabolism to pyruvate and lactate, i.e., glycogenolysis.** Glucose-6-P, glucose-6-phosphate; TCA, tricarboxylic acid; ETC, electron transport chain.

functional activity of complete glucose oxidation in these cells (9, 84).

The hypothesis proposed by Pellerin and Magistretti (85) that glutamate recycling in the glutamate-glutamine cycle is tightly coupled to astrocytic glycolysis converting glucose essentially quantitatively to lactate and that this lactate is transferred to neurons for complete oxidation [the astrocyte-neuron-lactate-shuttle (ANLS) has been a subject of considerable debate (84, 86, 87)]. The fact that astrocytes have a considerable oxidative metabolism of glucose (84) and that lactate can be transferred bi-directionally between neurons and astrocytes (88–91) is not in favor of ANLS being the unifying hypothesis of glucose metabolism and transfer of lactate to neurons to serve as the major energy substrate in these cells. Actually, it has been shown that glucose and not lactate is required to maintain glutamatergic activity in cultured cerebellar granule cells, a glutamatergic neuronal preparation (92–94). Additionally, Zielke et al. (95) have elegantly demonstrated that at least half of the extracellularly available glucose in the brain is accumulated by neurons and that lactate is metabolized by both neurons and astrocytes.

### *Glutamate as an energy substrate*

The demonstration that glutamate can be oxidatively metabolized to $CO_2$ in cultured astrocytes (18, 96, 97) provides strong support to the notion that the glutamate-glutamine cycle cannot operate in a stoichiometric fashion, i.e., that there is a 1:1 exchange of glutamate and glutamine (**Figure 4**). It is, however, compatible with a considerable pyruvate carboxylation enabling *de novo* synthesis of glutamine to compensate for loss of glutamate by oxidative metabolism (see above). That the carbon skeleton of glutamate to a considerable extent gets access to the TCA cycle can be deduced from experiments using [$^{13}$C]glutamate and NMR spectroscopy to study glutamate metabolism in cultured astrocytes. It could be demonstrated that glutamate-carbon labeled lactate derived from the TCA cycle via ME activity more extensively than glutamine formed in the GS catalyzed reaction (98). It should be emphasized that complete oxidation of glutamate via the combined action of the GDH reaction, pyruvate recycling and the TCA cycle generates 75% of the energy in the form of ATP that would be produced by oxidation of glucose. The significance of glutamate as an energy source is further discussed by Kreft et al. (81).

## CONCLUDING REMARKS

A recent comprehensive review on astrocyte physiology and pathophysiology (99) has provided compelling evidence to support the view which has emerged over the past several years that these cells constitute as aptly put by Hertz and Zielke (100): "The stars of the show." The present review has focused on the functional role of astrocytes in relation to their regulatory function in amino acid neurotransmission. The main conclusion is that these neurotransmission processes which operate in the vast majority of the synapses in the brain would be completely dysfunctional without astrocytic support and regulatory control. In this context it is important to note that astrocyte morphology and process ramification has undergone a dramatic increase in sophistication from small rodents to humans which by far exceeds the corresponding difference seen for nerve endings (101).

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**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

*Received: 31 May 2013; paper pending published: 22 June 2013; accepted: 31 July 2013; published online: 15 August 2013.*
*Citation: Schousboe A, Bak LK and Waagepetersen HS (2013) Astrocytic control of biosynthesis and turnover of the neurotransmitters glutamate and GABA. Front. Endocrinol. **4**:102. doi: 10.3389/fendo.2013.00102*
*This article was submitted to Frontiers in Cellular Endocrinology, a specialty of Frontiers in Endocrinology.*

frontiers in
ENDOCRINOLOGY

REVIEW ARTICLE
published: 27 May 2013
doi: 10.3389/fendo.2013.00059

# The glutamate–glutamine (GABA) cycle: importance of late postnatal development and potential reciprocal interactions between biosynthesis and degradation

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The gold standard for studies of glutamate–glutamine (GABA) cycling and its connections to brain biosynthesis from glucose of glutamate and GABA and their subsequent metabolism are the elegant *in vivo* studies by $^{13}C$ magnetic resonance spectroscopy (NMR), showing the large fluxes in the cycle. However, simpler experiments in intact brain tissue (e.g., immunohistochemistry), brain slices, cultured brain cells, and mitochondria have also made important contributions to the understanding of details, mechanisms, and functional consequences of glutamate/GABA biosynthesis and degradation. The purpose of this review is to attempt to integrate evidence from different sources regarding (i) the enzyme(s) responsible for the initial conversion of α-ketoglutarate to glutamate; (ii) the possibility that especially glutamate oxidation is essentially confined to astrocytes; and (iii) the ontogenetically very late onset and maturation of glutamine–glutamate (GABA) cycle function. Pathway models based on the functional importance of aspartate for glutamate synthesis suggest the possibility of interacting pathways for biosynthesis and degradation of glutamate and GABA and the use of transamination as the default mechanism for initiation of glutamate oxidation. The late development and maturation are related to the late cortical gliogenesis and convert brain cortical function from being purely neuronal to becoming neuronal-astrocytic. This conversion is associated with huge increases in energy demand and production, and the character of potentially incurred gains of function are discussed. These may include alterations in learning mechanisms, in mice indicated by lack of pairing of odor learning with aversive stimuli in newborn animals but the development of such an association 10–12 days later. The possibility is suggested that analogous maturational changes may contribute to differences in the way learning is accomplished in the newborn human brain and during later development.

**Keywords: aspartate aminotransferase, glutamine–glutamate cycle, postnatal metabolic enzyme development**

## GLUTAMATE AND GABA

The function of glutamate and γ-aminobutyric acid (GABA) as the key excitatory and inhibitory transmitters in mammalian brain was not realized until the second half of the twentieth century (Okamoto, 1951; Florey, 1956; Roberts, 1956; Curtis et al., 1960; Watkins, 2000). Relatively soon thereafter evidence was obtained that a cycle of neuronal-astrocytic interactions plays a major role in the production from glucose and the metabolism of both amino acid transmitters (van den Berg and Garfinkel, 1971; Benjamin and Quastel, 1972), and intense uptake of the transmitters, especially glutamate, was demonstrated and quantitated in astrocytic preparations (McLennan, 1976; Schousboe et al., 1977; Hertz et al., 1978a,b). Although the importance of glutamatergic/GABAergic activation of endocrine responses was suggested already at that time (Ondo and Pass, 1976; Ondo et al., 1976), the full consequence of the involvement of the amino acid transmitters only became realized during the last decade. More recently, direct evidence is emerging that astrocytes may also account for much of glutamate degradation (Bauer et al., 2012; McKenna, 2012; McKenna, present Research Topic; Whitelaw and Robinson, present Research Topic), and that production and degradation pathways may interact (Hertz, 2011a). These recent conclusions and observations place an increased focus on identification of the enzymes(s) carrying out the undisputed initial conversion of glutamate to α-ketoglutarate (α-KG) (almost certainly mainly transamination) and, especially *vice-versa*. This paper will deal with these questions and discuss a possible interaction between the pathways mediating synthesis and degradation of the two amino acid transmitters. It will also discuss an observed late maturation of the metabolic processes involved. Many of the developmental observations were made decades ago, but their full importance can only now be understood after the realization in the living rodent and human brain of the huge glutamine–glutamate (GABA) cycle flux determined in the brain *in vivo* and described below.

## THE GLUTAMINE–GLUTAMATE (GABA) SHUTTLE AND ITS RELATION TO GLUCOSE METABOLISM

**Figure 1** is a cartoon of selected parts of glucose metabolism in astrocytes (right) to neurons (left). They are connected by a flow of glutamine (produced directly from glutamate, generated as

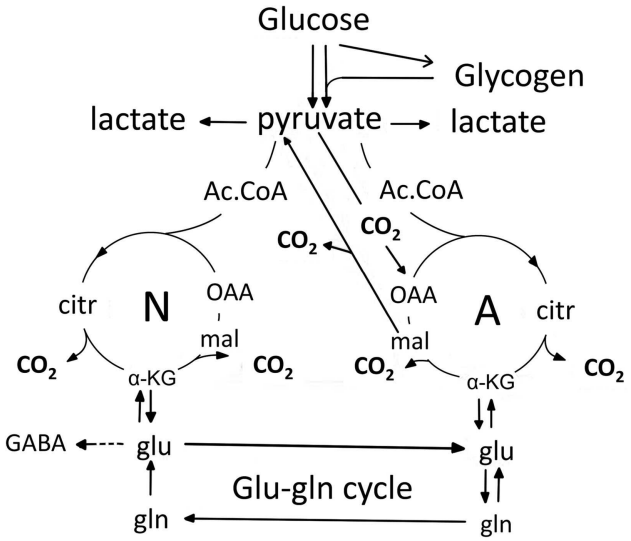


**FIGURE 1 | Cartoon of glucose metabolism via pyruvate in neurons (left – N) and astrocytes (right – A) and of glutamine–glutamate (GABA) cycling**. In both cell types pyruvate metabolism via acetyl Coenzyme A (ac.CoA) leads to formation of citrate by condensation with *pre-existing oxaloacetate* (OAA) in the tricarboxylic acid (TCA), an end-result of the previous turn of the cycle. Citrate oxidation in the TCA cycle includes two decarboxylations, leading to re-formation of oxaloacetate, ready for another turn of the cycle, and to production of large amounts of energy (ATP). Pyruvate carboxylation creates a *new molecule* of oxaloacetate, which after condensation with acetyl Coenzyme A, derived from a second molecule of pyruvate, forms a new molecule of citrate. This process can be used for replacement of worn TCA cycle intermediates. More important in the present context is that α-ketoglutarate (α-KG), one of the intermediates of the TCA cycle can leave the cycle to form glutamate (glu) and, catalyzed by the cytosolic and astrocyte-specific enzyme glutamine synthetase, glutamine (gln). After release from astrocytes glutamine is accumulated in glutamatergic and GABAergic neurons [lower line ($V_{gln}$ in the $^{13}$C-NMR studies) of the glutamine–glutamate (GABA) cycle (glu–gln cycle), converted to glutamate (and in GABAergic cells onward to GABA)] and released as transmitter. Released glutamate is almost quantitatively re-accumulated in astrocytes, together with part of the released GABA [upper line ($V_{cyc}$ in the $^{13}$C-NMR studies) of the glutamine–glutamate (GABA) cycle (glu–gln cycle)] and re-accumulated in the astrocytic cytosol. Here, about 85% is converted to glutamine and re-enters the glutamine–glutamate (GABA) cycle. The remaining 15% is oxidatively degraded after re-conversion via α-ketoglutarate to malate, exit of malate to the cytosol, decarboxylation to pyruvate by the already mentioned cytosolic malic enzyme and further pyruvate oxidation in the TCA cycle via acetyl Coenzyme A. Combined astrocytic formation and oxidation of glutamate creates almost as much ATP as direct oxidation of glutamate (Hertz et al., 2007).

discussed below) from astrocytes to neurons. In the neurons glutamate is converted to transmitter glutamate and GABA. However, after their release as transmitters most glutamate and a considerable amount of GABA are returned to astrocytes. This is the glutamine–glutamate (GABA) cycle. Elegant $^{13}$C-NMR analysis (*in vivo* injection of labeled glucose, or in some cases acetate, and determination of labeled metabolites) has shown that the glutamine flux in the cycle, $V_{gln}$ in the $^{13}$C-NMR studies, is slightly greater than the flux, $V_{cyc}$ in the $^{13}$C-NMR studies, of released transmitter glutamate and GABA in the opposite direction (Rothman et al., 2011), and that GABA fluxes account for up to 20% of total flux in $V_{cyc}$ (Patel et al., 2005; Chowdhury et al., 2007a). The reason for the slightly smaller $V_{cyc}$ than $V_{gln}$ may mainly be that some GABA is re-accumulated in GABAergic neurons (Schousboe, this Research Topic), where it can be oxidized (Yu, 1984). However, as discussed above, a considerable amount of GABA is also transferred to astrocytes, where it is taken up (Hertz et al., 1978a), transaminated to succinic acid semialdehyde (SSA), oxidized to succinate and then either (i) exits the tricarboxylic acid (TCA) cycle as malate (**Figure 1**); or (ii) is converted via α-ketoglutarate and glutamate to glutamine and returned to neurons in the glutamine–glutamate (GABA) cycle. Cytosolic malate is decarboxylated by the astrocyte-specific (Kurz et al., 1993), remarkably active (McKenna et al., 1995; Vogel et al., 1998) cytosolic malic enzyme to pyruvate, which can then be completely oxidized in the TCA cycle. Using this route, released glutamate is almost quantitatively taken up by astrocytes (Danbolt, 2001), and either converted to glutamine and reintroduced in the glutamine–glutamate (GABA) cycle, or metabolized to α-ketoglutarate by glutamate-dehydrogenase (GDH) or aspartate–glutamate transferase (AAT), followed by α-ketoglutarate oxidation after malate exit and decarboxylation. Glutamate oxidation is intense in cultured astrocytes (Yu et al., 1982; Hertz et al., 1988; McKenna, 2012), and increases with increasing glutamate concentration (McKenna et al., 1996). *In vivo* ∼85% of the accumulated glutamate is converted to glutamine and re-used, whereas the last 15% is oxidatively degraded (Rothman et al., 2011). *The close quantitative correlation between $V_{cyc}$ and rate of glucose oxidation suggests that over 80% of neuronal oxidative ATP production is coupled to neuronal signaling even in the absence of specific stimulation* (Rothman et al., 2011).

The operation of glutamine–glutamate (GABA) cycle in one direction only is a result of the *astrocyte*-specific (probably *not glia*-specific) localizations of the enzymes pyruvate carboxylase, PC (Yu et al., 1983; Shank et al., 1985; Hutson et al., 2008) and glutamine synthetase, GS (Norenberg and Martinez-Hernandez, 1979; Derouiche, 2004). Pyruvate carboxylase is the enzyme catalyzing formation of oxaloacetate (OAA in **Figure 1**) from pyruvate. This is the only enzyme catalyzing net synthesis from glucose of a *new* TCA intermediate. Cytosolic malic enzyme normally only operates toward decarboxylation. The ubiquitously expressed pyruvate dehydrogenase (PDH) carries pyruvate, via pyruvate dehydrogenation and formation of acetyl Coenzyme A, into the TCA cycle in both neurons and astrocytes, but no new TCA cycle intermediate is generated by the action of this enzyme *alone*. This is because the citrate (citr), which is formed by condensation of acetyl Coenzyme A with pre-existing oxaloacetate in the TCA cycle loses two molecules of $CO_2$ during the turn of the cycle, which leads to re-generation of oxaloacetate. This mechanism allows addition of another molecule of pyruvate in the next turn of the TCA cycle to continue the process, but it does not provide a *new* molecule of a TCA cycle intermediate that the cycle can afford to release and convert to glutamate. In contrast *joint* activity of PDH and PC activity creates a *new* molecule of citrate (**Figure 1**), which via α-ketoglutarate can be converted to glutamate, and by the aid of glutamine synthetase converted to glutamine. The pyruvate carboxylase is activated by enhanced brain function, as shown by an increase in $CO_2$ fixation with brain activity in the awake rat brain (Öz et al., 2004). After termination of increased brain activity this

effect may be reversed by increased glutamate degradation (see also below). Pyruvate can also be formed glycogenolytically from glycogen, previously generated from glucose (not shown), but glycogen turnover and glycogenolysis are slow processes (Watanabe and Passonneau, 1973; Dienel et al., 2002; Öz et al., 2012), except perhaps for occasional rapid bouts of glycogenolysis during very short time periods (Hertz et al., 2003). Glycogenolysis seems thus to be incapable of contributing much to metabolic fluxes, although blockade of glycogenolysis during sensory stimulation of awake rats does increase glucose utilization (Dienel et al., 2007). As discussed below, glycogenolysis seems mainly to serve as a fuel for signaling pathways, which are activated by stimulation of glycogenolysis, either by increased extracellular $K^+$ concentrations (Hof et al., 1988) or transmitter effects (Magistretti, 1988; Subbarao and Hertz, 1990).

As illustrated in **Table 1** the rate of flux in the glutamine–glutamate (GABA) cycle *in normal rat brain cortex* is only slightly lower than that of neuronal glucose oxidation (Sibson et al., 1998; Rothman et al., 2011, 2012; Hyder et al., 2013). Publications by these authors also show that the slight difference between the two fluxes is due to the persistence during deep anesthesia of a small amount of glucose oxidation but no glutamine–glutamate (GABA) cycling, whereas there is an approximately 1:1 ratio between the two parameters under all other conditions. This includes brain stimulation (Chhina et al., 2001; Patel et al., 2004).

Stimulated brain activity is accompanied by a small immediate increase in glutamate content, associated with a quantitatively similar decrease in content of aspartate and with a slower decrease in content of glutamine (Dienel et al., 2002; Mangia et al., 2007; Lin et al., 2012). The matched increase in glutamate and decrease in aspartate may suggest an activity-induced alteration in relative distribution of these two amino acids in their association with the malate–aspartate shuttle (MAS) (Mangia et al., 2012). However, a larger increase in glutamate content without concomitant decrease in aspartate observed in an epileptic patient almost certainly represents increased *de novo* synthesis (Mangia et al., 2012). The same probably applies to a short-lasting increase in glutamate, together with a similar increase in glutamine (**Figure 2**) and aspartate (not shown) during learning (Hertz et al., 2003; Gibbs et al., 2007). The rapid subsequent return to normal amino acid levels is most likely brought about by enhanced degradation.

Oxidative metabolism in astrocytes is a *sine qua non-for* operation of the glutamine–glutamate (GABA) cycle. Pioneering studies early in this century (Gruetter et al., 2001; Lebon et al., 2002) showed that neurons account for up to 75% of oxidative glucose metabolism in the living brain and that astrocytes contribute most of the rest. These studies have been consistently and repeatedly confirmed in both human and rodent brain, and many of the rates are tabulated by Hertz (2011b). Since the volume occupied by astrocytes is similar to, or smaller, than the relative contribution of these cells to energy metabolism, their rate of oxidative metabolism per cell volume must be as high, if not higher, than that of neurons (Hertz, 2011b). This conclusion is consistent with an at least similarly high expression of most enzymes involved in oxidative metabolism of glucose in astrocytic as in neuronal cell fractions freshly obtained from the mouse brain (Lovatt et al., 2007).

**Table 1 | Approximate metabolic rates in the non-anesthetized brain cortex from a multitude of $^{13}$C-NMR studies cited in text.**

| Parameter | μmol/g wet wt per min |
|---|---|
| Rate of glucose oxidation | 0.7 |
| Rate of brain glucose utilization in astrocytes | 20% of 0.7 = 0.14 |
| Rate of glutamine–glutamate (GABA) cycle | 0.6 |
| Pyruvate carboxylase-mediated flux | 50% of 0.14 = 0.07 |
| Rate of glycogenolysis (Öz et al., 2012) | 0.003 |

*Except for glycogenolysis, measured in humans, the values apply to the rat brain. For more details, see references cited in the text.*

## THE GLUCOSE-TO-GLUTAMATE PATHWAY

In cultured cerebellar astrocytes conversion of glutamate to α-ketoglutarate at least mainly occurs via a transamination (Westergaard et al., 1996). This is consistent with a recent *in vivo* study by Pardo et al. (2011), which established that the contents of glutamate and glutamine in cultured astrocytes increase by ∼50% in the presence of aspartate at a concentration of ≥100 μM, but not in the presence of alanine or leucine. On the basis of this finding the authors suggested the pathway shown in **Figure 3A**, according to which glucose-derived α-ketoglutarate leaves the astrocytic TCA cycle in exchange with malate, generated via oxaloacetate (OAA), which in turn had been formed from aspartate *in a transamination process*. Subsequently OAA is reduced to malate (MAL), with concomitant oxidation of NADH to $NAD^+$. The entire process requires operation of the α-ketoglutarate/malate exchanger (OGC in **Figure 3A**), but not of the aspartate/glutamate exchanger

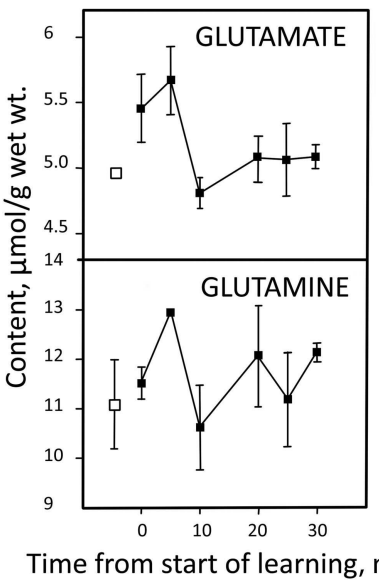


**FIGURE 2 | Learning-induced changes in glutamate and glutamine content in the equivalent of the mammalian brain cortex in day-old chicken**. Pre-learning contents are indicated by open symbols and post-learning contents with filled-in symbols. From Hertz et al. (2003).

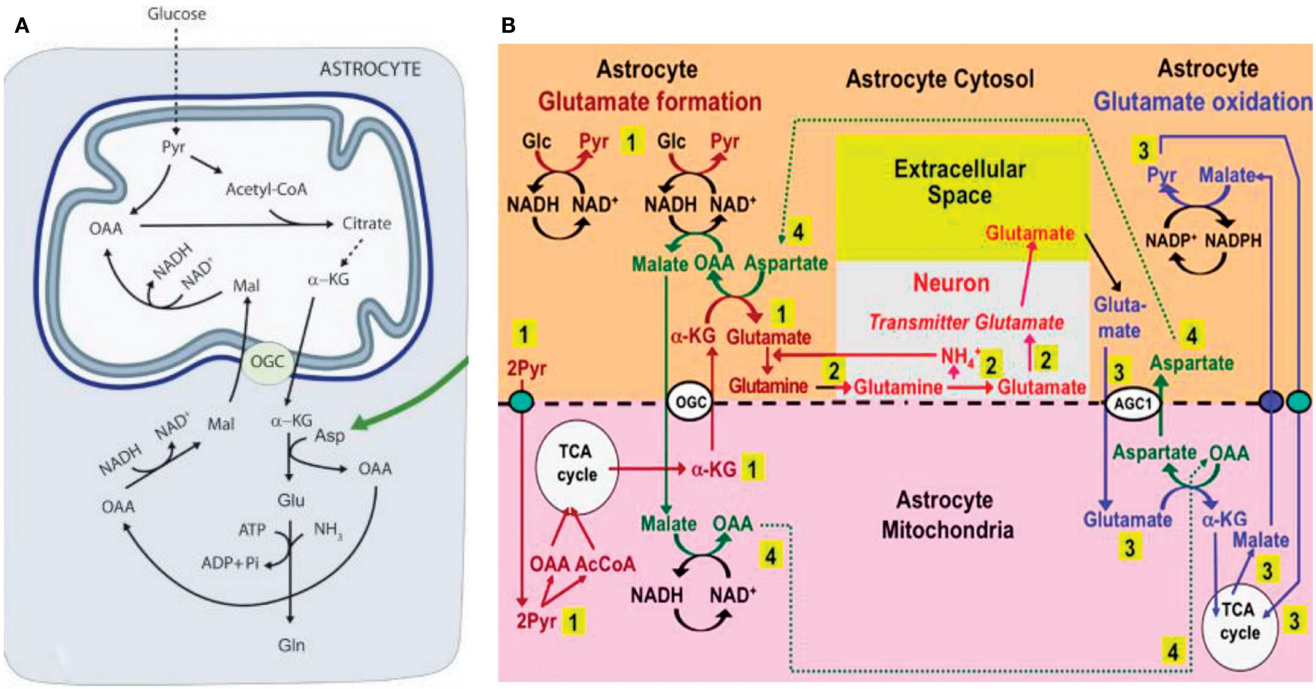


**FIGURE 3 | (A)** Cartoon describing metabolic pathway from pyruvate to glutamate/glutamine in astrocytes, as suggested by Pardo et al. (2011). Joint pyruvate carboxylase and pyruvate dehydrogenase activation generates a "new" molecule of citrate as described above. Citrate-derived α-ketoglutarate exiting the mitochondrial membrane leaves the astrocytic TCA cycle and is transaminated with aspartate to form glutamate, with concomitant oxaloacetate (OAA) formation from aspartate. The mitochondrial exit of α-ketoglutarate occurs via the ketoglutarate/malate exchanger, generally acknowledged to be expressed in astrocytes, and the cytosolic malate with which is exchanged, is generated via NADH-supported reduction of aspartate-generated oxaloacetate. No aralar-requiring aspartate/glutamate exchanger (Slc1) activity is involved. **(B)** Proposed expansion by Hertz (2011a) of the model shown in **(A)**. The expanded model shows astrocytic production of glutamine (pathway 1), its transfer to glutamatergic neurons (without indication of any extracellular space, because there is no other function for extracellular glutamine than astrocyte-to-neuron transfer) and extracellular release as the transmitter glutamate (pathway 2), and subsequent reuptake of glutamate and oxidative metabolism in astrocytes (pathway 3), with connections between pathways 1 and 3 shown as pathway 4. Biosynthesis of glutamine is shown in brown and metabolic degradation of glutamate in blue. Redox shuttling and astrocytic release of glutamine and uptake of glutamate are shown in black, and neuronal uptake of glutamine, hydrolysis to glutamate, and its release is shown in red. Reactions involving or resulting from transamination between aspartate and oxaloacetate (OAA) are shown in green. Small blue oval is pyruvate carrier into mitochondria and small purple oval malate carrier out from mitochondria. AGC1, aspartate/glutamate exchanger, aralar; α-KG, α-ketoglutarate; Glc, glucose; Pyr, pyruvate; OGC, malate/α-ketoglutarate exchanger. It should be noted that (i) aralar activity is required initially for reversal of cytosolic $NAD^+$/NADH changes occurring during the one oxidative process occurring during pyruvate formation, but subsequently not in astrocytes until the oxidation of glutamate, probably allowing rapid glutamate synthesis, and (ii) all reactions are stoichiometrically accounted for **(A)** From Pardo et al. (2011); **(B)** From Hertz (2011a).

AGC, in brain AGC1. On the basis of their own and previous immunocytochemical observations in brain tissue by themselves and others (Ramos et al., 2003; Berkich et al., 2007), Pardo et al. (2011) regarded this exchanger as absent or sparsely expressed in astrocytes because of deficient expression of aralar, a necessary component of AGC1.

Subsequently Hertz (2011a), suggested that (i) the reduction of oxaloacetate to malate was a necessary compensatory consequence of the reduction of $NAD^+$ to NADH during the one oxidative process during glycolysis (glyceraldehyde-3-phosphate to 1-3-biphosphoglycerate), without which normally no production of α-ketoglutarate can occur from glucose, and (ii) that aspartate, *formed from OAA in astrocytes when glutamate during its oxidation is transaminated to α-ketoglutarate, supplied the needed aspartate*, as illustrated in **Figure 3B**. The latter suggestion required exit to the cytosol of mitochondrially located aspartate via the aralar-dependent AGC1 in the MAS. The involvement of the MAS during glutamate oxidation, but not during its synthesis (**Figure 3A**) might contribute to the development of MAS-based alteration in glutamate/aspartate ratio during brain activation suggested by Mangia et al. (2012). The suggestion of malate–aspartate participation in **Figure 3B** was felt to be justified by the finding by Lovatt et al. (2007) of equal expression of mRNA for aralar, determined by microarray analysis, in freshly isolated astrocytes and neurons. Moreover, it was calculated (based on data by Berkich et al., 2007) that the aralar expression found by Pardo et al. (2011) sufficed to produce enough aralar for the proposed model to function, although malate–aspartate cycle activity needed for synthesis of α-ketoglutarate at the beginning of the process increase demands. Equally high levels of mRNA aralar expression is astrocytes were later confirmed, *and its protein expression* (**Figure 4**) shown in freshly separated astrocytes and neurons from isolated

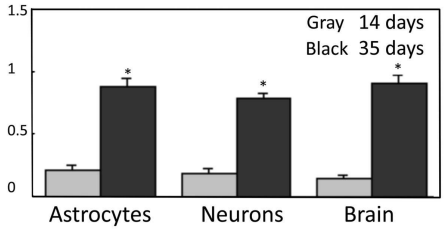


**FIGURE 4 | Protein expression of aralar in neuronal and astrocytic cell fractions are similar and develop at identical rates**. Neuronal and astrocytic cell fractions were gently isolated from two mouse strains, one expressing a neuronal marker with a specific fluorescence and the second expressing an astrocytic fluorescent signal (Lovatt et al., 2007). Samples were applied to slab gels of 12% polyacrylamide, separated by electrophoresis, and transferred to nitrocellulose membranes. After blocking with 5% skim milk powder and washing, these membranes were incubated for 2 h at room temperature with the primary anti-aralar antibody sc-271056 (from Santa Cruz Biotechnology, CA, USA) after dilution (1 × 100), shown not to react with the related carrier citrin, and followed by incubation with a goat anti-mouse HRP-conjugated secondary antibody, also from Santa Cruz Biotechnology (dilution: 1 × 200) for 2 h at room temperature. Anti β-actin (Sigma, St. Louis, MO, USA) was applied in the same lanes as the aralar antibody to use β-actin as a housekeeping protein providing an internal control for protein load. The intensities of both bands on the blots were scanned, and the ratios between aralar and β-actin were calculated and shown in the Figure. From Li et al. (2012b).

cell fractions (Li et al., 2012b). The separation procedure used selects astrocytes indiscriminately, but among neurons it mainly isolates glutamatergic projection neurons. These experiments also demonstrated *remarkably large differences in aralar expression in young and mature animals.* This finding was replicated in cultured astrocytes, whereas homogeneous neuronal cultures are too short-lived to provide meaningful results.

The model suggested in **Figure 3B** is consistent with the important $^{13}C$ labeling data in the study by Pardo et al. (2011) in young aralar$^{-/-}$ animals, showing incorporation of [$^{13}C$]glucose into glutamate but not into glutamine. This is because the absence of aralar does not exclude mitochondrial glutamate synthesis, especially if a substantial amount of α-ketoglutarate is supplied from non-glucose source in these young animals. However, in aralar$^{-/-}$ animals de-amidation of glutamine *in neurons* may be impaired as will be discussed below, which may prevent glutamine synthesis in astrocytes.

Formation of glutamate from glucose requires glycogenolysis, both in the intact chicken brain (Gibbs et al., 2007) and in cultured astrocytes (Sickmann et al., 2009). Absence of glycogen phosphorylase in oligodendrocytes (Richter et al., 1996) therefore is a powerful argument against functioning pyruvate carboxylase activity in oligodendrocytes. The rate of glycogenolysis in brain (**Table 1**) is not high enough that pyruvate derived from glycogen could be used by the astrocytes as the sole source of pyruvate for carboxylation. Most, although probably not all glucose oxidation in astrocytes proceeds via glutamate formation, astrocytes account for 20% of total glucose oxidation rate, or 0.14 μmol/g per min (in the rat), and one half of glutamate formation (0.07 μmol/g per min) occurs *via* pyruvate carboxylation (with the other one half mediated by PDH). This exceeds the rate of glycogenolysis by at least 10 times. Rather, as in the case of other astrocytic processes requiring activation of specific signaling pathways (Xu et al., 2013), glycogenolysis seems to be required for signaling processes needed to activate pyruvate carboxylase activity. Glycogenolysis is stimulated by even very small increases in extracellular $K^+$ concentrations above their normal level (Hof et al., 1988), and in astrocyte cultures pyruvate carboxylation is increased by an elevation of the $K^+$ concentration in the medium (Kaufman and Driscoll, 1993). Pyruvate carboxylation at least in other cell types (Garrison and Borland, 1979) is also stimulated by noradrenaline, as is astrocytic glycogenolysis (Magistretti, 1988; Subbarao and Hertz, 1990). This does not mean that a very brief increase in glutamate content, as shown in **Figure 2** might not, at least partly, be derived from glycogen, which showed a simultaneous precipitous and large fall (Hertz et al., 2003).

Formation of glutamine from glutamate in the astrocytic cytosol is in agreement with the astrocyte-specific expression of glutamine synthetase (Norenberg and Martinez-Hernandez, 1979), with probable lack of expression in oligodendrocytes confirmed by Derouiche (2004). In cultured astrocytes reduced function of the glutamine synthetase after administration of its inhibitor, methionine sulfoximine (MSO), causes an increase in glutamate and aspartate formation, the latter probably reflecting increased glutamate oxidation, when glutamine synthesis is inhibited (Zwingmann et al., 1998). Increased content of aspartate in brain slices during MSO inhibition has also been shown by Nicklas (1983). Aspartate production by this route might under adverse conditions supplement the aspartate needed for transamination, when α-ketoglutarate is converted to glutamate (**Figures 3A,B**). Chronic infusion of MSO into rat hippocampus increases glutamate content, specifically in astrocytes, by almost 50%, but has remarkably little effect on glutamate in synaptic endings (Perez et al., 2012). The animals develop seizures, and the authors suggested that the extracellular brain glutamate concentration had become increased, perhaps due to excessive release of glutamate and/or decreased extracellular clearance.

Glutamine can travel between gap-coupled astrocytes, and the distance it reaches increases during brain activation (Cruz et al., 2007). Different transporters have been proposed to *direct* its transport from astrocytes to neurons, but it now appears well established that glutamine release occurs via the amino acid transporter SN1. This transporter is densely expressed in astrocytic processes abutting glutamatergic and GABAergic neurons (Boulland et al., 2002). Efflux through SN1 is increased by acidic extracellular pH and by increased intracellular $Na^+$ concentrations (Bröer et al., 2002). Uptake of $Na^+$ in astrocytes during re-accumulation of excess extracellular $K^+$ from the extracellular space after neuronal excitation (Xu et al., 2013) might therefore increase glutamine release. Extracellular glutamine is taken up into neurons by SAT1,2 (Kanamori and Ross, 2006; Blot et al., 2009; Jenstad et al., 2009). This topic is discussed in detail in the paper by Chaudhry et al. in this Research Topic.

Although not shown in **Figure 3B** (for the sake of simplicity), the subsequent de-amidation of glutamine to glutamate appears to be somewhat complex, probably reflecting the subcellular localization of the phosphate-activated glutaminase (PAG). In cultured

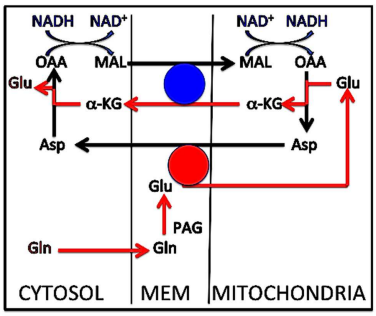


**FIGURE 5 | Metabolic pathway for conversion of glutamine to glutamate in cultured cerebellar granule neurons**. Glutamine enters the intermembranaceus space from the cytosol (red arrow at bottom of Figure). Here glutamate is formed by phosphate-activated glutaminase (PAG), but instead of returning to the cytosol, it enters the mitochondrial lumen and is transaminated to aspartate, coupled with transamination of oxaloacetate (OAA) to α-ketoglutarate (α-KG). As in the malate–aspartate shuttle α-KG exits the mitochondrial membrane in exchange with incoming malate, and intramitochondrial malate is oxidized to oxaloacetate. The mitochondrially generated aspartate is the source of the cytosolic malate exchanging with α-KG after it has been transaminated to oxaloacetate and reduced to malate. Two transmitochondrial carriers are involved, (1) the glutamate/aspartate exchanger AGC1, shown by a red circle, which requires aralar [undisputed presence in neurons, except in the aralar$^{-/-}$ mice studied by Pardo et al. (2011), where glutamate synthesis may have been abrogated], and (2) the α-ketoglutarate/malate carrier (OGC), shown by a blue circle. As mentioned in the text this process is similar to that operating in the malate–aspartate shuttle, with the exception that glutamate in the latter originates in the cytosol, not in the mitochondrial intermembranaceus space. The interactions between oxaloacetate and malate must be coupled to conversion of NADH to NAD$^+$ in the cytosol and the reverse change in the mitochondria (top of Figure). This contributes to the quantitative correlation between glucose flux and glutamine/glutamate (GABA) cycle activity at different activity ranges. Modified from Palaiologos et al. (1988).

glutamatergic neurons inhibitor studies have suggested the pathway indicated in **Figure 5** (Palaiologos et al., 1988). This Figure shows conversion of glutamine to glutamate by PAG, followed by a process similar to that occurring in the MAS, with the only exception that the glutamate molecule involved does not originate in the cytosol, but from PAG-activated de-amidation of glutamine in the intermembranaceus space of the mitochondrion. This mechanism implies a concomitant mitochondrial reduction of NAD$^+$ to NADH, associated with malate oxidation to OAA, cytosolic oxidation of NADH to NAD$^+$, and reduction of oxaloacetate to malate. Evidence that a similar process occurs in freshly isolated mitochondria (Bak et al., 2008) and description of perhaps even more complicated processes in GABAergic neurons are discussed by Schousboe et al. in the present Research Topic.

$NH_4^+$ released during the glutaminase reaction (and/or the small amount of $NH_3$ present at physiological pH) may easily traverse the outer, permeable mitochondrial membrane to reach the neuronal cytosol. A large number of studies have attempted to investigate ammonia transport from here to the astrocytic cytosol, where it is needed for continuous glutamine production. Many of these have focused on potential amino acid shuttles, capable of mediating this transport, but a recent review by Rothman et al. (2012) has shown too little transport capacity of these cycles in the brain *in vivo* to be entirely responsible for this function. This does not mean that they could not have a back-up function. This conclusion may re-focus attention on channel- and/or transporter-mediated contributions to efflux from neurons and influx into astrocytes (Benjamin, 1987). A major ion extruder in neurons is the $K^+$–$Cl^-$ co-transporter KCC2 (Chamma et al., 2012; Löscher et al., 2013), and Marcaggi and Coles (2000) has shown rapid ammonia exit from neurons in the bee retina via a co-transporter. Fittingly, in cultured astrocytes Nagaraja and Brookes (1998) showed that channel- and NKCC1-mediated $NH_4^+$ uptake together accounted for an uptake, which was similar in magnitude to the glutamate uptake in similar cultures. At 1 mM extracellular $NH_4Cl$ it amounted to 30 nmol/mg protein per min, which *in vivo* (100 g protein/g wet wt.) would equal 3 μmol/g wet wt per min, or about one half of the *in vivo* rate of the glutamine–glutamate (GABA) cycle (**Table 1**). At the same time the cytosol became acidic. Reversal of intracellular acidosis by NHE1 and NBCe1 acid extruders (Song et al., 2008, 2012) would create extracellular acidosis, stimulating SN1-mediated glutamine release. $Na^+$,$K^+$-ATPase activity is required both to support NKCC1 function, since NKCC1 operates as a secondary active transporter supported by $Na^+$,$K^+$-ATPase-generated ion gradients, and to maintain conditions allowing inward channel-mediated $NH_4^+$ transport. Inhibition of glutamine formation and retention in rat brain slices by ouabain (Benjamin, 1987) might therefore result from impairment of channel and transporter activity.

Regardless of detailed mechanisms involved, *ATP requirement for glutamine synthesis and ammonia uptake in astrocytes and for glutamine (or GABA) uptake in neurons makes neuronal transmitter supply from astrocytes an approximately two–three times more expensive process than neuronal production or uptake from the extracellular fluid would have been.*

## RETURN AND RE-USE OF RELEASED GLUTAMATE

It is well established that virtually all released transmitter glutamate is accumulated specifically into astrocytes by the two transporters GLAST (EAAT1) and Glt1 (EAAT2) (Danbolt, 2001). Claims to the contrary can generally be discounted as due to artifacts, e.g., homo- or hetero-exchange with intracellular amino acids. There is also no doubt that intact brain tissue can oxidize glutamate. Brain slices show higher respiratory activity during incubation in a medium containing L-glutamate as the only substrate than in the absence of any substrate (Dickens and Greville, 1935; Abadom and Scholefield, 1962). Nevertheless, according to most authors (Lipsett and Crescitelli, 1950; Ghosh and Quastel, 1954) rodent brain slices incubated with L-glutamate show a lower rate of oxygen consumption than during incubation with glucose alone. These findings are consistent with glutamate being a metabolic substrate that can be utilized by some, but not all cells, in the tissue. A recently demonstrated ability of glutamate to decrease the rate of glucose oxidation in incubated rat hippocampi (Torres et al., 2013) is reproduced in **Figure 6** and shows that oxidation of glutamate can supply energy supporting not only the increased demand created by its own uptake (e.g., McKenna, 2012), but also demands normally fueled by glucose oxidation. The relative

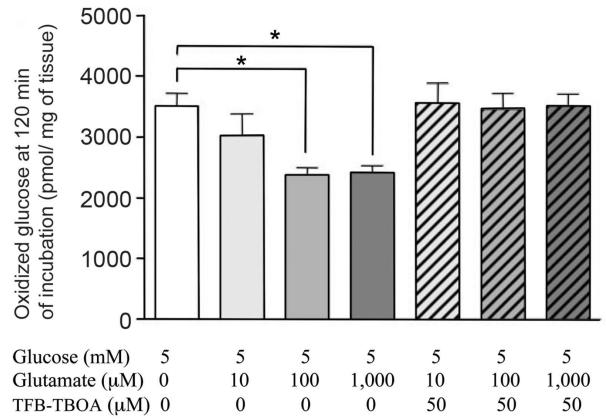


**FIGURE 6 | Glucose oxidation rates of intact rat hippocampi, incubated in tissue culture medium for 2 h with [U-$^{14}$C] glucose**. From Torres et al. (2013).

low rates of apparent glucose oxidation in such experiments is well known, partly due to the incubation *in vitro* and partly to the late turns of the TCA cycle during which some the labeled atoms are oxidized. Although it may be doubtful if glutamate oxidation is *necessary* metabolically, it may be functionally very important for complete disposal of glutamate in the brain *in vivo*, with continuous glutamate *de novo* synthesis and degradation and paucity/absence of other disposal or dilution routes.

In astrocyte cultures (Eriksson et al., 1995; Hertz and Hertz, 2003) L-glutamate is an excellent substrate for oxidative metabolism. The observation by Yu et al. (1982) that glutamate oxidation is insensitive to the transamination inhibitor AOAA and therefore initiated by GDH activity has been confirmed by virtually all authors with the exception of Hutson et al. (1998). However, this *in vitro* observation may not be valid for the brain *in vivo*, and in glutamate-dehydrogenase knock-out mice glutamate oxidation, determined in astrocyte cultures is reduced, but absolutely not abolished (Frigerio et al., 2012). Moreover, glutamate-dehydrogenase-mediated glutamate oxidation disagrees with the observation by Balazs (1965) that in brain mitochondria by far most of the glutamate conversion to α-ketoglutarate is catalyzed by the aspartate aminotransferase, an observation confirmed in both synaptic and non-synaptic mitochondria by Berkich et al. (2007). These observations raise the question whether lack of some of the many mechanisms regulating GDH activity (McKenna, 2011; Li et al., 2012a) may not function in isolated mitochondria, or whether use of isolated astrocytes without the possibility of metabolic interactions between glutamate synthesis ad glutamate oxidation may have caused the GDH dependency (see also below). Observations by Wysmyk-Cybula et al. (1991) in freshly isolated cerebral astrocytes and by Rao and Murthy (1993) in isolated cerebellar astrocytes that glutamate oxidation is mainly transaminase-dependent, support the latter conclusion. Additional support may be provided by the stimulation of glutamate *production* by aspartate shown by Pardo et al. (2011), since transactivation during glutamate *oxidation* can provide the needed aspartate (**Figure 3B**), but GDH-mediated α-ketoglutarate cannot. In contrast to glutamate oxidation via GDH, that by the aspartate transaminase also requires use of oxaloacetate, in the proposed model generated during glutamate production (labeled 4 close to the lower edge of **Figure 3B**).

Patients suffering from temporal lobe epilepsy, with sclerotic astrocytes and neuronal loss, often display a combination of highly elevated extracellular glutamate concentrations, also interictally, interictal hypometabolism, reduced glutamine synthetase activity and greatly reduced glutamine formation from glutamate (Petroff et al., 2002a,b; Eid et al., 2012). As during chronic perfusion of normal animals with MSO (Perez et al., 2012), the interictal elevated glutamate may largely reflect higher cellular glutamate levels within astrocytes, probably due to chronic impaired glutamine synthetase function and perhaps also impaired oxidation, since neuronal number and volume is reduced much more than total tissue glutamate levels (D. L. Rothman, personal communication). It would be interesting actually to determine extracellular glutamate concentration in the animals treated by Perez et al. (2012), since Exposito et al. (1994) previously showed that *acute* intrastriatal administration of MSO decreases the extracellular glutamate concentration.

Glutamate synthesis and degradation in differently located astrocytes was envisaged to present a possible problem in the suggested interacting pathways of glutamate production and oxidation with its exclusive use of aspartate aminotransferase (**Figure 3B**) rather than GDH (Hertz, 2011a). It was discussed that trans-astrocytic transport of co-factors and metabolites and lactate formation may alleviate this problem, but this may not always be sufficient, and lack of GABA return, and thus oxidation, in astrocytes will aggravate the situation. Pronounced expression in non-synaptic mitochondria of both AOAA- and non-AOAA-sensitive glutamate oxidation pathways (**Table 2**) may suggest a back-up function of the GDH. This would be consistent with the findings by Balazs (1965) and Wysmyk-Cybula et al. (1991) that transaminase-dependent glutamate oxidation accounted for most, but not all, glutamate oxidation. However, in GDH 1 knock-out mice (only humans express GDH 2) most functions, except the already mentioned reduced glutamate oxidation in cultured astrocytes, are remarkably intact (Frigerio et al., 2012). This applies to synaptic activity and long-term potentiation (LTP), an observation which may exclude that GDH in intact tissue should be essential for glutamate oxidation in astrocytes. However, glutaminase activity, glutamine content, and expression of the two astrocytic transporters Glt1 and GLAST were moderately increased in the knock-out animals. These alterations are consistent with, but do not prove, that glutamate production is rendered slightly more difficult, but certainly not abolished, when GDH is silenced.

Based on the sum of cited evidence it appears reasonable to suggest initiation of glutamate oxidation by transamination as the default pathway, but participation of GDH may occur in situations when an insufficient match between glutamate biosynthesis and degradation is not possible because of different rates, too long distances between biosynthesis and degradation sites, or prevailing conditions *in vitro*. The ability of the transaminase-mediated pathway to provide aspartate for glutamate synthesis

**Table 2 | Oxygen consumption rates as indications of kinetics of glutamate-dehydrogenase and aspartate–glutamate transferase activities in non-synaptic mitochondria from the rat.**

| | High-affinity Km (mM) | High-affinity *V*max (μmol $O_2$/min per g) | Low-affinity Km (mM) | Low-affinity *V*max (μmol $O_2$/min per g) |
|---|---|---|---|---|
| Glutamate + malate | 0.26 | 3.75 | 1.3 | 7.65 |
| Glutamate + malate + AOAA | 0.19 | 2.45 | | |

*From Lai and Clark (1976).*

is a strong argument for its function, and the increased aspartate content in brain slices after administration of MSO found by Perez et al. (2012) might partly reflect a decreased glutamate oxidation. In addition to the pathway suggested in **Figure 3A**, Hutson et al. (1998) has proposed a different pathway for aspartate transaminase-mediated glutamate oxidation. This model relies on many complex interactions, including branched chain amino acid cycling, but Rothman et al. (2012) concluded that it would be able to function in the mammalian brain *in vivo*, since *in vivo* data showed high enough fluxes and enzyme expression levels for this to be possible.

The total of four conversions of $NAD^+$ to NADH in the joint glutamate synthesis/oxidation process suggested [two in astrocytes during the production of α-ketoglutarate and one during glutamate oxidation (**Figure 3B**), together with one in neurons (**Figure 5**)] may explain the close to 1:1 ratio between rates of glucose oxidation and of glutamine/glutamate (GABA) cycle flux. The repeated $NAD^+$/NADH conversions also suggest much more complicated and repeated oxidation/reduction responses in brain cortex during neuronal and astrocytic activities than previously considered (e.g., Kasischke et al., 2004). It was also mentioned above that *over 80% of neuronal oxidative ATP production is coupled to neuronal signaling even in the absence of specific stimulation* (Rothman et al., 2011). What is peculiar, though, is that three of the four $NAD^+$/NADH conversions occur in astrocytes. The initial formations of α-ketoglutarate from glucose do require malate–aspartate cycle activity, but there seem to be agreement that only 15–20% of glutamine–glutamate (GABA) cycle activity is connected with *de novo* synthesis of glutamate. Could glutamate destined for oxidation and for glutamine synthesis be segregated, and the pathways suggested in **Figure 3A** apply only to the former? This question makes it so important to determine the pathway(s) for glutamate degradation not only in isolated astrocytes but also in intact brain tissue (see papers by McKenna and by Whitelaw and Robinson in this Research Topic). Would studies of metabolism of labeled glutamate (with appropriate receptor antagonists) at least in brain slices be useful? Also, could glutamate conversion to α-ketoglutarate be catalyzed by aspartate aminotransferase in one potential subfraction and by GDH in the other? These are critical questions. Finally, even if virtually all glutamate is oxidized in astrocytes, pyruvate formed from malate could be converted to lactate and transferred to neurons, but most evidence does not support lactate transfer from astrocytes to neurons. Nevertheless, astrocytes do contribute energetically to glutamine, glutamate, and GABA homeostasis (by uptake and glutamine synthesis) to a similar degree as neurons (cellular uptake and vesicular accumulation of glutamate and GABA). The same is the case for clearance of excess extracellular $K^+$ (astrocytic uptake followed by release and neuronal uptake after extracellular $K^+$ clearance, and they seem even to be responsible for the post-excitatory undershoot in extracellular $K^+$ concentration (Hertz et al., 2013). Since glutamate is the major excitatory transmitter, its release will cause efflux of neuronal $K^+$, followed by extracellular $K^+$ clearance and, after extensive stimulation, also post-excitatory undershoot in extracellular $K^+$ concentration. Thus, glutamate-mediated, $K^+$-associated excitatory activity may increase astrocytic energy demands as much as neuronal. One may wonder (i) if this dual uptake (and thus double metabolic billing), apparently of both glutamate and $K^+$ is the major reason for the extremely high energy metabolism in brain, and (ii) whether signaling possibilities especially exist during transport through the astrocytic syncytium.

## RETURN AND RE-USE OF RELEASED GABA

Although GABA contributes at most 10–20% of fluxes in the glutamine–glutamate (GABA) shuttle, GABAergic signaling is essential in the regulation of endocrine functions and in brain information processing. It is therefore an important question whether oxidation of GABA in astrocytes might also be associated with the biosynthetic pathway. Pathways for such a potential interaction are suggested in **Figure 7**. The synthetic pathway is identical to that for glutamate up till GABA formation from glutamate, which is discussed by Schousboe et al. in this Research Topic. During metabolism the most uncertain point is how GABA enters the mitochondrion. The model suggests an exchange with glutamate, but no mitochondrial GABA/glutamate exchanger is known in mammalian cells, although an exchanger has been demonstrated in plants (Michaeli et al., 2011), and the cell membrane of prokaryotes (which have no mitochondria) express a glutamate–GABA exchanger, GadC (Ma et al., 2012). After its mitochondrial exit cytosolic glutamate follows a similar pathway as during glutamate oxidation. The relatively low content of glutamate in astrocytes (Storm-Mathisen and Ottersen, 1983; Storm-Mathisen et al., 1992), and thus also in astrocytic mitochondria, will not be affected, because mitochondrial glutamate is re-established via MAS-mediated uptake, initial transamination to α-ketoglutarate, coupled to transamination of oxaloacetate, and a second transamination coupled to GABA transaminase-mediated formation of SSA. The steps of GABA transaminations are conventional, as is its subsequent complete oxidation via succinate, malate, and pyruvate.

An increase in aspartate but a decrease in glutamate and glutamine contents and synthesis has been observed in brain cortex from 17-day-old succinic-semialdehyde dehydrogenase-deficient

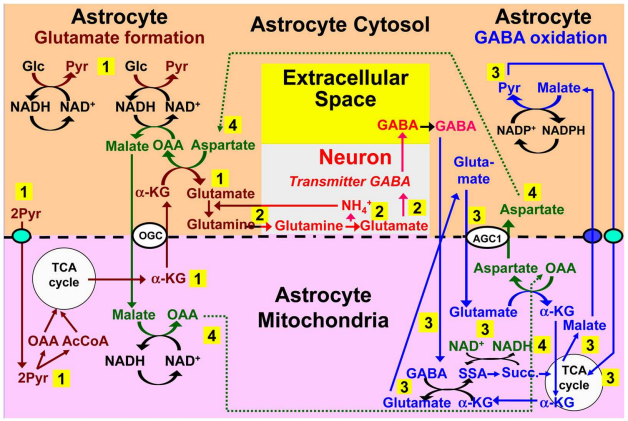


**FIGURE 7 | Proposed model for cytosolic–mitochondrial trafficking associated with astrocytic production of glutamine (pathway 1), its transfer to GABAergic neurons (without indication of any extracellular space, because astrocyte-to-neuron transfer is the only major function for extracellular glutamine), neuronal GABA formation via glutamate (without details), and extracellular release as transmitter GABA (pathway 2), with subsequent reuptake of GABA and oxidative metabolism in astrocytes (pathway 3), and connections between pathways 1 and 3 shown as pathway 4**. Biosynthesis of glutamine is shown in brown and metabolic degradation of GABA in blue. GABA is suggested to enter the mitochondria in exchange with glutamate, although no such exchanger is presently known, and subsequently be transaminated to succinic-semialdehyde and oxidized to succinate. α-KG is initially used for the transamination, but later re-generated during metabolism of glutamate entering the mitochondria and metabolized as previously suggested for transmitter glutamate, with the mitochondrial malate extruder shown as a small purple oval. Redox shuttling and astrocytic release of glutamine and uptake of GABA are shown in black, and neuronal uptake of glutamine, followed by GABA formation and release in red. Reactions involving or resulting from transamination between aspartate and oxaloacetate (OAA) are shown in green. Note that (i) aralar activity is required not only for the initial production of each of two molecules of pyruvate (light blue oval in pathway 1), but also during the re-entry of glutamate into the TCA cycle and for the final entry of pyruvate into the mitochondria (light blue oval in pathway 3), (ii) all reactions are stoichiometrically accounted for, although only when GABA synthesis and oxidation are integrated. AGC1, aspartate/glutamate exchanger, aralar; α-KG, α-ketoglutarate; Glc, glucose; Pyr, pyruvate; OGC, malate/α-ketoglutarate exchanger.

mice together with a pronounced decrease (40%) in glutamine synthetase expression, whereas GABA production was virtually unaffected (Chowdhury et al., 2007b). From **Figures 3A,B** follows that reduction of glutamate production, including initial transamination from α-ketoglutarate might explain both the increase in aspartate content and the reduced glutamine synthetase expression. On the other hand deficient GABA transamination should decrease aspartate production in the return route. Although degradation-synthesis coupling would be consistent with **Figures 3A,B**, it is also unexplained why metabolism of GABA should be so important for glutamate production, since GABA in adult animals contributes so relatively little to the return flux toward astrocytes ($V_{cyc}$). However, by necessity 17-day-old mice were used (the gene-deficient animals die around day 21). As will be discussed below, the glutamine–glutamate (GABA) cycle is not fully developed in these immature animals. Moreover, in rat brain slices both GABA and glutamate uptake and release rates show very rapid and pronounced quantitative fluctuations during early development (Schousboe et al., 1976), and GABA fluxes may have been considerable in 16-day-old animals.

Metabolism of GABA along the pathway suggested in **Figure 7** would eliminate a need for uptake of exogenous aspartate and its conversion to malate in astrocytes in an aspartate-dependent pathway model for GABA formation suggested by LaNoue et al. (2007). Such a pathway is not likely to operate, since aspartate itself must be synthesized in an astrocyte–neuron metabolic co-operation. Also, in contrast to glutamate, aspartate cannot sustain its own uptake in cultured astrocytes by oxidative metabolism (Peng et al., 2001), as it should have been able to do in order for the subsequent oxaloacetate-to-malate reduction suggested by LaNoue et al. (2007) to occur. These models were based on the assumption that astrocytes express no aralar. The repeated findings of aralar mRNA together with the additional demonstration of its protein expression in freshly isolated astrocytes probably mean that they can now be regarded as outdated.

## HOW DOES THE BRAIN MANAGE BEFORE THE DEVELOPMENT OF THE GLUTAMINE–GLUTAMATE (GABA) CYCLE?

The strikingly slow development of full aralar expression in rat brain and isolated brain cells (**Figure 4**) probably mainly reflects that gliogenesis in the rodent cerebral cortex is mainly postnatal (Altman, 1969b). A glial cell population, predominantly containing astrocytes, expands in the rodent brain cortex during the first 3 weeks of postnatal development, largely by local division (Ge et al., 2012). This is in contrast to a virtually completed neurogenesis at birth (Altman, 1969a; Bhardwaj et al., 2006). Mori et al. (1970) and Schousboe (1972) took advantage of the developmental difference between neurogenesis and gliogenesis to regard measured cell division in brain cortical tissue from postnatal day 6 and onward (by incorporation of label from [$^{14}$C]thymidine) as mainly reflecting formation of astrocytes. This appears justified, since simultaneous proliferation of oligodendrocytes and vascular cells probably contribute less to total volume in gray matter than astrocytes and may be less condensed time-wise. Both groups found similarly intense and virtually constant cell proliferation rate for slightly more than one subsequent week, followed by its abrupt termination around postnatal day 15 (**Figure 8A**). The cell proliferation is accompanied by huge increases in brain weight and total DNA, also stopping around day 14 (**Figure 8B**).

The postnatal gliogenesis is accompanied and followed by many biochemical alterations. As could be expected, the activity of core astrocyte-specific enzymes depend upon the formation of astrocytes, although some enzymes that later become astrocyte-specific are neuronal during early development. The activity of the pyruvate carboxylase is very low in 8-day-old rat brain and only reaches adult activity after >30 days (Wilbur and Patel, 1974) (**Figure 9**). A fivefold higher pyruvate carboxylase activity in adult mouse brain than in newborn mouse brain was confirmed by Yu et al. (1983), and Yu (1984), showed a 10- to 15-fold increase in the activity of this enzyme in astrocyte *cultures* between the ages of 1 and 3 weeks. This contrasts a much faster development of glutamate

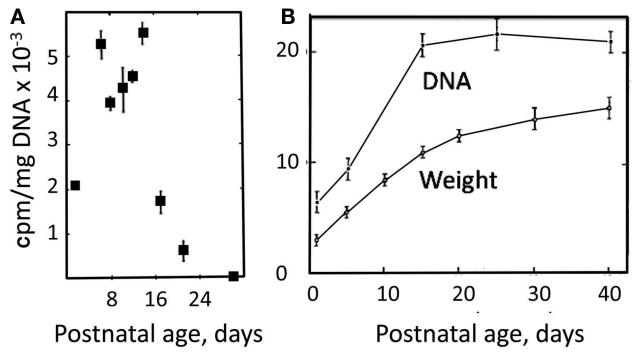


**FIGURE 8 | (A)** Rate of DNA synthesis, measured in unincubated brain slices by incorporation $^{14}C$ after [$^{14}C$]thymidine exposure 12 h earlier. From Schousboe (1972). **(B)** Developmental increase in weight and DNA content of rat brain. From Mori et al. (1970).

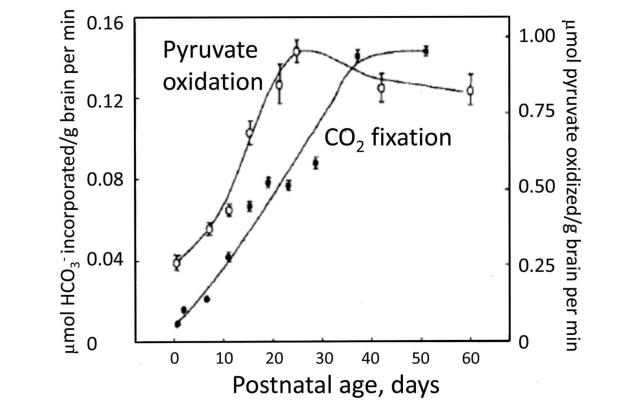


**FIGURE 9 | Decarboxylation of [l-14C]pyruvate by pyruvate dehydrogenase and the fixation of $HCO_3^-$ by pyruvate carboxylase in rat brain homogenates obtained from animals of different ages between birth and adulthood.** From Wilbur and Patel (1974).

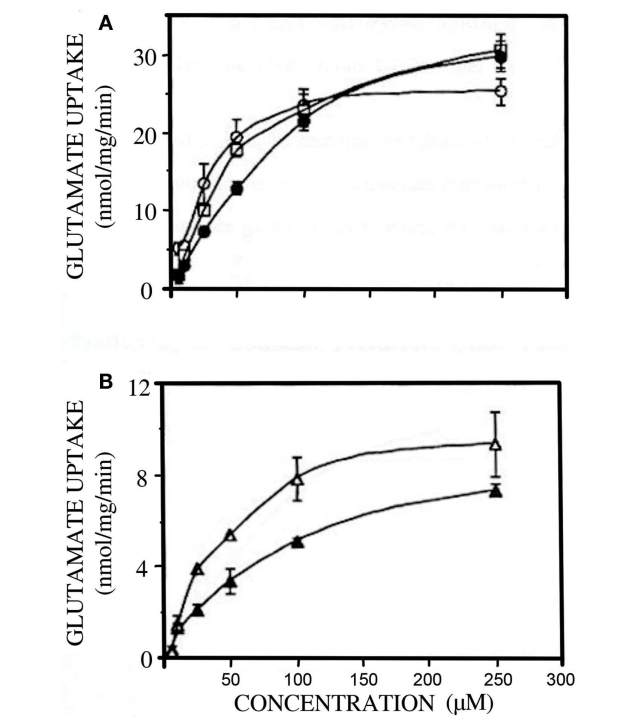


**FIGURE 10 | Developmental changes in glutamate uptake in primary cultures of mouse astrocytes (A) and cerebral cortical neurons (B).** **(A)** open circles, open squares and filled circles: 7, 18 and 30 days in culture; **(B)** open triangles 4 and closed triangles 8 days in culture. From Peng (1995).

uptake in cultured astrocytes, which is quite pronounced, although not mature, in 1-week-old cultures (**Figure 10A**). The activity of glutamine synthetase increases steeply during all the first 3 weeks of development both in *cultured* astrocytes and in brain *in vivo* (Hertz et al., 1978c; Patel et al., 1982). Glycogen content as well as activity of its degrading enzyme, glycogen phosphorylase, are low in brain at birth and increase during early postnatal development (Ferris and Himwich, 1946; Folbergrová, 1995).

The activities of many enzymes that are not specific for astrocytes also show drastic increases in activity during this period. This applies to glucose-metabolizing enzymes (e.g., hexokinase, aldolase, lactate dehydrogenase, phosphofructokinase, and PDH), which increase considerably in activity between the ages of ∼15 and ∼30 days (Takagaki, 1974; Wilbur and Patel, 1974; Land et al., 1977; Leong and Clark, 1984; – see also **Figure 9**). Synaptic mitochondria mature earlier (Almeida et al., 1995) than non-synaptic mitochondria (Bates et al., 1994), and cytosolic malate dehydrogenase (MDHc), which operates in the MAS but not in the TCA cycle, matures much more slowly than mitochondrial malate dehydrogenase (MDHm), which functions both in the MAS (**Figure 1**) and in the TCA cycle (Malik et al., 1993). Glutamate-metabolizing enzymes also show changes during the early postnatal period. Thus, the glutamate dehydrogenase activity falls between the ages of 2 and 3 weeks in astrocyte cultures, whereas that of aspartate aminotransferase increases (Hertz et al., 1978c). These observations point toward a slow postnatal development of the glutamine–glutamate (GABA) cycle, and perhaps decreased importance of GDH.

A three fourfold increase in cycle flux has been shown by $^{13}C$-NMR in rat brain cortex by Chowdhury et al. (2007a) between postnatal days 10 and 30, and energy metabolism in the glutamatergic and GABAergic neurons increased proportionately with cycle flux, leading to an ∼threefold increase in TCA cycle activity between postnatal days 10 and 30. Since, as was illustrated in **Figure 8B**, the amount of respiring brain tissue also increases hugely, *total* rate of energy metabolism in the rat brain cortex must increase about 10 times within these 20 days. That a small amount of astrocytic activity did occur, even at day 10 is indicated by the finding of detectable, although low incorporation of label from the astrocyte-specific substrate acetate into glutamate and GABA. Thus, postnatal day 10 must be close to the beginning of neuronal–astrocytic interactions involved in the glutamine–glutamate (GABA) cycle. Re-analysis of the acetate data may allow determination of the developmental pattern also of astrocytic TCA cycle flux (D. L. Rothman and K. L. Behar,

personal communication). Measuring *rapid* incorporation of $^{14}C$ from glucose into amino acids in rat brain, which also is an indication of glutamine–glutamate (GABA) cycle function, at many different developmental stages, Gaitonde and Richter (1966) and Patel and Balázs (1970) had also found a sharp increase between postnatal days 10 and 20, and that a maximum was not reached until around postnatal day 25. Maximum metabolic compartmentation between glutamate and glutamine, another indicator of glutamine–glutamate cycle activity was not found until a few days later. Thus old-style biochemical studies and cutting-edge $^{13}C$-NMR determinations have similarly identified the time period during which the glutamine–glutamate (GABA) cycle develops in the rat to between postnatal days 10 and 30.

Other aspects of TCA cycle function in brain are completed around postnatal day 15, indicated by maximum oxidative response in rat brain slices to stimuli at this age (Holtzman et al., 1982), and by sensory-evoked increases in brain glucose utilization *in vivo* by day 10 in barrel cortex (Melzer et al., 1994) and between days ∼13 to ∼18 in auditory and visual areas (Nehlig and de Vasconcelos, 1993). A functioning brain cortex is obviously working at that time, which is consistent with the completion of neurogenesis except at a few specific locations (Altman, 1969a). This is also exemplified by active GABAergic signaling at early neonatal stages (Lauder et al., 1986), and glutamatergic activation of synchronized spike waves in 3–5-day-old rats (Seki et al., 2012). Astrocytes are among the targets of glutamatergic signaling, which plays a role during astrocytic differentiation (Oppelt et al., 2004; Stipursky et al., 2012; Sun et al., 2013). Such an ontogenetic development from a purely neuronal nervous system to a neuronal–glial system also occurs during phylogenesis (Reichenbach and Pannicke, 2008). It is generally accepted that mammalian brain function should be studied in mammals, but far too often tissues from rats or mice younger than 4 weeks are studied.

In the absence of pyruvate carboxylase activity during early postnatal development (**Figure 9**) glutamate synthesis within the brain must be replaced by import of glutamate or a precursor. A relatively fast uptake of glutamine, glutamate, and GABA from the systemic circulation occurs across the blood-brain barrier at this age (Pardridge and Mietus, 1982; Al-Sarraf et al., 1997; Al-Sarraf, 2002), and it might be the source of neuronal amino acid transmitters. Also, during early postnatal development GLT1 is expressed in neurons (Shibata et al., 1996), although it later becomes astrocyte-specific. Glutamate oxidation by neurons occurs at much higher rates during early development, when little if any glutamate can be metabolized by astrocytes. This is illustrated in **Figure 10B**, showing a 50% reduction in rate of glutamate uptake in the glutamatergic cerebellar granule neurons between the ages of 4 and 8 days in culture (Peng, 1995). Similarly, other accounts of neuronal ability to oxidize glutamate may reflect the young age of the neurons (Olstad et al., 2007).

The large increase in metabolic demand during the first postnatal month may partly reflect that instead of purely neuronal uptake of glutamate and GABA during very early postnatal development, virtually all glutamate and some GABA now becomes accumulated twice. The first uptake is of glutamate and GABA into astrocytes, where a large fraction is converted to glutamine in an energy-requiring process, and the second uptake is that of glutamine into neurons. However, this alone cannot explain a 10-fold increase in energy demand, and many other processes may also become very costly in energy. Thus, $Na^+$,$K^+$-ATPase-mediated uptake of $K^+$ released during neuronal excitation into astrocytes seems also to precede neuronal re-accumulation of $K^+$ in *adult astrocytes and brain* (Xu et al., 2013). Since the astrocytic $K^+$ uptake also depends on glycogenolysis-activated signaling (to facilitate $Na^+$ access to the $Na^+$,$K^+$-ATPase's $Na^+$-sensitive site), it must also be absent in neonatal brain. Greatly increased growth and branching of *neuronal* processes also occur during the first year (Conel, cited by Altman, 1967), and synaptic density increases, in frontal gyrus peaking at 12–15 months of age (**Figure 11**) and before or during adolescence decreasing to similar levels as in the newborn (Huttenlocher, 1979; Huttenlocher and Dabholkar, 1997).

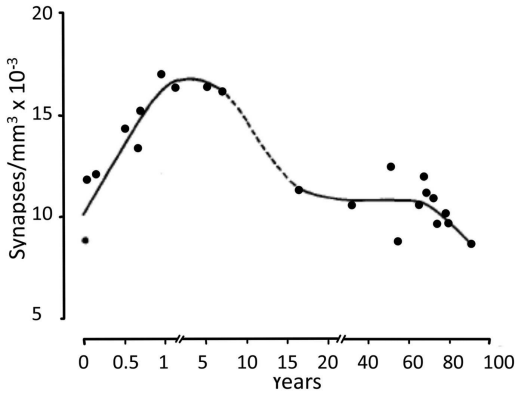


**FIGURE 11 | Developmental changes in synaptic density in frontal gyrus, peaking at 12–15 months of age, and before or during adolescence decreasing to similar levels as in the newborn**. From Huttenlocher (1979).

Since the early postnatal rat brain functions well on the much smaller budget and without astrocytic participation in glutamate and GABA biosynthesis, one may ask which advantages could be associated with the developing dependence on astrocytic functions. One potential answer may be enhanced precision and more autonomy from the periphery. The changes may affect mental function, including learning. The day-old chick is a precocious animal. Its brain contains glycogen, and both glycogenolysis and glutamate formation, events occurring during glutamate production in the combined neuronal–astrocytic system, are essential for learning of a one-trial aversive memory task (Gibbs et al., 2007). Moreover, learning is inhibited by a glial-specific metabolic inhibitor (Ng et al., 1992). In this task the bird learns to associate a specific color on an artificial bead tainted with an aversively tasting compound and as a result later refuses to peck at beads of this color, even when untainted. Noradrenaline, released from locus coeruleus, acts mainly on astrocytes during learning in the day-old chick (Gibbs et al., 2008), but in the non-precocious newborn rat pups odor learning occurs via a direct noradrenaline effect on the neuronal mitral cells of the olfactory glomerulus (Wilson and

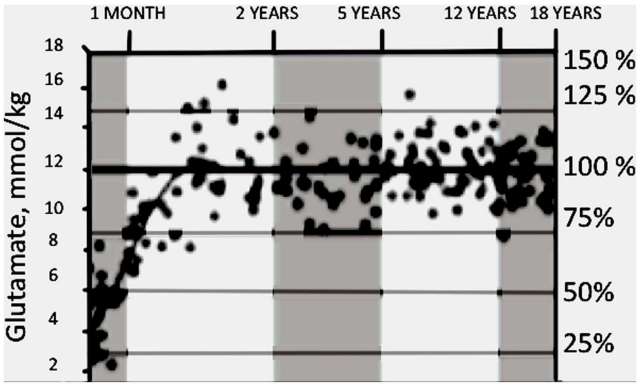


**FIGURE 12 | Developmental changes in glutamate content in parietal/occipital gray matter measured by MRI in young children.** On percentage scale 100% refers to ages between 2 and 18 years. From Blüml et al. (2013).

Sullivan, 1991). In odor learning, pairing with an aversive stimulus has no negative effect until postnatal day 10–12 (Haroutunian and Campbell, 1979; Camp and Rudy, 1988; Raineki et al., 2010). This coincides with incipient glutamine–glutamate (GABA) cycle function in the brain. It would be interesting to know if disruption of glutamate production, and thus of glutamine supply to neurons, by the inhibitor of glycogenolysis DAB would prevent the effect of pairing with an aversive stimulus, but not odor learning as such. Moreover, in the chick learning task, memory formation can also be inhibited by pharmacological disruption of astrocytic gap junction permeability, and trafficking of glutamine through the astrocytic syncytium might play a role in connecting the visual stimulus with the aversive gustatory signal. Would an inhibitor of astrocytic gap junction affect odor learning as such and odor learning paired with an aversive stimulus differently in 12-day-old rats?

In *human* brain cortex neurogenesis is also completed at birth in most brain regions (Bhardwaj et al., 2006), but as in many other animals, cortical gliogenesis occurs peri- and postnatally (Marn-Padilla, 2011). Recently glutamate content of different brain areas was studied in human children of different ages by magnetic resonance spectroscopy (MRS) in several brain regions (Blüml et al., 2013). Consistent with the late gliogenesis, the content of glutamate quadrupled between birth and 2 years of age (**Figure 12**) and subsequently remained stable at the adult value of about 12 mmol/kg. About one half of the change occurred during the first 3 months of life and most of the rest between 3 and 12 months of age.

Human learning *may* also provide a hint of functional gains that are time-wise, and *perhaps* causally, related to the change from a purely neuronal cerebral cortex to a cerebral cortex with the costly neuronal–astrocytic interaction, such as the glutamine–glutamate (GABA) cycle. Although disagreeing about mechanisms involved, both Rovee-Collier and Giles (2010) and Bauer and Nelson (Bauer et al., 2003; Bauer, 2006) similarly describe how early-maturing memory functions support gradual and exuberant learning of perceptual and motor skills, but memories are fragile and short-lived, as exemplified by our inability to recall events from early life. Only later-maturing modification(s) can support long-lasting representations of contextually specific events, relationships, temporal orders, and associations. Bauer (2006, 2008) as well as Nelson (1995) consider these differences as due to the operation of two different memory systems, implicit and explicit, with implicit memory being the unconscious memory function involved in motor skills. The development of explicit memory may occur via a pre-explicit system, and Nelson (1995) suggested that the development of explicit memory from implicit and pre-explicit memory may be associated with recruitment of additional specific brain structures. The simultaneous development and maturation of astrocytic functions and glutamine–glutamate (GABA) cycle operation together with the importance of astrocytic metabolic processes for aversive learning in the chick brain might suggest that the newly established, ubiquitous metabolic interactions between neurons and astrocytes could also play an important role in human learning.

## CONCLUDING REMARKS

The importance of the neuronal–astrocytic glutamine–glutamate (GABA) cycle in cerebral cortex is shown by the magnitude of this flux, equaling total rate of neuronal glucose oxidation. Although 85% of the cycle "only" serves to return previously released neurotransmitter glutamate and GABA, astrocytes contribute actively by accumulating the transmitters and converting them to glutamine, which can travel through gap junction-coupled astrocytes before its release. The astrocytic participation in the remaining 15% of the flux is even greater. They maintain an equilibrium between astrocytic biosynthesis and oxidative degradation, which perhaps also is astrocytic and is capable of establishing net synthesis or net degradation according to glutamate needs. The requirement of aspartate for maximum glutamate synthesis indicates that glutamate synthesis from α-ketoglutarate probably occurs by transamination, and pathway models for interaction between biosynthesis and oxidative degradation of both glutamate and GABA suggest that transamination may also be the default reaction for initiation of glutamate oxidation. Whether or not glutamate oxidation mainly occurs in astrocytes is perhaps the most important question, both for the viability of the suggested models (**Figures 3B** and **7**) and for the functional importance of the glutamine–glutamate (GABA) cycle. Although the late development and maturation of the cycle has been realized for more than 40 years, the full consequences of this late and profound alteration in brain metabolism remain to be fully understood. They represent a development from purely neuronal processes to integrated neuronal and astrocytic activities, which may be functionally extremely important in brain function, including human mental activities. In practical terms it also means that mature brain function should not be investigated until full maturation has occurred, which in mice and rats is at a postnatal age of 3–4 weeks.

## ACKNOWLEDGMENT

Mr. Goncalo Vargas, Medicine Production Office, Frontiers is thanked for his great help with the Figures.

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**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

*Received: 28 March 2013; accepted: 02 May 2013; published online: 27 May 2013.*

*Citation: Hertz L (2013) The glutamate–glutamine (GABA) cycle: importance of late postnatal development and potential reciprocal interactions between biosynthesis and degradation. Front. Endocrinol. **4**:59. doi: 10.3389/fendo.2013.00059*

*This article was submitted to Frontiers in Cellular Endocrinology, a specialty of Frontiers in Endocrinology.*

frontiers in
ENDOCRINOLOGY

REVIEW ARTICLE
published: 28 October 2013
doi: 10.3389/fendo.2013.00156

# Metabolic flux and compartmentation analysis in the brain *in vivo*

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Through significant developments and progresses in the last two decades, *in vivo* localized nuclear magnetic resonance spectroscopy (MRS) became a method of choice to probe brain metabolic pathways in a non-invasive way. Beside the measurement of the total concentration of more than 20 metabolites, $^{1}$H MRS can be used to quantify the dynamics of substrate transport across the blood-brain barrier by varying the plasma substrate level. On the other hand, $^{13}$C MRS with the infusion of $^{13}$C-enriched substrates enables the characterization of brain oxidative metabolism and neurotransmission by incorporation of $^{13}$C in the different carbon positions of amino acid neurotransmitters. The quantitative determination of the biochemical reactions involved in these processes requires the use of appropriate metabolic models, whose level of details is strongly related to the amount of data accessible with *in vivo* MRS. In the present work, we present the different steps involved in the elaboration of a mathematical model of a given brain metabolic process and its application to the experimental data in order to extract quantitative brain metabolic rates. We review the recent advances in the localized measurement of brain glucose transport and compartmentalized brain energy metabolism, and how these reveal mechanistic details on glial support to glutamatergic and GABAergic neurons.

**Keywords: brain energy metabolism, neurotransmitter metabolism, neurotransmission, mathematical modeling, MRS**

## INTRODUCTION

Localized magnetic resonance spectroscopy (MRS) is a powerful tool to investigate brain metabolism *in vivo*. MRS detection of $^{1}$H nuclei is widely employed because it takes advantage of this being the most sensitive nucleus in nuclear magnetic resonance (NMR). At high magnetic field it allows detection of a neurochemical profile of about 20 metabolites, particularly glucose, lactate, alanine, glutamate, glutamine, and aspartate, which are involved in energy metabolism and neurotransmission (1). Similarly, MRS of $^{31}$P provides a way of detecting non-invasively the phosphorus-containing metabolites, including the high energy phosphate compounds ATP and phosphocreatine, whose peaks can be used to determine the rate of creatine kinase that composes a brain's energy buffering system [e.g., Ref. (2)]. NMR detects the non-radioactive, stable isotope $^{13}$C that occurs at a natural abundance of 1.1%, while the most abundant carbon isotope is $^{12}$C (98.9%), a NMR inactive nucleus. Nevertheless, the low natural abundance of $^{13}$C becomes an advantage when $^{13}$C-enriched substrates are administered and the rates of isotopic incorporation into specific carbon positions within different brain metabolites are dynamically detected (3–7). Upon employment of adequate mathematical models describing brain metabolism, this $^{13}$C incorporation rates can then be used to estimate fluxes through important metabolic pathways.

**Abbreviations:** ATP, adenosine-5′-triphosphate; FE, fractional enrichment; fMRI, functional magnetic resonance imaging; MRS, magnetic resonance spectroscopy; NMR, nuclear magnetic resonance; TCA, tricarboxylic acid.

The application of dynamic $^{13}$C MRS to the study of brain energy metabolism and its coupling to neurotransmission has provided important insights on mechanisms supporting brain function but it also raised controversy on modeling approaches, metabolic assumptions in the models, and some extracted results. The significant developments on NMR technology over the last decade provided increase sensitivity and $^{13}$C MRS is now being performed with substantial improvements in spectral, spatial, and/or temporal resolution (8–11). This high quality data allowed determination of metabolic fluxes with better precision, but also made discrepancies between fitted curves and experimental data more apparent, suggesting that current models of brain metabolism lack a number of metabolic features necessary to fit experimental data (10, 12).

This review covers the approaches to design mathematical models of brain metabolism that allow quantification of metabolic fluxes from MRS data. Progress and controversies in the realm of brain metabolic modeling will be discussed. At last, we will discuss how $^{13}$C MRS data acquired at high-field support: (1) the role of glial metabolism in sustaining glutamatergic and GABAergic neurotransmission and (2) the coupling of the malate-aspartate shuttle with mitochondrial metabolism through the TCA cycle.

## DESIGNING A METABOLIC MODEL

An essential step to link the measured labeling time courses with biochemical quantities is the analysis with a metabolic model to derive quantitative metabolic fluxes. The most common models are the so-called multi-compartmental models.

A compartment is defined as an idealized store of molecules that exhibit the same behavior in a tracer experiment. Examples of compartments are the neuronal and glial compartments used in the $^{13}$C MRS modeling of brain glucose metabolism. In each compartment, molecules are physically or kinetically separated in metabolite pools. In the model, these are also called labeling pools and represent the major chemical intermediates involved in the metabolism of a particular substrate (e.g., glucose, acetate).

In $^{13}$C MRS experiments, for example, all isotopomers (i.e., isomers of isotope atoms) of a particular chemical species can appear in a metabolic pool, which in turn can be located in different compartments or physical environment (13). The number of labeling pools is reduced by lumping the chemical pools with similar characteristics and behaving identically into a limited set of pools.

The objectives of a metabolic model are the following:

1. identification of the structure of the system (pools and fluxes)
2. estimation of internal metabolic parameters
3. prediction of the response of the model to external factors

Briefly, a labeling pool that represents a molecule $P$ labeled at a chemical position $x$ is characterized by the total concentration $P$ of the considered molecule, the concentration $P_x^*$ of those molecules labeled at the position $x$ as well as by the set of inflows and outflows of the pool (**Figure 1**). The concentrations are usually expressed in micromoles per gram (μmol/g) of tissue, while the fluxes are given in micromoles per gram per minute (μmol/g/min).

For a given product $P$, the variation of total concentration of the product follows the mass balance equation (7, 13):

$$\frac{dP(t)}{dt} = \sum_i^n V_i^{\text{in}} - \sum_j^m V_j^{\text{out}} \tag{1}$$

This equation is a mathematical expression of the fact that the variation of the quantity of molecules in the pool is the difference between what is entering the pool at a given time and what is exiting. In this general case, we consider $n$ influxes and $m$ effluxes. A simplification is often made by assuming that the biochemical system is at metabolic steady-state. In this case, the total concentration of the labeling pools as well as the metabolic fluxes between the pools are assumed constant over the duration of the measurement, which is a good approximation in many physiological conditions, for example for the glutamate and glutamine labeling pools in the case of [1-$^{13}$C] or [1,6-$^{13}C_2$]glucose infusion (7, 14). At steady-state,

$$\sum_j^n V_i^{\text{in}} - \sum_j^m V_j^{\text{out}} = 0 \tag{2}$$

The labeling dynamics of a metabolic pool $P$ is governed by the isotope balance equation, which determines how the concentration of labeled molecules changes over time, as a function of the influxes and effluxes:

$$\frac{dP_x^*(t)}{dt} = \sum_i^n V_i^{\text{in}} \frac{S_i^*(t)}{[S_i]} - \sum_j^m V_i^{\text{out}} \frac{P_x^*(t)}{[P]} \tag{3}$$

where we assumed a general elementary unit $P$ of a metabolic model, as presented in **Figure 1**. The pool $P$ has $i$ influxes from $i$ substrates $S_i$ (with total concentration $[S_i]$ and labeled concentration $S_i^*(t)$) and $j$ outfluxes. The terms $S_i^*(t)/[S_i]$ and $P^*(t)/[P]$ can be understood in a probabilistic way as the probability that a molecule leaving the substrate pool $S_i$ is labeled and the probability that a molecule leaving the product pool is labeled, respectively. These dimensionless terms are called fractional enrichment (FE) and vary between 0 and 1.

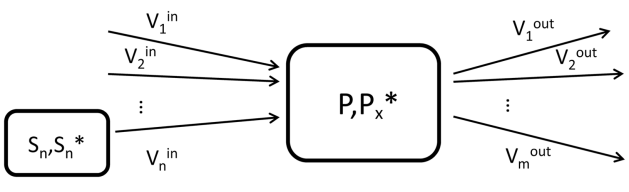


**FIGURE 1 | Schematic view of the elementary unit (labeling pool) used in compartment modeling**. $P$ is the total concentration of molecules in the labeling pool, while $P_x^*$ represents the concentration of labeled molecules at the molecular position $x$. In the general situation, the pool can have n influxes from $n$ substrate pools $S_n$ and $m$ outfluxes.

## MODEL FOR A SINGLE METABOLIC POOL

In many $^{13}$C labeling studies, the protocol of glucose infusion (bolus followed by a continuous infusion) is chosen so that the pyruvate enrichment has a shape close to a step function (7). The pyruvate enrichment is therefore approximated by:

$$\begin{cases} \frac{S^*(t)}{S} = 0 \text{ for } t < 0 \\ \frac{S^*(t)}{S} = \text{constant for } t \geq 0 \end{cases} \tag{4}$$

The time course of the precursor is called the input function and plays the role of a boundary condition of the system of ordinary differential equations. Since metabolic modeling is governed by linear differential equations, this will typically lead to exponential or multi-exponential solutions. If the input function is not a step function, then the exponential solutions are convoluted with the input function, which makes the system more complicated to analyze. In the case of bolus injections, which are widely used in positron emission tomography (PET) labeling experiments, a good knowledge of the exact shape of the input function is not always achievable, which can significantly affect the precision and accuracy of the derived fluxes (15). The mass balance equation for the one-product pool presented in **Figure 2** is given by:

$$\frac{dP(t)}{dt} = V_{\text{in}} - V_{\text{out}} \tag{5}$$

The labeling equation for the one-product pool is given by:

$$\frac{dP^*(t)}{dt} = V_{\text{in}} \frac{S^*(t)}{[S]} - V_{\text{out}} \frac{P^*(t)}{[P]} \tag{6}$$

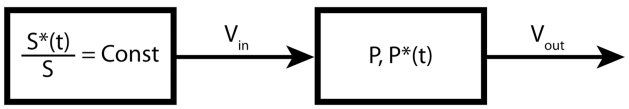


**FIGURE 2 | One-product pool model**. In this example, the substrate pool is forced to maintain a constant fractional enrichment.

The general solution of $P^*(t)$ is:

$$P^*(t) = \int_0^t V_{\text{in}} \frac{S^*(t')}{[S]} \exp\left(\frac{V_{\text{out}}}{[P]}(t'-t)\right) dt' \quad (7)$$

The integral expression in Eq. 7 is a convolution product in the sense of the Laplace transform. In fact, Eq. 7 is the convolution of the input function $S^*(t')/[S]$ with the impulse response of the system (in our case the metabolic system). With a steady-state enrichment of the precursor, i.e., with a step function as input, Eq. 7 simplifies to:

$$P^*(t) = \int_0^t V_{\text{in}} C_{\text{FE}} \exp\left(\frac{V_{\text{out}}}{[P]}(t'-t)\right) dt' \quad (8)$$

where $C_{\text{FE}}$ is the constant fractional enrichment of the precursor.

Using the mass balance Eq. 5 in metabolic steady-state conditions (no net change in total concentration), we have $V_{\text{in}} = V_{\text{out}}$. The solution for the fractional enrichment of the product pool is therefore given by:

$$\frac{P^*(t)}{[P]} = C_{\text{FE}}\left(1 - \exp\left(-\frac{V_{\text{out}}}{[P]}t\right)\right) \quad (9)$$

This is a typical expression for the labeling turnover of a pool with a step input function. In the field of linear differential systems, it is called the step response of the system. It is interesting to mention that the slope of this exponential curve at $t = 0$ is equal to $C_{\text{FE}}\, V_{\text{out}}/[P]$ (in this simple case, we have $V_{\text{in}} = V_{\text{out}}$).

### MONTE CARLO SIMULATIONS FOR TESTING THE RELIABILITY OF FLUX ESTIMATIONS

Given the complexity of metabolic models, it is essential to ascertain that the analysis is robust and that the fit is not unstable, a situation that happens typically when a model is described with too many degrees of freedom compared to the available experimental data (7). Estimates of the standard deviation of the fitted parameters can be obtained from the fitting algorithm, by the calculation of the covariance matrix. The estimation of the covariance matrix by the calculation of the information matrix (16) is related to several assumptions on the variance of the data. However, for many MRS experiments, noise is not vanishingly small but frequently on the order of 10–30% of the steady-state labeling intensity. It was shown that the distribution of the fitted parameters can deviate strongly from the usually admitted normal distribution that is assumed by the fitting algorithms (6), which can lead to misinterpretation of the results and to false conclusions.

One solution to the problem of non-negligible noise is the implementation of Monte Carlo simulations (17, 18). In principle, Monte Carlo approaches are simpler than analytical approaches but more computationally expensive. The objective of Monte Carlo simulations is to obtain the probability distribution of each estimated parameter. Briefly, the model is first fitted to the experimental data to obtain the most probable estimate of the model parameters, using non-linear regression. This set of parameters is then used to generate a "perfect" noise-free dataset, by simulating the enrichment curves with the metabolic model. This "perfect" dataset can be subtracted from the experimental data to estimate the noise level in each of the experimental labeling curves. In a second step, noise with the same distribution as the experimental noise is randomly added to each labeling curve of the "perfect" dataset to create a simulated noisy dataset. Finally, the model is fitted to this new artificial data to estimate the metabolic rates (**Figure 3**).

The process of generating and fitting artificial noisy datasets is repeated several hundred times to generate a list of simulated fitted parameters that are used to create a histogram of the distribution of the estimated parameter values. For each parameter, this histogram not only gives an estimation of its standard deviation but also information about the dissymmetry of its probability distribution (6, 7). The more Monte Carlo iterations are performed, the more accurate the probability distribution will be. In general, the initial guess for the estimated parameters used in the non-linear regression is also varied from one to the next Monte Carlo iteration. This avoids the optimization process converges to a local minimum.

In $^{13}$C MRS experiments, a set of $^{13}$C time courses is obtained from each subject and can be analyzed to obtain individual metabolic rates and their variation across the sample of the selected population. However, since it is known that the accuracy in flux estimation is inversely proportional to the noise level of experimental data, i.e., increases with reduction in the noise of $^{13}$C enrichment curves (19), $^{13}$C enrichment curves are often averaged across all subjects rather than fitting individual time courses. These averaged curves are then used for mathematical modeling and determination of metabolic fluxes. In this case, the variance across subjects can only be inferred from experimental $^{13}$C time courses, while the uncertainty of estimated fluxes is provided by Monte Carlo analyses.

## ONE-COMPARTMENT MODEL OF BRAIN ENERGY METABOLISM

In the past two decades, $^{13}$C MRS labeling studies raised a strong interest for the study of brain energy metabolism. Early studies were essentially undertaken by infusion of [1-$^{13}$C]glucose (14, 20–22) and were analyzed with one-compartment models of brain metabolism.

The one-compartment model was the first metabolic model proposed to fit the glutamate C4 enrichment curves obtained following the infusion of [1-$^{13}$C]glucose (20, 22). In this model, no distinction is made between neuronal and glial cells. Since most of glutamate is located in the neuronal compartment, the one-compartment model has been assumed to reflect primarily the neuronal TCA cycle rate. This model, depicted in **Figure 4**, enables

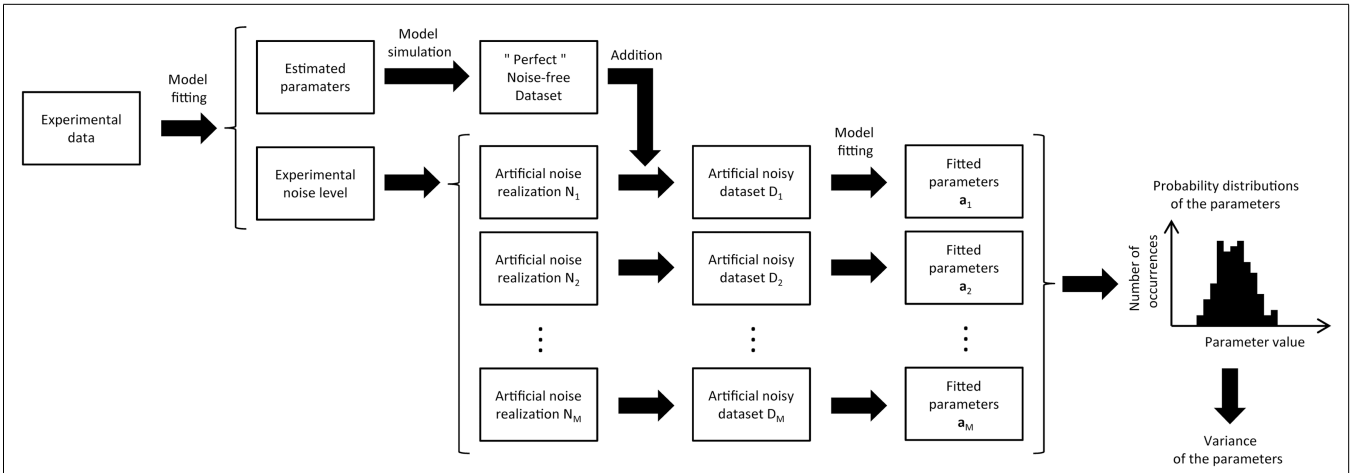


**FIGURE 3 | Schematic view of the Monte Carlo analysis used in metabolic modeling to estimate the variance of the adjusted parameters**. The metabolic model is first fitted to the experimental data, giving an optimal parameter set and the related model turnover curves that best describe the data. From the fit residual, the noise level of the different turnover curves is estimated. Artificial datasets (typically several hundreds) are then generated by adding different noise realizations to the turnover curves obtained from the best fit. For each turnover curve, the artificial noise realizations are generated with the same noise level as in the corresponding experimental curves. Finally, the metabolic model is successively fitted to all the synthetic datasets, resulting in a distribution of fitted values for every free parameter of the model. This distribution characterizes the precision of each adjusted parameter, taking directly into account the experimental noise level.

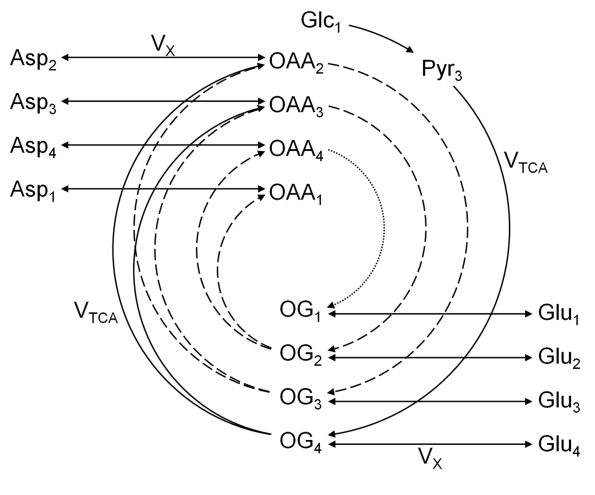


**FIGURE 4 | Scheme of the one-compartment TCA cycle model**. The one-compartment model is characterized by the total TCA cycle flux $V_{TCA}$ and the transmitochondrial flux $V_X$. The splitting of the arrows after 2-oxoglutarate (OG) represents the symmetry at the succinate level. Glc, Pyr, OG, Glu, OAA, and Asp stand for glucose, pyruvate, 2-oxoglutarate, glutamate, oxaloacetate, and aspartate, respectively. The indexes represent the carbon positions that get labeled in each metabolite. The first, second, and third turns of the TCA cycle are represented by solid, dashed, and dotted lines, respectively. The model presented here corresponds to a [1-$^{13}$C]glucose or [1,6-$^{13}C_2$]glucose infusion experiment.

the measurement of the overall (glial and neuronal) TCA cycle rate and the transmitochondrial flux $V_X$, summarizing the glutamate to 2-oxoglutarate conversion and its transport across the mitochondrial membrane.

[1,6-$^{13}C_2$]Glucose is a widely used NMR tracer to probe mitochondrial metabolism. After transport across the blood-brain barrier (BBB), two molecules of [3-$^{13}$C]pyruvate are generated from one molecule of labeled glucose through the glycolysis. When infusing [1-$^{13}$C]glucose, only one molecule of [3-$^{13}$C]pyruvate is produced, while the second pyruvate molecule generated by the glycolysis is unlabeled. The fate of the labeling from pyruvate C3 in the one-compartment model is depicted in **Figure 4**. Briefly, $^{13}$C from [1-$^{13}$C] or [1,6-$^{13}C_2$]glucose enters both glial and neuronal TCA cycles at the position C4 of citrate. In the first turn of the TCA cycle, $^{13}$C reaches the position C4 of 2-oxoglutarate, which exchanges label with cytosolic glutamate. This transmitochondrial label exchange transfers label from the carbon position C4 of 2-oxoglutarate to the position C4 of glutamate. Due to the symmetry of the succinate molecule, the second turn of the TCA cycle brings half of the labeled carbons of the position C4 of 2-oxoglutarate to the position C3 of 2-oxoglutarate and the other half to the position C2 of 2-oxoglutarate. Through the transmitochondrial exchange, [3-$^{13}$C]glutamate is formed from [3-$^{13}$C]2-oxoglutarate and [2-$^{13}$C]glutamate from [2-$^{13}$C]2-oxoglutarate. In the third turn of the TCA cycle, half of the labeled carbons of the position C3 of 2-oxoglutarate reach the position C2 of the same molecule, while the other half remains at the position C3. At the same time, $^{13}$C from the position C2 of 2-oxoglutarate is transferred to the position C1, labeling further the position C1 of glutamate. The carboxyl position C1 of glutamate is usually not simultaneously measurable with the positions C4, C3, and C2 using $^{13}$C MRS, due to the large chemical shift of the C1 carbon position compared to the other resonances (23).

In some cases, the neurotransmission process is modeled in the one-compartment model in a simplified way by a glutamine exchange rate $V_{Gln}$ (22, 24). Together with this flux, additional dilution of $^{13}$C enrichment at the level of glutamate from unlabeled glutamine was used to allow different fractional enrichments in glutamate C4 and C3 at steady-state, as observed

experimentally. Although devoid of major effects in modeling $^{13}$C data from short experiments where isotopic steady-state is not reached, the inclusion of $V_{\mathrm{Gln}}$ and dilution from glutamine are crucial for reliable determination of $V_{\mathrm{TCA}}$ and $V_X$ in one-compartment modeling. This model was employed in recent studies to estimate fluxes from $^{13}$C curves of glutamate C4 and C3 (25–28).

Due to its intrinsic low sensitivity, $^{13}$C MRS only enables the measurement of metabolites that occur at sufficiently high concentration (typically >1 mM), most often allowing detection of the labeling positions of glutamate, glutamine, and aspartate. The pool size of oxaloacetate and 2-oxoglutarate is typically on the order of 0.1 μmol/g, while the pool sizes of glycolytic and TCA cycle intermediates are assumed to be small and without effect on the labeling dynamics of the observed amino acids (29). Thus, since the intermediate products of glycolysis and TCA cycle are present in too low concentrations to result in substantial delays in the labeling of the measured amino acids (i.e., small turnover times for these intermediates), the model can be simplified to retain only the labeling pools representing the MRS detectable amino acids and the intermediate pools at chemical branch points, such as oxaloacetate and 2-oxoglutarate. It was recently shown that the intermediate pools can be eliminated from the equations describing the one-compartment model, without affecting the dynamics of the glutamate uptake curves (30).

#### THE $V_X/V_{\mathrm{TCA}}$ RATIO

The labeling of the carbon positions in glutamate is the result of two processes: the TCA cycle ($V_{\mathrm{TCA}}$) and the transmitochondrial exchange ($V_X$). These two fluxes are therefore intrinsically coupled to each other in the labeling equations describing glutamate $^{13}$C turnover. For the sake of argument, we analyze the relation between $V_{\mathrm{TCA}}$ and $V_X$ in the simple one-compartment model with exclusion of aspartate and glutamine (**Figure 4**). After simplification of the TCA cycle intermediates (30), the $^{13}$C labeling curves of the C4 and C3 positions of glutamate ($\mathrm{Glu}_4$ and $\mathrm{Glu}_3$) are given by:

$$\frac{d\mathrm{Glu}_4(t)}{dt} = \frac{V_X \cdot V_{\mathrm{TCA}}}{V_X + V_{\mathrm{TCA}}}\left(\mathrm{FE}(\mathrm{Pyr}_3) - \frac{\mathrm{Glu}_4(t)}{[\mathrm{Glu}]}\right) \tag{10}$$

$$\frac{d\mathrm{Glu}_3(t)}{dt} = \frac{V_X \cdot V_{\mathrm{TCA}}}{(2V_X + V_{\mathrm{TCA}})(V_X + V_{\mathrm{TCA}})} \times \left[V_{\mathrm{TCA}}\,\mathrm{FE}(\mathrm{Pyr}_3) + V_X\frac{\mathrm{Glu}_4(t)}{[\mathrm{Glu}]} - (V_X + V_{\mathrm{TCA}})\frac{\mathrm{Glu}_3(t)}{[\mathrm{Glu}]}\right] \tag{11}$$

where $\mathrm{FE}(\mathrm{Pyr}_3)$ is the enrichment of the C3 position of pyruvate, the direct precursor of the TCA cycle, which is assumed to reach steady-state faster than the glutamate. [Glu] is the total glutamate concentration in the tissue (labeled and unlabeled).

Equation 10 highlights the fact that the labeling of the C4 position of glutamate is a combined effect of the TCA cycle activity and transmitochondrial transport, characterized by a composite flux $V_{\mathrm{gt}}$ (20, 30).

$$V_{\mathrm{gt}} = \frac{V_X \cdot V_{\mathrm{TCA}}}{V_X + V_{\mathrm{TCA}}} \tag{12}$$

The solutions of these equations for a constant precursor enrichment $\mathrm{FE}(\mathrm{Pyr}_3)$ are given by:

$$\mathrm{Glu}_4(t) = \mathrm{FE}\left(\mathrm{Pyr}_3\right)[\mathrm{Glu}]\left(1 - \exp\left(-t\frac{V_{\mathrm{gt}}}{[\mathrm{Glu}]}\right)\right) \tag{13}$$

$$\mathrm{Glu}_3(t) = \mathrm{FE}\left(\mathrm{Pyr}_3\right)[\mathrm{Glu}]\left[1 + \exp\left(-t\frac{V_{\mathrm{gt}}}{[\mathrm{Glu}]}\right) - 2\exp\left(-t\frac{V_{\mathrm{gt}}}{[\mathrm{Glu}]}\frac{(V_X + V_{\mathrm{TCA}})}{(2V_X + V_{\mathrm{TCA}})}\right)\right] \tag{14}$$

The labeling equation of $\mathrm{Glu}_4$ only carries information on the value of $V_{\mathrm{gt}}$. **Figure 5** illustrates the fact that an infinite number of pairs ($V_{\mathrm{TCA}}$, $V_X$), distributed on a hyperbolic curve, result in the same value of $V_{\mathrm{gt}}$. Therefore, when fitting the $\mathrm{Glu}_4$ curve alone, the values of $V_{\mathrm{TCA}}$ and $V_X$ are not separately accessible.

However, the separate determination of $V_{\mathrm{TCA}}$ and $V_X$ is possible when fitting the model also to $\mathrm{Glu}_3$. This is directly related to the fact that the labeling equation of $\mathrm{Glu}_3$ does not depend only on $V_{\mathrm{gt}}$ (Eq. 14). $V_{\mathrm{TCA}}$ and $V_X$ have a distinct role in the labeling dynamics through the dilution term $V_X/(V_X + V_{\mathrm{TCA}})$.

The value of the $V_X/V_{\mathrm{TCA}}$ ratio is still a matter of controversy. In early studies, the value of $V_X$ was reported to be much higher than the value of $V_{\mathrm{TCA}}$ (20, 22, 31, 32). In several following studies, it was therefore assumed that $V_X \gg V_{\mathrm{TCA}}$, which results in a simplification of the model, since in this case, $V_{\mathrm{gt}} = V_{\mathrm{TCA}}$ (see Eq. 12). $V_{\mathrm{TCA}}$ could be therefore directly extracted from the fitting of the $\mathrm{Glu}_4$ curve. However, later *in vivo* and *in vitro* studies (9, 24, 29, 33–35) provided evidence that the value of $V_X$ is on the same order of magnitude as $V_{\mathrm{TCA}}$. In this context, using the assumption of a very large $V_X$ value leads to an underestimation of $V_{\mathrm{TCA}}$ by a factor of two, since for $V_X \cong V_{\mathrm{TCA}}$, we obtain $V_{\mathrm{gt}} \cong V_{\mathrm{TCA}}/2$.

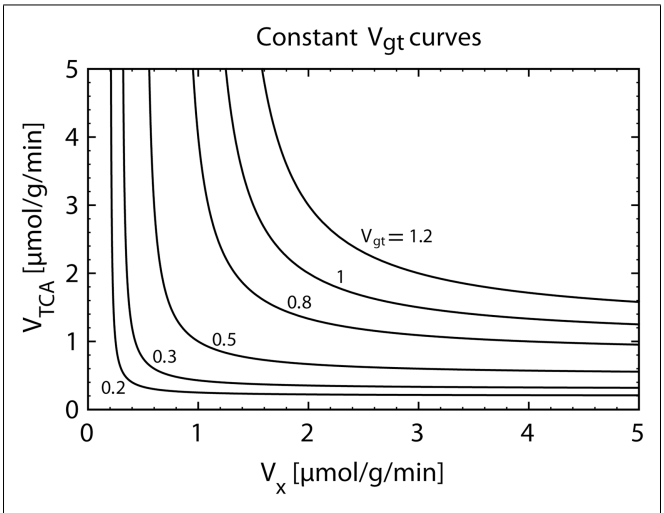


**FIGURE 5 | Plot of the hyperbolic relationship between $V_X$ and $V_{\mathrm{TCA}}$ for a constant composite flux value $V_{\mathrm{gt}}$ (Eq. 12).** The knowledge of $V_{\mathrm{gt}}$ alone only allows the calculation of $V_{\mathrm{TCA}}$ under an assumption for the value of $V_X$. However, the magnitude of the assumed $V_X$ flux can have a strong impact on the estimated $V_{\mathrm{TCA}}$. When assuming $V_X \gg V_{\mathrm{TCA}}$, we have $V_{\mathrm{TCA}} = V_{\mathrm{gt}}$. If $V_X$ is almost equal to $V_{\mathrm{TCA}}$, we obtain $V_{\mathrm{TCA}} \cong 2{\cdot}V_{\mathrm{gt}}$.

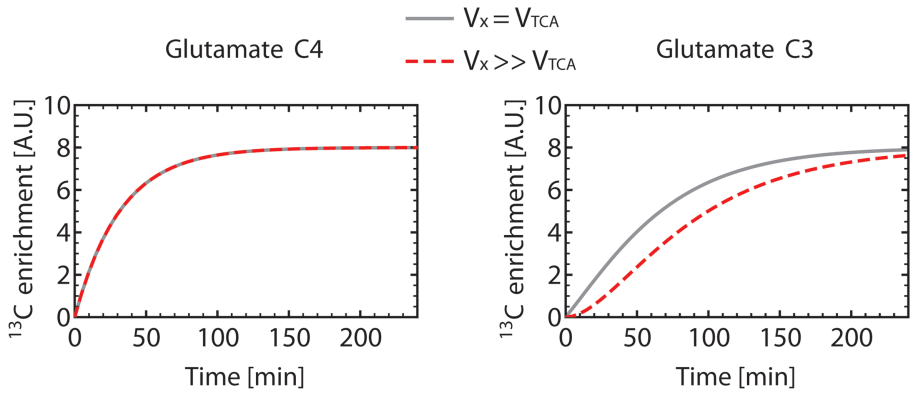


**FIGURE 6 | Turnover curves of the carbon position C4 and C3 of glutamate as simulated using the one-compartment model (Figure 4) with a constant $V_{gt}$.** For the gray turnover curves, $V_X$ was set equal to $V_{TCA}$ ($V_X = V_{TCA} = 0.5\,\mu$mol/g/min), resulting in a non-zero slope of the glutamate C3 curve at $t = 0$. For the dashed red turnover curves, $V_X$ was set much larger (about 200 times) than $V_{TCA}$ ($V_X = 50\,\mu$mol/g/min, $V_{TCA} = 0.251\,\mu$mol/g/min), with the same value for $V_{gt}$. This results in a sigmoidal-shaped glutamate C3 turnover curve, while the glutamate C4 curve remains unchanged. This observation illustrates the necessity to measure the C3 turnover curve to determine both $V_X$ and $V_{TCA}$. In these simulations, dilution at the level of glutamate was not included, resulting in the same enrichment level for C4 and C3 at steady-state.

**Figure 6** shows how $Glu_3$ is affected by the value of the $V_X/V_{TCA}$ ratio. When $V_X \gg V_{TCA}$, the initial slope of $Glu_3$ is zero. When $V_X \cong V_{TCA}$, the $Glu_3$ curve loses its sigmoid shape, presents a non-zero initial slope and, furthermore, reached higher enrichment than for $V_X \gg V_{TCA}$.

## COMPARTMENTALIZED BRAIN ENERGY METABOLISM MEASURED FROM AMINO ACID TURNOVER

Metabolic compartmentation consists in the co-existence of separate pools of a given metabolite that are kinetically different and do not equilibrate rapidly with each other. Compartmentation of cerebral energy metabolism was initially identified by observing that certain radiolabeled tracers could lead to higher enrichment of glutamine than of its precursor glutamate (36, 37). In addition, different metabolic activities were observed in microdissected (38, 39) and cultured (40, 41) neurons and glial cells, and then glia-specific enzymes involved in intermediary metabolism were discovered in the brain tissue, namely glutamine synthetase (42, 43) and pyruvate carboxylase (44). The concept of exchange of metabolites between these compartments was also developed (45, 46) and resulted in the proposal of a glutamate-glutamine cycle linking glutamatergic neurons and astrocytes (47, 48).

Many studies followed and led to a growth of knowledge on the metabolic network underlying the interrelation between neurons and astrocytes. Nevertheless, we are still far from fully understanding the complex regulation of energy metabolism in the living brain. In this realm, the impressive development of localized $^{13}$C MRS *in vivo* since its first application to the head (49) has been much appreciated (4). Currently, the direct detection of $^{13}$C at high magnetic field provides a large amount of specific information regarding pathways of intermediary metabolism. In particular, $^{13}$C MRS is now able to quantify not only the incorporation of $^{13}$C into all aliphatic carbons of amino acids such as glutamate, GABA, glutamine, and aspartate, but also some multiplets resulting from the homonuclear coupling between adjacent carbons of these molecules, i.e., isotopomers (8, 50, 51). Alternatively, specific pathways can be addressed by providing certain labeling patterns to other carbons of glutamate and glutamine through administration of specifically labeled substrates (52). With this substantial increase in the amount of information from $^{13}$C MRS experiments at high magnetic field, higher accuracy has been achieved in the estimation of metabolic fluxes. However it is also becoming evident that state-of-the-art compartmental models of brain energy metabolism are unable to fully describe obtained experimental data (10, 12). Hereafter we describe and discuss the metabolic fluxes included in models of brain energy metabolism and suggest possible directions to improve the description of experimental data, namely by including sub-cellular compartments for particular metabolic pools.

### $^{13}$C MRS STUDIES AND MODELING OF METABOLIC COMPARTMENTATION

When $^{13}$C glutamine was first detected *in vivo* in $^{13}$C glucose infusion studies (21, 53), it became natural to model the neuronal and glial TCA cycles and their interaction through the glutamate/glutamine cycle using a two-compartment model (29, 54), as shown in **Figure 7**.

Distribution of metabolic pools within these major compartments has been assumed based on data collected *in vitro*. It is well established that at least one small and one large glutamate pools exist in the glial and neuronal compartments respectively (55, 56). In contrast, glutamine has been mostly attributed to glia, where it is synthesized, while glutamate resides in neurons. Since $^{13}$C isotopes of the astrocyte-specific substrate acetate lead to labeling of glutamine to a greater extent than glutamate [e.g., Ref. (57)] this assumption seems to be valid. In a recent $^{1}$H-[$^{13}$C] MRS study based on the infusion of glial-specific [2-$^{13}$C]acetate (35), the glutamate pool distribution between the glial and neuronal compartments could be determined directly *in vivo* and supports the presence of a small glial glutamate pool accounting for about 5% of total glutamate.

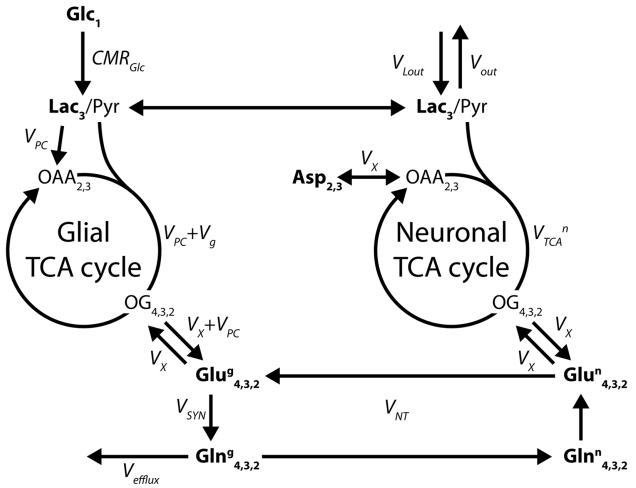


**FIGURE 7 | Two-compartment model of compartmentalized brain metabolism, as proposed by Gruetter et al. (29).** The model consists of the glial and neuronal TCA cycles, linked by the glutamate/glutamine cycle. Glc, glucose; Lac, lactate; Pyr, pyruvate; OAA, oxaloacetate; OG, oxoglutarate; Asp, aspartate; Glu, glutamate; Gln, glutamine. The system is characterized by the following fluxes: $CMR_{Glc}$, cerebral metabolic rate of glucose; $V_{PC}$, pyruvate carboxylase; $V_g$ glial TCA cycle rate; $V_X$, transmitochondrial exchange; $V^n_{TCA}$, neuronal TCA cycle rate; $V_{SYN}$, glutamine synthesis; $V_{NT}$, apparent neurotransmission rate; $V_{efflux}$, loss of glutamine from the glial compartment; $V_{out}$ and $V_{Lout}$, label dilution and exchange of lactate across the blood-brain barrier. The indexes represent the labeled carbon positions. Boldface indicates NMR-measurable metabolites.

However, $^{13}$C MRS measures the sum of all pools of a given metabolite. The detection of multiple compartments in the brain is only possible due to the presence of pyruvate carboxylation and glutamine synthesis in glia but not in neurons (42, 44) that leads to different label distribution in glutamate and glutamine. Pyruvate carboxylase is the main anaplerotic enzyme in the brain (58), leads to $CO_2$ fixation and generates oxaloacetate that can be further used to generate new glutamate molecules. Pyruvate carboxylase brings $^{13}$C from [3-$^{13}$C]pyruvate to the position C3 of oxaloacetate, which further labels the position C2 of 2-oxoglutarate and glial glutamate. On the other hand this reaction brings unlabeled $^{12}$C to the position C3 of glial glutamate. This glial dilution effect is one of the features that make it possible to distinguish glial and neuronal intermediary metabolism using glutamate and glutamine $^{13}$C time courses following infusion of [1-$^{13}$C]- or [1,6-$^{13}C_2$]glucose. In addition, modeling of high-field $^{13}$C MRS data suggested that pyruvate pools may become differently labeled in neurons and astrocytes (10, 59, 60). In line with this, previous reports have frequently introduced glial and neuronal dilution fluxes at the level of lactate to account for this effect (61).

The glutamate-glutamine cycle is a major biochemical pathway *in vivo*, directly involved in the glutamatergic neurotransmission process (47, 48), and results from the compartmentation of glutamine metabolizing enzymes: glutamine synthetase is located exclusively in astrocytes (43), while the glutamate to glutamine conversion through phosphate activated glutaminase (PAG) occurs mostly in neurons (62). Furthermore, most of the glutamate is in the neurons, while glutamine is essentially located in the glial cells (56). Both glutamine and glutamate are 5-carbon chains differing by an amino group at the carbon position 5 of glutamine. In the glutamate-glutamine cycle, the carbon positions are maintained, which means that a carbon located at the position C4 of glutamate will reach the position C4 of glutamine and *vice versa*, and similarly for all positions of glutamate and glutamine.

The adjusted parameters of the two-compartment model (**Figure 7**) are the glial and neuronal TCA cycle fluxes $V_g$ and $V^n_{TCA}$, the transmitochondrial flux $V_X$ that describes the combined effect of glutamate dehydrogenase, aspartate transaminase, and transport across the mitochondrial membranes, the apparent neurotransmission flux $V_{NT}$ and $V_{PC}$, the rate of pyruvate carboxylation in the glia. A dilution $V_{out}$ at the level of pyruvate is generally included to take into account the metabolism of other substrates, such as lactate or glycogen. Moreover, the glial acetyl-CoA, at the entrance of the TCA cycle, is diluted by alternative energetic fuels that glial cells can metabolize, such as acetate and fatty acids (63, 64). Glucose transport across the BBB is usually modeled using a Michaelis–Menten modeling approach (29), although more complex models have been described (65). In more recent *in vivo* studies, the two-compartment description of brain energy metabolism was further used for $^{13}$C labeling experiments using other substrates, such as [2-$^{13}$C]acetate (35, 66, 67) or even adapted to describe the uptake curves measured by $^{11}$C positron emission when infusing [1-$^{11}$C]acetate (68), bridging the gap between two major bioimaging modalities by proposing a common metabolic modeling approach.

Upon administration of $^{13}$C-enriched glucose, glutamine carbons become less enriched than those of glutamate and, to account for this discrepancy, dilution fluxes were placed in glutamine pools accounting for $^{13}$C loss by exchange with unlabeled pools ($V_{ex}$), which can allow different compartment enrichments (33). Alternatively, other modeling studies assume different dilution fluxes at the level of pyruvate in each compartment, i.e., loss of labeling by exchange with unenriched pools of lactate (61). This accounts for possible $^{13}$C dilution by unlabeled brain glycogen or blood-born lactate or alanine.

There is now consensus that the interpretation of $^{13}$C incorporation curves from substrates into brain metabolites is never complete if mathematical models disregard metabolic compartmentation. This is particularly true when the fate of $^{13}$C is measured at high magnetic field where high spectral resolution allows for quantification of $^{13}$C in an increased number of amino acid carbons. Indeed, elegant simulations by Shestov et al. (19) demonstrated that increasing temporal resolution and decreasing noise level in $^{13}$C incorporation curves lead to increased accuracy in metabolic flux estimation. In addition, higher detail can be introduced in compartmentalized models of brain metabolism as more experimental curves are measured and for a longer period (19, 60).

## SUB-CELLULAR COMPARTMENTATION

Increased sensitivity in measuring $^{13}$C enrichment curves provides insight into sub-cellular compartmentation, which has been proposed in a plethora of studies *in vitro* (69–71). Sub-cellular compartmentation has been disregarded in most studies *in vivo*.

As explained above, carboxylation of [3-$^{13}$C]pyruvate via PC labels C2 and dilutes C3 in glutamate/glutamine, resulting in observed relative enrichments of C4 > C2 > C3. In $^{13}$C MRS experiments at high magnetic field, with infusion of [1,6-$^{13}C_2$]glucose, glutamine enrichment in C2 was similar to that in C4 when approaching isotopic steady-state (9). The enrichment of glutamine C2 approaching that of the C4 carbon is consistent with high PC relative to PDH or with glial-specific dilution of acetyl-CoA, or a combination of both effects. Because metabolic modeling shows that such labeling patterns cannot be fully explained by pyruvate carboxylation, an additional glial-specific dilution flux $V_{dil}$ has been introduced (9) to represent the utilization of glial-specific substrates that enter brain metabolism at the level of acetyl-CoA in astrocytes but not in neurons. The rate of $V_{dil}$ was in the range of the rate of utilization of acetate *in vivo* (67, 72). This further ensures that pyruvate carboxylase and pyruvate dehydrogenase can carry different enrichment levels from pyruvate into the TCA cycle, as if distinct substrate sources would feed each pathway. This is also consistent with the presence of at least two distinct pyruvate pools in the glial compartment.

In cultured neurons, simultaneous incubation with [1,2-$^{13}C_2$]glucose and [3-$^{13}$C]lactate revealed the existence of at least two cytosolic pools of pyruvate that do not equilibrate rapidly (73). While one of the pools was derived from glycolysis, the other was associated to lactate metabolism. Also in cultured astrocytes, labeling of alanine from $^{13}$C-enriched glucose has been observed to be lower than that of lactate, suggesting sub-cellular compartmentation of pyruvate (34, 74). One of the pyruvate pools could be specifically formed from TCA cycle intermediates. Indeed, in cultured astrocytes, the decarboxylation of malate by malic enzyme can represent a small fraction of the total pyruvate synthesis. This pyruvate formed via malic enzyme must then re-enter the TCA cycle to be oxidized, thus completing the pyruvate recycling pathway. This pathway has been proposed by $^{13}$C MRS of extracts from studies *in vitro* (34, 75–77) and *in vivo* (57, 78). This pathway is likely to be more active when cells need to dispose of glutamate and glutamine by oxidative degradation (77), which is an alternative to glutamine efflux from the brain that is generally modeled in $^{13}$C MRS data *in vivo* ($V_{efflux}$, equivalent to $V_{PC}$). The latter is however a recognized mechanism for ammonia disposal (79, 80).

However, if pyruvate recycling would be the main form of losing a four carbon intermediate from the TCA cycle to balance glutamate oxidation in the astrocyte, additional peaks would be observed in glutamate C4 in the $^{13}$C NMR spectrum. As discussed in Duarte et al. (9), while [1,6-$^{13}C_2$]glucose originates pyruvate labeled in C3, pyruvate recycling (coupled to glutamate oxidation) would generate pyruvate labeled in C2 or simultaneously in C2 and C3, which via pyruvate dehydrogenase originates 2-oxoglutarate and glutamate labeled in C5 or simultaneously C4 and C5. These were not detectable *in vivo* (9). Furthermore, labeling from pyruvate C2 is incorporated in lactate C2. In brain extracts at the end of the experiment, FE of brain lactate C2 was at least 20 times smaller than C3. Since this labeling patterns could also be observed in plasma lactate, probably resulting from peripheral metabolism rather than brain release, there is no convincing evidence that glutamate oxidation and pyruvate recycling are measurable *in vivo* upon infusion of [1,6-$^{13}C_2$]glucose (9, 12).

In addition, when steady-state of $^{13}$C enrichment was reached for carbons of glutamate and aspartate that have major pools in neurons, a significant and continuous increase in glutamine enrichment occurred (9, 12). This could be caused by increase of total glutamine concentration during glucose infusion, as was observed under hyperammonemia (81). However, brain glutamine concentration was not altered in similar experimental conditions (82, 83). While pyruvate formation from four carbon TCA cycle intermediates and further carboxylation could eventually explain such effect in C3 and C2, glutamine C4 only receives labeling from acetyl-CoA and thus should reach the same steady-state enrichment as carbons in the neuronal compartment (where PC is absent) and with which it exchanges via glutamate-glutamine cycle. This continuous increase in brain glutamine enrichment upon $^{13}$C-enriched glucose administration was observed in studies using dynamic $^{13}$C MRS *in vivo* in both rodents (9, 10, 12) and humans (84), and suggests that the existent mathematical models of brain metabolism are incomplete and may particularly benefit from inclusion of features like sub-cellular metabolic compartmentation or a multitude of astrocytes with heterogeneous metabolic rates. Accordingly, astrocyte morphology is compatible with the existence of different functional domains [reviewed by Pellerin and Magistretti (85)], there is mitochondrial heterogeneity and glutamine synthesis from multiple glial TCA cycles (69, 71) and, in cultured astrocytes, intracellular, and released glutamine were found to display distinct labeling patterns from metabolism of $^{13}$C-enriched glucose and lactate (70).

Upon this evidence supporting the existence of multiple glial glutamine pools, in our most recent $^{13}$C MRS study (12), two distinct pools of glutamine were modeled within the glial compartment, in addition to the pools in glutamatergic and GABAergic neurons. One of these glial glutamine pools was non-metabolizable and was in direct exchange with the other that was involved in the glutamate-glutamine cycle. Although this substantially improved fitting to the experimental data, the additional glutamine pool could as well be introduced in any other metabolic compartment and provide similar approximation to the experimental data. This was however a simple approach to tackle sub-cellular compartmentation in astrocytes. Although a more realistic model would rather include more than one glial compartment, the increasing number of unknown variables (fluxes) in the model would lead to increased uncertainty in flux estimation.

The inclusion of a vesicular glutamate pool in the neuronal compartment has also been proposed and seems to improve fitting of $^{13}$C multiplet data and increase accuracy in $V_{NT}$ estimation (60). However, since this pool is actively involved in the glutamate-glutamine cycle, it does not allow for different curve shapes for the labeling of glutamate and glutamine.

## METABOLIC PATHWAYS COUPLED TO BRAIN ACTIVITY

Over the last two decades, systems neuroscience was revolutionized by blood oxygen level dependent (BOLD) functional magnetic resonance imaging (fMRI). BOLD fMRI measures the global hemodynamic response, i.e., changes in local cerebral blood flow, volume, and oxygenation, that are related to neuronal activity in the brain. Although the underlying mechanisms of cerebral hemodynamic control remain to be firmly established, the link between

the modifications in neuronal activity and the observed hemodynamic response is known to require metabolic involvement of a multitude of cells, including astrocytes. In fact, due to the particular cytoarchitectural relation to both neurons (forming the tripartite synapse) and blood vessels (mostly arterioles and capillaries), astrocytes are ideally positioned to detect neuronal activity, transmit signals to neighboring vascular cells and regulate supply of energy substrates to neurons. Indeed, the link between cerebral activity and metabolic fluxes for production of energy has been suggested since early studies of brain energy metabolism (45).

#### NEUROTRANSMISSION AND THE RATE OF GLUCOSE OXIDATION

Above isoelectricity, glutamate-glutamine cycle increases ∼1:1 with glucose oxidation that has been proposed to occur mostly in neurons, thus providing coupling between functional neuroenergetics and glutamatergic neurotransmission (32, 86–88). Such analyses have however disregarded glial glucose oxidation, even though conversion of neurotransmitter glutamate to the electrophysiological inactive glutamine in astrocytes involves energy metabolism (89–91). Furthermore, this relation does not appear to stand for GABAergic neurotransmission, which relies on astrocytic oxidation of GABA (12, 87). Therefore, a more complete analysis of the metabolic fluxes involved in sustaining neurotransmission is required.

$^{1}$H MRS has been used to report the time course of brain metabolites, mostly lactate, during focal activation. Increase in lactate concentration was reported upon sustained visual cortex stimulation first by Prichard et al. (92), suggesting a stimulation-induced increase in cerebral metabolic rate of glucose ($CMR_{Glc}$). This observation was further confirmed in humans and rodents (93–97) and suggested to be associated to other metabolic modifications like a decrease in glucose concentration, as detected by *in vivo* $^{1}$H MRS (93, 98, 99), and in phosphocreatine *versus* inorganic phosphate in $^{31}$P MRS experiments (95, 100). In line with this, both glucose transport and consumption were found substantially reduced in the rat brain under isoelectricity (83, 101, 102).

With the increase in sensitivity and spectral resolution at high magnetic field, $^{1}$H MRS in the human visual cortex allowed detection of an increase in glutamate and a putative decrease in aspartate during stimulation, in addition to lactate and glucose modifications (97, 103), suggesting modifications in the flow through the malate-aspartate shuttle, possibly linked to adjustments in redox potential upon increased cerebral glucose consumption. Similar alterations of lactate, glutamate, and aspartate concentrations as well as increased alanine levels have been reported in the cortex of conscious rats upon sensory stimulation (104) and rats under light α-chloralose anesthesia upon trigeminal nerve stimulation (96). However, using *in vivo* $^{1}$H MRS in rats under α-chloralose anesthesia, Xu et al. (105) found that sensorial stimulation could lead to an increase in glutamine and a decrease in glutamate, *myo*-inositol, and phosphocreatine to creatine ratio in the focally activated primary sensory cortex, albeit the modifications in lactate and glucose concentrations upon cerebral activation were not detected. $^{1}$H-[$^{13}$C] NMR spectroscopy studies in rodents upon infusion of $^{13}$C-enriched glucose measured an increased tricarboxylic acid cycle flux ($V_{TCA}$) in focally activated primary sensory cortex during forepaw stimulation (106–108) that is certainly linked to the increased $CMR_{Glc}$ and oxygen $\left(CMR_{O_2}\right)$. Similar observations appeared in studies in humans (109). Altogether, these studies indicate that the BOLD fMRI signal-change is associated with an increase in oxidative metabolism.

#### GLIAL METABOLISM SUPPORTS NEUROTRANSMISSION

However, specific pathways of brain energy metabolism that support neurotransmission remain to be elucidated. Sibson et al. (86) suggested that in $^{13}$C MRS experiments, $V_{NT}$ correlated with the neuronal fraction of $CMR_{Glc}$ above isoelectricity, which has been confirmed in a large number of studies across different laboratories (32, 86–88). Other metabolic pathways may be linked to $V_{NT}$ but remain to be analyzed. **Figure 8** aims at elucidating how energy metabolism is related to both glutamatergic and GABAergic synaptic transmission. In contrast to previous analyses (88), now we exclusively selected $^{13}$C MRS studies in which metabolic modeling was performed with fully independent flux estimation (9, 10, 12, 29, 33, 35, 102). Interestingly this allowed identification of a correlation not only between $V_{NT}$ and glucose oxidation (86, 88) but also between $V_{NT}$ and $V_{PC}$. Oxidative metabolism in neurons was correlated with $V_{NT}$ only when GABAergic compartment is not included in the model. Correlation between glial TCA cycle and $V_{NT}$ was noticeable in rats. Perhaps with additional experimental data sets from the human brain, we could depict a species-dependent relation of glial $V_{TCA}$ to $V_{NT}$.

Since its introduction, the astrocyte-neuron lactate shuttle model (89) has found additional confirmatory experimental data. Nevertheless it remains matter of debate, mostly because it considers that while neuronal metabolism is mainly oxidative, astrocytes exhibit a mainly glycolytic phenotype (85). In other words, glucose and oxygen are mostly consumed in astrocytes and neurons, respectively, and since brain hexokinase operates near it maximum rate assayed *in vivo* (65), only the neuronal TCA cycle can initially respond to the metabolic demand during increased brain activity [even though upon reduction of glucose-6-phosphate levels, there is loss of feedback inhibition and concomitant gain in hexokinase activity (110)].

The analysis in **Figure 8** indicates that changes in glutamatergic neurotransmission, i.e., the glutamate-glutamine cycle, are as well coupled to glial pyruvate carboxylation and glial TCA cycle fluxes. This analysis agrees with simulations by Jolivet et al. (111) predicting that an important fraction of $O_2$ utilization in astrocytes is dedicated to neurotransmission in the living brain. Furthermore, it is interesting to note that the four fluxes depicted in **Figure 8** are significantly different from zero in the brain *in vivo* under isoelectricity ($V_{NT} = 0$), which would be associated to housekeeping tasks and other non-signaling brain processes. Recent estimations of energy budgets for the brain in awake state considered a 25 or 50% of the total energy budget for non-signaling tasks in either the cortical gray matter or the whole brain, respectively (112). If one considers that non-signaling and signaling energy metabolism takes place mostly in glia and neurons respectively, these data agrees with glial oxidative metabolism much larger than 25% of total energy consumption in the case of the whole brain. Thus, energy consumption for tasks unrelated to neurotransmission could account for up to ∼50% of total energy expenditure in

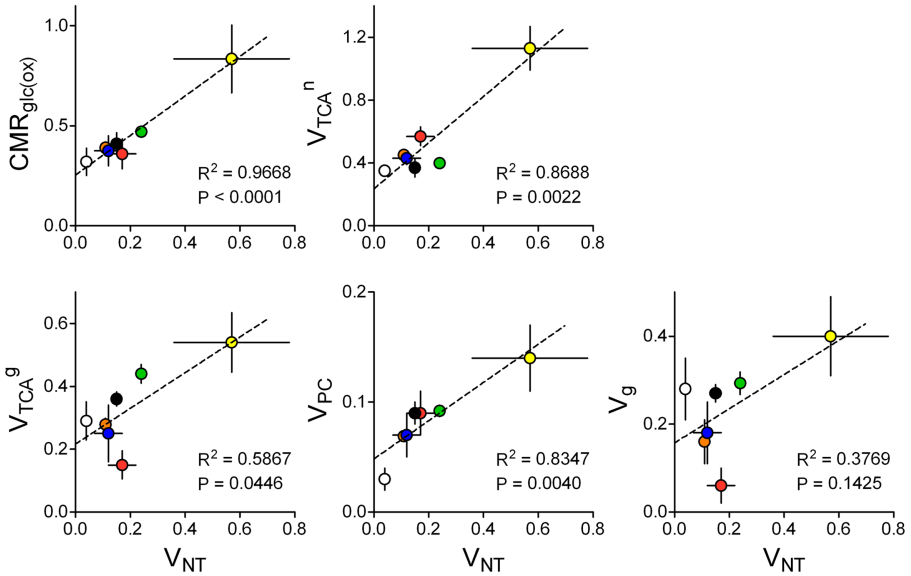


**FIGURE 8 | Relation of estimated metabolic fluxes to glutamate/GABA-glutamine cycle, i.e., glutamatergic plus GABAergic neurotransmission (total VNT), across several studies using similar methodology in the rat or human brain: rat brain under pentobarbital-induced isoelectricity [white; Ref. (102)] and under light anesthesia [orange, blue, green, and black are data from Ref. (9, 10, 12, 35), respectively], human cortex [red; Ref. (29)], awake rat brain [yellow; Ref. (33)].** Metabolic fluxes are shown in micromoles per gram per minute with associated SD. *P*-value for the slope and $R^2$ are shown for each linear regression.

the whole rat brain [discussed by Howarth et al. (112)], and may largely occur in glial cells.

Since only a fraction of $V_{TCA}^{g}$ is associated with neurotransmission $V_{NT}$, the remaining may be associated to housekeeping tasks. In line with this, there is no clear relation between $V_g$ and $V_{NT}$. $V_g$ is a flux accounting for the difference between glial $V_{TCA}$ and pyruvate carboxylase in the two-compartment model (see above). In the models where the GABAergic compartment is included and glial cells are oxidizing GABA, $V_g = V_{TCA}^{g} - V_{PC} - V_{shunt}^{g}$ [see Ref. (12)]. Since $V_{PC}$ matches the efflux of glutamine and $V_{shunt}^{g}$ is equivalent to the shuttling of glutamine to the GABAergic neurons, $V_g$ denotes the flux through glial pyruvate dehydrogenase corresponding to the complete oxidation of pyruvate. Therefore, in mathematical models designed as proposed in this manuscript, one can assume that pyruvate carboxylation and/or GABA oxidation are the main drive for the relation between $V_{shunt}^{g}$ and $V_{NT}$.

Most recent publications reporting multi-compartmental models of brain energy metabolism constrained metabolic fluxes in astrocytes to those in neurons [discussed in Ref. (113)]. In particular, $V_{TCA}$ in astrocytes and/or $V_{PC}$ were systematically constrained as $V_{PC} = 0.2\ V_{GS}$ and $V_{TCA}^{g} = 0.15 \cdot V_{TCA}^{total}$ (61, 114). Other studies determined $V_{TCA}$ in the astrocytic compartment from experiments upon [2-$^{13}$C]acetate infusion and the obtained flux is used as constraint to the metabolic modeling of $^{13}$C-labeled glucose experiments for simultaneous determination of fluxes in glutamatergic and/or GABAergic neurons (115). In contrast, in studies that considered all metabolic fluxes as independent parameters, there was a fairly linear relation between pyruvate carboxylation rate ($V_{PC}$) and the glutamate/GABA-glutamine cycle (**Figure 8**). In fact, the activity-induced increase in glial anaplerosis through $V_{PC}$ is consistent with increased influx of bicarbonate into astrocytes upon neuronal release of $K^+$ (116) that may stimulate pyruvate carboxylation (117). Flux through pyruvate carboxylation has been evaluated using [2-$^{13}$C]glucose (for comparison with labeling from [1-$^{13}$C]glucose see Ref. (54)). Interestingly, $V_{PC}$ was not significantly altered in high neuronal activity upon by bicuculline-induced seizures (118), suggesting that pyruvate carboxylase and *de novo* glutamine synthesis are not required to support dysfunctional glutamatergic activity in this pathological condition.

Notably, in models that include GABAergic and glutamatergic neurons rather than a single neuronal compartment (12, 61, 119), correlation of total $V_{TCA}$ in neurons with $V_{NT}$ is not maintained because GABA-glutamine cycle relies on glial GABA oxidation in the TCA cycle [**Figure 8**, see also review by Hyder et al. (87)]. In line with this, nearly half of GABA produced in GABAergic neurons is further oxidized in astrocytes through the glial GABA shunt and half of GABA synthesis relies on glutamine provided by astrocytes (12), which is in accordance to observations in cultured cortical neurons supplied with $^{13}$C-enriched glutamine (120).

## MITOCHONDRIAL MEMBRANE TRANSPORT IS COUPLED TO METABOLIC DEMAND IN NEURONS

Labeling of brain glutamate from a $^{13}$C-enriched oxidative substrate requires transfer of the label from the mitochondrial TCA cycle intermediate 2-oxoglutarate to cytosolic glutamate, which also sustains the transfer of reducing equivalents from cytosol to mitochondria. In the compartments where the GABA shunt occurs, i.e., glia and GABAergic neurons (12, 61), a substantial

part of 2-oxoglutarate transamination to glutamate occurred with GABA, yielding succinic-semialdehyde that is further metabolized by the TCA cycles. Mitochondrial transport of $^{13}$C label in glia, which includes GABA transamination (in models with GABAergic compartment) and net glutamate synthesis, was much lower than the neuronal counterpart. This is in agreement with the finding that carriers exchanging aspartate and glutamate in the malate-aspartate shuttle are predominantly expressed in neurons rather than astrocytes (121–123). In contrast, comparable amounts of Aralar protein, the main mitochondrial carrier for aspartate/glutamate in the brain, were found in freshly isolated neurons and astrocytes (124), suggesting that the transfer of reducing equivalents across the mitochondrial membrane is not a limiting step for adaptation to altered metabolic or energetic demand in astrocytes. It is plausible that $V_X$ varies with increased metabolic activity as higher glycolysis requires transference of reducing equivalents into the mitochondria. However, this may be a small effect because of the inability to substantially increase hexokinase activity (discussed above).

As discussed above, in the absence of measurable $^{13}$C turnover curves for 2-oxoglutarate, the fluxes $V_{TCA}$ and $V_X$ are intrinsically coupled (see **Figure 5**) and are of difficult determination when few $^{13}$C enrichment curves are taken for metabolic modeling. Although the rate of label transfer from 2-oxoglutarate to glutamate is frequently thought to occur at a more rapid rate than that of TCA cycle intermediate oxidation (22, 86, 125), metabolic modeling of $^{13}$C MRS data from humans (29) and rats (9, 10, 12, 33, 35, 102) with independent flux estimation found mitochondrial exchange fluxes on the order of the TCA cycle rate. In line with this, $^{13}$C-enriched 2-oxoglutarate but not glutamate was detected in the rat brain *in vivo* by $^{13}$C MRS upon infusion of hyperpolarized $^{13}$C-acetate (126). With further methodological development of these pioneering experiments, one can envisage that dynamic detection of labeling in both 2-oxoglutarate and glutamate will allow to reliably measure $V_X$.

The fact that $V_X$ and $V_{TCA}$ are on the same order of magnitude suggests that higher brain metabolic activity may require increased $V_X$, especially in neurons. Interestingly, in mathematical models of brain energy metabolism where $V_X$ and $V_{TCA}$ were determined independently from high resolution $^{13}$C MRS data, $V_X$ was related to $V_{TCA}$ within the same compartment (**Figure 9**). This analysis was not performed for the glial compartment because most studies assumed identical $V_X$ for neurons and astrocytes. Moreover, when glial $V_X$ was independent, conversion of 2-oxoglutarate to glutamate is driven by *de novo* glutamine production, which is generally set to match $V_{PC}$. Neuronal $V_X$ was also related to $V_{NT}$ for the respective compartment, i.e., either glutamatergic or GABAergic neurons.

This subject requires further research, especially in conditions of high brain activity, since very few experimental data sets are available for analysis and most were acquired under anesthesia. In addition, since different models were generally used to estimate metabolic fluxes in different studies, this sort of analyses (**Figures 8** and **9**) should be considered cautiously. Employment of the same mathematical model to experiments under a range of brain activities and identical physiological conditions is required (86).

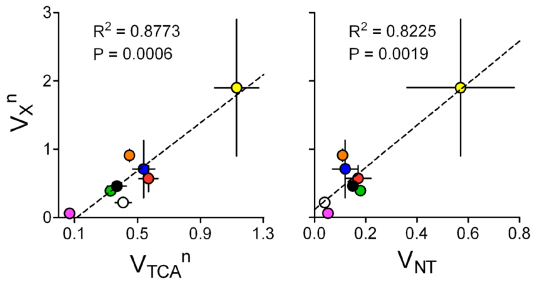


**FIGURE 9 | $V_X^n$ and $V_{TCA}^n$ are on the same order of magnitude and related (left)**. Accordingly, $V_X^n$ is also linearly related to glutamatergic or GABAergic $V_{NT}$ (right). Data was collected from the same experiments as in **Figure 8**, which determined both fluxes in an independent manner: rat brain under pentobarbital-induced isoelectricity [white; Ref. (102)]; rat brain under light α-chloralose anesthesia [orange, blue, and black are from Ref. (9, 10, 35)]; rat brain under light α-chloralose anesthesia but data modeled with three metabolic compartments [pink/green are GABAergic/glutamatergic compartments in Ref. (12)]; human cortex [red; Ref. (29)]; awake rat brain [yellow; Ref. (33)]. Metabolic fluxes are shown in micromoles per gram per minute with associated SD. *P*-value for the slope and $R^2$ are shown for each linear regression.

## SUBSTRATE TRANSPORT AND UTILIZATION

Proton MRS is emerging as an important tool for diagnosis and therapy monitoring as it provides biomarkers that offer fingerprints of neurological disorders in translational and preclinical neuroscience research (1). In addition, *in vivo* $^1$H MRS can be applied dynamically to evaluate cerebral cellular mechanisms that involve modification of metabolite concentrations, such as homeostasis disruption by pharmacological interventions (127) and substrate uptake and utilization (83).

Glucose transport kinetics as measured *in vivo* by $^1$H MRS has been mostly determined under steady-state conditions [e.g., Ref. (101, 128)]. Steady-state transport measurement from brain glucose content does not allow measuring glucose transport independently from glucose consumption but a ratio between the apparent maximum transport rate ($T_{max}$) and the $CMR_{Glc}$ is determined. Other studies determined glucose transport kinetics from variations of brain glucose content measured by $^1$H MRS upon a rapid change in plasma glucose concentration, which allowed quantifying both $T_{max}$ and $CMR_{Glc}$ (83, 129, 130). However, in these studies, different kinetic mechanisms have been defined for the glucose carriers at the BBB [discussed by Duarte and Gruetter (65)].

Initial modeling studies on glucose transport were based on the standard Michaelis–Menten kinetics to describe unidirectional fluxes across the membranes composing the BBB. Hexokinase, the rate-limiting step for glycolysis in the brain, operates close to saturation at physiological glucose levels. Thus, such standard Michaelis–Menten model predicted a maximum level for brain glucose, namely that brain glucose should be below 5 μmol/g for plasma glucose concentrations up to 30 mM.

However, brain glucose concentrations detected non-invasively by MRS are typically ∼9 μmol/g at plasma glucose concentrations of 30 mM in both humans and rodents [compared by Duarte et al. (128)]. At such high plasma glucose concentrations, glucose

efflux from the brain is substantial and inhibits its own carrier-mediated uptake. Brain glucose concentrations above the $K_M$ of GLUT1, the main glucose carrier at the BBB, imply that product formation is not unidirectional, i.e., the reverse reaction may proceed at significant rate. Glucose binding to the transporter at the abluminal membrane may partially inhibit the influx from the blood stream. When the product formation is not unidirectional, reversible Michaelis–Menten kinetics are applicable, which can be interpreted as reduced affinity for glucose influx, when substantial brain glucose is present. At steady-state, the reversible Michaelis–Menten model results in a linear relationship between brain and plasma glucose (102, 128, 131, 132). Dynamic $^{13}$C MRS studies with $^{13}$C-enriched glucose also demonstrated that transport of glucose into the brain can be predicted by the reversible model in rodents (9) and humans (29).

In certain studies, deviation from this predicted linearity was observed at high glycemia (82, 133). This suggests that brain glucose can induce a certain degree of inhibition at the glucose carrier upon severe hyperglycemia, which is patent in experimental models of diabetes (82, 134, 135). This has been attributed to trans-acceleration or asymmetry that are not accounted for in Michaelis–Menten kinetics. Conversely, conformational four-state exchange kinetic models of solute carriers can simultaneous account for asymmetry, product inhibition, trans-acceleration, and multiple substrate competition (136, 137). Such kinetics was shown to efficiently describe brain glucose levels in multi-compartmental models of brain glucose transport (83, 128, 138). Unlike the reversible Michaelis–Menten model, this four-site exchange mechanism assumes that the free carrier after glucose release to the brain's interstice is not conformationally the same that binds glucose outside, and that equilibrium exists between the two states of the unloaded carrier (**Figure 10**). The four-state conformational model predicts reverse glucose transport (and thus impediment of glucose uptake by the occupied carrier, like the reversible model) and that the presence of substantial amounts of glucose in the interstice prevents the carrier from acquiring a conformation that binds to glucose from plasma. In this case, for nearly symmetric carriers, the net transport can be expressed as:

$$v_t = \frac{T_{\max}\left(G_{\text{plasma}} - G_{\text{brain}}\right)}{K_t + G_{\text{plasma}} + G_{\text{brain}} + \frac{G_{\text{plasma}}\,G_{\text{brain}}}{K_{\text{ii}}}} \qquad (15)$$

where $K_t$ is the apparent affinity constant and $K_{ii}$ denotes the iso-inhibition constant that reflects the ability of glucose to inhibit the translocation of carrier isoforms between the two faces of the membrane. As previously demonstrated (128), the conformational model is equivalent to the standard Michaelis–Menten model for $K_t$ close to $K_{ii}$, and to the reversible Michaelis–Menten model at physiological glucose levels when $K_{ii}$ largely exceeds $G_{plasma}$. Since $K_{ii}$ is indeed much larger than the concentrations of glucose typically observed in the brain (83, 128, 138), the transport mechanism based on the reversible Michaelis–Menten kinetics can fully describe glucose transport under normal physiological conditions. However, in metabolic conditions where high glucose concentrations are observed, namely under eventual uncontrolled diabetes (128, 135), the inhibition constant $K_{ii}$ may be important in describing glucose transport at the BBB.

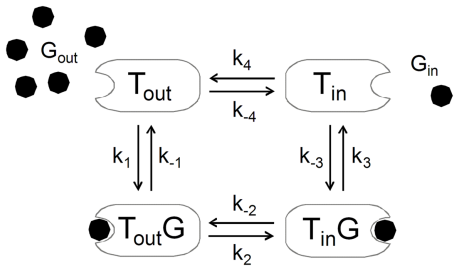


**FIGURE 10 | The alternating-conformation kinetics of the glucose carrier**. In the absence of glucose ($G_{out}$ or $G_{in}$), the carrier can exist in two inter-converting isomers that are ready to bind glucose either outside ($T_{out}$) or inside ($T_{in}$) the membrane. When loaded, the carrier can also assume two isomeric forms favoring glucose release to the outer ($T_{out}G$) or inner ($T_{in}G$) side of the membrane. The rate constants $k_1$ and $k_{-3}$ define glucose binding while $k_{-1}$ and $k_3$ define its dissociation from the carrier. The rate constants $k_2$ and $k_{-2}$ or $k_4$ and $k_{-4}$ reflect the isomerization of the loaded or unloaded carrier.

Magnetic resonance spectroscopy can be further extended to spectroscopic imaging with spatial resolution in the microliter range in rodents (139), being comparable to the spatial resolution of animal PET imaging with [$^{18}$F]fluorodeoxyglucose but with the advantage of detecting glucose directly and, simultaneously, other neurochemicals involved in energy metabolism (1). Such methods can eventually be used to map regional glucose transport (140).

Although glucose is the main substrate for the brain, other compounds like ketone bodies, acetate, or lactate can be used as source of energy to maintain cerebral functions when glucose supply is limiting. Because they can be easily detected by $^1$H MRS (1), similar approaches could be used to determine their transport rates across the BBB. Although the potential of $^1$H MRS to non-invasively evaluate homeostasis of substrates other than glucose remain to be investigated, the measurement of the brain transport kinetics for lactate or acetate were accomplished by $^{13}$C MRS upon infusion of $^{13}$C-enriched tracers in rodents (67, 72) and humans (141).

## GLYCOGEN METABOLISM

Brain glycogen levels exceed those of glucose and are measurable in a non-invasive way by localized $^{13}$C MRS after administration of $^{13}$C-enriched glucose (142–144). In the adult brain, glycogen is primarily located in glial cells (145–148) as the glycogen synthesis machinery is physiologically inactive and glycogen phosphorylase nearly absent in neurons (149–151).

In the rat brain *in vivo*, glycogen concentration was determined to range from 3 to 6 μmol/g (144, 152–154). Its turnover time was found to lie between 5 and 10 h under physiological conditions (142, 144, 154, 155). Similar glycogen turnover time was reported in the brains of mice (156) and humans (143).

Regulation of brain glycogen levels is complex and not completely understood. Brain glycogen content was suggested to increase with circulating insulin and with brain glucose concentration (152, 153, 157–159). While glycogen content is increased under anesthesia (152, 160, 161), somatosensory stimulation increases glycogenolysis rate (162, 163) and thus

reduces brain glycogen levels (164, 165). Although suggesting a direct role of glycogen metabolism in brain function, this was not observed under visual stimulation in both rodents (165) and humans (143). In the mouse hippocampus, glycogen was suggested to be involved in memory processing, being essential for long-term but not short-term memory formation (166).

Brain glycogen decreases upon insulin-induced hypoglycemia (152, 167, 168) due to enhanced glycogenolysis rate (157, 169, 170). It has been also proposed that recurrent hypoglycemia leads to increased substrate transport through the BBB, as well as to glycogen "supercompensation," in which the brain adapts to hypoglycemia by increasing glucose storage in the form of extra glycogen content (157, 168). Similarly, glycogen supercompensation was observed after depletion upon exhaustive exercise in several brain areas of the rat brain (171). This role for brain glycogen in buffering neuroglycopenia suggests its involvement in hypoglycemia unawareness, which is defined as impaired counter-regulatory hormonal responses to glycemia challenges and loss of the neurogenic (autonomic) warning symptoms of developing hypoglycemia. Unawareness of low blood glucose is eventually caused by recurrent hypoglycemia induced by intensive insulin therapy in diabetes, particularly type 1 diabetes mellitus. Glycogen supercompensation in the brain after episodes of hypoglycemia and its role in hypoglycemia unawareness have hitherto been matter of debate (133, 157, 167–169, 172, 173). However, a variety of inconsistent experimental protocols have been employed to tackle this question, and it is not excluded that regulation of glycogen levels is also affected by the duration of hypoglycemia insults or the glycemia levels in the immediate period after hypoglycemia.

In summary, although brain glycogen metabolism presents itself as having roles in glucose buffering upon limited glucose supply, in either physiological or pathological conditions, the exact mechanisms of glycogen metabolism regulation *in vivo* remain to be understood. Modeling of glycogen metabolism in the brain *in vivo* generally assume that the rates of binding and releasing glucosyl units from the glycogen molecule are equal and that all glycogen molecules display the same behavior independently of their size (142, 155, 169). The parameters extracted from such models are limited to glycogen concentration and turnover. However, due to its tight and complex regulation (174), study of glycogen metabolism *in vivo* may require an analysis with mathematical models including a more complete representation of the regulatory network, as has been done for other organs with metabolic control analysis [e.g., Ref. (175)].

## CONCLUSION

The recent developments in the field of *in vivo* dynamic MRS of brain metabolism have provided precious information on substrate transport and utilization as well as neurotransmission mechanisms. A quantitative interpretation of these data requires advanced metabolic modeling approaches based on the biochemical knowledge accumulated over several decades. The level of complexity of these mathematical models strongly depends on the amount of information accessible *in vivo* and limits the number and precision of measurable metabolic rates.

In this review, we explained and discussed the methodology applied in mathematical modeling of brain energy metabolism measured with dynamic MRS, the assumptions required for modeling, and the ways to estimate the robustness and adequacy of a model. Monte Carlo simulations proved to be a precious tool for this purpose. Although some metabolic flux values are still a matter of debate, compartmental modeling of brain metabolism of $^{13}$C-labeled energy substrates shined a new light on the understanding of neuronal and glial oxidative reactions and neurotransmission processes. Recent studies showed that substantial glial metabolism supports both glutamatergic and GABAergic neurotransmission. Even though the results obtained with two- and three-compartmental models in those works tend to come to a good agreement, some evidence supports the fact that the current models of brain energy metabolism fail to completely describe $^{13}$C MRS data.

The recent dynamic $^{13}$C isotopomer analysis using multiplets from homonuclear $^{13}$C coupling (10, 51) or the use of hyperpolarized $^{13}$C methods (59, 126) may help to solve several remaining questions concerning brain metabolic processes. Overall, the availability of high magnetic field NMR systems and the continuous improvements in the detection methods in both $^{13}$C and $^{1}$H MRS enable the non-invasive acquisition of metabolic data with a steadily increasing level of detail and precision, which will require improvement of current metabolic models but is also expected to provide new insight in the understanding of brain energy processes and brain function in the near future.

## ACKNOWLEDGMENTS

This work was supported by the Swiss National Science Foundation (grant 131087) and by Centre d'Imagerie BioMédicale (CIBM) of the UNIL, UNIGE, HUG, CHUV, EPFL, and the Leenaards and Jeantet Foundations.

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**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

*Received: 31 May 2013; accepted: 07 October 2013; published online: 28 October 2013.*
*Citation: Lanz B, Gruetter R and Duarte JMN (2013) Metabolic flux and compartmentation analysis in the brain in vivo. Front. Endocrinol. **4**:156. doi: 10.3389/fendo.2013.00156*
*This article was submitted to Cellular Endocrinology, a section of the journal Frontiers in Endocrinology.*

frontiers in
ENDOCRINOLOGY

OPINION ARTICLE
published: 15 October 2013
doi: 10.3389/fendo.2013.00149

# *De novo* synthesis of glial glutamate and glutamine in young mice requires aspartate provided by the neuronal mitochondrial aspartate-glutamate carrier aralar/AGC1

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***Edited by:***
*Leif Hertz, China Medical University, China*

**Keywords: AGC1, aralar, aspartate, glial glutamine, mitochondrial aspartate-glutamate carrier**

## INTRODUCTION

In brain, the glutamate-glutamine and the glutamate-glutamine-GABA cycles are essential for efficient glutamatergic and GABAergic neurotransmission. The interactions between neurons and astrocytes required for the operation of these cycles have received considerable attention since their discovery (1). In gray matter, glutamate released from glutamatergic neurons is mostly taken up into astrocytes (2, 3) where it is converted into glutamine which is sent back to the neurons for conversion to glutamate. This drain of glutamate released from neurons to astrocytes is compensated for by a flow of glutamine from astrocytes to neurons, thus closing the glutamate-glutamine cycle.

In addition to transcellular cycling, the glutamate-glutamine cycle faces losses of components, mainly glutamate, by oxidation, which is balanced by the anaplerotic synthesis of glutamate and glutamine in astrocytes. As shown by Rothman (4) and discussed by Hertz (5), *in vivo* about 85% of the glutamate cycles in the glutamate-glutamine cycle but 15% is oxidized. Oxidation may take place in astrocytes and neurons, and under basal conditions it does not appear to require glutamate dehydrogenase (GDH), a mitochondrial enzyme present mainly in astrocytes, as a brain-specific-disruption of the gene has small effects on brain glutamate levels (6). *De novo* synthesis of glutamate takes place in astrocytes thanks to pyruvate carboxylase (PC) (7, 8), which is expressed in astrocytes but not in neurons (9–11). This process generates a new molecule of oxaloacetate, which may condense with acetyl-CoA to provide net synthesis of α-ketoglutarate, from which glutamate can be formed by transamination (12). Subsequently, glutamine will be synthesized from glutamate via glutamine synthetase, which is exclusively present in the cytosol of astrocytes (13, 14). However, this general picture of the glutamate-glutamine cycle may be oversimplified, and there may be important variations, at least, during postnatal development. Recent metabolic studies in the neonatal rat brain have shown that the flow of glutamate from neurons to astrocytes is negligible when compared to the adult brain (15), while, paradoxically, the flow of glutamine from astrocytes to neurons is the same or even larger than in adults. This may suggest that astrocytes are slowly supplying glutamine to neurons so as to build up the neuronal pools of glutamate, aspartate, and N-acetylaspartate (NAA), which are lower than in the adult brain (15), and also, that a molecule other than glutamate is being transferred from neurons to astrocytes.

## BRAIN ASPARTATE, N-ACETYLASPARTATE, GLUTAMINE AND GLUTAMATE LEVELS, AND THE SYNTHESIS OF GLUTAMINE *IN VIVO* ARE REDUCED IN ARALAR-DEFICIENT MICE

Aralar/AGC1/Slc2512 is an isoform of the mitochondrial carrier of aspartate-glutamate (AGC), a component of the malate-aspartate shuttle (MAS) and it is regulated by calcium from the external side of the inner mitochondrial membrane (16). MAS activity allows the reoxidation of cytosolic NADH which is required to maintain glycolysis coupled to pyruvate oxidation in mitochondria rather than lactate formation. Cytosolic calcium sensed by EF-hands in the N-terminal extensions of aralar carrier regulates MAS activity in neurons (17) and β-cells (18) and $Ca^{2+}$-activated respiration in intact neurons (19).

The deficiency in aralar results in a drastic fall in brain aspartate and NAA levels, in a fall in NAA production by neurons in culture, and global hypomyelination both in mice (20–22) and humans (23). Hypomyelination has been attributed to a lack of neuronal NAA which, after transaxonal transport and cleavage by aspartoacylase, is required to supply acetyl groups to developing oligodendrocytes for myelin lipid synthesis (20, 24), energy metabolism (25), or other essential functions [reviewed in Ref. (22)].

Aralar deficiency also results in a large fall in brain glutamine content but only a modest decrease in brain glutamate levels, which is not associated to decreases in neuronal glutamate content or changes in synaptosomal glutamate release (26). None of the activities of the three main enzymes involved in the glutamine metabolism [phosphate-activated glutaminase, GDH, and mitochondrial aspartate aminotransferase (mAST)] are changed in the brain of the aralar-KO mice (26). The synthesis of glutamate and glutamine *in vivo* was also analyzed in aralar-deficient mice at P18-20 after labeling with (1,2-$^{13}$C)acetate or (1-$^{13}$C) glucose. Interestingly, labeled glutamine in the brain of aralar-KO mice was found to be below detection limit, regardless of whether ($^{13}C_2$)acetate or (1-$^{13}$C)glucose were used as cerebral substrates. These results clearly pointed out to a role for neuronal aralar in glial glutamine production in mouse brain.

## EXPRESSION AND FUNCTIONAL RELEVANCE OF ARALAR IN BRAIN CELLS

A decrease in brain glutamine synthesis in aralar-KO mice is puzzling, in the face of the prominent expression of aralar in neurons rather than in brain astrocytes

(26–30). This preferential expression in neurons was surprising, as aralar, and also citrin/AGC2/Slc25a13, is definitely present in cultured astroglia (26, 27, 31) and aralar mRNA has been reported to be present in brain astrocytes (31, 32). However, whether aralar is actually used in astrocytes in culture is uncertain, as the lack of aralar in cultured astrocytes did not cause the metabolic alterations characteristic of its deficiency in neurons [increased utilization of external pyruvate, decrease in aspartate levels; (26)]. So far, the most accurate determination of the localization of aralar in brain was that obtained by electron microscopy and immunogold-particle labeling of neuronal and astrocytic mitochondria, especially as the specificity of the labeling was contrasted with the use of aralar-KO mouse brains. These studies showed that neurons contained 94% of the labeled profiles whereas glial cells (both astrocytes and oligodendrocytes) contained only about 7%, close to the background level (26). These data clearly indicate that, at the protein level, aralar is localized preferentially, if not exclusively, in neurons.

## ROLE OF ASPARTATE IN GLUTAMINE SYNTHESIS BY ASTROCYTES

The most striking defect in the aralar-deficient neuron is the complete loss of aspartate. In addition, the levels of other amino acids which give rise to pyruvate also decrease, in response to a failure of MAS which results in a limitation in pyruvate supply to mitochondria. As the defect in glutamine synthesis is necessarily astrocytic, a metabolic limitation in the synthesis of glutamate and glutamine was suspected to happen in brain astrocytes. As the levels of α-KG were also maintained in the KO brains, the drop in glial glutamate-glutamine synthesis was attributed to a shortage of the amino-group donor (26). It is known that the *de novo* glutamate and glutamine production in astrocytes requires the supply of one or two ammonia groups, respectively, and neurons are thought to supply one or both (33). This would depend on whether glutamate oxidation occurs in astrocytes, which would maintain the amino group of glutamate in the astroglial cell, or in neurons, which would not. In this case, neurons would need to supply two amino groups to neighboring astrocytes per newly made glutamine.

It is currently believed that most of the glutamate oxidation occurs in astrocytes (4). Indeed, photoreceptors in the retina avoid the oxidation of glutamate through a tight regulation of the fate of α-KG exerted by the $NAD^+$/NADH ratio (34). However, glutamate is also oxidized in photoreceptors and neurons. For example, under hypoglycemia glutamate oxidation takes place through the truncated tricarboxylic acid cycle and aspartate becomes the end product (35–38). The relative importance of astrocytes versus neurons in the oxidation of glutamate in the intact brain both at rest and in conditions of activation is still unknown.

Astrocytes take up aspartate through the same plasma membrane transporters that take up glutamate and with the same affinity (39). In astrocytes, $^{15}$N-aspartate is rapidly taken up from the medium (40, 41), and its amino group is transferred to glutamate in the aspartate aminotransferase reaction, which then gives rise to glutamine. The fate of the carbon skeleton of aspartate in astrocytes has been also addressed in metabolic studies with U-$^{13}$C-aspartate. The results indicate that after an initial transamination, to give rise to oxaloacetate and glutamate, oxaloacetate enters the TCA and is largely incorporated into glutamine. Another large fraction of oxaloacetate is converted into lactate through pyruvate recycling (42–44). These facts suggested that aspartate produced by neurons may be required for glutamate and glutamine production in astrocytes, at least during postnatal development.

To test the role of aspartate in astrocytic glutamate and glutamine synthesis, astrocytes were incubated in the presence of glucose and different amino acids. The supply of 50–200 μM aspartate during 1 h to astroglial cultures resulted in glutamate and glutamine levels significantly higher than those obtained with all the other amino acids tested including alanine, BCAAs (leucine), and GABA, which, in fact, did not enhance glutamate or glutamine synthesis. Of note, this effect of external aspartate was identical in astrocytes derived from wild type or aralar-KO mice. These findings showed that aspartate is the main amino-group donor for *de novo* glutamate synthesis in astrocytes and explain why the lack of aspartate produced in neurons causes an impairment in the synthesis of glutamine in aralar-KO mice (26). On the basis of these results we proposed a transcellular traffic of aspartate from neurons to astrocytes which is summarized in **Figure 1**.

L-Aspartate was proposed to act as a neurotransmitter (46), but this hypothesis has been largely abandoned. However L-aspartate can be taken up in synaptic vesicles and exocytotically released form nerve terminals (47). Whether this is the mechanism utilized for the transcellular flux of aspartate from neurons is unknown. Another possibility for the traffic of aspartate from neurons to astrocytes may involve NAA, as the neuronal transporters of NAA are well established, as is its uptake by astrocytes and subsequent cleavage by aspartoacylase to release acetate and aspartate [reviewed in Ref. (22)].

The neuron-to-astrocyte aspartate efflux pathway described in **Figure 1** may provide a means to transfer NADH/$NAD^+$ redox potential to astrocyte mitochondria an alternative transcellular shuttle system. Indeed, aspartate uptake coupled to OAA production provides a substrate for cytosolic malate dehydrogenase resulting in NADH consumption in the cytosol and malate formation. As the α-ketoglutarate–malate carrier is equally represented in neuronal and glial mitochondria (28), aspartate utilization in glutamate formation in astrocytes will be stoichiometrically related to reducing equivalent transfer to mitochondria. In this way, transcellular aspartate traffic would result in malate oxidation by astrocytic mitochondria. Malate formed in astroglial cytosol may be also transferred back to neurons, as malate is released to a higher extent from cultured astrocytes than from cultured neurons (45).

Alternatively, Hertz (5, 48) has suggested that aralar/AGC1 in brain astrocytes, even at very low levels, could play a role in a modified aspartate-malate shuttle to oxidize reducing equivalents in mitochondria. In this modified shuttle OGC and AGC1 are involved in two different functions: the OGC, in glutamate formation (as shown in **Figure 1**) and the AGC1 in glutamate oxidation.

## CONCLUDING REMARKS

The role of neuronal aspartate as precursor of glutamate synthesis in astrocytes explains the alterations found in the brains of aralar/

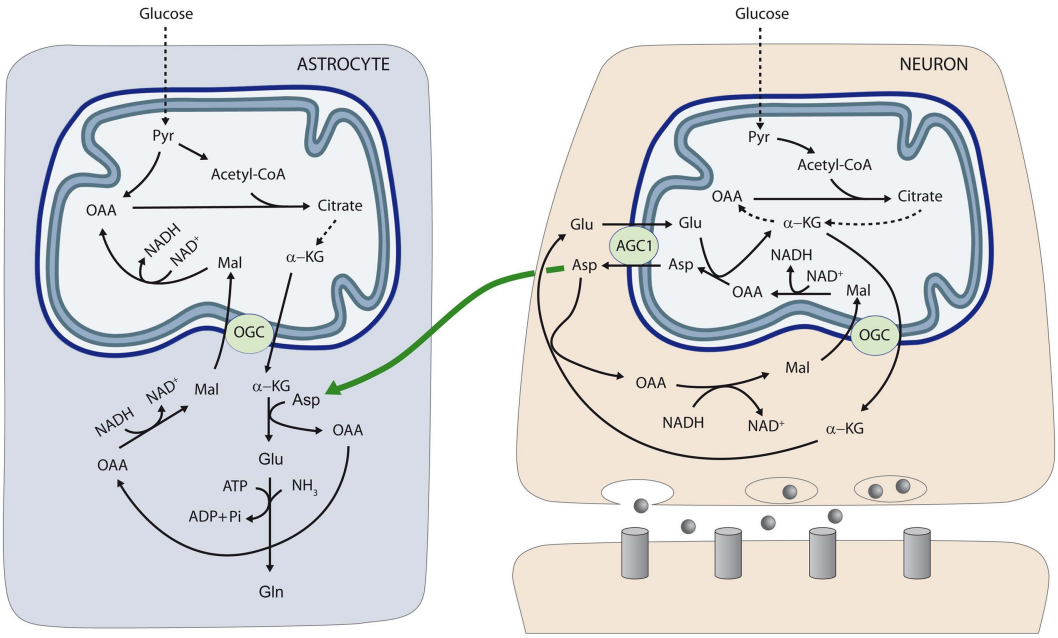


**FIGURE 1 | Neuron-to-glia transcellular aspartate efflux pathway for glial glutamate synthesis.** Neuronal mitochondria are provided with aralar/AGC1/Slc25a12 and the oxoglutarate carrier/OGC/Slc25a11 and carry out the malate-aspartate shuttle to transfer NADH reducing equivalents to the mitochondrial matrix. AGC1 is irreversible in polarized mitochondria and the main pathway of glutamate supply to the mitochondrial matrix. As cAST functions in the direction of glutamate formation in cells with an active MAS, mitochondria are the only site where aspartate is produced (in the mitochondrial aspartate aminotransferase reaction), and aspartate leaves the matrix through AGC1 to reach the cytosol. *De novo* glutamate synthesis in astroglial cells takes place in the cytosol in the cAST reaction with aspartate as amino-nitrogen donor to α-KG. A second amino group (possibly arising from ammonia itself formed in neurons in the phosphate-activated glutaminase reaction, or imported from the blood stream) is acquired in the glutamine synthetase reaction and glial glutamine is now transferred to neurons along the glutamate–glutamine cycle (not shown). Oxaloacetate (OAA) arising from the cAST reaction is converted to malate, and malate entry in glial mitochondria along the OGC provides an alternative pathway for redox transfer to mitochondria, which partly compensates for the lack of a malate-aspartate shuttle in brain astrocytes. In this way, equivalent transfer to astroglial mitochondria is stoichiometrically related to *de novo* glutamate production. Alternatively, malate formed in astroglial cytosol may be transferred back to neurons, as malate is released to a higher extent from cultured astrocytes than from cultured neurons (45) (not shown). The diagram does not address the regulation of the two fates of aspartate in the neuron, MAS (as depicted) or the transfer to the astrocyte (as also depicted), which are obviously mutually exclusive. Thus, transfer of aspartate to astrocytes is associated with glutamate oxidation in neurons, possibly through a truncated TCA cycle, rather than the operation of MAS. AGC, aspartate–glutamate carrier; Asp, aspartate; Gln, glutamine; Glu, glutamate; α-KG, α-ketoglutarate; Mal, malate; OAA, oxaloacetic acid; OGC, α-ketoglutarate–malate carrier; Pyr, pyruvate. Gray circles at presynaptic neuron represent neurotransmitter released; and gray columns at postsynaptic neuron the corresponding receptors [reproduced from Ref. (26)].

AGC1 KO mice, specifically, the impressive drop in the synthesis of glutamine. However, these findings were obtained at 18–20 days postnatal, at a time where the full development of the glutamate/glutamine cycle has not yet taken place. Further studies in adults will be required to fully understand the role of this transcellular pathway.

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*Received: 30 September 2013; accepted: 02 October 2013; published online: 15 October 2013.*

*Citation: Pardo B, Contreras L and Satrústegui J (2013) De novo synthesis of glial glutamate and glutamine in young mice requires aspartate provided by the neuronal mitochondrial aspartate-glutamate carrier aralar/AGC1. Front. Endocrinol. **4**:149. doi: 10.3389/fendo.2013.00149*

*This article was submitted to Cellular Endocrinology, a section of the journal Frontiers in Endocrinology.*

frontiers in
ENDOCRINOLOGY

PERSPECTIVE ARTICLE
published: 18 November 2013
doi: 10.3389/fendo.2013.00179

# Quantitative analysis of neurotransmitter pathways under steady state conditions – a perspective

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In a contribution to this Research Topic Erkki Somersalo and Daniela Calvetti carried out a mathematical analysis of neurotransmitter pathways in brain, modeling compartmental nitrogen flux among several major participants – ammonia, glutamine, glutamate, GABA, and selected amino acids. This analysis is important because cerebral nitrogen metabolism is perturbed in many diseases, including liver disease and inborn errors of the urea cycle. These diseases result in an elevation of blood ammonia, which is neurotoxic. Here, a brief description is provided of the discovery of cerebral metabolic compartmentation of nitrogen metabolism – a key feature of cerebral glutamate–glutamine and GABA–glutamine cycles. The work of Somersalo and Calvetti is discussed as a model for future studies of normal and pathological cerebral ammonia metabolism.

**Keywords: branched-chain amino acid branched-chain keto acid shuttle, GABA–glutamine cycle, glutamate–glutamine cycle, metabolic compartmentation, cerebral nitrogen metabolism**

The concept of compartmentation of cerebral nitrogen metabolism originated with the classic work of Berl et al. (1). These authors infused large (non-tracer) doses of [$^{15}$N]ammonia into cats and determined the relative enrichment of $^{15}$N in cerebral glutamate, glutamine (amine), and glutamine (amide). Glutamine synthetase (GS) catalyzes the conversion of ammonia and glutamate to glutamine (Eq. 1). A portion of this glutamate was labeled with $^{15}$N as a result of the action of glutamate dehydrogenase (GDH) (Eq. 2). Thus, if the brain were acting as a single metabolic compartment, the relative enrichment of label should have been greater in glutamate precursor than in glutamine (amine). However, the relative enrichment of $^{15}$N was found to be: glutamine (amide) > glutamine (amine) > glutamate. This finding prompted Berl et al. (1) to suggest that in the brain, glutamate is rapidly turned over to glutamine in a small compartment that is distinct from a larger compartment of more slowly turning over glutamate. We later verified cerebral compartmentation using intracarotid and intraventricular infusion of *tracer* quantities of [$^{13}$N]ammonia into rats (2). Importantly, Norenberg and colleagues showed that in brain parenchyma GS is present exclusively in astrocytes (3, 4). [See also the article in this Research Topic by Derouiche and Anlauf.] Thus, ammonia entering the brain largely by diffusion of ammonia free base ($NH_3$) (2, 5, 6) is rapidly converted to glutamine (amide) in the small compartment (i.e., astrocytes). The large compartment approximately represents the neurons, which contain a portion of the GDH activity of the brain.

$$\text{L-Glutamate} + NH_3 + \text{ATP} \rightleftarrows \text{L-glutamine} + \text{ADP} + P_i \quad (1)$$

$$\alpha\text{-Ketoglutarate} + NH_4^+ + \text{NAD(P)H} \rightleftarrows \text{L-glutamate} + \text{NAD(P)}^+ + H_2O \quad (2)$$

Compartmentation plays a pivotal role in the cerebral glutamate–glutamine cycle. Thus, glutamate released during neurotransmission is taken up in the small compartment (astrocytes), where it is converted to glutamine by GS. Glutamine is released from the astrocytes to the large compartment (neurons) where it is hydrolyzed by glutaminase (phosphate-activated glutaminase; PAG; Eq. 3) to glutamate and ammonia, completing the cycle (7, 8).

$$\text{L-Glutamine} + H_2O \rightarrow \text{L-glutamate} + NH_3 \quad (3)$$

The glutamate–glutamine cycle is not strictly stoichiometric and is "leaky." For example, net uptake of many amino acids occurs across the human blood–brain barrier (BBB) (9). The nitrogen obtained from this source contributes to the astrocytic pool of nitrogen in glutamate and glutamine. Moreover, not all astrocytic glutamine is released to the neurons. To maintain nitrogen balance cerebral glutamine is released to the extracellular fluids and circulation (10). Nevertheless, the concept of a glutamate–glutamine cycle has been useful in furthering our understanding of nitrogen homeostasis in the brain. The flux through this cycle is extremely rapid, estimated to be about 80% the rate of glucose oxidation (11).

[$^{13}$N]Ammonia has proved useful in positron-emission tomography (PET) studies of cerebral ammonia metabolism in controls, cirrhotics with non-overt encephalopathy, and cirrhotics with overt encephalopathy (12, 13). The main finding is that cerebral ammonia metabolism is enhanced due to increased blood ammonia in cirrhotic patients, but the kinetics of cerebral ammonia uptake and metabolism are not affected by hyperammonemia. Thus, owing to the higher concentration of ammonia in the blood, more ammonia will enter the brain in hyperammonemic patients than in normoammonemic individuals, despite the fact that tracer uptake by the brain is similar in the two groups. Because metabolic trapping of [$^{13}$N]ammonia is similar in both cases, the findings suggest that the concentration of ammonia in astrocytes is below

the $K_m$ value exhibited by GS toward ammonia even in the hyperammonemic individuals. In this regard, the whole brain ammonia concentration in the rat is estimated to be ∼180 μM (10), a value identical to the $K_m$ for ammonia exhibited by ovine brain GS (14). Moreover, as discussed below, the ammonia concentration in astrocytes is likely to be even lower than that of intact brain. Some evidence suggests that the specific activity of cerebral GS is decreased in portacaval-shunted rats (a model of chronic liver disease) (15, 16). Nevertheless, increased influx of ammonia in hyperammonemic individuals will result in increased synthesis of astrocytic glutamine. Owing to the uncertainty of the specific activity of [$^{13}$N]ammonia in the astrocytes it is not yet possible to assign an absolute value for glutamine synthesis in the brains of normal and cirrhotic patients using PET. Nevertheless, the trapping of blood-derived [$^{13}$N]ammonia in human brain is consistent with a rapid nitrogen flow through the glutamate–glutamine cycle.

A GABA–glutamine cycle also operates in the brain. Inhibitory neurotransmitter GABA released by neurons is taken up in part by astrocytes. In the astrocytes, GABA is transaminated to succinic semialdehyde, which is oxidized to succinate by succinic semialdehyde dehydrogenase, allowing the 4-carbon unit of GABA to enter the tricarboxylic acid (TCA) cycle (the GABA shunt). Glutamine is then released to the neurons and converted to glutamate. A portion of this glutamate is decarboxylated to GABA, thereby completing the GABA–glutamine cycle (17, 18). In the brain, acetate is metabolized exclusively in astrocytes (19). Studies with [$^{13}$C]acetate suggest that flux through the GABA–glutamine cycle in the neocortex of vigabatrin-treated rats is about 6% the rate through the TCA cycle (20). In a more recent study, van Eijsden et al. (21) calculated the flux through the glutamate–glutamine cycle, GABA–glutamine cycle, and GABA shunt in rat brain to be $0.274 \pm 0.023$, $0.033 \pm 0.005$, and $0.025 \pm 0.006$ μmol/min/g, respectively.

Most of the ammonia destined for glutamine (amide) synthesis in astrocytes is likely derived from ammonia taken up from the blood/cerebrospinal fluid (CSF) and from ammonia-generating reactions in both astrocytes and neurons. But how is the astrocytic glutamate (amine) pool maintained? In the simplest explanation, glutamate released from the neurons is stoichiometrically converted to glutamine in the astrocytes, but this is unlikely. Despite the fact that the GDH reaction is reversible (accounting for labeling of brain glutamate in tracer studies with [$^{13}$N]ammonia/[$^{15}$N]ammonia) it is likely that the *net* direction of the GDH reaction in astrocytes (22) and in whole brain is in the direction of glutamate oxidation (23). Thus, α-ketoglutarate generated by the GDH reaction in astrocytes must be converted back to glutamate to maintain glutamate balance (24, 25). This conversion can be accomplished by transamination of α-ketoglutarate. However, in order to maintain nitrogen homeostasis the amino acid transamination partner must be imported into astrocytes from the neurons or from the blood/CSF. As discussed below, various neuron-to-astrocyte amino acid shuttles have been proposed.

Waagepetersen and colleagues (26–29) suggested that ammonia is incorporated into glutamate in neurons via the GDH reaction (forward direction of Eq. 2) and thence into alanine by means of the alanine aminotransferase (ALAT)-catalyzed reaction (Eq. 4). Alanine is then transported into the astrocytes and transaminated with α-ketoglutarate to regenerate glutamate nitrogen (Eq. 5). In support of this notion both glutamine and alanine are greatly elevated in cerebral extracellular fluid in hyperammonemic patients with fulminant hepatic failure (30) and in a rat model of this disease (31). Carbon balance may possibly be maintained by transfer of lactate from astrocytes to neurons (the lactate–alanine cycle). Redox balance is maintained because GDH is proposed to catalyze net reductive amination of α-ketoglutarate in neurons (Eq. 2, forward reaction) and net oxidation of glutamate in astrocytes (Eq. 2; back reaction). However, tracer studies with $^{15}$N-labeled alanine, ammonia, and glutamine revealed no direct coupling of the glutamate–glutamine and lactate–alanine shuttles in cerebellar cocultures (27). Moreover, the existence of an astrocyte-to-neuron lactate shuttle is controversial (32). Therefore, the following discussion will focus only on the part of the pathway suggested to transfer alanine nitrogen from neurons to astrocytes, which is referred to as the alanine shuttle.

Previous work has shown that (1) the specific activity of cerebral ALAT is relatively low (33), (2) even after a 20-min intracarotid infusion of [$^{13}$N]ammonia into the rat brain, label cannot be detected in alanine despite labeling of glutamate (23), and (3) even after brain GS is inhibited 85% by the GS inhibitor L-methionine-*S*,*R*-sulfoximine (MSO) and the rats are hyperammonemic, relatively little label derived from intracarotid [$^{13}$N]ammonia is incorporated into brain glutamate (2, 23). Thus, under normoammonemic conditions an alanine shuttle [i.e., ammonia → glutamate (neurons) → alanine (neurons) → alanine (astrocytes) → glutamate (astrocytes)] is likely to be of minor importance for replenishing glutamate nitrogen in astrocytes (23). Bak et al. (27), however, suggest that the alanine shuttle may be prominent under hyperammonemic conditions.

$$\text{Pyruvate} + \text{L-glutamate} \rightarrow \text{L-alanine} + \alpha\text{-ketoglutarate (neurons)} \quad (4)$$

$$\text{L-Alanine} + \alpha\text{-ketoglutarate} \rightarrow \text{pyruvate} + \text{L-glutamate (astrocytes)} \quad (5)$$

Another nitrogen shuttle is the branched-chain amino acid/branched-chain α-keto acid (BCAA/BCKA) shuttle, first proposed by Hutson et al. (34, 35). These authors pointed out the unique distribution of the branched-chain aminotransferase (BCAT) isozymes in brain. The cytosolic isozyme (BCATc) is widely distributed in tissues, including brain, whereas the mitochondrial isozyme (BCATm) is confined to brain, placenta, and ovaries (34). In the brain, BCATc is more prevalent in neurons, whereas BCATm is more prevalent in the astrocytes (34). The presence of both BCAT isozymes in the brain suggests that they play an important role in maintaining cerebral BCAA homeostasis (34, 35). In the BCAA/BCKA shuttle, BCATc catalyzes the transamination of a BCKA, such as α-ketoisocaproate (KIC, the α-keto acid analog of leucine) with glutamate in neurons (Eq. 6). The resulting BCAA is taken up by astrocytes, where BCATm catalyzes the transfer of nitrogen from the BCAA to α-ketoglutarate, regenerating

glutamate (Eq. 7). The KIC generated in the astrocytes is returned to the neurons conserving carbon balance.

$$\text{L-Glutamate} + \alpha\text{-ketoisocaproate} \rightarrow \alpha\text{-ketoglutarate} + \text{L-leucine (neurons)} \quad (6)$$

$$\text{L-Leucine} + \alpha\text{-ketoglutarate} \rightarrow \alpha\text{-ketoisocaproate} + \text{L-glutamate (astrocytes)} \quad (7)$$

The notion that astrocytes metabolize leucine has strong support. Leucine is taken up across the human BBB (9) and has a relatively high brain uptake index in rats (36). Moreover, Berl and Frigyesi (37, 38) previously showed that leucine is metabolized in the small compartment in cat brain. As pointed out by Yudkoff et al. (39, 40), transfer of leucine nitrogen to α-ketoglutarate is favorable in brain, especially in astrocytes. Isoleucine, another BCAA, is of interest because its metabolism will not only replenish glutamate nitrogen in astrocytes, but also generate the TCA cycle intermediates succinyl-CoA and acetyl-CoA (41). However, although isoleucine provides some cerebral glutamate nitrogen this contribution is likely modest (41). Nevertheless, an analysis by Rothman et al. (25) suggests that the BCAA/BKCAA shuttle in brain is feasible. But, as pointed out by Somersalo and Calvetti (42) the proposed BCAA/BCKA shuttle is problematic because the GDH reaction is suggested to proceed in the direction of reductive amination of α-ketoglutarate in the neurons and oxidation of glutamate in the astrocytes. As noted above, relatively little label derived from intracarotid [$^{13}$N]ammonia is incorporated into brain glutamate in MSO-treated rats even when GS is inhibited 85% and the animals are hyperammonemic. Under these conditions, compartmentalization of ammonia metabolism in the brain is disrupted such that blood-derived [$^{13}$N]ammonia, which would normally have been efficiently trapped as glutamine (amide) in astrocytes, freely mixes with the neuronal ammonia pool. If the GDH reaction were important for the *net* synthesis of glutamate in neurons considerable label should have been present in brain glutamate in the MSO-treated rats. The fact that this was not observed suggests that the GDH reaction is not important for the net synthesis of glutamate in neurons even under hyperammonemic conditions. Thus, although transfer of leucine and other BCAAs between neurons and astrocytes is feasible and much evidence suggests that leucine is transaminated in astrocytes, the GDH reaction is unlikely to play a major role in any BCAA/BCKA shuttle.

As the above discussion attests, glutamate/glutamine homeostasis in astrocytes is still not fully understood. Somersalo and Calvetti (42) offer a mechanism for balancing nitrogen and carbon metabolism in the brain by suggesting that alanine derived from transamination of pyruvate with glutamate in the neurons (Eq. 4) is taken up by astrocytes, where the reverse reaction transfers the amino group from alanine to α-ketoglutarate (Eq. 5). Concomitantly, α-ketoglutarate is transaminated with leucine in the neurons to generate glutamate and KIC by the action of BCATc. This KIC is taken up by the astrocytes where it is transaminated with glutamate to generate leucine and α-ketoglutarate via a reaction catalyzed by BCATm. [The direction of nitrogen flow is the opposite of that shown in Eqs 6 and 7.] According to their Fig. 10 the combined action of the alanine shuttle and the GDH reaction replenishes the ammonia pool in the astrocytes.

The Somersalo and Calvetti model will be useful for further studies of cerebral nitrogen metabolism. The results of our $^{13}$N tracer studies suggest that nitrogen shuttles requiring operation of the GDH reaction in opposite directions in astrocytes and neurons are unlikely. In the model suggested by Somersalo and Calvetti the GDH reaction proceeds in the direction of oxidative deamination of glutamate in *both* compartments in agreement with our previous findings (see their Fig 10). The Somersalo and Calvetti model also requires a net movement of ammonia from neurons to astrocytes, consistent with our results obtained with [$^{13}$N]ammonia. A major source of ammonia in the neurons is the PAG reaction. However, there are many additional enzyme-catalyzed metabolic reactions that can generate ammonia in both compartments, including the GDH reaction (10). Tracer studies with blood-derived [$^{13}$N]ammonia clearly show that the two metabolic pools of ammonia in the rat brain do not readily mix except under hyperammonemic conditions in which the GS reaction is strongly inhibited (2). Despite the presence of two kinetically distinct ammonia compartments in the normal brain the ammonia generated in the neuronal compartment must be disposed of eventually. Since the GDH reaction in neurons is unlikely to participate in the net removal of ammonia and the brain does not have a complete urea cycle, the overwhelming route for removal of neuron-derived ammonia is the GS reaction in astrocytes. Evidently, under normoammonemic conditions the GS reaction removes ammonia efficiently from the astrocyte pool at a rate that ensures a net movement/gradient of ammonia from neurons to astrocytes, preventing uniform mixing of ammonia in the small and large compartments. In this regard, previous work has shown that ammonia is passively and actively rapidly transported into astrocytes in culture (43).

An unresolved issue is the nature of the transamination partners required for net conversion of α-ketoglutarate to glutamate in astrocytes. As noted above, neuronal-derived alanine is a possible source (42), but the contribution of alanine may be quantitatively minor under normoammonemic conditions. Another possibility is neuron-derived aspartate (Eqs 8 and 9) as suggested by Pardo et al. (24). In many respects, aspartate is a more attractive transamination partner in astrocytes than is alanine as a source of glutamate nitrogen. Aspartate aminotransferase (ASPAT) is extremely active in most tissues and the components of the reaction are thought to be at or near thermodynamic equilibrium in brain (44, 45) and liver (46). As a result, nitrogen is exchanged extremely rapidly between glutamate and aspartate in these tissues (23, 45, 47). Moreover, ASPAT is present in cytosolic and mitochondrial compartments. Thus, any model of nitrogen flux among amino acids that are transaminated with α-ketoglutarate in the brain must take into account nitrogen exchange between glutamate and aspartate in mitochondria and cytosol. A variant of the model proposed by Pardo et al. (24) has been suggested by Hertz (48). In the Hertz model ASPAT-catalyzed transamination of aspartate with α-ketoglutarate in astrocyte cytosol is retained. However, the "missing" aspartate is generated in the astrocytic mitochondria by transamination of glutamate with oxaloacetate rather than from the mitochondrial ASPAT reaction in neurons as

envisaged by Pardo et al. (24).

$$\text{L-Glutamate} + \text{oxaloacetate} \rightarrow \alpha\text{-ketoglutarate} + \text{L-aspartate (neurons)} \quad (8)$$

$$\text{L-Aspartate} + \alpha\text{-ketoglutarate} \rightarrow \text{oxaloacetate} + \text{L-glutanate (astrocytes)} \quad (9)$$

Finally, the importance of linked aminotransferase reactions in the brain should be emphasized. The capacity of the brain to catalyze α-ketoglutarate- and glutamate-linked aminotransferase reactions with a large number of amino acids/α-keto acids is considerable (33). As noted above, the brain has the capacity to take up many amino acids from the circulation, including BCAAs. These amino acids can then contribute to the astrocytic pool of glutamate nitrogen via α-ketoglutarate-linked aminotransferase reactions.

A diagram of the major pathways contributing to cerebral nitrogen homeostasis is shown in **Figure 1**. Summary: (1) Ammonia for glutamine synthesis in astrocytes is obtained by diffusion from blood and CSF, and from numerous endogenous enzyme-catalyzed reactions, but principally the PAG reaction (in neurons) and the GDH reaction (in both astrocytes and neurons). (2) To maintain nitrogen balance glutamine is exported to blood/CSF. (3) The net GDH-catalyzed conversion of glutamate to α-ketoglutarate and ammonia in astrocytes results in a drain on glutamate in these cells that cannot be replenished by the glutamate–glutamine cycle (or GABA–glutamine cycle). (4) Conversion of α-ketoglutarate back to glutamate in astrocytes can be accomplished by α-ketoglutarate-linked aminotransferases, which are well represented in brain. (5) Transamination partners in the astrocytes include neuron-derived alanine (less important) and aspartate (more important). (6) ASPAT is crucial in maintaining

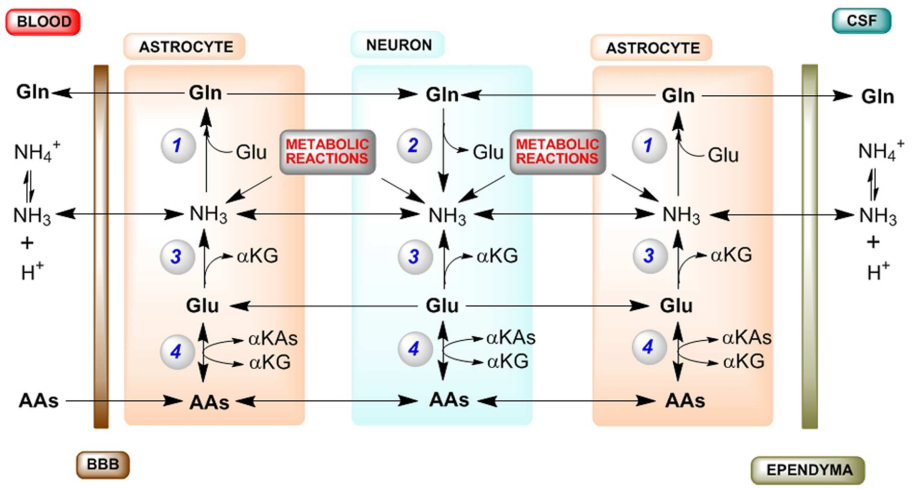


**FIGURE 1 | Major routes for nitrogen homeostasis in the brain with emphasis on the central importance of ammonia**. Abbreviations: AA, amino acid; αKA, α-keto acid; αKG, α-ketoglutarate; A–V, arterial–venous; BBB, blood–brain barrier; CSF, cerebrospinal fluid; GDH, glutamate dehydrogenase; GS, glutamine synthetase; PAG, phosphate-activated glutaminase; PC, pyruvate carboxylase. Enzyme reactions: (1) GS, (2) PAG, (3) GDH, (4) various αKG/glutamate-linked aminotransferases. Tracer studies with [$^{13}$N]ammonia have shown that about 25–40% of the tracer is taken up in a single pass through the brain (2, 5, 6). Most of this blood-derived [$^{13}$N]ammonia (as well as CSF-derived [$^{13}$N]ammonia) is rapidly trapped in the astrocyte compartment as L-[*amide*-$^{13}$N]glutamine by the action of GS. The cerebral trapping of blood-derived [$^{13}$N]ammonia suggests that there should be an A–V difference for ammonia across normal brain. However, the concentration of ammonia in blood is relatively low (<40 μM in normal human blood and <80 μM in normal rat blood). As a result, a cerebral A–V difference for ammonia is difficult to measure in normoammonemia, although it is well documented to occur during hyperammonemia [reviewed by (10)]. A net cerebral uptake of many AAs has been demonstrated for healthy human volunteers in the post absorptive state (9). In order to maintain cerebral nitrogen balance some glutamine is released to the blood/CSF, a process that is more pronounced during hyperammonemia [reviewed by (10)]. As a result, the concentration of glutamine in the normal CSF/extracerebral fluid is relatively high and much higher than that of any other amino acid (49). Many endogenous reactions contribute to the cerebral ammonia metabolic pool, but especially the PAG and GDH reactions (10). As a result of rapid removal of this ammonia as glutamine there is an ammonia concentration gradient from the neurons to the astrocytes, maintained by diffusion of $NH_3$ and active transport of $NH_4^+$ [c.f. (43)]. During the glutamate–glutamine cycle, approximately one equivalent of nitrogen enters the astrocytes as glutamate and approximately two equivalents exit as glutamine. One equivalent of nitrogen in glutamine is readily obtained from ammonia, which is incorporated into the amide position. The other nitrogen in glutamine is derived from glutamate. However, a portion of this glutamate is metabolized to αKG and ammonia by the action of GDH. Moreover, some carbon/nitrogen originating in glutamate is lost when glutamine exits the brain. Glutamate carbon may be replenished in astrocytes by anaplerotic mechanisms, most notably pyruvate carboxylase (PC). [PC activity is present exclusively in astrocytes, and its activity has been estimated to be about 37% that of the cerebral glutamine synthesis rate (50).] Glutamate nitrogen is replenished in the astrocyte by transamination reactions between αKG and a suitable AA, generating the corresponding αKA. Leucine is an especially favorable aminotransferase partner in astrocytes, because (1) it is readily taken up across the BBB (9), (2) blood-derived leucine is metabolized in the small compartment (i.e., astrocytes) (37, 38), and (3) both astrocytes and neurons contain appreciable branched-chain amino acid aminotransferase activity (34, 35). Another possible aminotransferase partner in the astrocytes is aspartate (24, 48). Note: (1) for simplicity, the GABA–glutamine cycle is not shown as it is considerably slower than the glutamate–glutamine cycle; (2) endogenously generated ammonia is depicted as $NH_3$ (but represents $NH_3 + NH_4^+$); (3) enzyme cofactors are not shown; (4) tracer studies show that the cerebral GDH reaction is reversible, but as discussed in the text the net direction of the GDH reaction in both astrocytes and neurons is likely in the direction of glutamate oxidation to αKG and ammonia; and (5) possible movements of αKAs between neurons and astrocytes (required for carbon balance) are not shown.

cerebral nitrogen balance. (7) Additional transaminase partners in the astrocytes include amino acids taken up across the BBB, most notably the BCAAs. Evidently, much work remains in elucidating mechanisms contributing to cerebral nitrogen flux/homeostasis.

## ACKNOWLEDGMENTS

I thank Dr Leif Hertz and Dr Boris Krasnikov for helpful suggestions. Work cited from the author's laboratory was supported by NIH grant RO1 DK 16739.

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**Conflict of Interest Statement:** The author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

*Received: 27 September 2013; paper pending published: 12 October 2013; accepted: 04 November 2013; published online: 18 November 2013.*
*Citation: Cooper AJL (2013) Quantitative analysis of neurotransmitter pathways under steady state conditions – a perspective. Front. Endocrinol. **4**:179. doi: 10.3389/fendo.2013.00179*
*This article was submitted to Cellular Endocrinology, a section of the journal Frontiers in Endocrinology.*

frontiers in
ENDOCRINOLOGY

REVIEW ARTICLE
published: 17 April 2013
doi: 10.3389/fendo.2013.00048

# GABA and glutamate uptake and metabolism in retinal glial (Müller) cells

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Müller cells, the principal glial cells of the retina, support the synaptic activity by the uptake and metabolization of extracellular neurotransmitters. Müller cells express uptake and exchange systems for various neurotransmitters including glutamate and γ-aminobutyric acid (GABA). Müller cells remove the bulk of extracellular glutamate in the inner retina and contribute to the glutamate clearance around photoreceptor terminals. By the uptake of glutamate, Müller cells are involved in the shaping and termination of the synaptic activity, particularly in the inner retina. Reactive Müller cells are neuroprotective, e.g., by the clearance of excess extracellular glutamate, but may also contribute to neuronal degeneration by a malfunctioning or even reversal of glial glutamate transporters, or by a downregulation of the key enzyme, glutamine synthetase. This review summarizes the present knowledge about the role of Müller cells in the clearance and metabolization of extracellular glutamate and GABA. Some major pathways of GABA and glutamate metabolism in Müller cells are described; these pathways are involved in the glutamate-glutamine cycle of the retina, in the defense against oxidative stress via the production of glutathione, and in the production of substrates for the neuronal energy metabolism.

**Keywords: retina, Müller cells, glutamate, GABA, recycling, retinal pathology**

The vertebrate retina contains two types of neuron-supporting macroglial cells, astrocytes and Müller cells. Astrocytes are restricted to the innermost layers of vascularized retinas (**Figure 1**). Müller cells are specialized radial glial cells which span the entire thickness of the neural retina (**Figure 1**). Müller cells support the functioning and metabolism of retinal neurons and are active players in normal retinal function and retinal degeneration (Bringmann et al., 2006). Müller cells provide trophic substances to neurons and remove metabolic waste (Newman, 1994; Tsacopoulos and Magistretti, 1996), mediate the retinal potassium, water, and acid-base homeostasis (Bringmann et al., 2006) and the maintenance of the blood-retinal barrier (Shen et al., 2012), and regulate the retinal blood flow (Metea and Newman, 2006). Müller cells act as living optical fibers which guide light toward the photoreceptors (Franze et al., 2007; Agte et al., 2011). Their processes function as soft, compliant embedding for neurons which supports synaptic plasticity and neurite outgrowth (Lu et al., 2006). Müller cells support the synaptic activity by neurotransmitter recycling, the supply of neurons with precursors of neurotransmitters, and the release of gliotransmitters which affect neuronal activity (Newman, 2004). Müller cells become activated upon virtually all pathogenic stimuli. Reactive Müller cells support the survival of photoreceptors and neurons, but may also contribute to neuronal degeneration (Bringmann et al., 2009). Currently, the many roles of Müller cells in the regulation of retinal function are still not elucidated, and are subject of intensive research.

The precise shaping of synaptic activity depends upon the kinetics of both the presynaptic neurotransmitter release and the re-uptake of the transmitter into the cells. In the retina, photoreceptor cells, neurons, and macroglial cells express high-affinity transporters for neurotransmitters. Müller cells express uptake and exchange systems for various neurotransmitters including glutamate and γ-aminobutyric acid (GABA). This review gives a survey of the present knowledge regarding the involvement of Müller cells in the uptake and metabolism of GABA and glutamate, the relationships between the glial transmitter recycling and the various other functional roles of Müller cells, and the contribution of Müller cell's transmitter recycling to the neuroprotective and detrimental effects of gliosis.

## GABA UPTAKE AND METABOLISM

γ-Aminobutyric acid is the main inhibitory neurotransmitter in the vertebrate retina, used by subclasses of horizontal, amacrine, ganglion, bipolar, and interplexiform cells. The termination of the synaptic action of GABA is achieved by its uptake into presynaptic neuronal terminals and into the surrounding glial cell processes. In addition to neurons such as amacrine and interplexiform cells (Moran et al., 1986; Pow et al., 1996) and, in the fish retina, horizontal and bipolar cells (Nelson et al., 2008), Müller cells and, at least under pathological conditions, astrocytes and microglia take up GABA (Sarthy, 1982; Osborne et al., 1995). It was suggested that in the retinas of most lower vertebrates and birds, GABA removal is almost exclusively mediated by neurons, whereas in the mammalian retina, neurons, and Müller cells remove extracellular GABA (Yazulla, 1986; but, see below). In mammals, GABA is taken up predominantly by amacrine and Müller cells in the inner retina, and almost exclusively by Müller cells in the outer retina (Marc, 1992; but, see below).

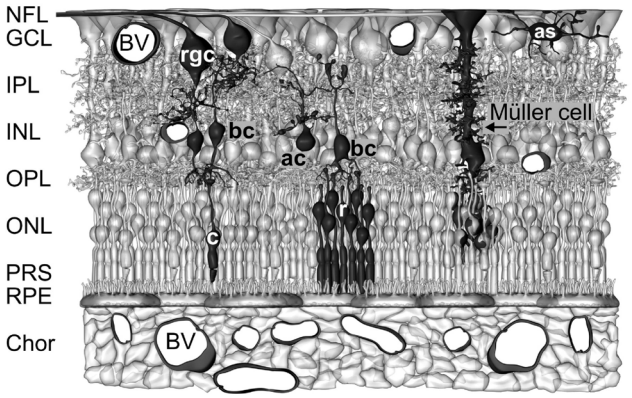


**FIGURE 1 | Müller cells span the entire thickness of the neuroretina.** Schematic drawing of the cellular constituents and basic neuronal circuits of a human retina. The inner retina contact the vitreal cavity (*top*) and contains the following retinal layers: nerve fiber layer (NFL), ganglion cell layer (GCL), inner plexiform layers (IPL), and inner nuclear layer (INL). The outer retina is directed to the outer surface of the eye and contains the following retinal layers: outer plexiform layer (OPL), outer nuclear layer (ONL), the subretinal space containing the photoreceptor segments (PRS), and the retinal pigment epithelium (RPE). The outer retina is supplied with oxygen and nutrients by the blood vessels (BV) in the choroid (Chor), while the inner retina is supplied by intraretinal vessels. Astrocytes (as) are localized in the NFL/GCL. The perikarya of Müller cells are localized in the INL. From the perikaryon, two stem processes of Müller cells extend toward both surfaces of the neuroretina. The funnel-shaped endfeet of Müller cells form (in association with a basement membrane) the inner surface of the retina. In the OPL and IPL, side branches which form perisynaptic membrane sheaths originate at the stem processes. In the ONL, the stem process of Müller cells forms membraneous sheaths which envelop the perikarya of rods (r) and cones (c). Microvilli of Müller cells extend into the subretinal space. ac, amacrine cell; bc, bipolar cell; rgc, retinal ganglion cell.

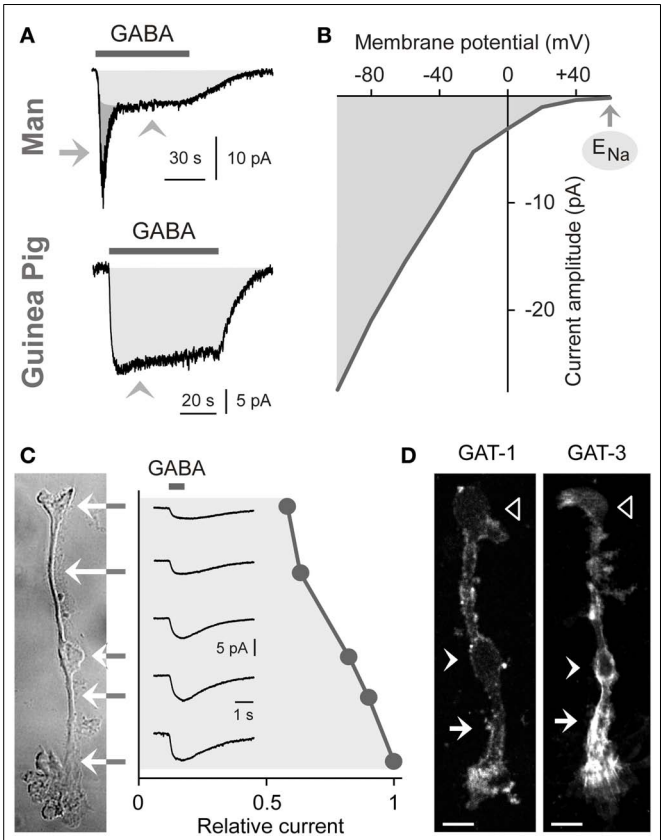


**FIGURE 2 | Electrogenic GABA transport in Müller cells.** The GABA-evoked membrane currents were recorded in freshly isolated Müller cells from man **(A)** and guinea pigs **(A–C)**. **(A)** Extracellular administration of GABA (100 μM) evokes two kinds of inward currents in a human Müller cell: a transient, rapidly inactivating chloride current mediated by $GABA_A$ receptors (*arrow*) and a sustained current mediated by electrogenic GABA transporters (*arrowhead*). In guinea pig Müller cells (which lack $GABA_A$ receptors), extracellular GABA (1 mM) evokes only a transporter-mediated inward current. The holding potential was −80 mV. **(B)** Current-voltage relation of the GABA transporter currents in guinea pig Müller cells. The amplitude of the transporter currents is zero near the equilibrium potential of sodium ions ($E_{Na}$) (when recorded with symmetrical chloride concentration at both sides of the membrane). GABA was administered at a concentration of 100 μM. **(C)** Subcellular distribution of the GABA transporter currents. The distribution of the currents was determined by focal ejections of GABA (1 mM) onto the following membrane domains of Müller cells: endfoot, inner stem process, soma, inner and outer parts of the outer stem process. **(D)** Distribution of GAT-1 and GAT-3 immunoreactivities in isolated Müller cells of the guinea pig. *Filled arrows*, outer stem process. *Filled arrowheads*, cell soma. *Unfilled arrowheads*, cell endfoot. Bars, 10 μm. With permission from Biedermann et al. (2002, 2004).

## GLIAL GABA UPTAKE

The uptake of GABA by Müller cells is mediated by sodium- and chloride-dependent high-affinity GABA transporters (GATs). Per transport step, two sodium ions and one chloride ion are co-transported with one GABA molecule (Qian et al., 1993; Biedermann et al., 2002). The transport process causes inwardly directed membrane currents (**Figure 2A**) and cellular depolarization. The GAT currents are voltage-dependent. The current amplitude increases (**Figure 2B**) and the affinity of GABA to the transporter molecules decreases with cellular hyperpolarization (Biedermann et al., 2002). The GAT currents in guinea pig Müller cells display their largest amplitude at the end of the outer stem processes which envelop the terminals and somata of photoreceptor cells (**Figure 2C**) (Biedermann et al., 2002). GABA at 100 μM is fully cleared from the extracellular space around one Müller cell after 70 ms (Biedermann et al., 2002). Due to this high efficiency of the GABA uptake, Müller cells were suggested to be involved in the rapid termination of the GABAergic transmission in the mammalian retina.

## EXPRESSION OF GABA TRANSPORTERS

To date four GAT subtypes have been described (GAT1-4) in addition to the vesicular transporter (VGAT) (Schousboe, 2003). The expression of GAT subtypes in Müller cells varies among the species. Müller cells of the guinea pig express GAT-1 and GAT-3 (Biedermann et al., 2002). The transporter proteins show elevated expression in the outer stem process of the cells (**Figure 2D**) (Biedermann et al., 2002). Müller cells of the chick and rat also express GAT-1 and GAT-3 (Brecha and Weigmann, 1994; Honda et al., 1995; Johnson et al., 1996) while Müller cells of the rabbit express GAT-3 (Hu et al., 1999). On the other hand, Müller cells of the bullfrog express GAT-1 and GAT-2 (Zhao et al., 2000), and Müller cells from other lower vertebrates such as tiger salamander and

salmon apparently do not express GAT proteins (Yang et al., 1997; Ekström and Anzelius, 1998).

Cultured avian Müller cells, but not avian Müller cells *in situ*, accumulate GABA (Marshall and Voaden, 1974; Pow et al., 1996; Calaza et al., 2001). However, a failure in demonstrable GABA uptake in Müller cells of distinct lower vertebrates and birds should be considered with caution. It can not be ruled out that GABA is rapidly converted by the GABA transaminase in Müller cells, resulting in a lack of detectable GABA in Müller cells. It has been shown, for example, that turtle Müller cells display a very little GABA transport activity but high levels of GABA transaminase (Sarthy and Lam, 1978).

Whether or not neuronal cells participate in the GABA clearance within the outer mammalian retina is unclear and may depend on the species investigated. Horizontal cells of guinea pigs and mice do not express GATs (Guo et al., 2010; Deniz et al., 2011) while rod bipolar and horizontal cells of monkeys express GAT-3 (Lassová et al., 2010).

### GABA METABOLISM

When GABA enters the Müller cell interior, it is metabolized by the mitochondrial enzyme GABA transaminase which catalyzes the formation of glutamate from 2-oxoglutarate, coupled to a conversion of GABA to succinate semialdehyde (**Figure 3**). Due to the efficiency of the GABA transaminase reaction, Müller cells display a very low level of intracellular GABA (Marc et al., 1998a). Under diabetic and ischemic conditions, GABA rapidly accumulates in Müller cells (Ishikawa et al., 1996; Napper et al., 2001) due to a decrease in the GABA transaminase activity (Barnett and Osborne, 1995; Ishikawa et al., 1996).

## GLUTAMATE UPTAKE AND METABOLISM

### GLUTAMATE UPTAKE

Glutamate is the main excitatory neurotransmitter in the retina, used in the forward transmission of visual signals by photoreceptors, bipolar, and ganglion cells. In the outer retina, glutamate is continuously released from photoreceptor terminals in the dark; this release is inhibited by light. In the inner plexiform layer, ON-bipolar cells release glutamate during light exposure whereas OFF-bipolar cells release glutamate in the dark.

#### *Importance of glial glutamate uptake*

In the neural retina, photoreceptors, neurons, and macroglial cells express high-affinity glutamate transporters (GLT) (Rauen and Wiessner, 2000). In the inner retina, Müller cells are responsible for the bulk of glutamate uptake (White and Neal, 1976; Harada et al., 1998). By their glutamate uptake, Müller cells are involved in setting the signal-to-noise ratio of synaptic transmission and the spatial resolution of light-induced signaling. Under pathological conditions, when the transport into Müller cells is reduced, more glutamate is transported into inner retinal neurons (Barnett et al., 2001; Holcombe et al., 2008). In the outer retina, the bulk of glutamate released from photoreceptor terminals was suggested to be removed by presynaptic transporters of photoreceptor cells (Hasegawa et al., 2006) and possibly by postsynaptic transporters at horizontal and bipolar cells (Rauen et al., 1996). Here, Müller cells take up glutamate which diffuses out of the synaptic clefts; this prevents the lateral spread of the transmitter and ensures visual resolution (Rauen et al., 1996). However, a recent study showed that inhibition of the main GLT of Müller cells (GLAST; see below) is highly effective in blocking the synaptic transmission in the outer plexiform layer, where photoreceptor terminals are localized (**Figure 1**) and in inducing a permanent electroretinogram deficit, whereas inhibition of GLT-1 (see below) caused no permanent electroretinogram changes (Levinger et al., 2012).

The glutamate uptake by Müller cells in the inner retina contributes to the rapid termination of the postsynaptic action of glutamate in non-spiking retinal neurons and in ganglion cells (Matsui et al., 1999; Higgs and Lukasiewicz, 2002). Here, Müller cell-provided glutamate uptake is an active player in synaptic transmission. When the retinal glutamate transport was blocked, the amplitude and duration of ganglion cell EPSCs increased dramatically whereas when only the neuronal transport was blocked, little change in synaptic currents was observed (Higgs and Lukasiewicz, 2002).

There are further data supporting the assumption that glutamate uptake and metabolism by Müller cells is more directly involved in the regulation of the activity of inner retinal neurons than that of photoreceptors. For example, the precursor of the glutamate synthesis in bipolar and ganglion cells, glutamine, is derived almost exclusively from Müller cells whereas photoreceptor cells synthesize only a part of their glutamate from Müller cell-derived glutamine (Pow and Robinson, 1994) while the other parts are derived from the re-uptake by the presynapse (Hasegawa et al., 2006) and, possibly, the transamination of α-ketoglutarate (Pow and Robinson, 1994). In addition, a significant amount of GABA in amacrine cells is synthesized from glutamate after uptake of Müller cell-derived glutamine (Pow and Robinson, 1994).

The clearance of synaptic glutamate by Müller cells is required for the prevention of neurotoxicity. After experimental inhibition of the glial glutamate uptake, even low concentrations of extracellular glutamate became neurotoxic (Izumi et al., 1999). Alterations in the activity of glial GLTs might be also involved in the regulation of the glial support of the neuronal signal transfer from photoreceptors to retinal ganglion cells. Repetitive light stimulation of the guinea pig retina induces a glial calcium response that occurs simultaneously throughout the whole length of Müller cells (Rillich et al., 2009). This response is induced by photoreceptor-to-glia signaling which evokes a hyperpolarization-induced influx of extracellular calcium (Rillich et al., 2009). The hyperpolarization is induced by decreases in the subretinal potassium and in the activity of electrogenic GLTs due to the light-induced reduction in photoreceptor activity and by the action of zinc ions (Rillich et al., 2009) which are released from photoreceptors (Redenti et al., 2007) and which directly inhibit GLTs (Spiridon et al., 1998). The glial forward signaling may include the opening of calcium-dependent potassium channels to increase the potassium buffering capacity, and a calcium-dependent release of glutamate to prevent hypoosmotic Müller cell swelling (Wurm et al., 2011; Slezak et al., 2012). Thus, alterations in the activity of glial GLTs may regulate glial neuron-supporting functions via, among other mechanisms, regulation of the membrane potential of Müller cells.

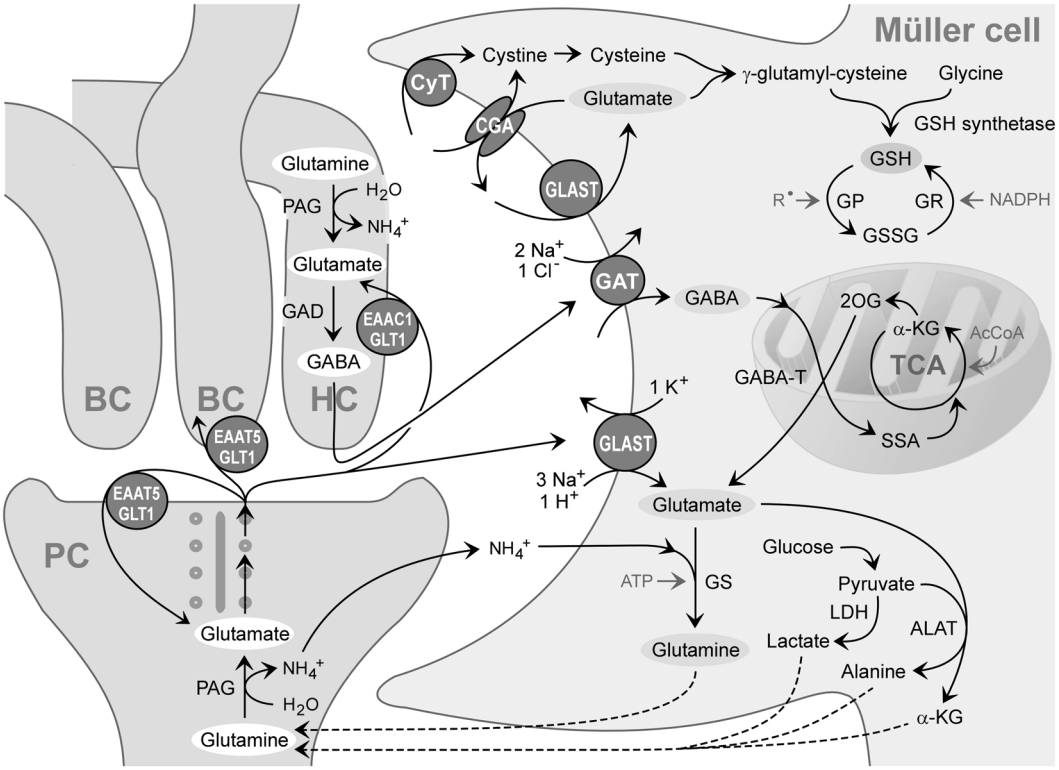


**FIGURE 3 | Recycling of amino acid neurotransmitters in the outer plexiform (synaptic) layer of the mammalian retina.** The ribbon synapse of a photoreceptor cell (PC) synthesizes glutamate which is continuously released during darkness. The postsynaptic elements are dendrites of bipolar (BC) and horizontal cells (HC). Horizontal cells release GABA which is formed from glutamate. The synaptic complexes are surrounded by Müller cell sheets; the right side shows neurotransmitter uptake systems and some metabolization ways of Müller cells. Glutamate, GABA, and ammonia ($NH_4^+$) are transported into the Müller cell and transformed to glutamine and α-ketoglutarate (α-KG). Glutamine is released from Müller cells and serves as precursor for the transmitter synthesis in neurons (glutamate-glutamine cycle). Lactate, alanine, pyruvate, α-ketoglutarate, and glutamine are utilized by neurons as substrates for their energy metabolism. The mitochondrial enzyme GABA transaminase (GABA-T) catalyzes the formation of glutamate from 2-oxoglutarate (2OG), coupled to a conversion of GABA to succinate semialdehyde (SSA). Another metabolic way is the production of reduced glutathione (GSH) which is an intracellular antioxidant, released from Müller cells and taken up by neurons under oxidative stress conditions. AcCoA, acetyl coenzyme A; ALAT, alanine aminotransferase; CGA, cystine-glutamate antiporter; CyT, cystine transporter; EAAC1, excitatory amino acid carrier 1; EAAT5, excitatory amino acid transporter 5; GAD, glutamic acid decarboxylase; GAT, GABA transporter; GLAST, glutamate-aspartate transporter; GLT-1, glutamate transporter-1; GP, glutathione peroxidase; GR, glutathione reductase; GS, glutamine synthetase; GSSG, glutathione disulfide; LDH, lactate dehydrogenase; PAG, phosphate-activated glutaminase; *R*•, free radicals; TCA, tricarboxylic acid cycle.

### *Glial glutamate transporters*

Müller cells regulate retinal glutamate levels via sodium-dependent and -independent uptake systems (Sarthy et al., 2005). The sodium-dependent uptake involves at least five excitatory amino acid transporters (EAAT1-5) (Kanai and Hediger, 2004). EAATs mediate the transport of L-glutamate, L-aspartate, and D-aspartate. The major GLT of Müller cells is the electrogenic, sodium-dependent, high-affinity glutamate-aspartate transporter (GLAST or EAAT1) (Rauen, 2000). In Müller cells of the mouse, approximately 50% of glutamate is taken up via GLAST, another 40% through electroneutral, sodium-dependent GLTs, and 10% via sodium-independent transporters or exchangers (Sarthy et al., 2005). The presence of further EAATs in Müller cells of various species has been described: glutamate transporter-1 (GLT-1 or EAAT2; goldfish, rat, man), excitatory amino acid carrier 1 (EAAC1 or EAAT3; carp, bullfrog, rat, man), EAAT4 (rat, cat), and EAAT5 (rat) (Rauen, 2000; Vandenbranden et al., 2000; Zhao and Yang, 2001; Kugler and Beyer, 2003; Fyk-Kolodziej et al., 2004; Ward et al., 2005).

Rat Müller cells express, in addition to normally spliced GLAST, the splice variants GLAST1a and 1b which lack exon 3 and 9, respectively (Macnab et al., 2006; Macnab and Pow, 2007). While GLAST is localized throughout the Müller cell membrane, GLAST1a is localized preferentially to the endfeet and inner stem processes, suggesting a selective regulation of GLAST function in different membrane domains of the cells (Macnab et al., 2006).

Glutamate-aspartate transporter is essential for the maintenance of normal synaptic transmission. Knockout or antisense knockdown of GLAST results in a marked suppression of the electroretinogram b-wave, which reflects the depolarization of ON-bipolar cells, and oscillatory potentials, whereas GLT-1 knockout mice exhibit minimal compromise of retinal function (Harada et al., 1998; Barnett and Pow, 2000). In GLAST knockout mice, the total retinal levels of glutamate and GABA, which is produced

from glutamate (**Figure 3**), is increased about twofold compared to that in the wild-type (Sarthy et al., 2004). While the retinas of GLAST and GLT-1 knockout mice show a benign phenotype, retinal injury after ischemia is exacerbated, suggesting that both transporters play a neuroprotective role (Harada et al., 1998).

### *Ion dependency of the glial electrogenic glutamate transport*

The transport of glutamate by EAATs involves the co-transport of three sodium ions and one proton, and the counter-transport of one potassium ion, with each glutamate anion (Kanai and Hediger, 2004). The coupling of the glutamate transport with ion transport allows a transport of glutamate into cells against a concentration gradient. The transport of sodium ions into the cell generates inward currents (**Figure 4A**) and cellular depolarization (Brew and Attwell, 1987; Barbour et al., 1988).

The amplitude of the electrogenic glutamate transport is voltage-dependent (**Figure 4B**); a very negative membrane potential is essential for the efficient uptake of glutamate (Brew and Attwell, 1987). Cell depolarization, for example by increases in extracellular potassium as occurring in ischemia and glaucoma, substantially decreases the uptake rate. EAATs are GLTs and chloride channels (**Figure 4A**) (Ryan et al., 2004). The chloride conductance of GLAST is relatively low when compared to EAAT4/5 (Grewer and Rauen, 2005). The glutamate-induced anion conductance observed in Müller cells from GLAST knockout mice (**Figure 4A**) might be mediated by EAAT5 (Sarthy et al., 2005).

### *Reversal of glial glutamate transport*

Under conditions of severe depolarization, Müller cells release glutamate via reversal of electrogenic GLTs (Szatkowski et al., 1990; Billups and Attwell, 1996). The transporter-mediated release of glutamate from Müller cells may contribute to excitotoxic damage to neurons (Szatkowski et al., 1990; Billups and Attwell, 1996; Maguire et al., 1998). Müller cells may also release aspartate, an

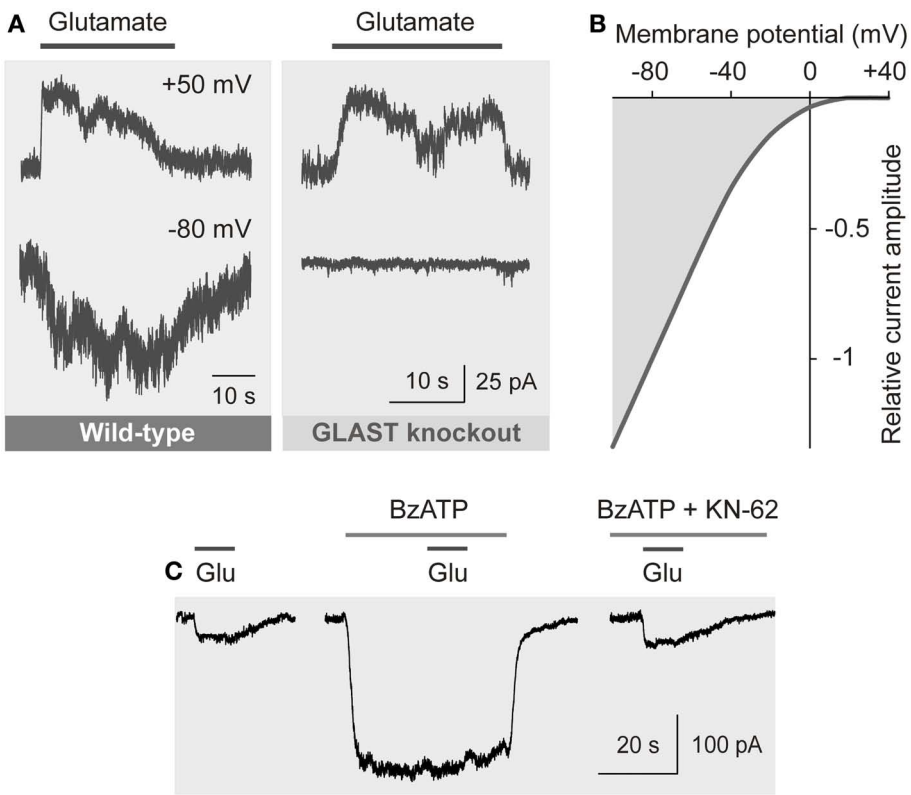


**FIGURE 4 | Electrogenic glutamate transport in Müller cells.** Whole-cell records of membrane currents were made in freshly isolated cells. **(A)** Administration of glutamate (1 mM) to a Müller cell of a wild-type mouse evoked inward currents at −80 mV, and outward currents at +50 mV. The inward currents were mediated by the sodium-dependent glutamate uptake, while the outward currents were mediated by the anionic conductance of the glutamate transporter. (The amplitude of the anionic conductance was increased by replacing extracellular chloride with thiocyanate.) In a cell of a GLAST knockout mouse, glutamate did not evoke inward currents, whereas outward currents remained. The absence of inward currents may suggest that at the resting membrane potential of murine Müller cells the electrogenic glutamate uptake is mediated by GLAST. The presence of the chloride conductance may suggest that EAAT5 (which has a large chloride conductance and minimal glutamate transport capability) is also expressed in the cells. **(B)** Current-voltage relation of the glutamate transporter currents in Müller cells of the guinea pig. (The anionic outward conductance is minimal because extracellular chloride instead of thiocyanate was used used to record the currents.) **(C)** Activation of ionotropic $P2X_7$ receptors (which mediate cation currents and thus a depolarization of the cells) decreases the electrogenic uptake of glutamate in human Müller cells. The uptake currents evoked by glutamate (Glu; 100 μM) were diminished in the presence of the $P2X_7$ receptor agonist 2′-/3′-*O*-(4-benzoylbenzoyl)-ATP (BzATP; 10 μM). Inhibition of $P2X_7$ activation by KN-62 (1 μM) suppressed the BzATP-evoked current (and cell depolarization), resulting in glutamate uptake currents similar in amplitude as under control conditions. With permission from The Society for Neuroscience (Pannicke et al., 2000) and Sarthy et al. (2005).

agonist at *N*-methyl-D-aspartate (NMDA) receptors, via reversal of GLTs; glia-derived aspartate contributes to the activation of NMDA receptors during ischemia (Marcaggi et al., 2005).

#### *Cystine-glutamate antiport*

Another mechanism of non-vesicular glutamate release is the transport via the electroneutral, sodium-independent, and chloride-dependent cystine-glutamate antiporter (Kato et al., 1993). This antiporter mediates an uptake of cystine in exchange for glutamate; cystine is used for the production of the antioxidant glutathione (**Figure 3**) (see below). Because this antiporter transports cystine using the transmembrane gradient of glutamate as driving force (Bannai and Tateishi, 1986), the exchanger can also mediate an uptake of glutamate when the extracellular concentration of glutamate is high. Oxidative stress induces increased expression of the antiporter (Mysona et al., 2009). This antiporter may contribute to the release of glutamate from Müller cells under oxidative stress conditions when an elevated production of glutathione and, therefore, an increased uptake of cystine is required (Kato et al., 1993).

#### *Vesicular release of glutamate*

After its uptake in Müller cells, glutamate can be also concentrated in secretory vesicles. Müller cells of adult mice release glutamate by a calcium-dependent non-vesicular mechanism and by calcium-dependent exocytosis of glutamate-containing vesicles (Slezak et al., 2012). Müller cell-derived glutamate is part of an autocrine glutamatergic-purinergic signaling cascade that prevents osmotic swelling of the cells (Wurm et al., 2011; Slezak et al., 2012). Glutamate released from Müller cells may also modify the light-induced neuronal activity (Newman and Zahs, 1998).

#### *Regulation of GLAST*

The expression and activity of GLAST in Müller cells is regulated by the availability of glutamate. Extracellular glutamate increases the expression of GLAST (Taylor et al., 2003) while extended exposure to high glutamate induces a time-dependent internalization of the transporters (Gadea et al., 2004). Activation of protein kinase C, e.g., after activation of ionotropic glutamate receptors or of mGluR1 and mGluR5 (Lopez-Colome et al., 1993; Lopez et al., 1998), may decrease the glutamate uptake activity (Bull and Barnett, 2002). An enhanced expression or activity of GLAST in Müller cells was observed under certain pathological conditions (Reichelt et al., 1997a) such as ischemia (Otori et al., 1994). Further factors that increase GLAST expression are cyclic AMP (Sakai et al., 2006) and neurotrophic factors such as glial cell line-derived neurotrophic factor, brain-derived neurotrophic factor (BDNF), and pigment epithelium-derived factor (PEDF) (Delyfer et al., 2005a; Dai et al., 2012; Xie et al., 2012).

#### *Glutamate uptake – pathology*

Elevated extracellular glutamate causes neuronal loss in many retinal disorders including glaucoma, ischemia, diabetic retinopathy, and inherited photoreceptor degeneration (Lieth et al., 1998; Dkhissi et al., 1999; Delyfer et al., 2005b). The uptake of glutamate by Müller cells blocks the neurotoxic effect of the transmitter (Izumi et al., 1999). A malfunction of the glial glutamate transport thus contributes to the increase in extracellular glutamate up to excitotoxic levels. Retinal ischemia and diabetes do not significantly alter the expression of GLAST (Barnett et al., 2001; Pannicke et al., 2005, 2006; Ward et al., 2005) but reduce the efficiency of the glutamate transport into Müller cells (Barnett et al., 2001; Li and Puro, 2002). Under these conditions, a high amount of glutamate is transported into photoreceptor, bipolar, and ganglion cells (Barnett et al., 2001).

Experimental glaucoma causes a failure of GLAST activity that results in a decreased accumulation of glutamate in Müller cells and in a significant glutamate uptake by retinal ganglion cells; the failure of GLAST coincides with the excitotoxic damage to the retina (Holcombe et al., 2008). Deletion of GLAST in mice leads to optic nerve degeneration similar to normal tension glaucoma (Harada et al., 2007). Increased intraocular pressure causes retinal hypoxia that stimulates free radical formation in the mitochondria and lipid peroxidation which disrupts the glutamate transport into Müller cells. A similar mechanism, i.e., malfunction of the glutamate transport into Müller cells caused by free radicals formed in mitochondria, may explain the retinal ganglion cell death in Leber hereditary optic neuropathy (Beretta et al., 2006).

A major factor that decreases the efficiency of the electrogenic glutamate transport is the depolarization of Müller cells (see above) (Napper et al., 1999). Depolarization of Müller cells can be induced by inflammatory lipids such as arachidonic acid and prostaglandins which are produced under oxidative stress conditions (Birkle and Bazan, 1989; Landino et al., 1996). These inflammatory mediators inhibit the sodium-potassium-ATPase, resulting in cellular depolarization (Lees, 1991; Staub et al., 1994). Arachidonic acid directly inhibits also the electrogenic GLTs (Barbour et al., 1989). Increased membrane localization of the sodium-potassium-ATPase, as induced by interleukin-1 (Namekata et al., 2008), may counterregulate the decrease in glutamate uptake into Müller cells.

Efficient glutamate uptake by Müller cells depends on the very negative membrane potential, around −80 mV, constituted by ample expression of inwardly rectifying potassium (Kir) channels (Kofuji et al., 2000). Human Müller cells of patients with various retinopathies such as retinal detachment, proliferative vitreoretinopathy, diabetic retinopathy, and glaucoma display a depolarization as consequence of a functional inactivation or downregulation of Kir channels (Reichelt et al., 1997a). Inactivation of Kir channels and depolarization of Müller cells were also observed in animal models of various retinopathies including retinal detachment, ischemia-reperfusion, and diabetic retinopathy (Francke et al., 2001; Pannicke et al., 2004, 2006). On the other hand, human Müller cells from patients with the above-mentioned retinopathies display an increase in the density of the GLT currents when compared to cells from donors (Reichelt et al., 1997a). An increase in GLAST labeling was also observed in experimental retinal detachment (Sakai et al., 2001). The increased expression of GLTs may represent a counterregulation in response to long-lasting cellular depolarization. Chick and human Müller cells express $P2X_7$; activation of this receptor, e.g., by ATP released from Müller cells (Newman, 2003; Reichenbach and Bringmann, 2013), causes membrane depolarization which impairs the uptake of glutamate (**Figure 4C**) (Pannicke et al., 2000; Anccasi et al., 2013). High-glucose was shown to induce downregulation of GLAST

and Kir channels in cultured Müller cells (Zeng et al., 2010; Xie et al., 2012; but, see Mysona et al., 2009). Treatment with PEDF or taurine inhibited these effects (Zeng et al., 2010; Xie et al., 2012). Dietary taurine ameliorates diabetic retinopathy in part via increased GLAST expression (Zeng et al., 2009). Further factors which reduce the glutamate transport in Müller cells are a reduction of the extracellular pH, as occurring in ischemia, zinc ions released from photoreceptors, and an increase in intracellular glutamate as observed after retinal detachment and in experimental diabetic retinopathy (Billups and Attwell, 1996; Marc et al., 1998b; Spiridon et al., 1998; Gowda et al., 2011).

#### *Uptake of ammonia*

Ammonia is formed in glutamatergic and GABAergic neurons in the course of the generation of glutamate from glutamine (**Figure 3**). It is released and taken up by glial cells (Tsacopoulos et al., 1997). Ammonia induces an alkalinization of the Müller cell interior which stimulates the glutamate uptake through GLAST. The mechanism of ammonia uptake by Müller cells is unclear. Retinal glial cells of the bee take up ammonia via a chloride cotransporter (Marcaggi et al., 2004).

## GLUTAMATE METABOLISM

#### *Production of glutamine*

After being taken up by Müller cells, glutamate is amidated to glutamine by the enzyme, glutamine synthetase (**Figure 3**). Glutamine can be also transaminated to α-ketoglutarate which is released and taken up by neurons as a substrate for their oxidative metabolism, utilized for the production of glutathione, or loaded into secretory vesicles (**Figure 3**). In the neural retina, glutamine synthetase is localized in the cytosol of astrocytes and Müller cells (Riepe and Norenburg, 1977). Glutamine is released from Müller cells and taken up by neurons as a precursor for the synthesis of glutamate and GABA (**Figure 3**) (Pow and Crook, 1996). Alternatively, glutamine is transported into the mitochondria of Müller cells (see below). The shuttle of glutamate and glutamine, respectively, between neurons and Müller cells is known as glutamate-glutamine cycle.

The glutamine synthetase activity enhances the rate of the glutamate uptake by Müller cells (Shaked et al., 2002). Due to the efficiency of the glutamine synthetase, free glutamate in Müller cells can be demonstrated immunohistochemically only when the glutamine synthetase activity is inhibited pharmacologically or under pathological conditions (Pow and Robinson, 1994; Marc et al., 1998b). When the glutamine synthetase in Müller cells is pharmacologically blocked, bipolar and ganglion cells lose their free glutamate content, and the animals become rapidly, i.e., within 2 min, functionally blind (Pow and Robinson, 1994; Barnett et al., 2000). The lack of immunohistochemically detectable free glutamate in bipolar and ganglion cells after inhibition of the glutamine synthetase suggests that these neurons do not synthesize significant amounts of glutamate from other substrates than glutamine (Pow and Robinson, 1994). On the other hand, inhibition of the glutamine synthesis decreases, but not abolishes, the level of detectable free glutamate in photoreceptor cells (Pow and Robinson, 1994). This suggests that photoreceptor cells take up significant amounts of glutamate from the synaptic cleft (Hasegawa et al., 2006) and are able to synthesize glutamate by transamination of α-ketoglutarate (Pow and Robinson, 1994).

Müller cells possess enzymes that are involved in the *de novo* synthesis of glutamate from pyruvate, e.g., pyruvate carboxylase, that catalyzes the carboxylation of pyruvate to oxaloacetate as substrate of the Krebs cycle, and glutamate dehydrogenase, that converts α-ketoglutarate to glutamate (Gebhard, 1992; Ola et al., 2011a). Glutamate dehydrogenase is able to metabolize glutamate at relatively low pH (Zaganas et al., 2012) that prevails in glial cells following glutamate uptake (Bouvier et al., 1992). The activity of the malate-aspartate shuttle in Müller cells is low (LaNoue et al., 2001) due to the low expression of the aspartate aminotransferase (Gebhard, 1991) and of glutamate-aspartate exchangers (Xu et al., 2007). Thus, the bulk of free glutamate is converted to glutamine, and only a small fraction of glutamate is transported into the mitochondria (Poitry et al., 2000). However, under pathological conditions, when the expression of glutamine synthetase is decreased (see below), more glutamate enters the mitochondria of Müller cells. The loss of the glucocorticoid-mediated inhibition of the expression of the glutamate-aspartate exchanger (Ola et al., 2005) under such conditions (see below) may increase the importance of oxidative glutamate metabolism.

#### *Regulation of the glutamine synthetase*

The gene transcription of both GLAST and glutamine synthetase is stimulated by glucocorticoids (Gorovits et al., 1996). The upstream region of the glutamine synthetase gene contains a glucocorticoid response element (GRE) that can bind the glucocorticoid receptor protein (Zhang and Young, 1991). There is an inverse relation between the expression of glutamine synthetase and Müller cell proliferation in the developing and injured mature retina (Gorovits et al., 1996; Kruchkova et al., 2001). At early developmental stages, the c-Jun protein, which is a component of the AP1 complex of transcription factors that regulates cellular proliferation, is abundant in proliferating retinal cells. This protein renders the glucocorticoid receptor molecules transcriptionally inactive, and glucocorticoids cannot induce the expression of glutamine synthetase (Berko-Flint et al., 1994). Concomitant with a decline in cell proliferation and c-Jun expression, the developing retina acquires the capability to express glutamine synthetase in response to glucocorticoids.

#### *Glutamine synthetase – pathology*

The expression of the glutamine synthetase is regulated by glutamate. The expression of glutamine synthetase in Müller cells is reduced when the major glutamate-releasing neuronal population, the photoreceptors, degenerate, as observed in inherited photoreceptor degeneration, retinal light injury, and retinal detachment (Lewis et al., 1989; Grosche et al., 1995; Härtig et al., 1995). A decline in glutamine synthetase expression and activity was also observed under ischemic, inflammatory, and traumatic conditions, and in glaucoma (Nishiyama et al., 2000; Kruchkova et al., 2001; Moreno et al., 2005). Downregulation of the glutamine synthetase results in a depletion of neuronal glutamate (Gionfriddo et al., 2009). No alterations, or even a slight enhancement, in the glutamine synthetase expression in Müller cells was observed in diabetic retinopathy and after optic nerve crush (Mizutani et al.,

1998; Lo et al., 2001; Chen and Weber, 2002; Gerhardinger et al., 2005; but, see Yu et al., 2009). An increase in the glutamine synthetase expression was also observed under conditions of increased ammonia (Germer et al., 1997; see below).

Downregulation of the glutamine synthetase in the rat retina by using siRNA induces glial dysfunction which results in a breakdown of the blood-retinal barrier (Shen et al., 2010). This suggests that impairment of Müller cell's glutamate metabolism also disturbs the integrity of the blood-retinal barrier.

#### *Regulation of glutamine synthetase by soluble factors*

The decline in the glutamine synthetase expression in Müller cells under pathological conditions is induced, at least in part, by soluble factors such as basic fibroblast growth factor (bFGF) and interleukin-1ß(Kruchkova et al., 2001; Shen and Xu, 2009). These factors increase the level of c-Jun and inhibit the glucocorticoid-induced expression of the glutamine synthetase (Kruchkova et al., 2001; Shen and Xu, 2009). bFGF is rapidly released in the retina after detachment (Geller et al., 2001), and increasingly expressed under ischemic and various other pathological conditions (Miyashiro et al., 1988; Gao and Hollyfield, 1996; Kruchkova et al., 2001). Though bFGF is a major neurotrophic factor which supports neuronal survival (Faktorovich et al., 1990), the bFGF-induced downregulation of the glutamine synthetase might rather aggravate neuronal degeneration. The decrease in glutamine synthetase expression after retinal detachment is likely also a result of the interruption in the supply with PEDF after the separation of Müller cells from the pigment epithelium (Jablonski et al., 2001). PEDF also inhibits the interleukin-1ß-induced downregulation of the glutamine synthetase in diabetic retinopathy (Shen et al., 2011). In addition to PEDF, hydrocortisone, taurine, and BDNF increase the expression of glutamine synthetase (Zeng et al., 2010; Ola et al., 2011b; Dai et al., 2012). Cannabidiol preserves the glutamine synthetase activity by blocking tyrosine nitration that inhibits the enzyme in diabetes (El-Remessy et al., 2010).

#### *Ammonia-induced regulation of glutamine synthetase – hepatic retinopathy*

The expression of glutamine synthetase is also regulated by the availability of ammonia. Elevated ammonia induces upregulation of the enzyme (Reichenbach et al., 1995b; Germer et al., 1997). As the glutamine synthetase is the most important enzyme available for ammonia detoxification in the retina (**Figure 3**), this is an important additional function of neurotransmitter recycling by Müller cells.

Liver diseases cause hyperammonemia associated with an increase in the cerebral ammonia to toxic levels (Swain et al., 1992; Tofteng et al., 2006). Hyperammonemia is a key factor in the pathogenesis of hepatic encephalo- and retinopathy (Reichenbach et al., 1995b; Albrecht and Norenberg, 2006). The major complication of fulminant hepatic failure is the development of brain edema characterized by swelling of astrocytes (Willard-Mack et al., 1996). In the neural retina, pathological alterations are found primarily in Müller cells and astrocytes; these alterations include cellular swelling (**Figure 5A**), mitochondrial dysfunction (**Figure 5B**), vacuolization, and necrosis (Reichenbach et al., 1995a; Karl et al., 2011). Ammonia-induced glial cell swelling depends on glutamine synthesis rather than on ammonia *per se* (Willard-Mack et al., 1996; Karl et al., 2011). The glutamine-induced swelling of Müller cells is accelerated under hypoosmotic conditions (**Figure 5A**) (Karl et al., 2011). Such conditions occur *in situ*; osmotic gradients across the glio-vascular interface are a result of ionic disbalances in the blood caused by, for example, hyponatremia due to liver disease and renal impairment.

The detoxification of excess ammonia is mediated by the formation of glutamine (**Figure 3**). Excess cytosolic glutamine is transported into the mitochondria where it is hydrolyzed to glutamate and ammonia by the phosphate-activated glutaminase (**Figure 5C**). High ammonia levels cause mitochondrial permeability transition (**Figures 5B,C**) and generation of free oxygen radicals (Albrecht and Norenberg, 2006; Karl et al., 2011). This results in mitochondrial dysfunction, energy failure, and in activation of cytosolic enzymes that generate oxygen and nitrogen radicals, e.g., nitric oxide synthases (**Figure 5C**) (Karl et al., 2011). Free radicals stimulate the production of inflammatory lipids including arachidonic acid and prostaglandins (**Figure 5C**) (Landino et al., 1996; Offer et al., 2005). These lipids inhibit the sodium-potassium-ATPase which results in intracellular sodium overload, water influx, and cellular swelling (**Figure 5C**) (Lees, 1991; Staub et al., 1994). The energy failure due to mitochondrial dysfunction and the high energy consumption of the glutamine synthetase reaction contribute to the inhibition of the sodium-potassium-ATPase. The decrease in glutathione synthesis (**Figure 3**) due to the enhanced glutamate consumption contributes to oxidative stress conditions (Reichenbach et al., 1999).

#### *Production of glutathione*

The uptake of GABA and glutamate by Müller cells links neuronal excitation with the defense against oxidative stress. Müller cells provide photoreceptors and neurons with an antioxidative environment (Bringmann et al., 2009). The major glia-derived antioxidant is reduced glutathione produced from glutamate, cysteine, and glycine (**Figure 3**) (Pow and Crook, 1995). Under normal conditions, retinal glutathione is concentrated in glial and horizontal cells (Pow and Crook, 1995; Schütte and Werner, 1998). In response to oxidative stress, glutathione is rapidly released from Müller cells and provided to neurons (Schütte and Werner, 1998) where it acts as cofactor of enzymes which remove toxic peroxides and regulate protein function through thiolation and dethiolation, such as glutathione peroxidase, reductase, transferase, and glutaredoxin.

The production of glutathione in Müller cells is critically dependent on the availability of extracellular glutamate and cystine (**Figure 3**) (Reichelt et al., 1997b). Inhibition or knockout of GLAST decrease the glutathione production (Reichelt et al., 1997b; Harada et al., 2007). Extracellular cystine is taken up mainly via the cystine-glutamate antiporter (**Figure 3**); inhibition of the antiporter results in a huge decrease in retinal glutathione (Kato et al., 1993). Inhibition of the antiporter can also result from an increase in extracellular glutamate (see above). Downregulation of the glutamine synthetase as occurring under pathological conditions (see above) results in a depletion of neuronal glutamate which also causes a decrease in retinal glutathione (Gionfriddo et al., 2009). Müller cells from aged animals contain reduced

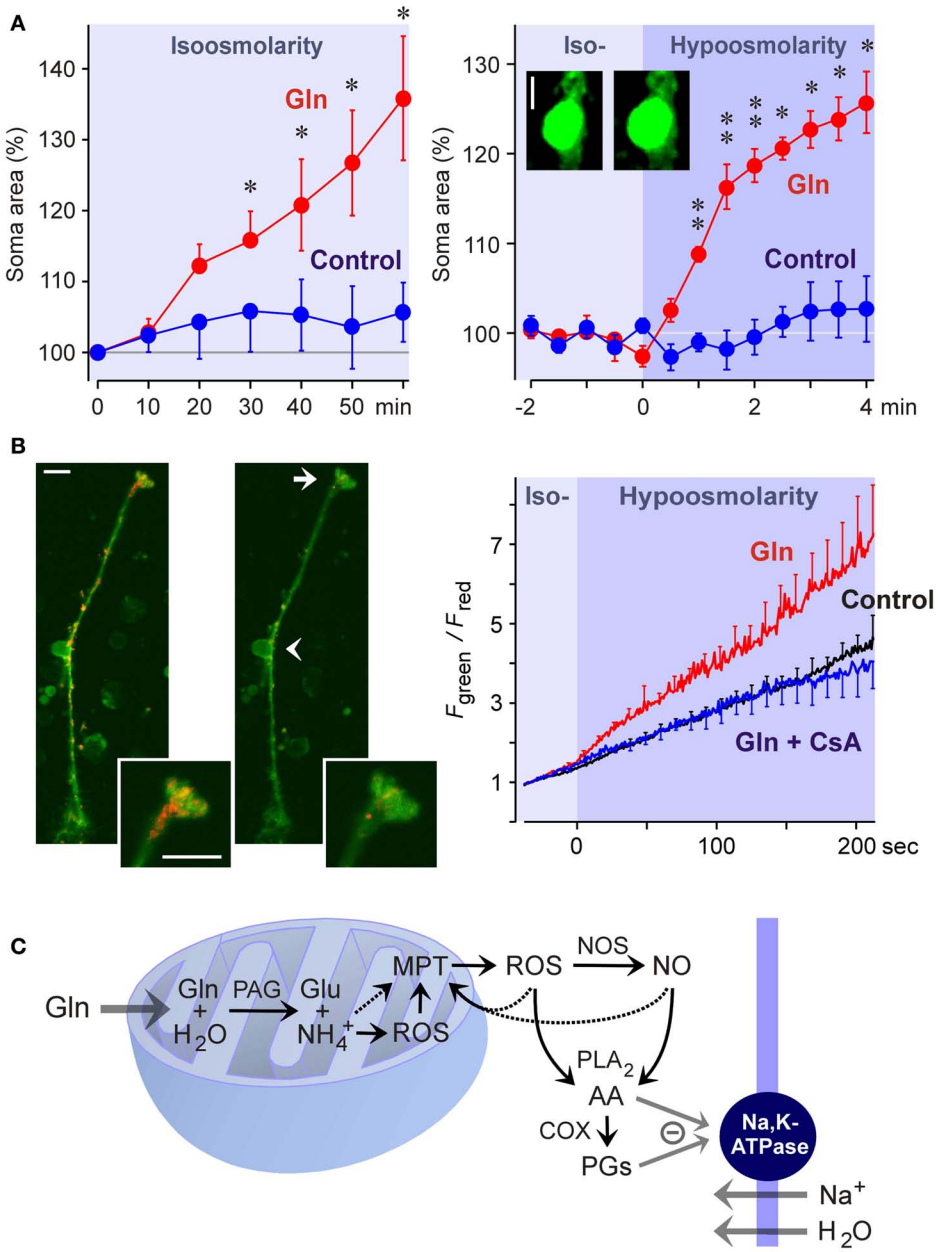


**FIGURE 5 | Glutamine (Gln) induces mitochondrial dysfunction and Müller cell swelling.** Experiments were carried out in retinal slices **(A)** and isolated Müller cells **(B)** of the rat. **(A)** Glutamine (5 mM) in isoosmotic extracellular solution induced a delayed swelling of Müller cells after 10 min of exposure (*left*) whereas glutamine (5 mM) in hypoosmotic solution (60% of control osmolarity) induced a rapid (within 1 min) swelling of Müller cells (*right*). The cross-sectional area of Müller cell somata was recorded and is expressed in percent of the value obtained before superfusion of the slices with the solutions (100%). The *images* display original records of a Müller cell soma obtained before (*left*) and during (*right*) superfusion with the glutamine-containing hypoosmotic solution. Scale bar, 5 μm. Significant difference vs. control: $*P < 0.05$; $**P < 0.01$. **(B)** Glutamine induces a dissipation of the mitochondrial membrane potential in Müller cells. The potential was recorded from the endfeet of freshly isolated cells by using the mitochondria-selective potentiometric dye JC-1. Mitochondrial depolarization is indicated by an increase in the green-to-red fluorescence intensity ratio. *Left side:* Example of a JC-1-loaded cell which was recorded before (*left*) and during (*right*) superfusion of a hypoosmotic solution containing glutamine (5 mM). *Insets*, cell endfoot at higher magnification. Note the decrease in the red fluorescence. *Arrow*, cell endfoot. *Arrowhead*, cell soma. Bars, 10 μm. *Right:* Time-dependent change in the ratio of the green-to-red fluorescence of the JC-1 dye. The fluorescence was recorded in the absence and presence of glutamine (5 mM), before and during the transition of an isoosmotic to a hypoosmotic extracellular solution. Glutamine-induced a faster dissipation of the mitochondrial membrane potential as compared to control. The inhibitor of the mitochondrial permeability transition, cyclosporin A (CsA; 1 μM), prevented the glutamine-induced dissipation of the mitochondrial potential. **(C)** Hypothetical scheme of the mechanism of glutamine-induced Müller cell swelling. Cytosolic glutamine is transported into the mitochondria and is hydrolyzed there to glutamate (Glu) and ammonia ($NH_4^+$) by the action of the phosphate-activated glutaminase (PAG).

*(Continued)*

**FIGURE 5 | Continued**
High ammonia levels stimulate the mitochondrial production of reactive oxygen species (ROS) and induce mitochondrial permeability transition (MPT) that leads to mitochondrial dysfunction, energy failure, and enhanced free radical production. Mitochondria-derived free radicals activate cytosolic enzymes that generate reactive oxygen and nitrogen species, e.g., nitric oxide synthases (NOS). These radicals stimulate the activity of phospholipases $A_2$ ($PLA_2$), that produce arachidonic acid (AA), lipoxygenases, and cyclooxygenases (COX) which generate prostaglandins (PGs). Arachidonic acid and prostaglandins inhibit the sodium-potassium-ATPase resulting in intracellular sodium overload, water influx, and cellular swelling. The energy failure due to mitochondrial dysfunction may contribute to the inhibition of the sodium-potassium-ATPase. With permission from Karl et al. (2011).

levels of glutathione; this is associated with mitochondrial damage, membrane depolarization, and reduced cell viability (Paasche et al., 2000).

### *Metabolic support of photoreceptors and neurons*

The metabolization of glutamate in Müller cells is tightly coupled to the nutritive function of glia. In the retina, glucose uptake and metabolism occurs predominantly in the inner processes of Müller cells, localized to the inner plexiform and ganglion cell layers (Poitry-Yamate et al., 2013) where glutamatergic signaling occurs. Müller cells produce substrates for the oxidative metabolism of photoreceptors and neurons including glutamine, lactate, pyruvate, alanine, and α-ketoglutarate (**Figure 3**) (Poitry-Yamate et al., 1995; Tsacopoulos et al., 1997). These substrates are used by photoreceptors and neurons in periods of metabolic stress, e.g., in the dark. The production of lactate in Müller cells is stimulated by glutamate and ammonia (Poitry-Yamate et al., 1995; Poitry et al., 2000). Energy substrates are formed from glutamate also by other metabolic pathways, e.g., transamination of pyruvate (**Figure 3**) (Poitry et al., 2000). The conversion of glutamate into α-ketoglutarate is reduced in Müller cells of diabetic rats; this results in increased intracellular glutamate which impairs the glutamate uptake by Müller cells (Gowda et al., 2011).

## CONCLUDING REMARKS

Müller cells contribute to the removal of extracellular GABA and glutamate. The uptake and metabolization of the transmitters by Müller cells is part of the glutamate-glutamine cycle, and is linked to various other functions of the cells including the metabolic support of photoreceptors and neurons, the defense against oxidative stress, the shaping and termination of the synaptic neurotransmitter action, the release of gliotransmitters, and the detoxification of excess ammonia. Müller cells remove the bulk of extracellular glutamate (Rauen, 2000); this may be important for setting the signal-to-noise ratio of glutamatergic transmission.

Though research of the last two decades yielded a huge increase in our knowledge regarding the functional roles of Müller cells, there remain many open questions. A fundamental issue is the relative contribution of neuronal vs. glial GLTs in the shaping of excitatory synaptic responses. Likewise, the role of glial GATs in controlling retinal neurotransmission is presently unclear. The role of glial transporters in shaping of synaptic transmission may vary in dependence on the type of synapses. Photoreceptor terminals are suggested to directly remove glutamate from the synaptic cleft, with a minimal contribution of glial glutamate uptake (Hasegawa et al., 2006). On the other hand, recent evidence suggests a crucial role of GLAST-mediated glutamate uptake in the synaptic transmission within the outer plexiform layer (Levinger et al., 2012). Whether the opposing data reflect species differences remains to be clarified. It was suggested that Müller cells act as diffusion barriers for synaptically released glutamate in the outer plexiform layer that separates individual synapses from each other (Rauen et al., 1996). However, the recent finding of a glutamate spillover between cone photoreceptors (Szmajda and Devries, 2011) questions the existence of such a diffusion barrier. Glial transporters may play a more active role in shaping the excitatory responses in the inner retina (Matsui et al., 1999; Higgs and Lukasiewicz, 2002). More information concerning the functional roles of neuronal and glial GLTs at different retinal synapses is needed.

There are various other points which remain to be resolved. For example, it is not known to what extent Müller cells in retinas of birds and lower vertebrates take up GABA. The relative contribution of electrogenic vs. electroneutral transporters (Sarthy et al., 2005) in the glutamate uptake by Müller cells also remains an open issue. What are the specific functional roles of the different types and splice variants of GLTs in Müller cells? Which neuro- and gliotransmitters regulate the neurotransmitter recycling by Müller cells? How ammonia is taken up by the cells?

Dysregulation of Müller cell-provided glutamate recycling is a common phenomenon in retinopathies, and is associated with increased extracellular glutamate. The pathogenic mechanisms resulting in impaired glial glutamate uptake are incompletely understood. Inflammation and oxidative stress are major causative factors. The decrease in uptake might be caused by the downregulation/inactivation of the glutamine synthetase, by mitochondria-derived free radicals, which inactivate the transporter molecules, as well as by cell depolarization resulting from high extracellular potassium, energy failure due to mitochondrial dysfunction, downregulation/inactivation of potassium channels, and inhibition of the sodium-potassium-ATPase and GLTs through inflammatory lipid mediators. The depolarization of Müller cells may be strong enough to reverse the operation mode of GLTs resulting in an efflux of glutamate that contributes to excitotoxic neuronal damage. A significant portion of glial glutamate might be also released through the cystine-glutamate antiporter and vesicular exocytosis. Understanding the cellular mechanisms involved in the impairment of glial glutamate recycling may help to find novel targets for the development of neuroprotective agents. An increase in glutamine synthetase in Müller cells may represent such an approach which has been shown to protect against neuronal degeneration in the injured retinal tissue (Gorovits et al., 1997).

## ACKNOWLEDGMENTS

Some of the work presented in this review was conducted with grants from the Deutsche Forschungsgemeinschaft (GRK 1097/1, RE 849/10-2, and RE 849/12-2 to Andreas Reichenbach; PA615/2-1 to Thomas Pannicke).

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**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

*Received: 19 February 2013; accepted: 04 April 2013; published online: 17 April 2013.*

*Citation: Bringmann A, Grosche A, Pannicke T and Reichenbach A (2013) GABA and glutamate uptake and metabolism in retinal glial (Müller) cells. Front. Endocrinol.* ***4****:48. doi: 10.3389/fendo.2013.00048*

*This article was submitted to Frontiers in Cellular Endocrinology, a specialty of Frontiers in Endocrinology.*

frontiers in
ENDOCRINOLOGY

REVIEW ARTICLE
published: 25 November 2013
doi: 10.3389/fendo.2013.00176

# Methodological limitations in determining astrocytic gene expression

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Traditionally, astrocytic mRNA and protein expression are studied by *in situ* hybridization (ISH) and immunohistochemically. This led to the concept that astrocytes lack aralar, a component of the malate-aspartate-shuttle. At least similar aralar mRNA and protein expression in astrocytes and neurons isolated by fluorescence-assisted cell sorting (FACS) reversed this opinion. Demonstration of expression of other astrocytic genes may also be erroneous. Literature data based on morphological methods were therefore compared with mRNA expression in cells obtained by recently developed methods for determination of cell-specific gene expression. All Na,K-ATPase-α subunits were demonstrated by immunohistochemistry (IHC), but there are problems with the cotransporter NKCC1. Glutamate and GABA transporter gene expression was well determined immunohistochemically. The same applies to expression of many genes of glucose metabolism, whereas a single study based on findings in bacterial artificial chromosome (BAC) transgenic animals showed very low astrocytic expression of hexokinase. Gene expression of the equilibrative nucleoside transporters ENT1 and ENT2 was recognized by ISH, but ENT3 was not. The same applies to the concentrative transporters CNT2 and CNT3. All were clearly expressed in FACS-isolated cells, followed by biochemical analysis. ENT3 was enriched in astrocytes. Expression of many nucleoside transporter genes were shown by microarray analysis, whereas other important genes were not. Results in cultured astrocytes resembled those obtained by FACS. These findings call for reappraisal of cellular nucleoside transporter expression. FACS cell yield is small. Further development of cell separation methods to render methods more easily available and less animal and cost consuming and parallel studies of astrocytic mRNA and protein expression by ISH/IHC and other methods are necessary, but new methods also need to be thoroughly checked.

**Keywords: astrocyte culture, BAC transgenic animals, fluorescence-assisted cell sorting, GFAP, immunohistochemistry, *in situ* hybridization**

## INTRODUCTION

Enzymes and transporters, involved in production and degradation of glutamate and GABA are expressed in astrocytes. Some have been known for a long time to be astrocyte-specific, e.g., glutamine synthetase (GS) (1), pyruvate carboxylase (2), and cytosolic malic enzyme (3). However, identification of either mRNA or protein expression in astrocytes in the brain *in vivo* or intact brain tissue, such as brain slices, is difficult on account of its extreme anatomical complexity. Conventional immunohistochemical methods seem occasionally to fail in demonstrating the expression of certain genes, as recently demonstrated in the case of aralar, a glutamate/aspartate exchanger operating in the malate-aspartate-shuttle (MAS). Its abundant mRNA (4) and protein (5) expression has been demonstrated by usual biochemical methods in freshly isolated mouse astrocytes and in well differentiated cultured astrocytes (5). However, gene expression of the aralar gene has repeatedly been found to be absent or sparsely expressed when morphology-based immunochemical methods were used, as will be described in Section "Determination of the Expression of Genes Involved in Different Pathways using Different Methodologies."

Traditional morphology-based methods for studying cell type-specific gene expression in brain *in vivo* or in excised brain tissues are *in situ* hybridization (ISH) and immunohistochemistry (IHC), analyzing mRNA and protein expression, respectively. Immunocytochemistry (ICC) is used for the same purpose in isolated cells. A technique, which has been newly established for adult brain, fluorescence-assisted cell sorting (FACS) yields highly purified populations of different types of brain cells (4, 6). It uses insertion of a fluorescent compound into the cell by aid of the promoter of an astrocyte-specific gene, such as glial fibrillary acidic protein

**Abbreviations:** AGC, glutamate/aspartate exchanger; BAC, bacterial artificial chromosome; BGT, low-affinity GABA transporter; CNT, Concentrative nucleoside transporters; COX, cytochrome oxidase; DMS, direct mRNA sequencing; dBcAMP, dibutyryl cyclic AMP; EAAT, glutamate transporters; ENT, equilibrative nucleoside transporters; FACS, fluorescence-assisted cell sorting; Fgfr, fibroblast growth factor receptor; GATs, high-affinity subtypes GABA transporters; GFAP, glial fibrillary acidic protein; GS, glutamine synthetase; α-KG, α-ketoglutarate; MAS, malate-aspartate-shuttle; OAA, oxaloacetate; OGC, malate/α-ketoglutarate exchanger; PI, propidium iodide; RT-PCR, reverse transcription-polymerase chain reaction; TCA, tricarboxylic acid.

(GFAP) or S100β, a principle developed for insertion of green fluorescent protein (7), and thus depends upon preservation of an intact cell. This and related methods are often used for determination of mRNA expression by microarray analysis, a quantitatively less accurate method, as evident from **Tables 1** and **2**, where different results quite often were obtained by different authors and from results by Hertz et al. (8), where consistency between different samples in some cases was poor. Nevertheless it is useful because of its requirement for very little tissue. This is a necessity for simultaneous determination of the expression of multiple genes, since the cell yield by FACS is small. The microarray analysis provides numbers, not a direct indication whether a specific gene is expressed or not. Therefore a numerical analysis is needed. In the paper by Lovatt et al. (4) the authors interpret several results as indicating whether a gene is expressed or not, but in their published Table (9) only numbers are presented. In reference (9), they also present a Table showing a comparison with non-astrocytes in the case of Lovatt et al. (4), and neurons in the case of Cahoy et al. (6) and indicate fold-enrichment and *P* values. This table has been used in connection with the Lovatt (4) and Doyle (10) data in **Tables 1** and **2** in the present paper, whereas the Cahoy data is based upon the numbers in the comprehensive Table provided by (6). This is because astrocyte expression, not enrichment is the topic of this paper.

The microarray analysis by Doyle et al. (10) also provides numbers. However, their analysis was not based on FACS but was based on generation of transgenic mice that expressed the ribosomal protein L10a in a cell-specific manner. Since this protein had been tagged with enhanced green fluorescent protein (EGFP), they could check proper cell-specific labeling by the green fluorescence and simultaneously use anti-GFP antibodies for immunopurification to enrich ribosome-associated, actively translated mRNAs. This procedure allowed them to bypass the cell separation procedure and instead use a total cell homogenate. The astrocytic-specific *Aldh1L1* (6) was used for their studies of astrocytic gene expression. The methodology employed in each of these studies is described in more detail in Section "Cell Sorting Based on Recognition of Cell-Specific Proteins." Here it suffices to note that in spite of the different procedures employed in these three studies, and although Lovatt et al. (4) and Doyle et al. (10) used adult mice, and the oldest mice studied by Cahoy et al. (6) were 17-day-old, the results are relatively, although far from completely, similar. In **Table 1** the two methods are therefore indicated close to each other, with all microarray results in red.

**Table 1 | Expression of astrocytic genes determined by different methodologies**.

| Protein title | Gene | mRNA | | | | Protein | | Gene |
|---|---|---|---|---|---|---|---|---|
| | | ***In situ* hybridization** | **FACS RT-PCR** | **Microarray** | **Culture RT-PCR** | **Immuno-histochemistry** | **Culture** | |
| Aralar | *Slc25a12* | | Present (5) | + (4) + (6) + (10) | Present (5) | Absent (11, 12)<br>Present (13) | Present (5) | *Slc25a12* |
| GLAST | *Slc1a3* | Present (14, 15) | | + (4) + (6) + (10) | | Present (16, 17) | Present (18) | *Slc1a3* |
| GLT-1 | *Slc1a2* | Present (19, 20) | | + (4) + (6) + (10) | | Present (21) | Present (22, 23) | *Slc1a2* |
| GAT1 | *Slc6a1* | Present (24, 25) | | + (4) + (6) + (10) | | Present (26) | | *Slc6a1* |
| GAT 2 | *Slc6a13* | Present (24, 26) | | − (4) − (6) − (10) | | Present (26, 27) | | *Slc6a13* |
| GAT 3 | *Slc6a11* | Present (26, 27) | | + (4) + (6) + (10) | | Present (28, 29) | | *Slc6a11* |
| BGT 1 | *Slc6a12* | | | − (4) − (6) − (10) | Present (30, 31) | | | *Slc6a12* |
| ENT1 | *Slc29a1* | Present (32, 33) | Present (34) | − (4) + (6) + (10) | Present (35) | | | *Slc29a1* |
| ENT2 | *Slc29a2* | Present (32) | Present (34) | − (4) + (6) + (10) | Present (35) | | | *Slc29a2* |
| ENT3 | *Slc29a3* | | Present (34) | − (4) + (6) + (10) | Present (35) | | | *Slc29a3* |
| ENT4 | *Slc29a4* | | Present (34) | − (4) − (6) + (10) | | Absent (36) | | *Slc29a4* |
| CNT1 | *Slc28a1* | | Absent (34) | | Present (37) | | | *Slc28a1* |
| CNT2 | *Slc28a2* | Absent (33) | Present (34) | − (4) − (6) + (10) | Present (35) | | | *Slc28a2* |
| CNT3 | *Slc28a3* | Low expression (33) | Present (34) | − (4) | Absent (35) | | | *Slc28a3* |
| Na,K-ATPase α1 | *Atp1a1* | Present (38–40) | | − (4) + (6) − (10) | | Present (41) | Present (41) | *Atp1a1* |
| Na,K-ATPase α2 | *Atp1a2* | Present (40, 42) | Present (43) | + (4) + (6) + (10) | | Present (41) | Present (41) | *Atp1a2* |
| NKCC1 | *Slc12a2* | Present (42, 44, 45) | | − (4) + (6) + (10) | | | Present (46) | *Slc12a2* |

*The data for aralar, Na,K-ATPase, and the nucleoside transporters are supposed to be comprehensive, whereas those for transporters involved in glutamate/GABA turnover are rather representative, at least with respect to immunohistochemistry and in situ hybridization. Genes consistently found to be expressed by all methodologies are shown in blue, those only found absent in one study in green, and results from studies using FACS-isolated astrocytes in red. As described in the text, microarray data were assigned as either positive or negative based on the number values in the microarray analysis itself [(6) http://www.stanford.edu/group/exonarray/cgi-bin/plot_selector.pl] and fold-enrichment and significance [6] and [10] as shown by Lovatt and Nedergaard (9), but using less stringent criteria with respect to fold enrichment (≥ 1.5) than in the original publications. This was found to be justifiable, because the microarray data here are used together with other data, but in the original publications as the only indication of gene presence. Reference numbers given in this Table refer to those in the reference list.*

**Table 2 | Drug effects on gene expression and editing in astrocytes are identical in freshly isolated cells from treated animals and primary cultures of astrocytes, but the expression is often not recognized by the microarray analyses, indicatest as + or −.**

| Protein | Gene | Drug | FACS | Culture | Microarray | | | |
|---|---|---|---|---|---|---|---|---|
| | | | | | (4) | (6) | (10) | [4*] |
| 5-$HT_{2B}$ receptor expression | *Htr2b* | Fluox | Up | Up | − | − | − | |
| 5-$HT_{2B}$ editing | *Htr2b* | Fluox | Up | Up | | | | |
| 5-$HT_{2c}$ receptor expression | *Htr2c* | Fluox | Unchanged | Unchanged | − | − | − | |
| $cPLA_{2a}$ | *Pla2g4a* | Fluox | Up | Up | − | − | − | + |
| $sPLA_2$ | *Pla2g2a* | Fluox | Unaltered | Unaltered | − | − | − | |
| ADAR2 | *Adarb1* | Fluox | Up | Up | − | − | − | − |
| GluK2 expression | *Grik2* | Fluox | Up | Up | + | + | − | − |
| GluK2 editing | *Grik2* | Fluox | Up | Up | | | | |
| GluK4 expression | *Grik4* | Fluox | Unchanged | Unchanged | − | − | − | − |
| cfos expression | *cFos* | Fluox | Up | Up | − | + | − | + |
| fosB expression | *Fosb* | Fluox | Up | Up | − | + | − | + |
| NBCe1 | *Slc4a4* | Cbz | Up | Up | + | + | + | + |
| GluK2 | *Grik2* | Cbz | Down | Down | | | | |
| $cPLA_2$ | *Pla2g4a* | Cbz | Up | Up | | | | |

*The Table shows all experiments in which we have compared drug [fluoxetine (Fluox) or carbamazepine (Cbz)] effects in cultured astrocytes and in astrocytes obtained by FACS. Complete agreement was found between these two preparations, but the correlation was poor with expression of the same genes obtained using microarray analysis and with the cultures, different from ours, studied by Cahoy et al. using microanalysis (4*). The FACS and culture data are from (47) and (48). Microarray data show only gene expression and are from Lovatt et al. [4], Cahoy et al. [6] and Doyle et al. [10] Cahoy et al. (6) also presented data for cultures different from ours, shown under [4]. As in* ***Table 1****, expression is shown as either present (+) or absent (−) based on the number values in the microarray analysis itself [2 and 4] or fold-enrichment and significance [1] and [3] shown by Lovatt and Nedergaard (9).*

If only expression of a single or a few genes are analyzed, FACS-isolated cells can also be used for a more accurate determination of mRNA by reverse transcription-polymerase chain reaction (RT-PCR), requiring larger cell samples, and even of protein by Western blot followed by reaction with a specific antibody, needing still more tissue. Gene expression in primary cultures of astrocytes has also been utilized as a method to determine astrocytic characteristics. Some recent papers criticizing this technique (49, 50) have unjustifiably failed to recognize that many types of astrocytic cultures exist and that they are not all identical. The cultures used by ourselves differ vastly both in methodology and characteristics from those previously used by Harold Kimelberg and from those discussed by Foo et al. (50). In contrast to those used by Foo et al. (50) they are grown in the presence of serum and they are differentiated by treatment with dibutyryl cyclic AMP (dBcAMP), necessary for the development of certain specific features, e.g., $K^+$-induced glycogenolysis (51) a well established phenomenon in intact brain tissue (52). In contrast to many other astrocyte cultures prepared from rats they are obtained from mice (53). Peculiarly enough, this does make a difference (54), e.g., in membrane conductance, which these authors found comparable only in mouse cells with the high membrane conductance characteristic of astrocytes in the brain *in vivo*.

The discrepancy regarding expression of the aralar gene between freshly isolated astrocytes and those studied in intact tissue by IHC raises the question whether expression of other important genes may be underestimated in the literature. This will be discussed in the present review by a comparison between results of determination of astrocytic gene expression in intact tissues, freshly isolated cells and cultures, presented in Section "Determination of the Expression of Genes Involved in Different Pathways using Different Methodologies." Some additional enzymes involved in glucose metabolism will be discussed in Section "Expression of Genes of Enzymes Catalyzing Glucose Metabolism," a few of those involved in the glutamate-glutamine cycle in Section "Enzymes and Transporters Operating in Glutamate and GABA Turnover," several of those involved in astrocytic ATP signaling (nucleoside transporter and adenosine kinase expression) in Section "Nucleoside Transporters and Adenosine Kinase," and those involved in $K^+$ homeostasis in Section "Expression of Genes of Transporters Involved in $K^+$ Clearance from the Extracellular Space." The two former are directly relevant for the present Research Topic. The two latter represent other important astrocytic functions, and are discussed, although more briefly, to better evaluate potential failures of some methodologies to disclose astrocytic gene expression. These sections will be followed by more detailed descriptions of individual methodologies in Section "Description of Methodologies Used for Determination of Gene Expression."

## DETERMINATION OF THE EXPRESSION OF GENES INVOLVED IN DIFFERENT PATHWAYS USING DIFFERENT METHODOLOGIES

### EXPRESSION OF GENES OF ENZYMES CATALYZING GLUCOSE METABOLISM

#### *Lack of astrocytic aralar expression in immunohistochemical studies versus prominent mRNA and protein expression in freshly isolated cells*

Oxidative metabolism is needed by astrocytes for two major purposes, (i) to supply ATP for energy-consuming processes, and

(ii) sto produce glutamate from glucose. This glutamate production is crucial for the production of transmitter glutamate and GABA, since glutamate does not easily enter the brain from the systemic circulation (55). The occurrence of one oxidative process during glycolysis, which generates NADH from $NAD^+$ and the inability of $NAD^+$ and NADH themselves to cross the mitochondrial membrane require that "reducing equivalents" are transferred across the mitochondrial membrane. Some shuttle mechanisms exist that are capable of doing this, but only the MAS is expressed in brain at a significant level as discussed by Dienel and Hertz (56). In the cytosol, reduction of oxaloacetate (OAA) to malate enables cytosolic NADH oxidation to $NAD^+$, and malate can be transferred across the mitochondrial membrane for re-oxidation (**Figure 1**). In MAS it enters the mitochondria in exchange with α-ketoglutarate (α-KG), using the malate/α-ketoglutarate exchanger (OGC – *Slc25a11*). OGC expression is similar in synaptic and non-synaptic mitochondria (12), it is functional in astrocytes ((13) – see also Pardo et al. in this research Topic), and OGC is expressed in freshly isolated astrocytes (6). After its re-oxidation in the tricarboxylic acid (TCA) cycle to OAA, the latter becomes transaminated to the corresponding amino-acid, aspartate. Aspartate can exit across the mitochondrial membrane in exchange with glutamate, using the glutamate/aspartate exchanger (AGC). After its arrival in the cytosol glutamate is converted to OAA, closing the circle. Both mitochondrial and cytosolic aspartate aminotransferases (57–59) in brain are well established, and aspartate aminotransferase activity is high in cultured astrocytes (60, 61). Nevertheless, histochemistry failed to show expression of the enzyme by histochemistry in some studies (62, 63), although moderate astrocytic expression had previously been shown (64). This is peculiar in light of unhindered demonstration by the same authors of the expression of glutamate dehydrogenase (GDH), a mitochondrial enzyme which also metabolizes glutamate (63). Both the cytosolic (*Got1*) and the mitochondrial (*Got2*) aspartate aminotransferase gene have been demonstrated in freshly isolated astrocytes (4, 6).

There are two different AGC forms, in adult brain almost exclusively AG1 or aralar (*Slc25a12*), with only small clusters of citrin (*Slc25a13*) in a few neurons (65). MAS cycle activity is needed for formation of glutamate and GABA in brain, which is well known to occur readily and to depend upon pyruvate carboxylase-mediated (2, 66) glutamine formation in astrocytes. It therefore came as a big surprise when the operation of MAS in astrocytes *in situ* was questioned due to an observed absence of aralar (and citrin) in astrocytes *in situ* (11). This would make appropriate oxidative metabolism of glucose impossible. Ramos et al. reported that only little aralar expression is found in cultured astrocytes and even less in astrocytes of the adult brain. Berkich et al. (12) confirmed absence of astrocytic aralar using a different antibody. Finally, Pardo et al. (13), using an improved immunofluorescent assay with antigen retrieval and identifying astrocytes histologically, reported presence of aralar protein in astrocytes in the mouse brain, but only in relatively small amounts. However, in freshly isolated astrocytes and neurons obtained by fluorescence-activated cell separation (FACS) both microarray analysis (4) and RT-PCR (5) showed at least as much mRNA expression in adult astrocytes as in adult neurons, and the levels of expression were comparable to those in whole brain. Since expression of mRNA is not necessarily accompanied by protein expression, Li et al. (5) also determined protein expression of aralar, which was found to match its mRNA expression in cells from 35-day-old animals, whereas the expression was much lower in cells (both neurons and astrocytes) from 14-day-old animals (**Figure 2**). A similar slow development of aralar expression was shown in astrocyte cultures (**Figure 2**), and both the level of expression and the developmental course were similar in the freshly isolated cells and in the cultures used. Thus, there is no reason to doubt MAS function in astrocytes, although several immunohistochemical studies had indicated that this could not be the case.

The discrepancy between results obtained by IHC and FACS followed by RT-PCR and Western blot could be caused by: (i) use of GFAP to identify astrocytes in the studies by Ramos et al. (11) and Berkich et al. (12), since GFAP is absent from the fine processes that contain a large number of mitochondria (4); (ii) loss of antigenicity upon tissue fixation or tissue processing. That both points may be important is shown by the fact that Pardo et al. (13), the only authors who have demonstrated aralar immunohistochemically (i) relied on cell morphology, not GFAP presence to classify a cell as astrocytic, and (ii) also were the only ones to use antigen retrieval, a procedure that partly can overcome artifacts from fixation or tissue processing. Nevertheless, the consistency in the lack of ability to demonstrate aralar histochemically between several different groups underlines that this is not due to methodological

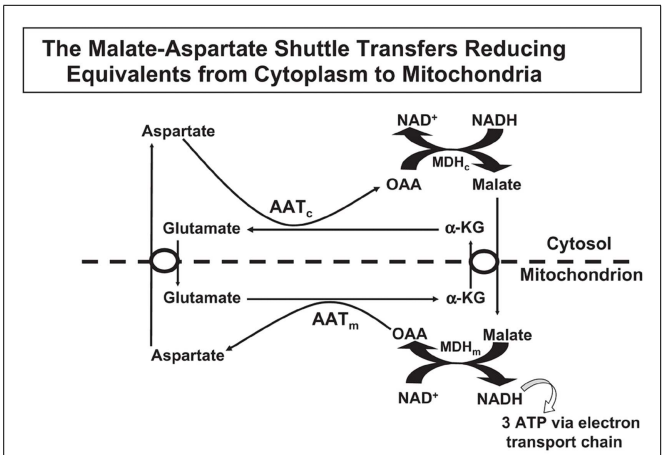


**FIGURE 1 | In the malate–aspartate shuttle (MAS) cytosolic malate dehydrogenase (MDHc) oxidizes NADH and converts oxaloacetate (OAA) to malate (top right of figure), which enters the mitochondria in exchange with α-ketoglutarate (α-KG)**. The mitochondrial malate dehydrogenase (MDHm) re-oxidizes malate to OAA, which is transaminated to aspartate by the mitochondrial aspartate aminotransferase (AATm). Aspartate leaves the mitochondria in exchange with glutamate, requiring ACG (aralar or citrin). In the mitochondria glutamate conversion to α-KG is essential for AATm activity forming aspartate from OAA and delivering α-KG for mitochondrial export. The glutamate imported into the mitochondria had been formed by cytosolic aspartate aminotransferase (AATc) from α-KG after its entry into the cytosol. Without MAS activity NADH formed in the cytosol during glycolysis would have been unable to enter the mitochondria for oxidation. Reprinted from Hertz and Dienel (69), with permission.

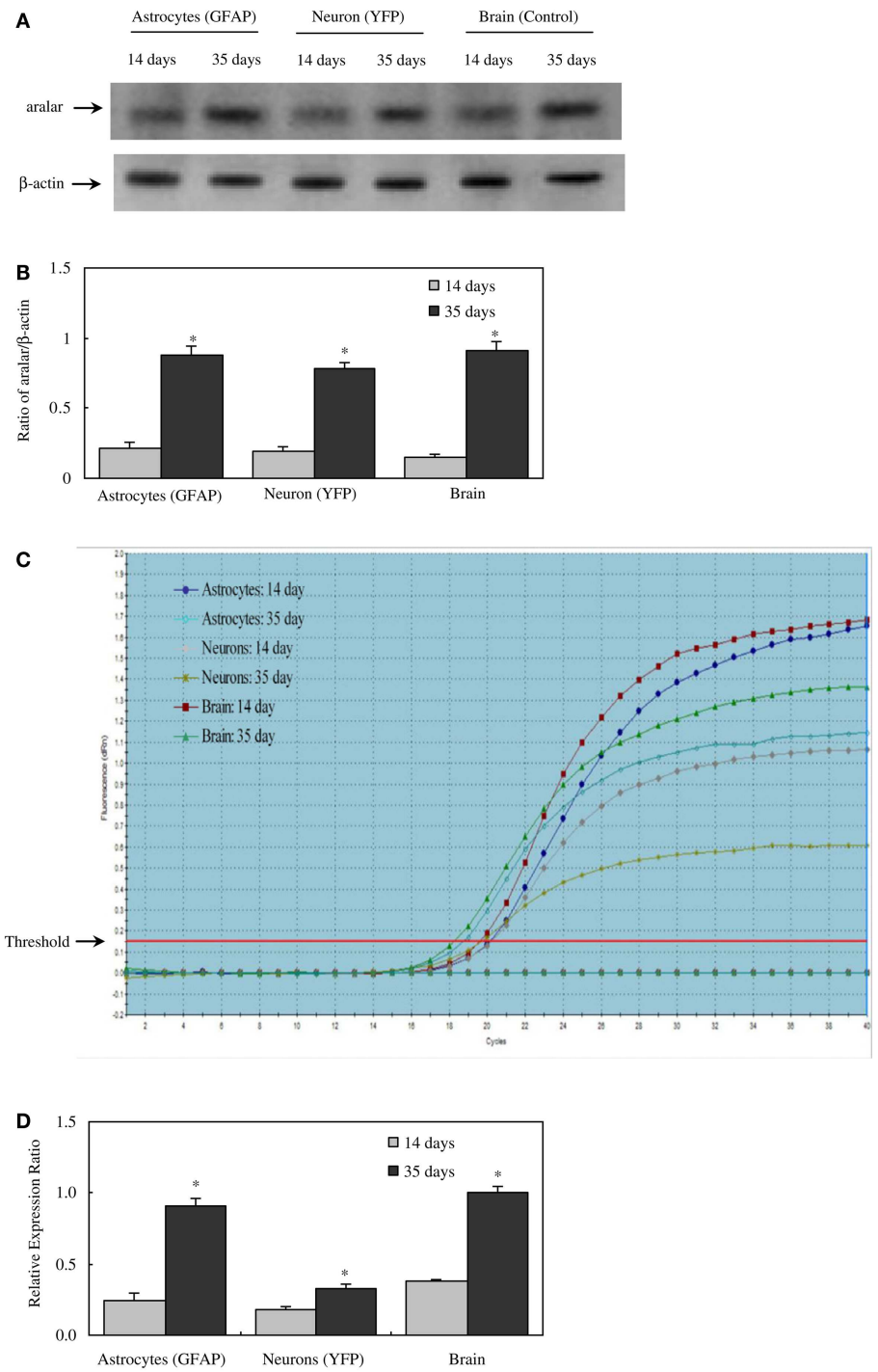


**FIGURE 2 | Protein and mRNA expression of aralar in astrocytes and neurons isolated by FACS from cerebral hemispheres of 14- and 35-day-old astrocyte-labeled [FVB/NTg(GFAP-GFP)14Mes/J] or neuron-labeled [B6.Cg-Tg(Thy1-YFPH)2Jrs/J] mice and in intact brain of adult CD-1 mice**. **(A)** A representative immunoblot showing protein expression for aralar and β-actin, used as a house-keeping protein. The size of aralar is 70 kDa, and of β-actin 46 kDa. Similar results were obtained from three independent experiments. **(B)** Means ± SEM of scanned ratios between aralar and β-actin. *Statistically significant ($P < 0.05$) difference from the same preparation from 14-day-old animals. **(C)** A representative amplification plot of aralar mRNA expression, determined by real-time PCR. Similar results were obtained from three independent experiments. For analysis of graph, see Xu et al., under revision. **(D)** Means ± SEM ($n = 3$) of the relative expression ratio of aralar. *Statistically significant ($P < 0.05$) difference from the same preparation from 14-day-old animals. From (5).

errors in a single study, but to systematic, unexplained deficiencies within the methodologies. For some reason demonstration of the expression of many astrocytic genes is enigmatic.

### *Expression of genes of other enzymes involved in glucose metabolism*

The discrepancy between results for aralar expression obtained by immunohistochemical analysis and by mRNA and protein determination raises the question whether a similar apparent failure of histochemistry to identify and accurately quantify expression of an important astrocytic gene also may apply to other genes. Lovatt et al. (4) carried out a microarray analysis of mRNA expression of a multitude of TCA cycle enzymes in FACS-isolated cells and found most of them to be expressed at higher levels in astrocytes than in neurons. In addition, these enzymes in freshly isolated astrocytes were functionally active, although to an undefined degree. However, Doyle et al. (10) reported much higher level of hexokinase gene expression in neurons than in astrocytes from bacterial artificial chromosome (BAC) transgenic mice, one of the newly established techniques (for more information see Fluorescence-Based Cell Sorting). This contrasts not only immunochemical studies (67), but also general concepts about energy metabolism in brain. Thus, Nehlig et al. (68) observed similar total glucose phosphorylation in astrocytes and neurons in intact brain tissue. Moreover, in the primary cultures used in our laboratory, the rate of glucose oxidative metabolism in astrocytes and cerebellar granule cells is quite similar, 1.2 and 1.0 nmol/mg protein per min [Table 6 in Ref. (69)], which is comparable with *in vivo* rates [reviewed by Ref. (70)]. One may therefore wonder if the animals used to study neuronal gene expression have shown more perfect labeling of their genome than those used for determination of astrocytically expressed genes. This should not conceal that many preparations of cultured astrocytes also show a very low rate of oxygen consumption (71), again emphasizing that not all astrocyte cultures are identical, and that many show characteristics making them unsuited as models for astrocytes *in vivo*. At least some enzymes involved in oxidative metabolism of glucose (and other substrates) have also been demonstrated immunohistochemically, since distinct astrocytic demonstration of cytochrome oxidase (COX) has been shown in sections from the monkey striate cortex (72). Astrocytes, but not neurons and probably also not oligodendrocytes show immunohistochemically determined expression of pyruvate carboxylase (2, 73), which is consistent with the operation of this enzyme in cultured astrocytes (66). The repeated demonstration of this enzyme in astrocytes is of crucial importance, since it is necessary for net synthesis of TCA cycle intermediates and thus for astrocytic production from α-KG of glutamate in astrocytes, needed for neuronal production of transmitter glutamate and GABA. Cultured astrocytes also express cytosolic, but not mitochondrial malic enzyme as shown both immunocytochemically (3) and by anion exchange chromatography to separate the cytosolic and mitochondrial isoforms of malic enzyme (74), whereas adult rat brain express the two isoforms about equally (suggesting neuronal localization of the mitochondrial form). There is consensus that cytosolic malic enzyme operates during complete oxidative metabolism of glutamate, converting it to pyruvate after its exit from the TCA cycle.

## ENZYMES AND TRANSPORTERS OPERATING IN GLUTAMATE AND GABA TURNOVER

Norenberg and Martinez-Hernandez (1) performed immunohistochemical analysis of GS in rat nervous system and showed that the enzyme was astrocyte-specific. The stain was confined to the cytoplasm and perivascular astrocytic processes. The intensity of staining varied between different brain locations, with highest level in hippocampus and cerebral cortex. GS expression has repeatedly been confirmed in astrocytic cultures [e.g., Ref. (75, 76)]. Presence of GS has also been claimed in oligodendrocytes in brain and spinal cord (77–79). However, oligodendrocytic manifestation was not described during the original demonstration of GS expression in astrocytes (1), and the absence of GS expression in oligodendrocytes has been confirmed by Derouiche (80) and again by Anlauf and Derouiche in the present Research Topic. The reader is referred to this paper for further discussion of this topic, which is important for understanding of both oligodendrocytic function and difficulties in correct demonstration of gene expression.

How glutamine transport is directed from astrocytes to neurons was long unknown, since no obvious differences were found between kinetics for glutamine uptake in astrocytes, cultured glutamatergic or GABAergic neurons, and neuronal perikarya prepared by gradient centrifugation (81). This problem has received its solution with the demonstration of different glutamine transporters in neurons and astrocytes. The bi-directional transporter SN1, also known as SNAT3 is abundantly expressed in astrocytic processes surrounding glutamatergic and GABAergic neurons and its expression is pronounced in the neocortex, cerebellum, olfactory bulb, and brain stem (82). The possibility that its absence from neuronal terminals could be due to insufficient antigen detection has been excluded by the demonstration that possible SN1/synaptophysin coexpression is rare (83). This system N transporter transfers glutamine in symport with $Na^+$ and in antiport with $H^+$, which is important for its role specifically in glutamine efflux from astrocytes (84). Its regulation by protein kinase C (PKC) is discussed in this Research Topic by Nissen-Meyer and Chaudhry. A potential role of its relative SN2 in some brain regions and its subcellular distribution have been discussed by Hamdani el et al. (85).

Released glutamate from glutamatergic neurons is mainly taken up by astrocytes ((86) – see also Zhou and Danbolt in this Research Topic), where it is either oxidative metabolized (∼20%) or converted to glutamine and re-transferred to neurons in the glutamine-glutamate cycle. Aspartate aminotransferase and/or GDH are involved in the conversion of glutamate to α-ketoglutarate. Aspartate aminotransferases were discussed above and astrocytic localization of GDH has been shown immunohistochemically by Aoki et al. (87) and Würdig and Kugler (63) and its gene expression has been demonstrated in FACS-sorted astrocytes by Lovatt et al. (4) and Cahoy et al. (6) using microarray analysis. It is reason for concern that astrocytic expression of this gene was not observed by Doyle et al. (10) in their study using BAC animals. In contrast, the genes shown to be expressed in **Table 1** were as well recognized in the study by Doyle et al. (10) as in the two other microarray studies. Thus this methodology seems for unknown reasons to have difficulty demonstrating only the expression of certain astrocytic genes.

The astrocytically located cytosolic malic enzyme discussed above is responsible for α-ketoglutarate's complete oxidation in the TCA cycle after conversion to malate and exit of malate to the cytosol. A larger fraction of released GABA than of released glutamate is re-accumulated in the neurons themselves but some is metabolized in the astrocytic TCA cycle after conversion to succinate via GABA transaminase (GABA-T) and succinic aldehyde dehydrogenase (SSADH) (see paper in this Research Topic by Schousboe et al.). GABA-T has been demonstrated histochemically in both neurons and astrocytes (88), consistent with GABA uptake in both cell types.

Among the five subtypes of glutamate transporters (excitatory amino-acid transporters; EAATs 1–5), astrocytes express L-glutamate/L-aspartate transporter (GLAST; EAATl) and GLTl (EAAT2). Lehre et al. (15) and Chaudhry et al. (16) stained brain slices for GLAST protein and concluded it was expressed in astrocytes. Later, Schmitt et al. (14) compared GLAST mRNA and protein expression in the CNS of rat. They found that GLAST mRNA was located in the cytoplasm of astrocytes. In Bergmann cells, GLAST mRNA stain also appeared in proximal processes. Protein stain showed similar pattern, but fine processes that were not labeled with GFAP were also stained for GLAST. In the retina GLAST expression even in the finest Müller cell processes had previously been shown by Derouiche and Rauen (17). GLAST expression varies in astrocytes in different brain regions. In cerebellum, Bergmann cells showed strong reaction, but the granule cell layer showed only faint astrocytic labeling (14). GLT-1 is expressed in astrocytes of the mature brain and spinal cord (19, 20). Although GLAST expression is distributed within all cortical layers, and strongly expressed throughout the granule cell layer of the dentate gyrus of the hippocampus, experiments with a GLT-1-preferring inhibitor, WAY-855 showed that GLT-1 was responsible for 80% glutamate uptake in isolated hippocampal tissue (89). Selective inhibition of GLT-1 with WAY-855 does not completely prevent glutamate build-up, but NMDA receptor-mediated neurotoxic effects remain, suggesting additional roles of other glutamate transporters in extracellular glutamate maintenance. However, an authoritative review by Danbolt (86) has also concluded that GLT-1 is the dominant glutamate transporter in cortical astrocytes and that GLT-1 and GLAST together account for the predominant astrocytic uptake of glutamate in the brain *in vivo*.

Kinetics for glutamate uptake has been determined by different authors in primary cultures of astrocytes, where the uptake can be extremely intense (90). Swanson et al. (91) and Schlag et al. (22) found that primary cultures of rat astrocytes express GLAST, but little or no GLT-1, and that treatment with dBcAMP, known to increase intracellular cAMP, enhanced the expression of both GLAST and GLT-l. This observation was made by both ICC and mRNA and protein determination. Nevertheless, GLT-1 expression was only a fraction of that observed in brain tissue (22). Co-culturing with neurons had a similar effect as the cAMP analog. dBcAMP treatment also increased $V_{max}$ for glutamate uptake (22), although it only reached about one half of its value in mouse cultures found by Hertz et al. (90). GLT-1 expression in cultured astrocytes can also be enhanced by activation of additional signaling pathways (23), and it would be extremely useful to establish an astrocyte culture with as high GLT-1 expression as in the brain *in vivo*.

Some released transmitter GABA is taken up by astrocytes, although the uptake rate in cultured astrocytes is much slower than that of glutamate (92) and also slower than in cultured neurons (93). GABA uptake is mediated by one of three high-affinity subtypes of GABA transporters (GATs), GATl, GAT2, and GAT3 or in some cases by the one low-affinity transporter BGTl. Astrocytes can express all four subtypes [for review, see Ref. (25)]. GAT1 is present in astrocytes in all brain regions, where IHC demonstrated punctate structures that were shown by electron microscopy to be located exclusively in the small cell processes (26). In cerebellum, GAT1 mRNA was detected in Bergmann cells [for review, see Ref. (25)]. In rat thalamus GAT1 and GAT3 proteins are mainly expressed in astrocytes and the stain of GAT3 is more intense than that of GAT1 (28). In the parabrachial and Kölliker-Fuse nuclei GAT3 was detected, whereas GAT1 was absent (29). Cerebellar mRNA and protein staining of GAT2 and GAT3 was also primarily glial, with GAT2 stain in the granule layer and GAT3 stain in the deep nuclei (24, 27). Nevertheless, FACS isolation followed by microarray analysis showed the GAT2 gene as not expressed in brain (6, 9) a similar result was reached in cerebellum using BAC transgenic mice (10). With respect to most other glutamate and GABA transporters there is virtual consensus by all methods that they are expressed in astrocytes. An exception is BGT-1 mRNA, which is observable in cerebellum of BAC transgenic mice (**Table 1**), although not with sufficiently high fold change for unequivocal demonstration (10). It has also has been reported in cultured astrocytes (30), although up-regulation of this betaine-GABA transporter did not affect GABA uptake (31).

## NUCLEOSIDE TRANSPORTERS AND ADENOSINE KINASE

There are two types of nucleoside transporters, the concentrative CNTs l–3, which are able to transport nucleosides against an intracellular/extracellular gradient of the nucleoside itself, and the equilibrative ENTs l–4. CNTs are necessary for termination of adenosine and guanosine transmitter effects by cellular uptake, regardless of cellular requirements. ENTs, like other equilibrative transporters, carry out transport only until equilibrium has been established between intra- and extracellular concentrations (94). Most nucleoside transporters are membrane-bound, but ENT3 is mainly intracellular (33), and may distribute adenosine into intracellular organelles (34).

mRNA expression of ENT1 and ENT2 mRNA, determined by ISH, is widespread in the brain (32), and the presence of both of these transporters has been reported in both astrocytes and neurons in a review by Parkinson et al. (33). The same review claimed that CNT2 and CNT3 were absent in astrocytes, and no information was provided about ENTs 3 and 4. Moreover, the impression was given that astrocyte expression of the remaining transporters was low. However, this information seems to be misleading, since mRNA for all nucleoside transporters except CNT1 are expressed to at least a similar degree (in relation to applied amount of RNA and to a house-keeping gene) in astrocytes as in neurons in freshly isolated astrocytes (34) (**Table 1**). Analysis using either FACS-sorted cells or BAC transgenic animals also demonstrates most of them although with some differences

between different investigators and especially good recognition by Doyle et al. (10). The astrocytic enrichment in ENT3 was dramatic (**Figure 3**), opening the possibility that gliotransmitter ATP may be synthesized in intracellular organelles, which has been confirmed by downregulation of ENT3 by siRNA (Xu et al., under revision). It is consistent with intense astrocytic but not neuronal formation of AMP, ADP, and ATP in cultured cells (95) that adenosine kinase, the enzyme converting adenosine to AMP in the adult brain, has been found by IHC to be selectively expressed in astrocytes (96). However, Parkinson et al. (97) reported very little nucleotide formation in astrocyte cultures, perhaps reflecting immaturity of the cells used, and again emphasizing that different astrocyte cultures may behave differently. As illustrated for ENT3 in **Figure 3**, the expression of ENTs and CNTs shown in freshly isolated cells has been replicated in our cultured cells (35, 43) with the exception of CNT3, which is sparsely expressed in brain.

## EXPRESSION OF GENES OF TRANSPORTERS INVOLVED IN $K^+$ CLEARANCE FROM THE EXTRACELLULAR SPACE

Two astrocytic membrane proteins mediate uptake of extracellular $K^+$ ($[K^+]_e$), Na,K-ATPase and NKCCl, which is an inwardly directed $Na^+$, $K^+$, $2Cl^-$ cotransporter expressed both in cultured astrocytes (98–100) and mature astrocytes *in vivo* (101, 102). The Na,K-ATPase operates alone below a total $[K^+]_e$ of ∼10 mM. At higher $[K^+]_e$, NKCC1 plays a dominant role, as shown by inhibition with bumetanide or furosemide, inhibitors of the cotransporter (103–105).

Na, K-ATPase contain α and β subunits. Three isoforms of its α subunits (α1–3) and 2 of its β subunits β1 and β2) are expressed in brain (38, 39). Immunofluorescent histochemistry showed that ATPase α1 protein is expressed both in neurons and glia, and its glial expression is obvious in co-cultures (41). Only Cahoy et al. (6) indicate expression of α1 mRNA after microarray analysis in spite of its demonstration by histochemistry, ISH (in cultures) and also after FACS separation followed by RT-PCR (43). The failure of both Lovatt et al. (4) and Doyle et al. (10) to show astrocytic expression of this gene in, respectively FACS-isolated astrocytes and astrocytes obtained from BAC mice treated with the astrocytic marker Aldh1L1 is unfortunate.

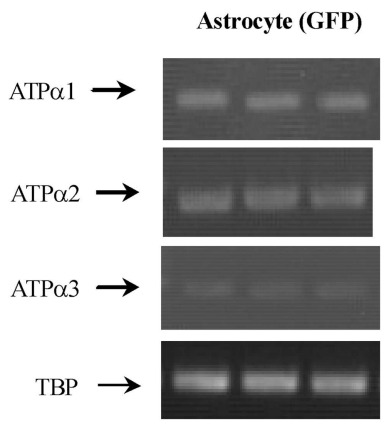


**FIGURE 3 | mRNA expression measured by RT-PCR of α1, α2, and α3 isoform of Na,K-ATPase in astrocytes isolated by FACS from cerebral hemispheres *in vivo* from adult mice [FVB/NTg(GFAP-GFP)14Mes/J].** A representative experiment showing mRNAs for α1, α2, and α3 isoform and for TBP, as a house-keeping gene. The sizes of the PCR products of α1 is 920 bp, α2 350 bp, α3 329 bp, and TBP 236 bp, $n = 3$. From (43).

Na, K-ATPase α2 expression is primarily in glia, and Na,K-ATPase α3 is only expressed in neurons (40, 44). Expression of α2 in astrocytes was first suggested by Watts et al. (42), who demonstrated its mRNA expression with ISH. Its labeling pattern was diffuse in hippocampus, neocortex, and brain stem. Later, Sweadner's group performed IHC on cerebellar brain slices and ICC on co-cultures of cerebellar granule cells and astrocytes (41) and consolidated their findings by ISH. Staining for α2 in the granular layer was observed in diffuse processes around granule cells (41) in a similar staining pattern as that found by McGrail et al. (40). In the co-cultures α2 staining was extensive in GFAP-positive astrocytes and it uniformly labeled the surface of the cells (41). Recent experiments using cells obtained by FACS have confirmed α2 expression in astrocytes (**Figure 3**) (43) in agreement with the previous observation by IHC, ICC, ISH, and cell culturing. Microassay analysis (**Table 1**) has also consistently shown expression of the gene of the α2 isoform of the Na,K-ATPase in astrocytes (4, 6, 10).

*In situ* hybridization showed NKCC1 mRNA located in granule layer and white matter tract of cerebellum (45). Attempts to demonstrate NKCC1 immunohistochemically in astrocytes has, however, also often provided negative results (M. Nedergaard, personal communication). Nevertheless functional responses to high extracellular $K^+$ concentrations (swelling or enhanced $K^+$ uptake and their inhibition by a NKCC1 inhibitor) have repeatedly identified the astrocytic location of this transporter both in the superfused monkey brain (106) and cultured astrocytes (104). Gene expression and phosphorylation of NKCC1 have been studied in cultured astrocytes under control condition and after trauma by increased barometric pressure by Jayakumar et al. (107). Silencing of NKCCl with siRNA led to a reduction in trauma-induced NKCC1 activity as well as in cell swelling. Microarray results by both Cahoy et al. (6) and Doyle et al. (10), but not those by Lovatt et al. (4), also indicate its expression in astrocytes (**Table 1**).

## COMPARISON OF RESULTS OBTAINED BY DIFFERENT METHODOLOGIES

Expression of some, but far from all genes in astrocytes seem to be impossible to demonstrate or quantitate with IHC and ISH (**Table 1**), even using optimized techniques, whereas their expression is clearly shown in intact cells freshly obtained from the brain. Biochemical analysis of these cells (determination of mRNA or protein) gives somewhat more consistent results than microarray analysis. There is no systematic explanation for the failure of IHC and ISH to show expression of certain genes, and the genes in question can be located either on the cell membrane (most of the nucleoside transporters) or intracellularly (aralar and ENT3). Results obtained by biochemical analysis in cultured astrocytes (see below) can be very similar to those obtained *in vivo*, but unfortunately great differences are found between results obtained in different cultures. The recently introduced BAC transgenic mice selecting genes co-expressed with the astrocyte-specific *Aldh1L1* gene seem also to have severe difficulties in recognizing several astrocytically expressed genes. There are no overlapping methodological mechanisms between this technique and IHC, but there

might some with ISH, perhaps helping to answer the question why demonstration of specifically astrocytically expressed genes is so enigmatic. It would also be interesting to compare astrocytic gene expression determined in BAC mice selected with a different astrocyte-specific gene.

## DESCRIPTION OF METHODOLOGIES USED FOR DETERMINATION OF GENE EXPRESSION

### IMMUNOHISTOCHEMISTRY AND IMMUNOCYTOCHEMISTRY

By determining the expression of protein, IHC and ICC should be the ideal tools for demonstrating gene expression in different cell types. IHC is a laboratory technique that uses specific antibodies to detect antigens in cells of a tissue section. ICC uses samples of intact cells without their surrounding extracellular matrix and contacts to other cells of different types, and is used for many types of cultured cells. The immunohistochemical study that did demonstrate aralar (13) used a polyclonal antibody, Ramos et al. (11) a previously prepared antibody by the same group, and Berkich et al. (12) a donated antibody, recognizing both citrin and aralar. In general, the binding site of the antibody is unknown except when a synthesized peptide is used as the antigen. Fixation is necessary and also difficult. Formaldehyde is the gold standard for fixation. Its basic mechanism is to form a product between the formalin and uncharged reactive amino groups (-NH or -NH2) by aid of cross-links and eventually change the three-dimensional structure of the proteins [for review, see Ref. (108)]. Overfixation produces false negative results. Antigen retrieval is usually used to reverse this conformation change, and among the papers studying aralar expression immunohistochemically it was used by Pardo et al. (13), but not in the other publications. In brain slices, GFAP is generally used as astrocytic marker, for example by Berkich et al. (12), but not by Pardo et al. (13), who relied on morphological characteristics. One problem is that GFAP is a cytoskeleton protein that is rarely distinguishable in all astrocytes (generally more after cell damage) and that its staining may not be representative of cytosolic and membrane proteins. Even when expressed in an astrocyte, GFAP is not present in the peripheral processes (4, 109) but only expressed in the main stem branching branches, and often even absent in the cell body. Using GFAP as a marker in IHC one may therefore significantly underestimate the abundance of genes of interest expressed in astrocytes (109). Many other genes and the proteins they code for are astrocyte-specific in adult animals, including GS, excitatory amino-acid transporter 2 (EAAT 2), and its rodent analog GLT-1, aquaporin 4, connexin 43 (ctx43), ALDH1L1 (6, 110) as well as the epithelial $Na^+/H^+$ exchanger regulatory factors NHERF1, and GLAST (EAAT-1) (111), with the latter showing pronounced species variability in the expression of its splice variants (112). An excellent marker for astrocytes seems to be EGFP introduced into transgenic mice under the control of the human GFAP promoter (7). This reporter molecule is a much better astrocytic marker than GFAP, since GFP is expressed even in the fine processes. GS is another astrocyte marker which is much better than GFAP (Anlauf and Derouiche, this Research Project). Thus, it may be possible to improve immunohistochemical demonstration of astrocytically expressed genes by discontinuing the use of GFAP as an astrocytic marker and rely more on other markers and/or morphology. The presence of antigen labeling present predominantly in the fine peripheral astrocyte processes, e.g., ezrin (113) and reelin may appear as a hazy "background." This can easily be overlooked, regardless which astrocyte-specific gene is used for cell identification and should be carefully checked for, although this can be difficult in intact brain.

### *IN SITU* HYBRIDIZATION

The ISH technique is performed in fixed tissue with all cellular relationships remaining intact. It determines anatomic localization of labeled or non-labeled RNA or DNA probes that hybridize to target complementary RNA or DNA sequences in the cell (114). These hybrids can be detected using either an isotopic probe and emulsion autoradiography or non-isotopic methods using specific antibodies to detect a hapten incorporated into the probe. Radioisotopic ISH is perceived as providing high sensitivity, quantitative labeling, and relatively unambiguous discrimination of signal versus background, whereas non-radioactive colorimetric ISH often provides better anatomic localization and discrimination between cells and is well suited to produce large amounts of data (114). ISH is limited by providing no information about translation and posttranslational processes on its own. This obviously also applies for expression of mRNA determined by different methodologies, for example FACS and BAC analysis. Taking advantage of automated high-throughput procedures and data acquisition ISH has been used to generate a digital atlas (the freely available Allen Brain Atlas) showing the expression patterns of approximately 20,000 genes in the adult mouse (115). Potential failure to identify expression of some astrocytic genes in an unpredictable manner in this excellent standard reference work would be most regrettable. Information in general about correlation between protein and mRNA expression would be valuable and an overall estimate of the frequency of differences would therefore be very valuable.

Several groups have conducted mRNA/protein correlation analyses, but the results are controversial. Guo et al. (116) carried out a correlation study for 71 genes using human circulating monocytes and showed an overall positive correlation. Gry et al. (117) investigated 23 human cell lines and 1066 genes and found that only one third of the genes showed significant correlation between mRNA and protein expression. Schwanhäusser et al. (118) found better correlation between mRNA and protein levels than previously thought.

## CELL SEPARATION TECHNIQUES, BAC TRANSGENIC MICE, AND METHODOLOGY FOR mRNA AND PROTEIN DETERMINATION

### MICRODISSECTION AND GRADIENT CENTRIFUGATION

Using the large Deiters' cell neurons and surrounding neuropil as well as microanalytic techniques Holger Hydén demonstrated more than 50 years ago that learning changes the base composition of nuclear RNA, in both neurons and glia. He also established that glial cells show more marked and earlier changes in RNA composition in Parkinson's disease than neurons. He had the vision and courage to suggest that "mental diseases could as well be thought to depend upon a disturbance of processes in glia cells as in the nerve cells," and showed that antidepressant drugs cause profound changes in glial RNA [for review, see Ref. (119, 120)].

There is no doubt that the neuropil samples constituted less pure glial samples than present-day astrocytic preparations, but nevertheless many of Hydén's observations are presently being confirmed. A different technique to separate neurons and glia, gradient centrifugation (121) was used in his laboratory by Hamberger and coworkers to show glial uptake of glutamate and GABA for the first time and many features of their subsequent metabolic fate (122, 123). Such studies are suitable for determination of $K_m$ values, but the cells are too damaged to evaluate $V_{max}$.

### RT-PCR FOLLOWING ELECTROPHYSIOLOGICAL SELECTION OF ASTROCYTES WITH SPECIFIC RECEPTOR EXPRESSION

The Steinhäuser group has carried out a series of studies combining patch clamp analysis with RT-PCR in studies of AMPA receptor subunit expression in astrocytes obtained from hippocampal slices (124–126). Facilitated by previous treatment with enzymes, glial cells expressing AMPA receptors were extracted after identification based on electrophysiological and immunocytochemical properties, including absence of action potentials. At the end of the recording, a negative pressure was applied to the pipette sucking in the cell, the tip of the pipette was broken off and the cell's contents were harvested under a microscope as originally described for Purkinje cells by Lambolez et al. (127). mRNA expression in the collected cells was analyzed with RT-PCR using repeated amplification and with emphasis on determination of AMPA receptor subtypes and astrocyte-specific compounds. The observed changes in splice variant expression and subunit assembly of AMPA receptors during cell maturation (125) is to be expected due to late generation of astrocytes (128) and profound alterations in gene expression and function in the mouse/rat brain during the first three postnatal weeks (129, 130).

### CELL SORTING BASED RECOGNITION OF CELL-SPECIFIC PROTEINS

#### *Fluorescence-based cell sorting*

The possibility to associate a specifically fluorescent drug to either neurons or astrocytes via their genetic promoters (7) and subsequently sort cells freshly dissociated from the brain according to their fluorescent characteristics can greatly improve the purity of the obtained cell fractions. While Fluorescence-activated cell sorting (FACS) has long been used in the immunology and cancer fields, its use in neuroscience was until recently limited to embryonic brain tissue, cultured cells, stem cells, or synaptosomes, because these cells or organelles lack or have fewer processes than adult neurons and astrocytes (131). Pioneering studies in Maiken Nedergaard's and Ben Barres's laboratories established this technique for use in adult (4) and adolescent (6) brain.

A genetically transformed (GFAP-S65T-GFP) mouse was originally generated by Zhuo et al. (132) from the Messsing group by inserting a 2.1 kb DNA fragment of the human GFAP promoter randomly into the mouse genome during oocyte injection of linearized transgene DNA for transgene generation. In many current references this mouse is called a GFAP-GFP or even GFAP-EGFP mouse, but it is a GFAP-S65T-GFP mouse. S65T-GFP is a modified and brighter fluorescent protein than the wild type GFP, but it is not as bright as EGFP (enhanced GFP) (7). The Nedergaard laboratory (4) used this mouse in combination with immunohistochemical labeling with anti GLT-1 antibodies to label astrocytes in adult mice for sorting by FACS. The combination of the two labels increases the purity of the isolated astrocytes.

Dissociated cells from the cerebral hemispheres of such mice were sorted and collected by a cell sorting system according to the wavelength of their fluorescent signal. Cell purity has been determined by mRNA expression of cell markers of astrocytes (*Gjb6, Gfap, Slc1a2, and Fgfr3*), and lack of mRNA from markers of neurons (*Gabra-l, Slc12a5, Snap25,* and *Syt1*), and oligodendrocytes (*Gjc2, Mag, Mog, and Mbp*). A relatively small number of cells (∼8%) die (become $PI^+$-positive) as a result of the procedure. Metabolic activity has been demonstrated in similar cells (4), but not quantitated. The astrocyte sample yield from FACS is about 1–2 μg RNA or 20 μg protein per brain which is sufficient for a multitude of microarray assays (4, 6) or for ∼10 RT-PCR determinations (5, 34, 43, 47, 133). Our aralar study (5) and further FACS studies were carried out using this technique, although without the GLT-1 antibodies, again with no contamination of the astrocyte samples with either neurons or oligodendrocytes (133). Our studies showed that determination of the expression of a moderate amount of genes (34, 43, 47) or of a single gene (5) by RT-PCR yields highly reproducible and comparable results, regardless whether "classical" RT-PCR or qRT-PCR (real-time RT-PCR) was used, as can be seen by comparison between **Figures 2C** and **2D**. The Barres laboratory (6) used FACS combined with immunopanning to isolate astrocytes from transgenic mice that express EGFP under the control of an S100β promoter.

#### *Cell sorting based on naturally expressed cell-specific genes*

A FACS-like method that does not depend on the use of transgenic animals has been developed for isolation of neurons and endothelial cells by Guez-Barber et al. (131). Specific fluorescent labeling of neuronal cells was obtained by treating the cells obtained after brain dissociation with a biotinylated NeuN antibody and subsequently with phycoerythrin-labeled streptavidin. Streptavidin has an extremely high affinity for biotin and phycoerythrin is a protein that produces a bright red-orange fluorescence, allowing conventional FACS methodology to be used for these cells, selected by Neu1 expression, and made fluorescent by the subsequent binding of phycoerythrin-labeled streptavidin. Jungblut et al. (134) used a related approach to obtain astrocytes from young postnatal brain. Their cell suspension was first labeled with the anti-GLAST antibody ACSA-1, conjugated to biotin, whereupon superparamagnetic MicroBeads coupled to an anti-biotin antibody were applied. The cells were resuspended in PBS with 0.5% BSA and the cell suspension was loaded onto an MS column (Miltenyi Biotec), which was placed in the magnetic field of a MiniMACSTM Separator from the same company. The magnetically labeled $GLAST^+$ cells were retained within the column and eluted after removing the column from the magnet. Viability of the cells was demonstrated by subsequent culturing, showing proliferation and the formation of a dense layer of GLAST/GFAP double-positive cells. However, the usefulness of the present modification is limited by the fact that astrocytes could only be successfully isolated from P1–P10 mice. When older animals were used, the presence of cell debris after tissue dissociation interfered with the separation

performance, lowered the purity of the isolated cells, and diminished their viability by at least 20%. Use of cells from very young animals can be gravely misleading because of important postnatal changes (129, 130).

#### *Cell sorting using bacterial artificial chromosome transgenic mice*

Heiman et al. (135) from the Heintz laboratory generated transgenic mice that expressed the ribosomal protein L10a tagged with EGFP. Using these mice one can achieve proper cell-specific labeling by green fluorescence and simultaneously use anti-GFP antibodies for immunopurification of ribosomes. Since the ribosomes carry mRNA for translation, mRNAs that are currently translated are co-purified. Accordingly, brains from mice of any age can be obtained and homogenized, and passage of the homogenate through a column with anti-GFP antibodies or immunoprecipitation will provide the currently translated mRNA in the cell type studied. In contrast to the FACS/immunopanning approaches cell viability is of no concern.

For their study of astrocytic gene expression Doyle et al. (10), also from the Heintz group, did not use the human GFAP promoter (as had been done in the Messing laboratory) but instead used a different astrocyte-specific (6, 110) marker, *Aldh1L1*. For proper transgenic labeling they used long stretches of genomic DNA (up to 100 MB in size), which can only be manipulated and amplified as BACs. In these DNA fragments the original gene is present, with probably all elements required for proper gene expression. When the open reading frame is replaced by EGFP, fluorescent cells are obtained [as in the study by Lovatt et al. (4)], and when it is replaced with L10a-EGFP ribosomes with adhering mRNA are obtained. Like the much shorter GFAP-EGFP construct the long BAC construct is injected into mouse oocyte for transgene generation. BAC transgenic mice have become popular, since there is a huge repository with BACs for almost all genes. In addition, there is a huge collection of transgenic mice generated by this approach (see www.gensat.org). The general assumption in the field is that BAC transgenes are more specifically expressed than the shorter promoter-using transgene constructs. For many cases, this is true, but there are also exceptions. *Aldh1L1* is a rather universal astrocytic marker, and it is almost unthinkable that *Aldh1L1*-expressing cells should not express the α1 unit of the Na,K-ATPase or GDH. It is therefore surprising (and disappointing) that genes for these proteins were not recognized in the study by Doyle et al. (10). Future studies of astrocytic gene expression using BAC mice will determine the general validity of this method for determination of astrocytic gene expression.

#### *Direct mRNA sequencing*

A newly developed technique, direct mRNA sequencing (DMS) (136), allows use of much smaller amounts and is well suited for determination of gene expression in astrocytic subcompartments (137). Like the methodologies used by Lovatt et al. (4) and Cahoy et al. (6) it requires initial isolation of the cells and the subcompartments to be investigated. It has generally been used together with microarray analysis but could also be used for RT-PCR (137) and thus enable accurate mRNA determination using smaller amounts of cells.

#### *Evaluation of the microarray data*

The studies by Lovatt et al. (4), Cahoy et al. (6), and Doyle et al. (10) all investigated astrocytic gene expression, although based on different astrocyte-specific genes. All used similar microarray procedures, but different sensitivities and purities are intrinsic to all of these methods. Most of the results probably overlap for up to 80% (see also **Table 1**), but particularly mRNAs with low abundance might be not detected in one or the other technique. However, the evasive nature of determination of astrocytic gene expression seems also occasionally apply to these newer methods. Among the relatively few genes shown in **Table 1**, expression of the aralar gene (*Slc25a12*) was not recognized in the study by Doyle et al. (10), which is worrisome. The lack of demonstration of expression of genes for ENT2 in the Doyle study and for ENT3 in the Lovatt study (in spite of its strong expression, determined by RT-PCR in both FACS-isolated cells and cultured cells shown in the present communication) is also reason for concern. Part of the problem might be the innate uncertainty of the microarray analysis, a problem that is obviously not solved by enrollment of additional gene identification methods. This concept is supported by results from **Table 2**, showing identical gene expression and editing determined in FACS-isolated and cultured astrocytes. However, expression of many of the same genes were not recognized by Lovatt et al. (4), using similar isolation procedure but determination of gene expression by microarray analysis. This applied also to the related studies by Cahoy et al. (6) and Doyle et al. (10), which also used microarray analysis. The safest procedure seems accordingly to be study of the expression of only one or a few genes of immediate interest and use of enough material (if needed, several animals) for an analysis by RT-PCR, repeated microarray analysis, or even better determination of protein by reaction with antibodies. Generally FACS-separated cells are not used for protein determination by Western blot and subsequent reaction with a specific antibody [only exception: aralar determined by (5)], since determination of multiple antigens (gene under study and house-keeping gene) by Western blot requires sample sizes between 25 and 75 μg protein (with some variability between the antigens of interest) for each determination. A single protein determination may therefore require brains from more than one animal.

## PRIMARY CULTURES OF ASTROCYTES

The pioneering technique of Booher and Sensenbrenner (138), allowing easy preparation of cerebral astrocytes, led to experiments providing many of the first hints of astrocytic characteristics and gene expression. A wealth of information was obtained, some of which stands, whereas others were shown to be incorrect, at least partly dependent upon the culturing method used. The value of cell culture studies in many different areas of astrocytic biology and pathology has been authoritatively reviewed by Lange et al. (139). The final version of our own cell cultures dates from 1978 (90). These cells are prepared from newborn male or female mice, and the methodology and functional characteristics have been described in some detail by Juurlink and Hertz (53), Hertz et al. (140), and Hertz (141).

The cultures can also be prepared from rats, but the rat astrocyte cultures are more contaminated with other cell types (41), and other differences, including much lower unidirectional $K^+$ influx

(reduced membrane conductance) have also been reported (41, 54) and remain unexplained. From the age of 14 days, 0.25 mM dBcAMP is included in the medium. This compound increases intracellular cyclic AMP and promotes differentiation in astrocyte cultures derived from newborn brain (142–144). The age of 2 weeks for its addition has been determined experimentally. This is consistent with the finding by Moonen and Sensenbrenner (145) that astrocytes need a certain stage of development in order to respond to dBcAMP, and that by Lodin et al. (146) that astrocytes de-differentiate *in vitro*, unless treated with this compound. Close similarities between the presently used cultures and freshly isolated astrocytes in not only levels of aralar protein and mRNA expression (5) but also rates of developmental changes support their validity as models of their *in vivo* counterparts. The same applies to gene induction following treatment with antidepressant or antibipolar disorder and their effects on not only gene up-regulation but also gene editing (47, 48), shown in **Table 2**. Neuronal cells are absent in these cultures. They contain <1% of non-parenchymal brain cells (e.g., meningeal or endothelial cells), a very small number of microglia (3%) and 95% of the cells are positive for GFAP and for GS (147). In contrast to the cells cultured by Foo et al. (50) from the astrocytic cell fraction obtained by their FACS technique from 1-week-old animals, our cultures survive well without growth factor addition to the media, but serum is routinely present, although at reduced amounts during later culturing stages. Foo et al. (50) successfully cultured the cells they obtained and showed that gene expression in the cultured cells mimicked that in FACS-isolated cells to a greater extent than the cultured cells (quite different from our cultured cells) they had previously studied (6).

There are many reasons that all cultured astrocytes are not identical. Differences in procedures used for preparation of astrocyte cultures by different authors include species (rats or mice), dissociation methods, amount of cells seeded, and medium used, but most procedures use new-borne mice or rats. That our cultures are so highly enriched in astrocytes that they require no subsequent cell separation, may be related to the small number of cells seeded and the small pore size used for filtering the cells. However, the most important difference between our astrocyte cultures and those used by other investigators is probably the addition of dBcAMP from the age of 2 weeks. One reason that we have chosen to supplement the culturing medium with dBcAMP is that most noradrenergic innervation from locus coeruleus (to a large extent contacting astrocytes) is reaching the forebrain at the time of birth (148). Since this is at the time the cells are harvested for culturing, they have not received the noradrenergic input which is important for brain development *in vivo* [e.g., Ref. (149)]. One may wonder if one of the reasons the astrocyte cultures established by Foo et al. (50) from 1-week-old astrocytes mimic astrocytes *in vivo* better than the cultured cells with which they were prepared is that they had received noradrenergic signaling at the onset of culturing. dBcAMP greatly enhances many characteristics, including gene expression of aralar (**Figure 2**). Expression of several proteins are altered (142, 143). In many cases it is obviously difficult to show that these changes make the cells more astrocytic, and many astrocytic features develop also without dBcAMP treatment. However, one important characteristic is that in the absence of dBcAMP treatment elevated concentrations of $K^+$ fail to stimulate glycogenolysis, a trait typical for astrocytes in normal brain tissue (52), whereas they do so after treatment with dBcAMP (51). dBcAMP was also found to increase the expression of GLT-1, but not to its level in brain. A goal of future research might be to establish similar GLT-1 gene expression in the cultures as in astrocytes in the brain *in vivo*. Increased use can be encouraged of good cultured cells, which by comparison with appropriately analyzed cell fractions have repeatedly shown to provide reliable results.

### SUMMARY

The expression of some astrocytic genes both in freshly isolated cells and in cultured astrocytes is undoubtedly real as shown by its repeated demonstration in different preparations using different techniques. Isolated preparations of astrocytes provide very useful information, but the intricate anatomic connections in adult brain represent a formidable barrier for preparation of freshly dissociated astrocytes from the adult brain in sufficient amounts. In spite of continuous progress in analytical methods, which may also decrease the amount of cellular material needed, the "bottleneck" for use of freshly isolated cells from adult animals by two different methods (4, 6) remains the lack of inexpensive and very gentle dissociation methods, preferably yielding large amounts of cells. Some, but not all, preparations of cultured astrocytes have shown themselves to closely resemble freshly isolated astrocytes in the expression of a multitude of genes. They have provided crucial information and appear also to be usable for further studies. The developmental signals to the cultures delivered by noradrenergic stimulation may be important determinants of their "astrocyticity." Nevertheless, they also have room for further improvement.

## CONCLUSION

Expression of many, but not all astrocytic genes can be appropriately recognized, at least qualitatively, by IHC and ISH. However, there are very important exceptions. The deficient aralar expression is probably no longer of major concern, since it has been contradicted by studies in both freshly dissociated astrocytes and in cultured cells. Moreover, lack of aralar expression is inconsistent with known astrocytic functions. The deficient qualitative and in some case quantitative expression of nucleoside transporters presently seems to represent a much bigger problem, requiring additional studies and changes of current concepts. Alternative methods may also fail. It is thus unfortunate that cell separation followed by microarray analysis in several cases provide different results in different studies and show negative results even when FACS sorting followed by determination of gene expression by RT-PCR and even IHC or ISH have indicated expression of a specific gene. Development of cheaper and gentler cell separation techniques is also urgently needed, but is bypassed when the method by Doyle et al. (10) is used. Gene expression determined in cultured cells can be reliable, but the cells used should have proven themselves capable of showing characteristics consistent with those of astrocytes obtained by other methodologies [**Table 2**; (50)]. The fact that several of the astrocytic genes expressed both in freshly isolated and cultured cells are neither recognized by IHC or ISH,

nor by the elegant newer cell-labeling techniques even in the hands of careful investigators is disturbing. It may be even worse that this seems to happen in a completely unpredictable manner. We have reviewed some of these genes but are in no doubt that expression of additional astrocytic genes may remain unrecognized.

## ACKNOWLEDGMENTS

We thank Frank Kirchhoff, University of Saarland, for his comments on the principles used for cell labeling in FACS and BAC procedures.

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**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

*Received: 06 June 2013; accepted: 31 October 2013; published online: 25 November 2013.*
*Citation: Peng L, Guo C, Wang T, Li B, Gu L and Wang Z (2013) Methodological limitations in determining astrocytic gene expression. Front. Endocrinol. **4**:176. doi: 10.3389/fendo.2013.00176*
*This article was submitted to Cellular Endocrinology, a section of the journal Frontiers in Endocrinology.*

frontiers in
ENDOCRINOLOGY

OPINION ARTICLE
published: 16 October 2013
doi: 10.3389/fendo.2013.00144

# Glutamine synthetase as an astrocytic marker: its cell type and vesicle localization

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**Keywords: astrocyte, oligodendrocyte, glutamate metabolism, immunocytochemistry, deconvolution**

The overall staining by GS clearly reveals astrocytes, including all cells of the astroglial family (1), i.e., Bergmann glia, Müller cells (2), tanycytes (3), and ependymal cells. The star shaped morphology from classical silver impregnations relates to cortical and hippocampal astrocytes, which display a comparable pattern in material stained for GFAP. However, the dense population of GS stained astrocytes found in all diencephalic and mesencephalic regions, known to display faint GFAP-labeling (unpublished observations) indicated that while apparently all astrocytes contain GS they have GFAP-ir filaments only in a region-dependent pattern. This is complicated by the emerging view that "astrocytes" constitute a heterogeneous population even within a given region. In the rat hippocampus, combined immunostainings have revealed that the "classical" GFAP-ir astrocyte constitutes a subpopulation of GS-ir astrocytes, which can also lack GFAP staining [direct double staining (4)]. In view of several astroglial subtypes and/or glial precursors present in the adult rodent brain, anti-GS appears to be the most general astrocyte marker, covering all subtypes. In addition, GS has been found early on to label exclusively astrocytic cells and no other glial or neuronal cell types *in situ* or in culture [reviewed by (5)]. GS has, thus, been applied as a reliable astrocyte marker in very many studies since.

"Complex cells" in rat hippocampus, initially assumed to be an astrocyte subtype (6) but now understood to belong to NG2 cells, a fourth glial type in the CNS (7), may display faint GS-ir in the soma but not its fine processes. Oligodendrocyte precursor cells, possibly also related to NG2 cells, were found to be devoid of GS-ir (8). The immunocytochemical profile and possible heterogeneity of NG2 cells is still under debate to date. Disputing the exclusion of non-astrocytic cells in GS staining, some authors have later reported GS$^+$ oligodendrocytes, although this has not been investigated systematically. Reports on non-astrocytic GS will be discussed in detail here.

## OLIGODENDROCYTES

The authors observing oligodendroglial GS localization rely mostly on the non-convincing morphology of "ovoid cells" in the gray matter, and only sometimes on the unambiguous alignment of interfascicular oligodendrocytes. Only one study is based on GS mRNA *in situ* hybridization (GS exclusively in astrocytes), and three studies on colocalization of GS-ir with oligodendroglial markers (see below). The reports on GS-ir in oligodendrocytes by three groups (9–11) can, however, not be reconciled, and might result from the use of different antisera and/or divergent interpretations of morphology. Thus, Cammer (9), applying a proprietary anti-sheep brain GS, observed clearly intrafascicular oligodendrocytes, but only faint white matter astrocytes in rat spinal chord. A similar pattern was evident in rat forebrain white matter (proprietary GS antiserum; specimens prepared by Dr. M. Lavialle). Anti rat liver GS (9) produced the most convincing intrafascicular oligodendrocytes displaying also immunoreactivity for CNPase, an established oligodendrocyte marker. However, in gray matter, oligodendrocytes but hardly astrocytes were detectable by anti-GS. Based on a different rabbit anti-sheep brain GS antiserum in cat brain, GS localization was found in an inverse relation, i.e., only in gray matter oligodendrocytes but not interfascicular oligodendrocytes (10). These cells were identified by light microscopic morphological criteria, most of them in perineuronal position. A localization of GS in gray (but not white) matter oligodendrocytes, mostly perineuronal and perivascular was confirmed by plausible ultrastructural criteria, using another rabbit anti-sheep brain GS in the mouse brain (11). In this context, the absence of a typical light microscopical pattern distinguishing astrocytes from oligodendrocytes in perineuronal position in cortex or hippocampus, and generally in non-telencephalic regions (where astrocytes are generally non-stellate) might be relevant. In these regions, the GS-ir gray matter oligodendrocytes observed by Miyake and Kitamura (11) were particularly abundant but "astrocytes" were hardly observed. GS-ir perineuronal oligodendrocytes were present in addition to astrocytes also in the cortex but not in the hippocampus, which would imply subclasses of perineuronal oligodendrocytes. Similarly, the figures provided by (10), of GS-ir gray matter oligodendrocytes in the cortex (perineuronal) and cerebellum (around Purkinje cells,) do not allow for clear differentiation from astrocytes or Bergmann glia. This applies particularly to the cerebellum where even an "oligodendrocyte-like astrocyte" has been described (12). In contrast, the GS-ir "ovoid cells" in the lizard mesencephalon were interpreted as astrocytic, since they were in alignment with radial glial fibers, and forming perivascular end feet (13). Non-astrocytic labeling by anti-GS might also be associated with

technical difficulties. Thus, Werner et al. (14), although colocalizing GS with CNPase in oligodendrocytes, depict GS-ir in the nucleus or putative perinuclear cytoplasm, but not in processes. They also find positive microglia, which has never been reported before, and would normally represent a negative control. Similarly, in the report on GS staining in over 50% of CNPase positive perineuronal oligodendrocytes (15), the GS-staining was not seen within the typical, ring-like CNPase$^+$ cell rim (as clearly shown for several other markers in the same publication). Although applying confocal microscopy, the images show occasional 3D overlay of incongruent shapes in the two channels, which might lead to misinterpretation particularly since the authors did not consider neighboring, perineuronal astrocytes. In cell culture, where glial cell type purity and technical preparation may lead to diverging results, GS can be induced in cells that are normally not GS$^+$, such as fibroblasts (16) or even chick brain neurons, which are GS$^-$ negative *in vivo* (17). GS induction in cultured oligodendrocytes, by corticoids or thyroid hormones was observed by some (18) but not others (17).

As evidence in favor of an astrocyte-restricted GS localization [reviewed by (5)], absence of oligodendrocyte labeling in white and gray matter has been reported by the group of Norenberg, in particular at the ultrastructural level (19, 20), and the group of Derouiche, who investigated vibratome sections from human (21), and rat (3), using a previously characterized anti-GS antiserum (16) or commercial GS antibodies (Chemicon-Millipore, Billerica, MA, USA: clone GS-6, MAB 302; Santa Cruz Biotechnology, CA, USA, Ab: sc-6640; unpublished observations). In particular, GS-ir in the conspicuous interfascicular oligodendrocytes would not have been overlooked in the studies mentioned, and very many others. Based on distribution and morphology, localization of GS mRNA in rat brain, although without cell identification, was in line with exclusive astrocytic labeling (22).

The clear distinction by GS-ir between astroglial cells and oligodendrocytes would be maintained in the tumors derived from these cells, since all astrocytomas and ependymomas but none of the oligodendrogliomas were GS$^+$ (23). Another astrocytic antigen, ezrin, which labels predominantly the fine, peripheral astrocyte processes of all astrocytic cells but not oligodendrocytes (24), has a corresponding, clear-cut specificity within the range of human glial tumors (25).

## NEURONS

Neuronal localization of GS has been undisputedly excluded by all studies, apart from two reports on human autoptic material from normal subjects, and individuals suffering from Alzheimer's disease (26, 27). In addition to astrocytes, the perikarya particularly of pyramidal cells were intensely labeled. Labeled neurons were observed in 2 (of 7) normal brains, and were highly variable in localization and quantity over cortical fields and layers also in all 10 cases from Alzheimer demented subjects (27). However, previous evidence and technical considerations suggest that these observations should be taken *cum grano salis*. Although there is no animal model for Alzheimer's disease, neuronal localization has not been reported in any of the many experimental neuropathology studies employing GS-ir. The finding also contrasts with previous human data (21) (4 cases), (23) (15), (28) (17). The only neuronal localization of GS *in situ* has been reported in a proteomic analysis of squid optic lobe synaptosomes, a definitely glia-free preparation (29). However, neuronal labeling similar to that reported (26, 27) has been observed in sections from rat brain (perfusion or immersion-fixed, vibratome) or human hippocampus (vibratome, paraffin), applying various anti-GS antisera in two laboratories (author's unpublished observations; Dr. M. Lavialle, personal communication). This neuronal labeling was regarded as spurious, since it occurred inconsistently after storage (exceeding 1 week), often without the expected glial staining, even in vibratome sections from the same block that has yielded the exclusive astroglial pattern in staining runs before. This might indicate the recognition of distinct epitopes displaying independent physicochemical properties. Interestingly, the anti-GS mAb (Chemicon) also used by (27, 28) has been found to cross reacts with a "GS-like protein" different from GS (30), however, its cellular localization in the brain has not been established.

## SUBCELLULAR GS LOCALIZATION

Anti-GS has been found to represent an ultrastructural marker completely "filling" astrocytic cytoplasm *in situ*, well suited to mark also the extremely fine glial processes (20, 31). However, it was noted that labeling might also be associated with vesicles (5), which could not be verified in the stainings based on the diffusible chromogen DAB (5). We further investigated this applying high-resolution fluorescence microscopy and deconvolution in primary astrocyte culture (32). Based on negative controls (**Figures 1A–J**), GS-ir using a polyclonal antibody made in rabbit (16) was concentrated in discrete structures resembling vesicles (**Figures 1K–O**), which were also double-labeled applying different GS antibodies simultaneously (**Figures 1K,N**). Note that there is no full "colocalization," i.e., full pixel superimposition of green and red channels. With the individual organelle as the unit of observation, however, the sparse, mostly incoherent pixels of the green channel [goat anti-GS (Santa Cruz sc-6640)] are mostly associated with the discrete, vesicle-like structures of the red channel (**Figures 1K,N**). This finding, possibly resulting from differential labeling efficiencies of the antibodies, can be referred to as vesicular colocalization. These double-labeled vesicular structures were present throughout the cell but concentrated at limited stretches of the cell boundaries (arrows in **Figures 1K,L**, also in **Figures 1E,H,J**), they were frequently arranged in rows (arrows in **Figures 1M,O**), and their structure as far as could be resolved was non-uniform, pleomorph (**Figures 1M,O**).

While these observations do not necessarily exclude the commonly assumed cytosolic presence of GS, they clearly suggest a vesicle-bound form. GS might be indirectly linked to vesicular membranes, as is known, e.g., from the glutamic acid decarboxylase isoform GAD65, which is attached to transmitter vesicles via the vesicular GABA transporter, to support neurotransmission (33). It will be interesting to further investigate whether these findings are important in the context of metabolic compartmentation, in particular in relation to glial glutamate uptake, metabolism, and release (34). Similarly, how would the GS$^+$ vesicles relate to those involved in vesicular exocytosis of glutamate from astrocytes?

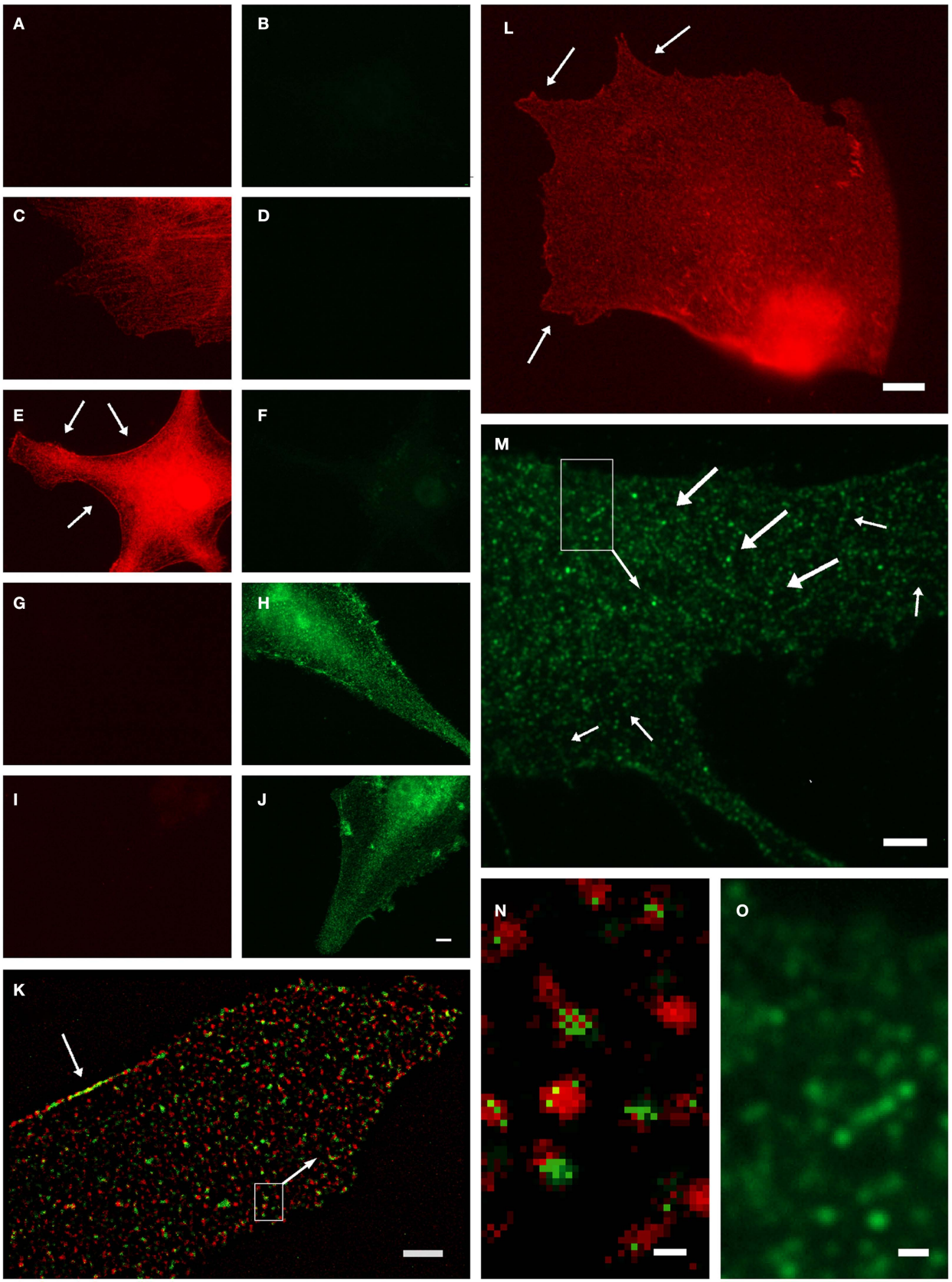


**FIGURE 1 | Continued**

**FIGURE 1 | Continued**
**Organelle-bound localization of GS-immunoreactivity, in primary culture of rat cortical astrocytes using the following anti-GS antibodies: (1) polyclonal made in rabbit (15), or (2) goat (Santa Cruz sc-6640), or (3) mouse monoclonal (Chemicon-Millipore, Billerica, MA, USA; clone GS-6, MAB 302).** **(A–J)** Controls for double-labeling (red and green) with two anti-GS antibodies, red channel always in left, green in right column. **(A,B)** Control for autofluorescence, no immunoreagents. **(C,D)** Control for fluorescence red-to-green bleed through: Single staining anti-GS (1) with secondary anti-rabbit antibody (red). **(E,F)** Control for detection system of green channel: Same as in **(C,D)**, in addition secondary anti-mouse antibody (green). **(G,H)** Control for fluorescence green-to-red bleed through: Single staining anti-GS (3) with secondary anti-mouse antibody (green). **(I,J)** control for detection system of red channel: Same as in **(G,H)**, in addition anti-rabbit antibody (red). **(K)** Double-labeling by antibodies (1) and (2) coincides on the same organelles, even at high magnification [**(N)**, from inset in **(K)**]. Antibody (1), red channel, labels the complete outline of organelles, whereas antibody (2), green channel only yields pixels within the extent of individual red labeled structures. Optical section 100 µm thick, after deconvolution. Single labeling by antibodies (3) **(L)** or (2) [**(M,O)**, from inset] yields comparable organelles. Scale 5 µm [in **(J)**, for **(A–J)**], 3 µm **(K,M)**, 4 µm **(L)**, 0.25 µm **(N)**, 0.5 µm **(O)**.

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*Received: 19 September 2013; accepted: 25 September 2013; published online: 16 October 2013.*
*Citation: Anlauf E and Derouiche A (2013) Glutamine synthetase as an astrocytic marker: its cell type and vesicle localization. Front. Endocrinol. 4:144. doi: 10.3389/fendo.2013.00144*
*This article was submitted to Cellular Endocrinology, a section of the journal Frontiers in Endocrinology.*

frontiers in
ENDOCRINOLOGY

MINI REVIEW ARTICLE
published: 13 May 2013
doi: 10.3389/fendo.2013.00054

# Metabolic aspects of Neuron-Oligodendrocyte-Astrocyte interactions

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Whereas astrocytes have been in the limelight of scientific interest in brain energy metabolism for a while, oligodendrocytes are still waiting for a place on the metabolic stage. We propose to term the interaction of oligodendrocytes with astrocytes and neurons: NOA (neuron–oligodendrocyte–astrocyte) interactions. One of the reasons to find out more about metabolic interactions between oligodendrocytes, neurons, and astrocytes is to establish markers of healthy oligodendrocyte metabolism that could be used for the diagnosis and assessment of white matter disease. The vesicular release of glutamate in the white matter has received considerable attention in the past. Oligodendrocyte lineage cells express glutamate receptors and glutamate toxicity has been implicated in diseases affecting oligodendrocytes such as hypoxic-ischaemic encephalopathy, inflammatory diseases and trauma. As oligodendrocyte precursor cells vividly react to injury it is also important to establish whether cells recruited into damaged areas are able to regenerate lost myelin sheaths or whether astrocytic scarring occurs. It is therefore important to consider metabolic aspects of astrocytes and oligodendrocytes separately. The present review summarizes the limited evidence available on metabolic cycles in oligodendrocytes and so hopes to stimulate further research interests in this important field.

**Keywords: energy metabolism, glucose, gray matter, white matter, pyruvate carboxylation, glycolysis, lactate, monocarboxylate transporters**

## INTRODUCTION

The body of research from the last century has established the present view of contiguous neurons in continuous, dynamic interaction with several types of glial cells (astrocytes, oligodendrocytes, microglia). The interactions between neurons and astrocytes characterized by the glutamate–glutamine(–GABA) shuttle have received considerable attention since its discovery in the 1970s (van den Berg and Garfinkel, 1971). This shuttle is necessary since neurons cannot make their amino acid neurotransmitters glutamate (excitatory, 90% of synapses), GABA (most abundant inhibitory), and aspartate without glutamine from astrocytes. In this interplay, glucose has a central role as the major (or exclusive) source of energy for the adult brain and the molecules used to synthesize glutamine and thus glutamate, GABA, and aspartate (McKenna et al., 2011).

In gray matter, glutamate released from neurons in glutamatergic neurotransmission is mainly taken up by astrocytes (Gegelashvili and Schousboe, 1997, 1998). This drain of glutamate is compensated for by a flow of glutamine from astrocytes to neurons, thus closing the glutamate–glutamine cycle (**Figure 1**). Net synthesis of tricarboxylic acid (TCA) cycle intermediates and related compounds like glutamate and glutamine depend upon entry of pyruvate *via* an anaplerotic pathway into the TCA cycle. In the brain, this is preferentially or exclusively achieved by pyruvate carboxylase (Patel, 1974a; Waagepetersen et al., 2001), which is not present in neurons but has been shown to be present in astrocytes (Yu et al., 1983; Shank et al., 1985; Cesar and Hamprecht, 1995). This process generates a "new" molecule of oxaloacetate, which may condense with acetyl-CoA to provide net synthesis of the TCA cycle intermediate α-ketoglutarate, from which glutamate can be formed by transamination (Westergaard et al., 1996). Subsequently, glutamine may be synthesized from glutamate (**Figure 1**) via glutamine synthetase, which like pyruvate carboxylase is exclusively expressed in astrocytes (Martinez-Hernandez et al., 1977; Norenberg and Martinez-Hernandez, 1979). Glutamine released by astrocytes can also function as precursor for the inhibitory neurotransmitter GABA via glutamate (Reubi et al., 1978; Sonnewald et al., 1993). Thus, the concept has been extended to a glutamate–glutamine(–GABA) cycle. The present review aims to further extend these cycles to include oligodendrocytes. There is a clear need for exploring the metabolism of oligodendrocytes in the context of inter-cellular interactions in the brain, involving the major neural cell types, neurons, astrocytes, and oligodendrocytes.

### TRI-CELLULAR COMPARTMENTATION OF BRAIN METABOLISM

Can the bi-cellular compartmentation of the central nervous system (CNS) described above be extended to a tri-cellular one including oligodendrocytes? The lack of knowledge available on the metabolic role of oligodendrocytes in the brain was highlighted recently with two publications in Nature (Funfschilling et al., 2012; Lee et al., 2012). These studies proposed, for the first time, a link between glycolytic metabolism in oligodendrocytes

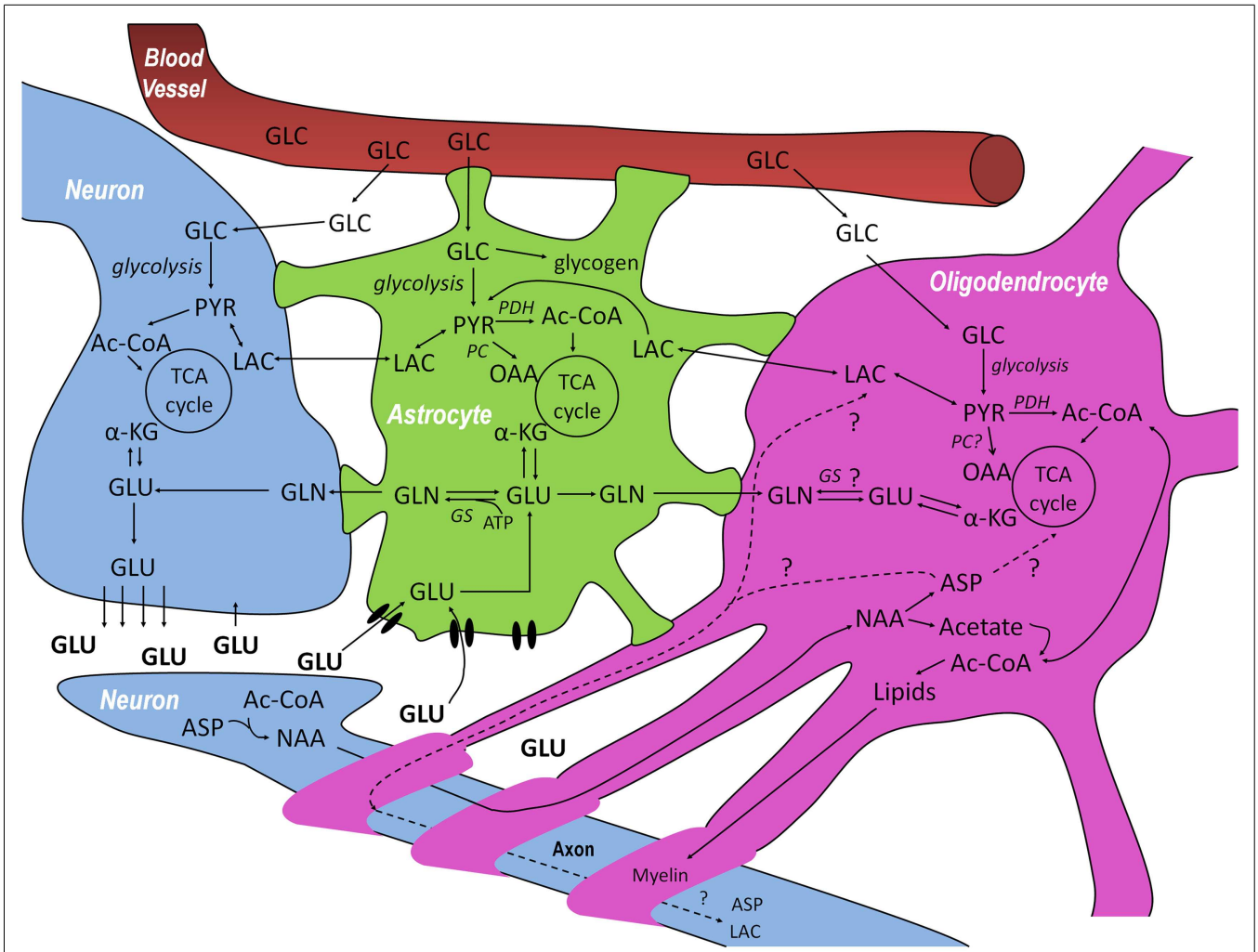


**FIGURE 1 | Schematic overview of the metabolic interactions between neurons–oligodendrocytes–astrocytes (NOA) discussed in this review**. Glucose (GLC) from the blood is taken up by neurons, astrocytes, and oligodendrocytes and can be metabolized via glycolysis, giving rise to pyruvate (PYR). In astrocytes, GLC can also be stored in the form of glycogen. PYR, the end product of glycolysis can be reduced to lactate (LAC) which can be released and taken up by cells with lower lactate concentration, be converted into alanine (not shown) or be converted into acetyl-CoA (Ac-CoA) and subsequently oxidized in the tricarboxylic acid (TCA) cycle. After synaptic release of glutamate (GLU) by neurons, astrocytes are responsible for most of its uptake via specific high-affinity glutamate transporters to prevent neuronal excitotoxicity, although some pre-synaptic re-uptake can also occur. GLU taken up by astrocytes can be converted to glutamine (GLN) by glutamine synthetase (GS) which can be transferred to neurons where it is transformed into GLU, making it available again for neurotransmission and, in this way, closing the GLU-GLN cycle. The close association between GLU, GLN, and TCA cycle metabolism is indicated in the three cell compartments: GLU can be additionally converted into α-ketoglutarate (α-KG) and be subsequently oxidized. Even though there are reports on the absence of glutamine synthetase and pyruvate carboxylase (PC) in oligodendrocytes, it is not totally clear whether or not they are capable of synthesizing GLN and performing anaplerosis. Finally, neurons are known to synthesize *N*-acetyl-aspartate (NAA) from aspartate (ASP) and Ac-CoA. NAA is thought to be transferred to oligodendrocytes where it is metabolized into ASP and acetate. Whereas the resulting acetate is thought to be extensively used for the synthesis of myelin lipids, the fate of the ASP is still unresolved since it can either be metabolized in oligodendrocytes or transported back to neurons, closing another potentially important metabolic cycle in the brain.

and axonal integrity and function. Analysis of hexokinase levels in the oligodendroglial fractions isolated from rat brain has shown that in comparison with whole brain or with isolated neurons or astrocytes, oligodendroglia express low levels of this enzyme (Snyder and Wilson, 1983). In agreement with this, Rinholm et al. (2011) suggest that lactate is transported from neurons to oligodendrocytes. Analyzing cell bodies and processes aligned with axons, which were presumed to be oligodendrocytes, a decrease in pH was shown when lactate was applied, thus suggesting that lactate was transported into the cells via MCTs that co-transported $H^+$. In a mouse model of globoid cell leukodystrophy (Krabbe disease) an increase in MCT1 was observed in the spinal cord (Meisingset et al., 2013). It should be noted that MCT1 is not only expressed by oligodendrocytes, and that its inhibition in brain has previously been associated with memory deficits due to astrocytic dysfunction (Suzuki et al., 2011). In the white matter, astrocytes are located close to oligodendrocytes and may have different tasks when compared to gray matter astrocytes which are

predominantly located near neurons. Disruption of white matter astrocyte–oligodendrocyte–neuronal interaction in multiple sclerosis is described in Cambron et al. (2012).

### TRI-CELLULAR COMPARTMENTATION OF NAA METABOLISM

Several reviews suggest that metabolism of *N*-acetyl-aspartate (NAA) has a tri-cellular compartmentation (Baslow, 2000; Moffett et al., 2007). Aspartate needed for NAA production can only be synthesized *de novo* in astrocytes (transported to neurons in the form of glutamine), NAA is then assembled and released from neurons and hydrolyzed to acetate and aspartate (**Figure 1**) by aspartoacylase, which is predominantly located in oligodendroglia (Madhavarao et al., 2004). Leukodystrophies are characterized by degeneration of myelin in the phospholipid layer insulating the axon of a neuron. Canavan's disease is characterized by an aspartoacylase deficiency and thus accumulation of NAA (Janson et al., 2006). Impairment of oligodendrocyte metabolism leading to accumulation of NAA was also shown in the spinal cord of Twi mice on postnatal day 30 (Meisingset et al., 2013). However, NAA production has also been reported in adult oligodendrocytes, depending on the culturing conditions (Bhakoo and Pearce, 2000). These authors suggest that mature oligodendrocytes in the adult brain synthesize NAA *in vivo*. However, at present there is a lack of consensus regarding oligodendrocyte-mediated NAA synthesis. This controversy needs to be resolved as it has important implications for the interpretation of $^{1}$HMRS data since NAA levels are commonly used as an indicator of neuronal viability.

### WHITE AND GRAY MATTER HAVE DIFFERENT ENERGY DEMANDS

There is a distinctive difference between white and gray matter astrocytes, moreover, the number of neurons and oligodendrocytes differs considerably between white and gray matter. From these observations it follows that energy demands will vary between these areas. The difference between white and gray matter metabolic intensity has attracted substantial interest. Most authors studying these differences agree that CNS white matter has a lower metabolic intensity than gray matter (Sokoloff, 1977). However, the values published for the cerebral metabolic rate of glucose (CMRglc) calculated from 2-deoxyglucose uptake or cytochrome oxidase activity measurements so far reported differ considerably. Using 2-deoxyglucose uptake in cortex CMRglc is 1–1.6 $\mu$mol (g min)$^{-1}$ whereas in white matter it is 0.3–0.4 $\mu$mol (g min)$^{-1}$ (references in McKenna et al., 2011). Applying cytochrome oxidase activity measurements to assess brain energy consumption it was proposed that the patterns found in the white matter were similar to those of 2-deoxyglucose uptake in conscious, "resting" animals, although some differences were detected (Hevner et al., 1995). For example, 2-deoxyglucose uptake was found to be approximately threefold higher in gray versus white matter (Sokoloff, 1977) whereas cytochrome oxidase activity showed an 8- to 12-fold difference. These and other discrepancies probably reflect basic technical differences between the two methods used. Compared to 2-deoxyglucose metabolism, the metabolism of cytochrome oxidase is more specific for oxidative metabolism and less so for glycolysis, and more reflective of overall neuronal functional activity occurring over longer time periods lasting hours and weeks, rather than minutes. Measuring oxygen uptake ratios in brain slices, the levels detected were in between those reported above (Hertz and Clausen, 1963). A disparity between glycolysis and oxidative metabolism of glucose in white matter has been found by (Morland et al., 2007). However, the usefulness of ratios is limited since they do not distinguish between axons and non-neuronal cells (oligodendrocytes and astrocytes). Glucose oxidation has been measured in neurons, astrocytes, and oligodendrocytes from the developing brain and these measurements showed that oligodendrocytes oxidized twice as much glucose per hour and mg protein as astrocytes in the TCA cycle and had only slightly less oxidation than neurons (Edmond et al., 1987). In this context it should be mentioned that oligodendrocytes are particularly sensitive to the effects of energy depletion as shown in case reports on patients exposed to CO poisoning (Grunnet and Petajan, 1976; Foncin and Le Beau, 1978; Egan et al., 2004). For example, Foncin and Le Beau (1978) reported an ultrastructural study of biopsy tissue, which showed well preserved and nearly normal cortex morphology, but extensive white matter injury, with disrupted or degenerated myelin and pycnotic oligodendroglia due to CO poisoning. In contrast, the appearance of axons, astrocytes, and capillaries had a nearly unchanged appearance. The destruction affecting oligodendroglia may have been caused by a special vulnerability of oligodendrocytes to glutamate. Similarly in the neonate, glutamate toxicity from hypoxia-ischemia during the perinatal period caused white matter injury and long-term motor and intellectual disability (Fields, 2010).

### THE OPERATION OF SEVERAL METABOLIC PATHWAYS REMAINS ELUSIVE IN OLIGODENDROCYTES

Another important question is whether oligodendrocytes also require glutamine from astrocytes. Can oligodendrocytes synthesize glutamine? Glutamine is thought to be an important component of proteins and an excellent energy substrate. Evidence suggests that glutamine synthetase is not present in oligodendrocytes (Pilkington and Lantos, 1982; Derouiche, 2004). Thus, it must be assumed that glutamine from astrocytes reaches the oligodendrocytes, possibly by passing through neurons.

Furthermore, neurons might supply glutamate to the oligodendrocytes or oligodendrocytes might regulate glutamate concentration in extracellular space of the white matter. Glutamate uptake has been shown in oligodendrocytes in the developing brain, but not the adult (DeSilva et al., 2009). Moreover, glutamate receptors are expressed in oligodendrocytes (Karadottir et al., 2005) and also vesicular glutamate release in the white matter has received considerable attention (Karadottir et al., 2005; Kukley et al., 2007; Ziskin et al., 2007).

Do oligodendrocytes express pyruvate carboxylase or are they dependent on aspartate from neurons (via NAA) for the synthesis of oxaloacetate? Aspartate is an essential member of the malate aspartate shuttle which is the major shuttle for reducing equivalents from NADH from glycolysis into the mitochondria (McKenna et al., 2006). It is important to note that aspartate production in neurons is only possible with the help of glutamine from astrocytes (see above). Acetyl-CoA from NAA degradation is used for lipid synthesis in oligodendrocytes and possibly energy production via TCA cycle activity (**Figure 1**). However, the fate of

aspartate from NAA is unclear. It might be (a) metabolized in the TCA cycle in oligodendrocytes, (b) sent back to neurons, or (c) degraded in both cell types (**Figure 1**). A pivotal question in this context is the localization of pyruvate carboxylase. This enzyme is a nuclear encoded homotetramer found in most eukaryotic and in many prokaryotic tissues and is a member of the family of biotin-dependent carboxylases (Wallace et al., 1998).

It is important to establish whether oligodendrocytes have the possibility to carboxylate pyruvate: if oligodendrocytes express PC they can afford to return aspartate from NAA to the neurons, if they do not, they cannot afford to do so since PC is the only anaplerotic enzyme in the brain (Patel, 1974b). There are a number of publications which propose that PC is exclusively expressed by astrocytes in the CNS (Yu et al., 1983; Shank et al., 1985; Kurz et al., 1993; Cesar and Hamprecht, 1995; McKenna et al., 1995, 2000; Vogel et al., 1998a,b). There is one report that has identified expression of PC in oligodendrocytes (Murin et al., 2009). However, the information in this publication has to be taken with caution since the oligodendrocyte cultures contained 10% astrocytes (Hirrlinger et al., 2002). Evidence for the lack of PC in oligodendrocytes comes from the lack of glycogen phosphorylase in those cells (Richter et al., 1996) since glycogenolysis is a prerequisite for glutamate formation (Gibbs et al., 2007; Sickmann et al., 2012; Xu et al., 2013) probably via dependency of glutamatergic signaling on pyruvate carboxylase (Hertz et al., 2013). Another unresolved question concerning oligodendrocytes is the extent of the pentose phosphate pathway activity. This glucose shunt is active in neurons and astrocytes (Kim et al., 2005; Brekke et al., 2012) and has its highest activity in oligodendrocytes in the developing brain (Edmond et al., 1987) but its activity in mature oligodendrocytes has yet to be investigated. Sykes et al. (1986) suggest that *de novo* synthesis of fatty acids and cholesterol by oligodendrocytes of neonatal rats should be closely geared to the activity of the pentose phosphate pathway in these cells.

## CONCLUSION

From the limited literature available it can be concluded that vital information about the metabolic capabilities of oligodendrocytes is still missing. With this review we would like to challenge the scientific community to devote time and effort to unraveling what we would like to call oligodendrocyte interaction with astrocytes and neurons (NOA:neuron–oligodendrocyte–astrocyte) interactions. Well-designed experiments are necessary to find answers to these and other questions concerning the referred metabolic pathways and cycles in oligodendrocytes.

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**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

*Received: 12 March 2013; accepted: 24 April 2013; published online: 13 May 2013.*

*Citation: Amaral AI, Meisingset TW, Kotter MR and Sonnewald U (2013) Metabolic aspects of Neuron-Oligodendrocyte-Astrocyte interactions. Front. Endocrinol. **4**:54. doi: 10.3389/fendo.2013.00054*

*This article was submitted to Frontiers in Cellular Endocrinology, a specialty of Frontiers in Endocrinology.*

frontiers in
ENDOCRINOLOGY

REVIEW ARTICLE
published: 02 October 2013
doi: 10.3389/fendo.2013.00138

# Protein kinase C phosphorylates the system N glutamine transporter SN1 (Slc38a3) and regulates its membrane trafficking and degradation

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The system N transporter SN1 (also known as SNAT3) is enriched on perisynaptic astroglial cell membranes. SN1 mediates electroneutral and bidirectional glutamine transport, and regulates the intracellular as well as the extracellular concentrations of glutamine. We hypothesize that SN1 participates in the glutamate/γ-aminobutyric acid (GABA)-glutamine cycle and regulates the amount of glutamine supplied to the neurons for replenishment of the neurotransmitter pools of glutamate and GABA. We also hypothesize that its activity on the plasma membrane is regulated by protein kinase C (PKC)-mediated phosphorylation and that SN1 activity has an impact on synaptic plasticity. This review discusses reports on the regulation of SN1 by PKC and presents a consolidated model for regulation and degradation of SN1 and the subsequent functional implications. As SN1 function is likely also regulated by PKC-mediated phosphorylation in peripheral organs, the same mechanisms may, thus, have impact on e.g., pH regulation in the kidney, urea formation in the liver, and insulin secretion in the pancreas.

**Keywords: SN1, Slc38, glutamine, glutamate, PKC, GABA, neurotransmitter replenishment, transporter**

## INTRODUCTION

Synaptic transmission at a chemical synapse is essential to many neuronal functions such as cognition, learning, and memory. It is based on exocytotic release of a neurotransmitter, its diffusion through the synaptic cleft and activation of specific receptors on the surface of the target cell. Glutamate is the major fast excitatory neurotransmitter in the central nervous system (CNS) undergirding the function of a wide range of synapses, while γ-aminobutyric acid (GABA) and glycine are the primary inhibitory neurotransmitters involved, among others, in synchronization of the principal neurons. In addition, monoamines, acetylcholine, neuropeptides, and other molecules sustain neuronal signaling at specific synapses [for review see Ref. (1)].

Sustained neurotransmission is dependent on replenishment of the neurotransmitters and efficient termination of the signal to reduce signal-to-noise ratio. In the case of the monoamines and acetylcholine, the neurotransmitters or choline (end-product of acetylcholine hydrolysis by acetylcholinesterase) are removed from the synaptic cleft by specific transporters on the nerve terminal membranes which also allow for their reuse in synaptic transmission (1). In contrast, the GABA transporter 3 (GAT3; and partially GAT1) and the major glutamate transporters (GLAST/EAAT1 and GLT-1/EAAT2) reside on surrounding astroglial cells, and a drain of these transmitters to the astroglial cells has been demonstrated (**Figure 1**) (2, 3). With the characterization of the members of the Slc38 family of amino acid transporters, showing that they may work in concert to shuttle glutamine from astroglial cells to neurons, the theory on a glutamate/GABA-glutamine cycle has been revitalized. In particular, the astroglial SN1, which releases glutamine, seems to be a major component of this cycle as it regulates extracellular concentrations of glutamine and shows dynamic membrane trafficking (4–6). In this review, we will discuss and consolidate recent data on the protein kinase C (PKC)-mediated regulation of SN1 and its functional implications.

## THE GLUTAMATE/GABA-GLUTAMINE CYCLE AND ITS CONTRIBUTION TO THE REPLENISHMENT OF THE NEUROTRANSMITTERS GLUTAMATE AND GABA

The considerable amount of transmitter steadily released from neuronal synapses demands a dependable mechanism for replenishment. The neurotransmitters glutamate and GABA cannot be generated from the tricarboxylic acid (TCA) cycle intermediates because neurons lack the ability for anaplerosis due to lack of pyruvate carboxylase (7). Indeed, glucose alone is insufficient to sustain neurotransmission in brain slices (8). Shuttling of monocarboxylates and TCA cycle intermediates from astroglial cells to neurons and contribution to the formation of the neurotransmitters has also been suggested (7, 9), but it

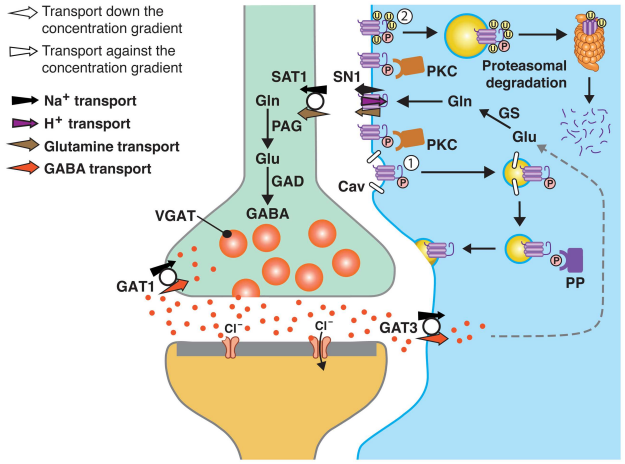


**FIGURE 1 | A model of SN1 membrane trafficking based on consolidated data on the regulation of SN1 activity by protein kinase C (PKC)**. The cartoon depicts a GABAergic synapse in adult rat brain where GABA is released exocytotically and acts upon specific post-synaptic receptors. The signal is terminated by removal of GABA from the synaptic cleft by transport of GABA back into the nerve terminal by the plasma membrane GABA transporter (GAT) 1. A substantial amount of GABA in the synaptic cleft is also transported into perisynaptic astroglial processes by GAT3 (and GAT1) and converted to glutamate, and then to glutamine catalyzed by glutamine synthetase (GS). Glutamine may then be released from the astroglial cells by the electroneutral and bidirectional system N transporter 1, SN1, and may subsequently be accumulated inside GABAergic neurons by the system A transporter SAT1. Here, glutamine is metabolized to yield glutamate and then GABA by the action of phosphate-activated glutaminase (PAG) and glutamic acid decarboxylase (GAD), respectively. Finally, GABA is translocated into synaptic vesicles by the vesicular GABA transporter (VGAT), wherefrom it is ready to be exocytotically released. Astroglial PKC may be activated upon stimulation of specific receptors on the astroglial membranes or e.g., by excessive amounts of $Mn^{2+}$. PKCα and PKCγ (and PKCδ) can phosphorylate SN1 on a serine at the 52 position. This results in caveolin (Cav)-dependent internalization of SN1 from the plasma membrane (1). The internalized SN1 may be relocated to the plasma membrane upon dephosphorylation by protein phosphatases (PP). PKC-mediated phosphorylation of SN1 also increases ubiquitination of SN1 (2). This may also internalize the protein into intracellular compartments and target it to the proteasomal degradation pathway. Similar regulation of SN1 activity also takes place at glutamatergic synapses. The same mechanisms are likely to be involved in the regulation of SN1 in hepatocytes, renal tubule cells, and the pancreatic B-cells.

remains to be demonstrated that they can undergird neurotransmitter synthesis and synaptic transmission. The prevailing hypothesis is therefore that the fast neurotransmitters shuttle through perisynaptic astroglial cells in order to sustain neurotransmission. According to this glutamate/GABA-glutamine cycle, glutamate and GABA are sequestered into astroglial cells and converted to glutamine. Astroglial cells then supply neurons with glutamine to fuel formation of glutamate and GABA.

There are several compelling findings supporting existence of the glutamate/GABA-glutamine cycle and that astroglial-derived glutamine is the primary precursor for the neurotransmitters glutamate and GABA (**Figure 1**). Astroglial cells ensheath synapses, furnish neurons with metabolic precursors and optimize conditions for neuronal function and signaling. They harbor GLAST, GLT-1, and GAT3 and quickly remove the neurotransmitters from the synaptic cleft and away from their receptors, by binding the neurotransmitters and subsequently transporting them into astroglial cells [(10–12), review (3)]. The sequestered glutamate and GABA are readily metabolized to glutamine by glutamine synthetase, which is enriched in astroglial cells and unique in being capable of synthesizing glutamine in the human body (13, 14). Glutamine transported into nerve terminals is catabolized by the phosphate-activated glutaminase (PAG), which is pronounced in nerve terminals, to resynthesize glutamate and GABA [for review see Ref. (15)]. Finally, the newly synthesized glutamate and GABA is accumulated inside synaptic vesicles by vesicular transporters prior to their exocytotic release (16, 17). Glutamine as a precursor for the neurotransmitters glutamate and GABA has been demonstrated beyond any doubts (7, 8, 18–22). However, how glutamine is shuttled from astroglial cells, where it is synthesized, into neurons for its utilization has been enigmatic.

## THE SYSTEM N AND SYSTEM A TRANSPORTERS SUSTAIN ASTROGLIAL-TO-NEURON TRANSPORT OF GLUTAMINE

The break-through in our understanding of the intercellular transport of glutamine and the glutamate/GABA-glutamine cycle was established by characterization of an orphan transporter homologous to the vesicular GABA transporter (VGAT): SN1 – a 504 amino acids long transporter with 11 putative transmembrane domains and a long intracellular N-terminal – transports glutamine, asparagine, and histidine consistent with the biochemically described system N activity (4). SN1 transport is coupled to $Na^+$ transport in symport and $H^+$ transport in antiport. Consequently, SN1 activity is associated with intracellular pH changes. The stoichiometric coupling of SN1 to $Na^+$ and $H^+$ running in opposite directions, makes the overall transport electroneutral and allows SN1 to work bidirectionally (4, 23–25). In addition to the coupled movement of $Na^+$ and $H^+$ ions, cations also penetrate SN1 in an uncoupled manner and enable SN1 to readily work in the release mode at physiological conditions. In the CNS, SN1 is localized on astroglial processes ensheathing synapses (5, 23, 26). During synaptic transmission and the subsequent depolarization of astroglial cells, ion and glutamine concentration gradients change and favor the release mode of SN1 (27). SN1 is therefore able to furnish nerve terminals with glutamine for neurotransmitter synthesis.

Interestingly, molecular identification of SN1 revealed a family of amino acid transporters (Slc38) including SN2 and the unidirectional system A transporters SAT1 and SAT2 [for review see Ref. (27, 28)]. The isoform-specific characteristics of these transporters together with their complementary localization enable these transporters to sustain intercellular transport of glutamine (**Figure 1**). SAT1 is pronounced in GABAergic neurons in the CNS, targeted to growth cones in developing neurons and to the same cellular compartments as VGAT in the mature intact neurons, indicating a role in glutamine uptake for GABA formation (29–31). In contrast, SAT2 is enriched in the somatodendritic compartments of glutamatergic neurons throughout the CNS and accumulates high levels of glutamine (32, 33). Upon stimulation of these neurons, glutamine is metabolized to generate glutamate which is released from their dendrites. Indeed, a pharmacologic

disruption of SAT2 abolishes retrograde signaling (33). Finally, the system N transporter SN2 is exclusively expressed on astroglial cell membranes and mediates electroneutral and bidirectional transport of several neutral amino acids (34, 35). SN2 participates in astroglial release of glutamine for neurotransmitter generation but adds on by releasing glycine for co-activation of NMDA receptors (35).

## A DIVERSE RANGE OF MECHANISMS ARE INVOLVED IN THE REGULATION OF THE SYSTEM A AND SYSTEM N TRANSPORTERS

As the Slc38 family of amino acid transporters sustains astroglial-to-neuron transport of glutamine, regulation of the system A and system N transporters may have impact on neurotransmitter replenishment and synaptic plasticity. A better understanding of molecular mechanisms involved in their function and regulation may reveal novel (patho-)functional roles of these transporters and the glutamate/GABA-glutamine cycle and unveil novel therapeutic targets. Eukaryotic cells have an entire range of possible regulatory mechanisms to regulate the activity and expression of their proteins. In principle, all steps of production, maturation, trafficking, and degradation of a cellular protein can be regulated to control its expression levels, in addition to all types of direct or indirect influence of protein activity mediated by interaction with molecules ranging from protons to macromolecular protein complexes. The glutamine transporters are no exception to this rule. Classical biochemical experiments early demonstrated adaptive, hormonal, and osmotic regulation of system A and N activities as measured by functional transport assays (36–38). A nutrition signaling cascade that includes activation of phosphatidylinositol 3-kinase (PI3K) and mammalian target of rapamycin (mTOR) has been shown to be important for upregulation of system A (39, 40). For SAT2, an amino acid response element regulating the promoter activity has been demonstrated (41).

The system N activity and SN1 is regulated at the transcriptional and translational levels (37, 42). Sophisticated regulation of SN1 by interacting proteins and ions has also been demonstrated. SN1 is e.g., targeted by the ubiquitin ligase Nedd4-2 (neural precursor cell expressed, developmentally down-regulated 4-2) which down-regulates SN1 activity in *Xenopus laevis* (*X. laevis*) oocytes (43). Insulin regulates expression of SN1 through the PI3K-mTor signaling cascade (44).

Protons regulate SN1 activity by competing with $Na^+$ at the sodium binding site of SN1 as shown by increasing $K_m$ for $Li^+$ (a substitute for $Na^+$) with little change in $V_{max}$ upon reducing extracellular pH (23). As $Na^+$ binding to the Slc38 family is a prerequisite for the binding of the amino acid prior to its translocation, extracellular pH changes have profound effect on SN1 activity (23, 24). Protons also regulate SN1 at the mRNA level. SN1 expression at normal conditions is restricted to the S3 segment of the proximal tubules of the kidneys. During chronic metabolic acidosis (CMA), SN1 is induced also in the S1–S2 segments of renal epithelium, thereby increasing glutamine metabolism and generation of bicarbonate to counteract acidosis (6). Induction of CMA in rats results in upregulation of SN1 by about 10-fold at the mRNA level and more than 5-fold at the protein level (6, 42, 45). A pH responsive element in the 3′ untranslated region (3′-UTR) of SN1 mRNA allows binding of specific proteins to the mRNA at low pH and thereby stabilizes the mRNA. As a result, SN1 expression is induced in the entire S1–S3 segments of the kidney (6).

## PKC-MEDIATED PHOSPHORYLATION IS CENTRAL FOR REGULATING MEMBRANE TRAFFICKING OF PLASMA MEMBRANE NEUROTRANSMITTER TRANSPORTERS

For proteins transporting neuroactive compounds, such as dopamine, serotonin (SERT), GABA, and glutamate, membrane trafficking regulated by phosphorylation/dephosphorylation events has been shown as a common denominator. Nedd4-2/serum and glucocorticoid inducible kinases 1 and 3 (SGK1 and 3) and protein kinase B (PKB) regulation have been described for the glutamate transporters EAAT1, 2, and 5 (46). However, the regulation by PKC stands out as a major mechanism. PKC isoforms and their numerous substrates regulate a variety of membrane proteins and in particular transporters [reviewed by (47, 48)]. In a comprehensive review, transporters for GABA (GAT1), SERT, dopamine (DAT1), and glutamate (EAAC1) were all shown to be regulated by PKC phosphorylation (48). For the three first transporters, PKC phosphorylation mediates internalization of the transporters, whereas dephosphorylation by Protein Phosphatase 2A (PP2A) or tyrosine phosphorylation mediates trafficking back into the cell membrane. For EAAC1, PKC seems to increase the surface expression together with PI3-kinase, whereas PP2A dephosphorylation elicits the internalization. Also the glycine transporter is being regulated by PKC (49–51). As PKC is ubiquitously expressed but strictly compartmentalized, its subcellular activation is differentially executed through a myriad of signal pathways, accounting for a large and diverse part of total phosphorylation phenomena in all cell types. Interestingly, PKC-signaling has been shown important for CNS processes like neuronal development, excitability, plasticity, and aging (52–54). Given the central regulatory role of PKC it is not surprising that the idea of PKC-regulation also of glutamine transporters was conceived by several different groups independently.

## EVIDENCE OF PKC-MEDIATED PHOSPHORYLATION OF SN1

Protein kinase C has recently been shown to regulate SN1 under physiological conditions by two groups (**Figure 1**), however, there is a difference in the reported mechanisms involved (55, 56). Balkrishna and co-workers report that treatment of *X. laevis* oocytes expressing rat SN1 with the phorbol ester phorbol 12-myristate 13-acetate (PMA) results in a rapid down-regulation of glutamine uptake in <20 min. As this PMA-induced reduction in glutamine uptake is prevented by the specific PKC-inhibitor bisindolylmaleimide (Bis) and could not be induced by an inactive form of PMA (4-α-PMA), the investigators conclude that PKC activation is involved. The specificity of the PKC action on SN1 was supported by lack of PMA-induced changes in the monocarboxylate transporter 1 (MCT1) activity in the same oocytes. The authors identify seven putative phosphorylation sites in the SN1 sequence, however, single or combined mutations of these sites have no impact on the PMA-induced down-regulation of SN1 activity. In an effort to further identify presence of particular regions or motifs on SN1 responsible for the observed PKC effect,

the SN1 cytosolic N-terminus was replaced with the N-terminal part of the homologous SAT1 protein. Treatment of the SAT1-SN1 hybrid with PMA still resulted in down-regulation of SN1, a result interpreted as proof that the targeting region is not localized in the SN1 N-terminus. Based on all these data it is concluded that SN1 is not directly phosphorylated by PKC; rather, the down-regulation of SN1 is mediated by some interaction with regulatory proteins endogenous to oocytes (55).

The authors also generate a fluorescently labeled construct of SN1, EGFP-SN1, and show that PKC-mediated down-regulation of glutamine uptake is caused by internalization of the fusion protein from the plasma membrane. The retrieval of SN1 from the plasma membrane is controlled by caveolin but remains dynamin-independent. Lastly, glutamine transport is challenged in the hepatocyte-derived HepG2 cells and cultured rat astrocytes; both hepatocytes and astrocytes have endogenous SN1 expression (26). PMA-treatment reduces glutamine uptake in cultured HepG2 cells but not in their cultured rat astrocytes, and this discrepancy is explained by differences in cell-specific regulatory mechanisms.

In the report by Nissen-Meyer and co-workers (56), we also detect a comparable time-dependent down-regulation of SN1 protein in the plasma membrane following PMA-treatment of mammalian cells stably transfected with SN1, and sequestration of the protein into intracellular reservoirs. The down-regulation is inhibited in the presence of Bis I, supporting mediation by PKC activation. However, we demonstrate PKC-mediated phosphorylation of SN1 in three different ways: first, direct phosphorylation of SN1 *in vitro* using a GST-fusion protein containing the N-terminal of SN1 and recombinant PKCα and PKCγ (56). Further, using site-directed mutagenesis to create unphosphorylatable SN1 mutants, we transfected cultured COS7 and PS120 cells with these mutant plasmids. Cells were then metabolically labeled with $^{32}$P-orthophosphate, stimulated with PMA and following immunoprecipitation and 2D-phosphopeptide mapping, we demonstrated that PKC-dependent phosphorylation in living cells was abolished selectively when a single serine residue was mutated (S52A) in the N-terminal of rat SN1, implicating this as the primary phosphorylation site.

Second, characterization of wild type and mutant SN1 in *X. laevis* oocytes electrophysiologically further corroborated our data on direct phosphorylation of SN1 by PKC. PMA-stimulation results in reduced SN1 activity as shown by abolished glutamine-induced inward currents. However, such reduction in the magnitude of the glutamine-induced inward currents perish when PKC is inhibited by Bis I. The unphosphorylatable S52A mutant resisted down-regulation in the presence of PMA, implicating that PKC isoforms phosphorylate SN1 at the S52.

Third, we also show direct phosphorylation of SN1 in cultured rat astroglial cells. By using specific affinity-purified antibodies selectively recognizing SN1 phosphorylated at the S52, we demonstrated that PKC stimulation results in increasing Bis I-sensitive phosphorylation of SN1 and that phosphorylated SN1 accumulates in intracellular compartments consistent with internalization of the protein. Such internalization of SN1 upon PKC-mediated phosphorylation is also supported by the fact that PKC activation significantly reduces $V_{max}$ of the glutamine-induced currents in *X. laevis* oocytes but has no effect on the $K_m$. Finally, our biochemical analyses suggest that SN1 may dynamically be recruited from these compartments upon dephosphorylation, however, prolonged activation of SN1 by PKC results in its degradation.

Interestingly, Sidoryk-Wegrzynowicz and co-workers recently also presented evidence that PKC is involved in the down-regulation of SN1 (57): $Mn^{2+}$ exposure upregulates the activity of both PKCα and PKCδ in cultured astrocytes. Both enzymes were activated by phosphorylation and PKCδ was in addition activated by caspase 3-dependent proteolysis. In their experiments, PMA-stimulation for 4 h significantly down-regulates system N-mediated glutamine uptake in cultured astrocytes, an effect which was inhibited by addition of the PKC-inhibitor Bis II. In harmony with our work, they show that PMA reduces the SN1-content of biotinylated surface membranes long before 4 h. Although they did not succeed in co-immunoprecipitating SN1 with PKCα, they did show that SN1 co-immunoprecipitates together with PKCδ at 0 and 2 h, but not at the later times investigated. Thus, these experiments also lend support to PKC being an important regulator of SN1 protein cell surface expression under physiological conditions, albeit not demonstrating direct phosphorylation of SN1. Thus, there are compelling evidence and some indications that SN1 is directly phosphorylated by PKCα, PKCγ, and PKCδ and that this is followed by caveolin-dependent internalization of SN1.

## IS PKC INVOLVED IN THE DEGRADATION OF SN1?

As shown above, PKC phosphorylates SN1 and regulates SN1 activity on cell membranes and thereby adjusts the transmembrane glutamine transport to comply with different (patho-)physiological demands for the neurotransmitter precursor. However, prolonged activation of PKC results in degradation of SN1 (**Figure 1**) (56, 57). SN1 interacts with Nedd4-2 when co-expressed in *X. laevis* oocytes and in astrocytes (43, 57). Moreover, stimulation of primary astrocytes with $Mn^{2+}$ induces ubiquitin/proteasome-mediated degradation of SN1 via the Nedd4-2/SGK1 signaling pathway, thus providing a partial explanation for $Mn^{2+}$-induced neurotoxicity (58). Consequently, SN1-mediated transport increases when SN1 is co-expressed with the SGK1 and 3, and PKB (43). Thus, this pathway could represent a link to PKC-regulation since phosphorylation by PKC frequently is a way to tag proteins for ubiquitination and further lysosomal or proteasomal degradation (**Figure 1**) (59, 60).

## WHAT IS THE FUNCTIONAL SIGNIFICANCE OF SN1 REGULATION FOR THE GLUTAMATE/GABA-GLUTAMINE CYCLE?

The glutamate transporters GLAST and GLT-1 and the GABA transporters GAT1 and GAT3 are enriched on the cell membranes of perisynaptic astroglial processes, capturing the exocytotically released neurotransmitters and translocating them into astroglial cells (10, 11). SN1 is also targeted to the same small glial processes (5, 26). Activation of the glutamate and GABA transporters will ensure that the local intracellular $Na^+$-concentration can be increased to a level where it can drive the SN1-mediated glutamine transport out of the cell (61), and as long as glutamine synthetase is present, glutamate and GABA imported will be

transformed to glutamine for export. Indeed, glutamate stimulates efflux of glutamine from astroglial cells (62).

Uwechue and co-workers have provided compelling evidence that glutamate evokes release of glutamine through a system N like activity in astrocytes juxtaposed to the glutamatergic calyx of Held synapse in the rat medial nucleus of the trapezoid body (MNTB). Subsequently, such glutamine release is sensed by MNTB principal neurons which express system A transporters consistent with an intact glutamate/GABA-glutamine cycle (63, 64). Similarly, studies on cultured Bergmann glia cells show that activation of glutamate transporters by D-aspartate results in release of glutamine (65). Altogether, these data strongly suggest functional coupling between the glutamate and glutamine transporters and existence of a glutamate/GABA-glutamine cycle. Taken together with the dynamic regulation of the membrane trafficking of SN1 activity (55–57) this suggests that SN1 may be one of the key regulators of neuronal supply of glutamine and thus the glutamate/GABA-glutamine cycle (**Figure 1**). In addition, inhibition of SN1 activity may stimulate targeting of glutamate, GABA, and glutamine for oxidation or increase trans-astrocytic glutamine fluxes with impact on surrounding regions (66).

## PKC-MEDIATED PHOSPHORYLATION MAY REGULATE A WIDE RANGE OF FUNCTIONS IN PERIPHERAL ORGANS

SN1 also sustains pivotal functions in peripheral organs. In the kidney, SN1 is localized on the basolateral membranes of the S3 segment of proximal tubules and is essential for glutamine metabolism. During CMA, $K^+$-deprivation, and/or high protein intake, the total levels of SN1 increase significantly in the kidney and SN1 is also induced in the S1–S2 segments of the nephron (6, 45, 67). In the endocrine pancreas, we have shown complementary expression of SN1 and SAT2 and suggested that they work in concert to regulate a local glutamate-glutamine cycle and secretion of insulin (68, 69). The liver has one of the highest cellular concentrations of SN1 and is suggested to mediate glutamine influx for urea formation in periportal hepatocytes and glutamine efflux from the perivenous hepatocytes for transport of glutamine to other peripheral organs for cellular metabolism (26, 27). Accordingly, SN1 expression in the liver is regulated during starvation and insulin secretion (44). Thus, SN1 is essential in several physiological processes and may be differentially regulated in different organs to optimize a wide range of functions. As PKC isoforms are ubiquitously expressed throughout the body, but differentially in different cellular and subcellular compartments, isoform-specific PKC-mediated phosphorylation of SN1 may have a range of physiological and pathological roles.

## CONCLUSION

Consolidated data from several papers on the regulation of SN1 (55–57) show that PMA-induced and Bis I-inhibitable retrieval of SN1 occurs in several mammalian cell types, including primary rat astrocytes, and in *X. laevis* oocytes (**Figure 1**). Such membrane trafficking is governed by specific phosphorylation of SN1 at S52, selectively by PKCα and PKCγ. Prolonged PMA-stimulation results in internalization by a caveolin-dependent and dynamin-independent mechanism and to degradation of SN1 through the Nedd4-2/ubiquitination pathway. PKC-mediated regulation of SN1 may, thus, be a key step in the regulation of the glutamate/GABA-glutamine cycle in the CNS and a wide range of pathophysiological processes in peripheral organs. Further studies are required for a better understanding of molecular mechanisms governing regulation of SN1 activity on the plasma membrane and its membrane trafficking and such studies may reveal novel mechanistic insight into a variety of physiological processes and to discovery of novel therapeutic targets.

## ACKNOWLEDGMENTS

We are grateful to Gunnar Lothe for help with preparation of the figures. The work was funded by the Research Council of Norway (RCN).

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**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

*Received: 02 August 2013; paper pending published: 10 August 2013; accepted: 16 September 2013; published online: 02 October 2013.*
*Citation: Nissen-Meyer LSH and Chaudhry FA (2013) Protein kinase C phosphorylates the system N glutamine transporter SN1 (Slc38a3) and regulates its membrane trafficking and degradation. Front. Endocrinol. **4**:138. doi: 10.3389/fendo.2013.00138*
*This article was submitted to Cellular Endocrinology, a section of the journal Frontiers in Endocrinology.*

frontiers in
ENDOCRINOLOGY

MINI REVIEW ARTICLE
published: 31 December 2013
doi: 10.3389/fendo.2013.00199

# The amino acid transporters of the glutamate/GABA-glutamine cycle and their impact on insulin and glucagon secretion

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Intercellular communication is pivotal in optimizing and synchronizing cellular responses to keep homeostasis and to respond adequately to external stimuli. In the central nervous system (CNS), glutamatergic and GABAergic signals are postulated to be dependent on the glutamate/GABA-glutamine cycle for vesicular loading of neurotransmitters, for inactivating the signal and for the replenishment of the neurotransmitters. Islets of Langerhans release the hormones insulin and glucagon, but share similarities with CNS cells in for example transcriptional control of development and differentiation, and chromatin methylation. Interestingly, CNS proteins involved in secretion of the neurotransmitters and emitting their responses as well as the regulation of these processes, are also found in islet cells. Moreover, high levels of glutamate, GABA, and glutamine and their respective vesicular and plasma membrane transporters have been shown in the islet cells and there is emerging support for these amino acids and their transporters playing important roles in the maturation and secretion of insulin and glucagon. In this review, we will discuss the feasibility of recent data in the field in relation to the biophysical properties of the transporters (Slc1, Slc17, Slc32, and Slc38) and physiology of hormone secretion in islets of Langerhans.

**Keywords: GABA, glutamate, glutamine, insulin, SAT2, Slc38a2, Slc38a3, SN1**

## INTRODUCTION

The endocrine cells of the pancreas and the cells of nervous system have different functions but also reveal interesting similarities. Islet cells of Langerhans secrete insulin and glucagon and are considered to be necessary for maintaining glucose homeostasis. However, neurons may also release insulin, and compelling evidence have been provided for a brain-centered glucoregulatory system that work in concert with the islet cells to regulate plasma levels of glucose (1–3). Interestingly, recent advances in the field reveal mechanistic similarities in the regulation of insulin and glucagon secretion in the islets as compared to neurotransmitter release. Amino acids, which play pivotal roles in fast neuronal signaling, have also been proposed to act as signaling molecules in the islets of Langerhans (4–7). This is consistent with the detection of significant changes in plasma glutamine concentrations in newly diagnosed diabetic patients (8) indicating that dysfunctional amino acid metabolism, signaling, and/or amino acid transporter function may precede and/or augment development of diabetes. In this review, we will discuss the significance of glutamate, GABA, and glutamine and their transporters in the regulation of insulin and glucagon secretion in the pancreas.

### AMINO ACID TRANSPORTERS AND NEURONAL SIGNALING

Classical neuronal signaling is a form of paracrine signaling where a neurotransmitter is released at the synapse from a neuron and the message is conveyed by activation of specific receptors on the surface of an adjacent neuron. Several amino acids play fundamental roles in synaptic transmission. Glutamate and GABA are the main fast excitatory and inhibitory neurotransmitters, respectively. After their release, the signal is partly inactivated by transport of the released neurotransmitter into astroglial cells and conversion to glutamine catalyzed by glutamine synthetase (GS) (9, 10). Glutamine may then be released from astroglial processes and shuttled back to neurons for regeneration of the transmitters by the neuronal phosphate-activated glutaminase (PAG) (11, 12). Existence of such a cycle, known as the glutamate/GABA-glutamine (GGG) cycle, is bolstered by the demonstration of glutamate and GABA transporters on synaptic vesicles and on perisynaptic astroglial plasma membranes (13–17), and by the characterization of the Slc38 family of amino acid (glutamine) transporters (18–20). We have demonstrated that the Slc38 family members SN1 (Slc38a3) and SN2 (Slc38a5) release glutamine from the astrocytes (18, 21, 22), while SAT2 (Slc38a2) imports glutamine into glutamatergic neurons and maintains neurotransmitter pools of glutamate involved in retrograde signaling (23). The Slc38 family member SAT1 (Slc38a1) is selectively localized in inhibitory neurons supporting uptake of glutamine for GABA formation (19, 24). Consistent with a role of SAT1 in the GGG cycle, the SAT inhibitor MeAIB reduces GABAergic inhibitory synaptic transmission (25, 26). However, the presence of these transporters

also in peripheral organs suggests important roles in cell-specific metabolism and/or in non-neuronal signaling (27–29).

### THE ENDOCRINE CELLS OF THE ISLETS OF LANGERHANS HARBOR PROTEINS INVOLVED IN CLASSICAL NEURONAL SIGNALING

The endocrine cells of the pancreas share many characteristics with the cells of the central nervous system (CNS). The two tissues share a similar transcription program (30) and have extensive similarities in global mRNA expression and chromatin methylation (31). In addition, transcription factors like Pax6 and Nkx6.1 are important for development and differentiation of both neurons and islet cells (32–34).

The endocrine islet cells also contain most of the components involved in synaptic transmission in the CNS. In addition to the hormone loaded secretory granules (SGs), islet cells contain synaptic-like microvesicles (SLMVs) resembling synaptic vesicles found in nerve terminals (35, 36). The exocytosis of SGs and SLMVs also share many similarities with the exocytosis of synaptic vesicles. As in the CNS, exocytosis is dependent on the opening of voltage-dependent $Ca^{2+}$ channels (37, 38), and on the complex assembly of SNARE proteins including SNAP25 and syntaxin coupled to the vesicle and to the target membrane (39). Furthermore, exocytosis and hormone secretion is in both regulated by myotrophin, and the same miRNA, miR-375, regulates translation of its gene in both tissues (40). In addition, the biogenesis of SLMVs in β-cells is dependent on the same adaptor protein complex as in GABAergic neurons (41).

There is co-release of glucagon and glutamate from α-cells (42), and SLMVs of β-cells contain high levels of glutamate (43). In β-cells, glutamate activation of AMPA and kainate receptors stimulates $Ca^{2+}$ influx and insulin secretion (44, 45), whereas mGluR stimulation, both inhibit and stimulate insulin secretion depending on the concentration of glucose (46, 47). In addition, glutamate stimulates GABA release from SLMVs in β-cells independently of insulin release (48). In α-cells, glutamate inhibits glucagon secretion. Thus, glutamate is involved in both paracrine and autocrine regulation of glucagon and insulin.

When it comes to GABAergic signaling, $GABA_A$ receptor activation is important for β-cell autocrine feedback (49), and it reduces glucagon secretion from α-cells (50, 51). Furthermore, the key enzymes involved in the metabolism of the neurotransmitters glutamate and GABA, e.g., PAG, glutamic acid decarboxylase (GAD), and GS, are all present in islet cells (4, 52, 53). In fact, islet β-cells contain GABA and GAD at the same levels as GABAergic neurons (54, 55), and β-cells have the highest GABA concentration outside the CNS.

### GLUCOSE AND AMINO ACIDS IN THE REGULATION OF INSULIN SECRETION

The main triggering signal for insulin release from β-cells is elevated plasma glucose levels. When plasma glucose levels rise postprandially, facilitated transport by the low affinity glucose transporter GLUT2 ($K_m = 15$–$20$ mM), the primary glucose transporter present on β-cells (56), is initiated (**Figure 1**). In β-cells, glucose is metabolized through the TCA cycle to yield ATP, the main stimulator of insulin secretion (37). ATP acts by closing potassium channels $\left(K^+_{ATP}\right)$ which leads to depolarization of the β-cell (37). Exocytosis of insulin containing SGs occurs when $Ca^{2+}$ levels rise after opening of voltage-gated $Ca^{2+}$-channels (37, 38).

Many amplifying signals modify islet secretion (57). There is support for amino acids influencing the endocrine function of the pancreas (4–6), and in particular glutamate, GABA, and glutamine are postulated to play a role in the regulation of hormone secretion (4, 7). Deletion of glutamate dehydrogenase (GDH), which catalyzes the conversion between glutamate and the TCA cycle intermediate α-ketoglutarate, decreases glucose-induced insulin secretion by approximately 40% (58). In addition, glutamine's ability to evoke insulin release is decreased in isolated islets from GDH knockouts (59). Furthermore, the very close proximity between the different cell types in human islets facilitates flux of substrates and paracrine signaling for the orchestration of islet secretion (**Figure 1**) (60, 61). Thus, there are compelling evidence for glutamate, GABA, and glutamine being involved in regulating islet hormone secretion.

## AMINO ACID TRANSPORTERS IN ISLET SECRETION

As described above, glutamate and GABA, their specific receptors and effects, as well as the enzymes involved in their metabolism have all been shown in the islet. However, the mechanisms involved in the transport of amino acids across plasma and vesicle membranes and mode of action in the islet cells are poorly understood. Interestingly, recent advances in the characterization of amino acid transporters in the islets reveal peculiar insight into novel mechanisms for signaling in the islets.

### VESICULAR GLUTAMATE AND GABA TRANSPORTERS ARE LOCALIZED ON SLMVs AS WELL AS SGs IN ISLET CELLS

Glutamate is transported into synaptic vesicles by the vesicular glutamate transporters (VGLUT1-3) of the Slc17 family (62), whereas VGAT (Slc32) transports the inhibitory neurotransmitters GABA and glycine (63, 64). In the CNS, the VGLUTs and VGAT may be localized on different subsets of synaptic vesicles or co-localized on the same vesicles (65–68). This differential localization of vesicular transporters allows for co-release or differential release of transmitters, as well as concerted action to increase packaging.

Islet cells express both VGLUTs and VGAT (**Figure 1**). In α-cells, VGLUT1 and VGLUT2 are found on glucagon containing SGs (43, 69), supporting the finding that there is co-release of glucagon and glutamate from α-cells (42). On the SLMVs of the α-cells, there is expression of the 57-kDa isoform of VGAT found in the CNS (55, 70–72), but in addition a novel 52.5 kDa isoform is found on the SGs (72). The expression levels of the 52.5-kDa VGAT isoform increase with increasing glucose levels, opposite to the regulation of the transcription and activity of VGLUT1 and VGLUT2 which is down-regulated by increasing glucose levels in islet cell cultures (73). Thus, granular loading of glutamate, GABA, and glycine changes with changing glycemic conditions, allowing for fine-tuning of hormone secretion.

In the β-cells, VGLUT3 is found on both SGs and SLMVs (**Figure 1A**) (43). However, the SGs contain little glutamate (43), and direct stimulation of granule secretion with sulfonylurea tolbutamide, which closes ATP-sensitive potassium channels, does not lead to glutamate release from β-cells (74), questioning the

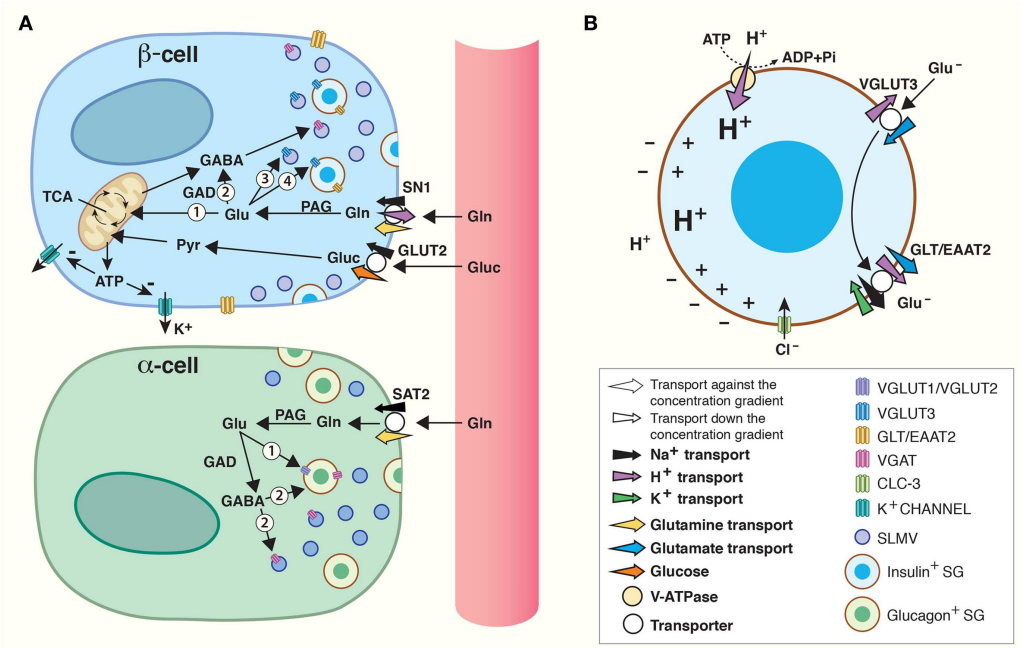


**FIGURE 1 | Amino acids in pancreatic endocrine signaling and insulin maturation**. **(A)** Amino acids are shuttled between cells and intracellular compartments and are involved in autocrine and paracrine signaling in the pancreatic islets of Langerhans. High levels of glucose (Gluc) are carried by the blood flow to the islet β-cells where glucose is accumulated by a glucose transporter (GLUT2). Through glycolysis, glucose is catabolized to pyruvate (Pyr) which is transported into mitochondria for oxidative phosphorylation. The product, ATP, inhibits $K^+$-channels. As a result, β-cells are depolarized and this stimulates fusion of insulin containing secretory granules (SGs) with the plasma membrane. Glutamine carried by blood can be accumulated inside β-cells by SN1 on their plasma membrane. Glutamine may be converted to glutamate by phosphate-activated glutaminase (PAG) and enter the TCA cycle inside mitochondria (1) to produce ATP which may regulate $K^+$-channels in the same way as glucose. Alternatively, glutamate may be translocated into synaptic-like microvesicles (SLMVs) (3) or insulin containing SG (4). Finally, glutamate may be metabolized to GABA (2) by glutamic acid decarboxylase (GAD) and accumulated inside SLMVs for secretion. In α-cells, SAT2 accumulates high levels of glutamine which may be metabolized to glutamate and/or GABA by PAG and GAD, respectively. The vesicular glutamate transporters (VGLUT) 1 and 2 and VGAT then transport glutamate (1) and GABA (2) into SLMVs and glucagon containing SG for exocytotic release. **(B)** VGLUT3-mediated transport of glutamate into SGs, followed by GLT/EAAT2-mediated transport out, contributes to maturation of insulin. A vacuolar (V)-ATPase generates a high electrochemical gradient for $H^+$. Flux of $H^+$ through VGLUT3 and down its electrochemical gradient energizes transport of glutamate ($Glu^-$) into SGs. Subsequently, the glutamate transporter GLT/EAAT2 translocates glutamate out of the SG, and this transport is coupled to 3 $Na^+$ and 1 $H^+$ transport in symport and 1 $K^+$ in antiport. The concerted action of VGLUT3 and GLT/EAAT2 results in a net movement of positive charge out of the SG. This counteracts inhibition of the V-ATPase and augments accumulation of $H^+$ inside SG. Such acidification stimulates conversion of pro-insulin to insulin. The chloride channel CLC-3 also resides on the membranes of SG. Transport of $Cl^-$ down its electrochemical gradient (into SG lumen) by CLC-3 also counteracts inhibition of the V-ATPase and increases the luminal acidification which stimulates maturation of insulin.

role of SG release of glutamate as important for signaling. Unlike β-cell SGs, SLMVs accumulate glutamate and express high levels of VGLUT3 (43). It has been hypothesized that the main function of SLMV exocytosis is to supply the plasma membrane with the proteins and lipids necessary to withstand the stress from substances like $Zn^{2+}$ secreted together with insulin (75). However, the presence of VGLUT3 on and glutamate in SLMVs might indicate that glutamate release from β-cells participates in feedback mechanisms regulating insulin secretion. This is supported by the fact that exocytosis of SLMVs and SGs is differentially regulated through two cAMP-dependent pathways (75). β-cells also express the 57-kDa isoform of VGAT on SLMVs (55, 71, 72), which probably primarily supports GABA release, as β-cells contain much higher levels of GABA compared to glycine (55).

Thus, both islet SGs and SLMVs have the machinery necessary to load them with glutamate and/or GABA and glycine, which can then be released in an exocytotic manner.

## PLASMA MEMBRANE GLUTAMATE TRANSPORTERS MAY TERMINATE FAST TRANSMISSION AS WELL AS CONTRIBUTE TO A NOVEL MECHANISM FOR THE MATURATION OF SGs

$Na^+$-dependent glutamate transport across the cell membrane in islet cells (76), probably facilitated by the glutamate transporter GLT/EAAT2 (Slc1) expressed on the plasma membrane of pancreatic β-cells (77), can terminate glutamate signaling (**Figure 1A**). In fact, glutamate transport by EAAT2 prevents glutamate induced excitotoxicity as down-regulation of EAAT2 lead to increased β-cell death, whereas up-regulation increased β-cell survival (77).

EAAT2 has also been shown to be strongly expressed on the membrane of SGs in β-cells (43), and co-localizes with both chromogranin, a neuroendocrine secretory protein in granules, and insulin in β-cells (77). As discussed above, the SGs also express VGLUT3, but there is very little concomitant release of glutamate and insulin (74) and glutamate levels inside SGs are insignificant (43). The presence of both of these transporters in the β-cells raises

the possibility of alternative mechanisms through which glutamate can act as an intracellular signaling molecule modulating the release of insulin.

Gammelsæter and co-workers postulated that glutamate transport might participate in the maturation of insulin in the SGs, and thus the regulation of insulin release (43). SGs contain a vesicular $H^+$-ATPase which pumps protons into the SGs (**Figure 1B**). Uptake of negatively charged glutamate by VGLUT3 sets up a counter-charge movement which decreases the granular membrane polarization allowing sustained transport by the $H^+$-ATPase. If glutamate leaves the SGs through EAAT2, three positively charged sodium ions and a proton will be exported, while one potassium ion will enter the SG. Thus, there will be a net export of positive charge, further counteracting inhibition of the $H^+$-ATPase, allowing for further acidification of the SG. Acidification is crucial for the conversion of pro-insulin to active insulin (78, 79), and it is thought to be important for the exocytotic process (79). Interestingly, perturbation of the granular acidification by genetic deletion of the chloride channel ClC-3, which resides on the SGs (**Figure 1B**), also results in abolished insulin release (80). Chloride ions also regulate or penetrate VGLUTs and VGAT (81, 82), and may acidify SG in multiple ways. Altogether, these data suggest that VGLUT3 probably works in consortium with EAAT2 to acidify the SGs through glutamate import and subsequent export, which further regulates the level of insulin released upon glucose stimulation.

### MEMBERS OF THE Slc38 FAMILY OF AMINO ACID TRANSPORTERS MIGHT BOTH SUPPLY GLUTAMINE FOR THE FORMATION OF GLUTAMATE AND GABA AND CONTRIBUTE TO PLASMA MEMBRANE DEPOLARIZATION IN THE ISLETS OF LANGERHANS

The islets of Langerhans express transcripts for several members of the Slc38 gene family (**Figures 1A** and **2**) (83). Immunofluorescence co-labeling with antibodies against SN1 and SAT2 with specific markers for the different cell populations of the islets showed SN1 at the plasma membrane of β-cells, whereas SAT2 was found on the membrane of α-cells. This distribution resembles the complementary expression pattern of SAT2 and SN1 in the CNS, where SN1 and SAT2 transport glutamine from astrocytes to glutamatergic neurons for the replenishment of glutamate (23).

SN1 translocates glutamine coupled to a symport of $Na^+$ (18). In addition, it is sensitive to pH, and SN1 both transports protons coupled to the transport of glutamine and allows for flux of protons not stoichiometrically dependent on the movement of substrate (21, 84, 85). Under most physiological conditions, the electroneutral SN1 may therefore transport glutamine bidirectionally (18, 21, 84–87). SN1 at the membrane of β-cells might therefore accumulate glutamine for glutamate and GABA synthesis (**Figures 1A** and **2**), but at different feeding status it may contribute to the release of glutamine (**Figure 2**), as discussed below.

Glutamine has been shown to have a profound effect on insulin secretion (4–6). Increased levels of glutamate and GABA in β-cells from replenishment by glutamine are not sufficient to explain this strong effect on secretion. It is therefore likely that glutamine contributes to the regulation of insulin secretion through additional mechanisms. First, glutamine taken up by SN1 might stimulate insulin secretion through the conversion of glutamine to glutamate by PAG (**Figure 1A**), which is present in β-cells (53). This is in agreement with earlier findings showing conversion of glutamine to glutamate in islet cells (88, 89). In the β-cell, glutamate can then enter the TCA cycle through the action of GDH, and stimulate insulin secretion in the same $K^+_{ATP}$-dependent way as glucose (53). This is supported by the fact that, in isolated islets from GDH knockouts there is an abrogation of insulin release evoked by glutamine (59). Alternatively, glutamate flux through VGLUT3 and EAAT2 on SGs might contribute to the maturation of insulin, as described above (**Figure 1B**). Glutamine transport by SN1 can also contribute to the depolarization of the membrane of β-cells. As already mentioned SN1 is sodium dependent and exhibits a channel-like activity, for e.g., protons, inducing inward currents (18, 21, 85). Due to these characteristics, SN1 can have a depolarizing effect upon transport of glutamine (21). The potassium channel $K^+_{ATP}$ is responsible for the depolarization initiated insulin release in response to glucose. In knockout mice with a loss of function mutation of the $K^+_{ATP}$, insulin secretion can be triggered by amino acids (4). This effect decreased 60% if glutamine was omitted, which was suggested to be due to the depolarizing effect of glutamine transport into the cell (4), and the conveyer may well be SN1 (83). Thus, glutamine uptake by SN1 due to elevated serum levels of glutamine may not only stimulate insulin secretion through conversion to glutamate, but also by amplifying the depolarization of the plasma membrane.

Lack of coupling to proton translocation enables the SATs to utilize both the electrical and the chemical gradients of sodium, creating higher glutamine concentration gradients compared to SN1 (19, 23, 90). In addition, SAT2 has higher affinity for glutamine than SN1 (90). According to our findings, SAT2 is expressed on α-cells (**Figures 1A** and **2**) (83). In α-cells, glutamate is exocytosed together with glucagon when blood glucose levels are low (42). As SAT2 is able to create a large concentration gradient of glutamine across the membrane, SAT2 might therefore ensure a constant supply of glutamine for the production of glutamate. Interestingly, when the interprandial serum levels of glutamine are low, flux reversal of SN1 might assure substrate for transport by SAT2 (**Figure 2**). Since system A transport depolarizes cells (19, 90), its activity might also stimulate glucagon secretion in a similar manner to the postulated role of SN1 in β-cell depolarization.

All in all, as SN1 changes direction of transport at glutamine concentrations well within physiological fluctuations in amino acid content of the blood (18), SN1 might be a convenient conveyer of plasma amino acid level stimuli for islet cell secretion. We have therefore proposed that SN1 works as a sensor of nutritional status (83). When the blood glutamine concentration is high it may mediate uptake of glutamine in β-cells. However, when blood glutamine concentration decreases between meals it may mediate release from β-cells. This will promote the ability of SAT2 to import glutamine into α-cells and thus stimulate glucagon secretion (**Figure 2**). Furthermore, insulin has been shown to regulate SN1 expression and function (28), allowing for possible additional feedback mechanisms.

## CONCLUSION

The main components of synaptic transmission are present in the islet cells implicating similarities in signaling pathways. This

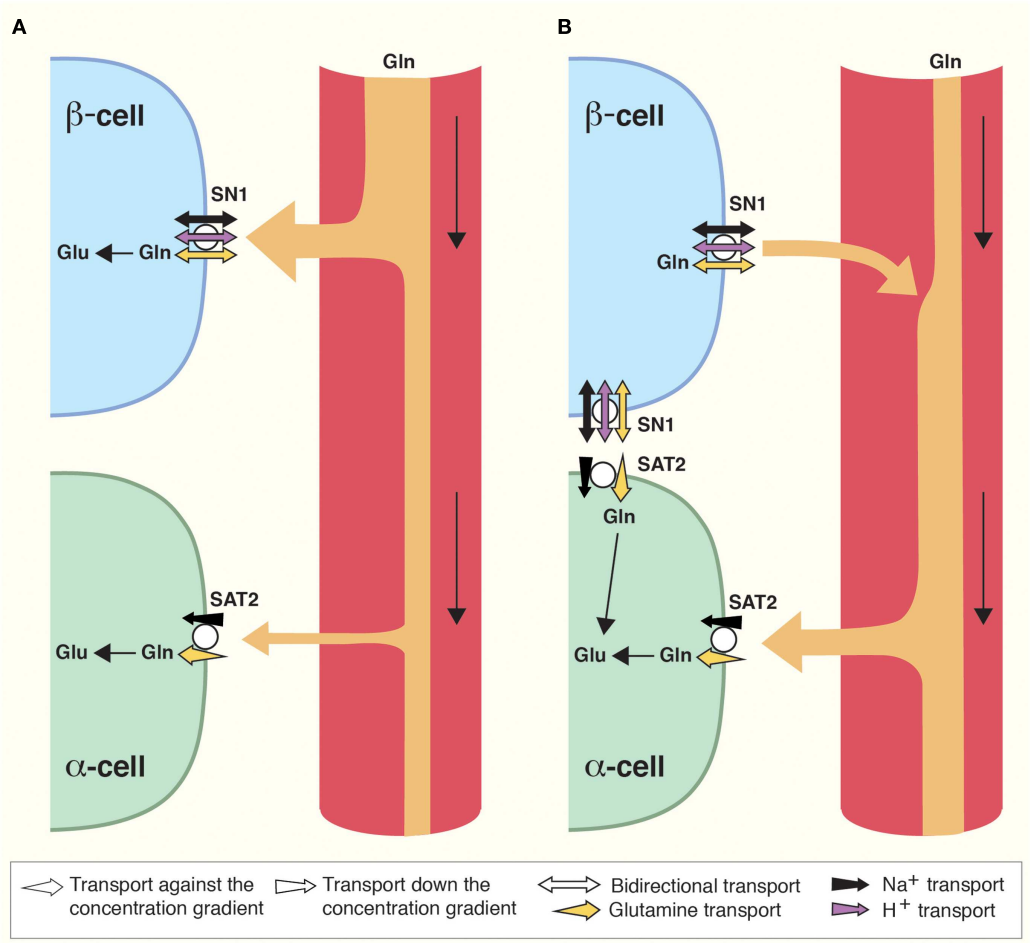


**FIGURE 2 | Direction of glutamine transport in the islet cells of Langerhans is differential and dependent on prandial status.** **(A)** Postprandially, blood supplied to the islets is enriched in glucose and amino acids such as glutamine (Gln). Gln is captured by SN1 and metabolized in β-cells to stimulate insulin secretion as described (**Figure 1**). As a result, blood glucose levels are reduced. **(B)** Interprandially, blood supplied to the islets has lower concentrations of Gln and glucose. Gln now preferentially activates SAT2 on α-cells due to its higher affinity for Gln. In addition, changes in the Gln gradient across the cell membrane of β-cells may stimulate SN1 to release glutamine which can then be taken up by adjacent α-cells. Enhanced Gln metabolism in α-cells stimulates secretion of glucagon which raises blood glucose levels.

notion is further reinforced by the fact that human neural progenitor cells may be differentiated to produce insulin (1). In the CNS, synaptic transmission is based on a tripartite synapse where the perisynaptic astroglial cells actively participate in fine-tuning and termination of the signal and in supplying precursors for the neurotransmitters. An analog to the tripartite synapse does not exist in the islets of Langerhans. However, secretion by the different islet cells is regulated by both paracrine and autocrine signaling, which again depends on glutamate and GABA. Moreover, the amino acid transporters involved in the GGG cycle in the CNS do have the corresponding and necessary localizations to support glutamine-mediated synthesis of glutamate and GABA, their exocytotic release and termination of the signal in the islets of Langerhans. Depolarization of the plasma membrane due to inward transport by SN1 upon elevated plasma glutamine concentration and/or its metabolism to glutamate and ATP might be important regulators of insulin secretion after protein rich meals. Similarly, glutamine from plasma or from β-cells upon reversed action of SN1 may activate SAT2 on α-cells (**Figure 2**). Such glutamine transport may depolarize α-cells and/or glutamine may be metabolized to glutamate to regulate glucagon secretion. This resembles the situation in the CNS where glutamine uptake through SAT1 depolarizes the nerve terminal and regulate transmitter release (19). The co-localization of EAAT2 and VGLUT3 on insulin containing SGs supports a cell-specific flux of glutamate through the granules, which may facilitate the acidification necessary for insulin maturation prior to secretion. Thus, there are several striking similarities, but there are also important differences in the functional roles of the amino acid transporters in these two tissues. Altogether, our data suggest that in addition to insulin secretion in the islets of Langerhans and the brain-centered glucoregulatory system (3), amino acid metabolism and amino acid transporters might also be potential therapeutic targets in diabetes.

## AUTHOR CONTRIBUTIONS

This review was written by Monica Jenstad and Farrukh Abbas Chaudhry in collaboration.

## ACKNOWLEDGMENTS

We would like to thank Gunnar Lothe for technical assistance in preparing the figures. This work has been supported by The Norwegian Diabetes Association, Johan Selmer Kvanes Endowment, and The Research Council of Norway.

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**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

*Received: 25 September 2013; paper pending published: 01 November 2013; accepted: 16 December 2013; published online: 31 December 2013.*
*Citation: Jenstad M and Chaudhry FA (2013) The amino acid transporters of the glutamate/GABA-glutamine cycle and their impact on insulin and glucagon secretion. Front. Endocrinol. **4**:199. doi:10.3389/fendo.2013.00199*
*This article was submitted to Cellular Endocrinology, a section of the journal Frontiers in Endocrinology.*

frontiers in
ENDOCRINOLOGY

ORIGINAL RESEARCH ARTICLE
published: 08 October 2013
doi: 10.3389/fendo.2013.00137

# Quantitative *in silico* analysis of neurotransmitter pathways under steady state conditions

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The modeling of glutamate/GABA-glutamine cycling in the brain tissue involving astrocytes, glutamatergic and GABAergic neurons leads to a complex compartmentalized metabolic network that comprises neurotransmitter synthesis, shuttling, and degradation. Without advanced computational tools, it is difficult to quantitatively track possible scenarios and identify viable ones. In this article, we follow a sampling-based computational paradigm to analyze the biochemical network in a multi-compartment system modeling astrocytes, glutamatergic, and GABAergic neurons, and address some questions about the details of transmitter cycling, with particular emphasis on the ammonia shuttling between astrocytes and neurons, and the synthesis of transmitter GABA. More specifically, we consider the joint action of the alanine-lactate shuttle, the branched chain amino acid shuttle, and the glutamine-glutamate cycle, as well as the role of glutamate dehydrogenase (GDH) activity. When imposing a minimal amount of bound constraints on reaction and transport fluxes, a preferred stoichiometric steady state equilibrium requires an unrealistically high reductive GDH activity in neurons, indicating the need for additional bound constants which were included in subsequent computer simulations. The statistical flux balance analysis also suggests a stoichiometrically viable role for leucine transport as an alternative to glutamine for replenishing the glutamate pool in neurons.

**Keywords: GABAergic, GABA cycling, flux balance analysis, Markov chain Monte Carlo, nitrogen metabolism**

## 1. INTRODUCTION

In the glutamate/GABA-glutamine cycle of neurotransmission, where the ammonium fixation is essential in the synthesis of glutamine in astrocyte, a natural question still waiting for a definitive answer is how the ammonium pool in astrocyte is replenished (1, 2). The tight coupling between reactions and transports involved in neurotransmitter synthesis and cycling within the complex metabolic network, and the distribution of the functions into different compartments makes it hard, if not impossible, to manually follow the fate of the metabolites and to identify in quantitative terms stoichiometrically feasible steady states. The computational challenge, hampering standard optimization schemes, is rooted in the indeterminacy of the governing system of mass balance equations, which allows a continuum of possible solutions, and is made worse by the need of introducing bounds on some of the flux rates, for example imposing positivity to exclude solutions that are either thermodynamically impossible or would require physiologically unfeasible conditions. The statistical sampling approach provides one viable approach for the *in silico* study of complex metabolic networks (3–7).

In order to shed some light on the nitrogen metabolism and shuttling of amino groups between astrocytes and neurons during inhibition, we propose a complex, eight compartment metabolic model which comprises astrocytes, glutamatergic, and GABAergic neurons, each equipped with separate cytosol and mitochondria. We perform a statistical flux balance analysis of the metabolic pathway during inhibitory activity and verify whether the amino group shuttling mechanisms proposed in the literature are supported by the results of our computational simulations.

The focus of the present *in silico* study is on the synthesis and cycling of the inhibitory neurotransmitter GABA, with the specific aim of elucidating the source and fate of amino groups and ammonia during the GABA cycle. Among the different reaction capable of generating ammonia in the brain, phosphate-activated glutaminase, predominantly expressed in neurons, is considered the major source of cerebral endogenous ammonia (1). Various mechanisms have been proposed for transporting ammonia from neurons, where it is released, to astrocytes, where it is needed for the glutamine synthesis, including a diffusive process of ammonia, the alanine-lactate shuttle, and the branched chain amino acid shuttle (1, 2). Although the basic mechanisms of the shuttles are well understood, by considering the joint action of them rather than each one of them separately and isolated from others, a different picture of their role may emerge.

## 2. THE PATHWAY MODEL

The template for the metabolic models that we test in this article is an eight compartment model, developed on the basis of the model in Calvetti and Somersalo (8), comprising separate cytosol and mitochondrial compartments for astrocytes, glutamatergic and GABAergic neurons, as well as blood and extracellular space (ECS) compartments, the latter one accounting also for the synaptic cleft, which in some models constitutes a separate compartment (9).

Each cell compartment is equipped with detailed cytosolic glycolytic pathway, including the reversible lactate dehydrogenase

(LDH), and mitochondrial tricarboxylic acid (TCA) cycle and oxidative phosphorylation (OxPhos).

The complete malate-aspartate shuttle (MAS) included in each cell consists of the oxoglutarate carrier (OGC) exchanging malate (Mal) and $\alpha$-ketoglutarate (AKG), and the aspartate-glutamate carrier (AGC1). To complete the shuttle, cytosol and mitochondria are equipped with aspartate aminotransferases (cAAT and mAAT, respectively), as well as with reversible malate dehydrogenases, converting oxaloacetate (OAA) to malate in cytosol (cMDH) or vice versa in mitochondria (mMDH).

Both glutamatergic and GABAergic neurons are equipped with mitochondrial malic enzyme (mME) (10), while in astrocyte malic enzyme is located in cytosol (cME) (11). Mitochondrial pyruvate carboxylase (PC) is included in astrocytes only (12).

Cytosolic glutamate (Glu) is packed in vesicles by presynaptic glutamatergic neurons and released to the synaptic cleft, from where it is quickly taken up by astrocytes. The astrocyte-specific enzyme glutamine synthetase (GS) located in the cytosol of astrocyte (13) catalyzes the amidation of glutamate to glutamine (14). The basic glutamate-glutamine cycle is completed by phosphate-activated glutaminase (PAG) which is believed to be located in the mitochondrial intermembrane (15). Since PAG replenishes the cytosolic glutamate pool, we include it in our model as a cytosolic enzyme in both glutamatergic and GABAergic neuron.

The inhibitory neurotransmitter $\gamma$-aminobutyric acid (GABA) is released by the GABAergic neurons into the synaptic cleft, and further taken up by the astrocytes. The GABA synthesis is catalyzed by the enzyme glutamic acid decarboxylase (GAD). This is a cytosolic enzyme with different isoforms (16) that are not distinguished in our analysis, and assumed to be present both in astrocytes and neurons.

Besides the neurotransmission, both glutamate and GABA play a role in the cell metabolism, thus complicating the tracking of amino groups in the network to determine their fate. GABA can enter the mitochondria through a mechanism not completely understood (17). In our model, the GABA permeation is assumed to take place through a GABA carrier, although a GABA/glutamate antiporter mechanism has been suggested (18), and recently identified and described in cell membranes of prokaryotes (19). After entering mitochondria, GABA can be transaminated by the enzyme GABA transaminase (GABA-T) to succinate semialdehyde (SSA), and subsequently converted by succinate semialdehyde dehydrogenase (SSADH) to succinate (Suc) (20). These reactions constitute the GABA shunt, providing an alternative route from $\alpha$-ketoglutarate to succinate bypassing some TCA reactions. By GABA-T, $\alpha$-ketoglutarate forms glutamate, which can be further transaminated by mAAT, transported into cytosol, or alternatively oxidized to $\alpha$-ketoglutarate by the enzyme glutamate dehydrogenase (GDH), which facilitates a bidirectional reaction; however, at the physiological ammonium concentrations and redox levels, the oxidative direction of the reaction is strongly favored (1, 21).

In addition to the AAT, glutamate may participate in other transaminase processes. We include in our model the bidirectional cytosolic alanine transaminase (ALT), in which alanine (Ala) is transaminated to pyruvate, while $\alpha$-ketoglutarate forms glutamate. This reaction, together with LDH, constitutes the lactate-alanine cycle (22, 23) proposed as a carrier of the ammonium ($NH_4^+$) between astrocytes and neurons.

Another important shuttle of the amino group identified in the brain tissue is the branched chain amino-transferase (BCAT), which is a cytosolic enzyme (BCATc) in neurons and mitochondrial (BCATm) in astrocyte (24). For simplicity, we use leucine (Leu) as a common representative of the three branched chain amino acids (BCAA) (leucine, isoleucine, and valine), and $\alpha$-ketoisocaproate (KIC) as the representative of the corresponding branched chain $\alpha$-ketoacids (BCKA). In mitochondria, $\alpha$-ketoisocaproate can be oxidized to acetyl-coenzyme-A (ACoA), although preferably reaminated back to BCAA (25). The BCAAs have been suggested to constitute an important alternative for shuttling ammonia between astrocyte and neuron.

As pointed out above, our model allows the passage of GABA through the mitochondrial membrane by a mechanism that has not been identified in mammalian brain. Cytosolic glutamate, on the other hand, has access to mitochondria through the ACG1 exchanger. Similarly, we include in our model a glutamate-hydroxyl carrier (GC) that allows the passage of glutamate through the mitochondrial membrane without tight coupling with aspartate (20). In addition, we allow the passage of malate through the mitochondrial membrane by the dicarboxyl carrier (26). Likewise, we assume a transport mechanism for branched chain amino acids and $\alpha$-ketoisocaproate across the astrocytic mitochondrial membrane.

In our model, the three cell types can uptake glucose (Glc) from the ECS compartment, and exchange oxygen ($O_2$), carbon dioxide ($CO_2$), ammonium, and lactate (Lac). Following Cooper (1), we do not distinguish between ammonium and ammonia, although a detailed modeling of the diffusion through the membranes would deserve more attention. We also assume a mechanism for passing branched chain amino acids, $\alpha$-ketoisocaproate and alanine between the cells and ECS. In contrast to our earlier models, aspartate is not included as an exchangeable substance because it is not believed to be an appropriate shuttle between the cells due to its excitatory nature (27, 28).

The substances exchanged between blood and ECS compartments include glucose, lactate, oxygen, carbon dioxide, ammonium, alanine, leucine, and glutamine. Although there is evidence of the presence of glutamate transporters at the blood-brain-barrier (BBB) (29), we assume here that the clearance by astrocytes is fast enough for the glutamate transport to be considered insignificant.

The energetic cost of neural activity is hard to estimate purely on a stoichiometric basis: The energetic needs of the cycling of the neurotransmitters constitute only a part of the total cost that includes the membrane potential homeostasis, signal propagation, and vesicle formation. In Attwell and Laughlin (30), a careful stoichiometric analysis of the energetic need of glutamate cycling between neurons and astrocytes leads to an estimate of approximately three ATP per each glutamate molecule. However, the authors point out that the total energetic need is higher. The energetic needs of astrocytes is even less well known. Part of the difficulty of incorporating a stoichiometrically justified figure for the energetic cost stems from the lumped nature of the model

which needs to integrate non-local ion translocation processes in a well-mixed compartment representation.

A semi-empirical approach has been suggested in the previous works of the authors (31, 32), and we will be adopt a similar approach in this paper. In Sibson et al. (33) it was empirically shown that in rat brain, the cerebral metabolic rate (CMR) of glucose in neurons is in almost 1:1 proportion to the glutamate flux, an observation that has been corroborated by several groups. A refined analysis was given by Hyder et al. (34), estimating that the total neurotransmitter cycle flux of glutamate is 68% of the total neuronal oxidative CMR of glucose, while the total neurotransmitter cycle flux of GABA is 21% of the total neuronal oxidative CMR of glucose. Estimating that each completely oxidized molecule of glucose produces 30–38 ATP molecules, depending on details included, we may conclude that the total cost for maintaining each unit of glutamate flux corresponds to 21–26 units of ATP converted to ADP + Pi (no GABAergic activity), and 6–8 units of ATP for maintaining one unit of GABA flux (no glutamatergic activity). As demonstrated in Calvetti and Somersalo (8), in spite of its coarseness, in simulations these flux estimates yield an energetic cost that correspond well to the data reported in Sibson et al. (33).

For simplicity, we include in our model a virtual vesicular compartment in the cytosol of glutamatergic and GABAergic neurons, and attach the total energetic cost in the flux of packing the neurotransmitters in the vesicular form, see **Table 1** for details.

The energetic cost of astrocytic neurotransmitter recycling is only partly known: the cost of glutamine synthetase and Na+/H+ extrusion after ion co-transport with glutamate is assessed at about 2ATP/glutamate in Attwell and Laughlin (30). In the present paper we use this stoichiometry for GABA recycling, understanding that it may underestimate the total energetic cost for astrocyte functions. In fact, experimental data suggest that astrocytes use from 15% to more than 25% of brain energy (35–37) for various functions, including $K^+$ clean-up and calcium signaling, neither of which is explicitly accounted for in the model. To counterbalance the underestimate, our model enables the cells to use energy for unspecified activities by including an ATP hydrolysis in each cell. As pointed out in Calvetti and Somersalo (8), the activity level of this flux, in particular in astrocytes, can be significantly elevated. Details will be given later when the computed examples are described.

A complete list of the reactions included in the model is given in **Table 1**. The configuration described by our model refers to well-mixed compartments in which the input variables are specified target levels of the cerebral metabolic rates of glucose and lactate, and the brain activity is simulated by specifying, in terms of target flux values, the efflux of the pertinent neurotransmitter, which in the present investigation is GABA. **Table 2** lists the transport fluxes of selected species between some of the compartments.

## 3. COMPUTATIONAL APPROACH

Under steady state hypotheses, the metabolic model is characterized by reaction fluxes and transport rates that must be in stoichiometric equilibrium. Each reaction $R_i$ within a specific compartment is assigned a reaction flux $\varphi_i$. Similarly, if a substrate is exchanged between two adjacent compartments through a transport $T_i$, the latter is assigned a transport rate $j_i$, expressed in the same units as the reaction fluxes. The reaction fluxes and transport rates are then collected in an array, the flux-transport vector $u$,

$$u = \begin{bmatrix} \varphi \\ j \end{bmatrix}, \text{ with } \varphi = \begin{bmatrix} \varphi_1 \\ \vdots \\ \varphi_n \end{bmatrix}, \quad j = \begin{bmatrix} j_1 \\ \vdots \\ j_k \end{bmatrix}, \tag{1}$$

where $n$ and $k$ are the total number of reactions and transports in the system.

Given a transport $T_i$ or a reaction $R_i$, we define the stoichiometric vector $s^i$ by specifying the number of units of each biochemical species in each compartment which are either produced or depleted if the transport or reaction runs alone for one time unit. Thus, the length of each stoichiometric vector equals the total number of species in the compartment model. Observe that a metabolite appearing in several compartments is counted as a different species in each compartment. After arranging the stoichiometric vectors as the columns of the stoichiometric matrix,

$$A = \begin{bmatrix} s^1 & s^2 & \cdots & s^{n+k} \end{bmatrix}, \tag{2}$$

the steady state equilibrium condition for the flux-transport vector can be expressed in the form

$$Au = r. \tag{3}$$

Above, the vector $r$ describes the transport rates of substances to and from outside the compartment model: its entries are all zeros for compartments that are not communicating with the outside world through convection or diffusion, while for the species exchanged through BBB and the blood flow, they represent the CMRs of the tissue sample that the model is describing. For further details, see **Table 3**.

The reaction fluxes and transport rates are subject to bound constraints: a flux $\varphi_i$ of a reaction $R_i$ that is thermodynamically possible only in one direction must respect the positivity condition $\varphi_i > 0$. Following the Bayesian paradigm, some of the positivity bounds may be purely *a priori* bounds, meaning that a positivity constraint may be implemented if there is a good reason to believe that a net flux of a bidirectional reaction or transport has a preferred direction, such as oxygen entering rather than exiting the tissue (38). Further, we may assume that all the reaction fluxes and transport rates must be bounded by some, possibly large, upper bound $V_{\max}$. The system of linear constraints is expressed in matrix form as

$$Cu \geq c, \tag{4}$$

where $c$ is a vector with as many entries as we have the inequality constraints, the matrix $C$ contains the coefficients of the linear expressions in the inequalities, and the matrix inequality is assumed to hold component-wise.

Methods for finding a feasible flux-transport vector satisfying equation (3) with bounds equation (4) are discussed in the classical flux balance analysis (FBA) literature, see, e.g., Kauffman et al.

**Table 1 | List of reactions included in the metabolic network model.**

| | Reaction | cA | mA | cGlt | mGlt | cGA | mGA |
|---|---|---|---|---|---|---|---|
| Glycolysis | $GLC + ATP \rightarrow G6P + ADP$ | × | | × | | × | |
| | $G6P + ATP \rightarrow 2GA3P + ADP$ | × | | × | | × | |
| | $GA3P + Pi + NAD^+ \rightarrow BPG + NADH$ | × | | × | | × | |
| | $BPG + 2ADP \rightarrow Pyr + 2ATP$ | × | | × | | × | |
| LDH | $Pyr + NADH \rightleftharpoons Lac + NAD^+$ | × | | × | | × | |
| ALT | $Pyr + Glu \rightleftharpoons Ala + AKG$ | × | | × | | × | |
| ATP-H | $ATP \rightarrow ADP + Pi$ | × | | × | | × | |
| GAD | $Glu \rightarrow GABA + CO_2$ | × | | × | | × | |
| PAG | $Gln \rightarrow Glu + NH_4^+$ | | | × | | × | |
| MDH | $OAA + NADH \rightleftharpoons Mal + NAD^+$ | × | | × | | × | |
| AAT | $Asp + AKG \rightleftharpoons OAA + Glu$ | × | × | × | × | × | × |
| ME | $Pyr + CO_2 + NADH \rightleftharpoons Mal + NAD^+$ | × | | | × | | × |
| GS | $Glu + NH_4^+ + ATP \rightarrow Gln + ADP + Pi$ | × | | | | | |
| GDH | $Glu + NAD^+ \rightleftharpoons AKG + NH_4^+ + NADH$ | | × | | × | | × |
| PDH | $Pyr + CoA + NAD^+ \rightarrow ACoA + NADH + CO_2$ | | × | | × | | × |
| PC | $Pyr + CO_2 + ATP \rightleftharpoons OAA + ADP + Pi$ | | × | | | | |
| TCA cycle | $ACoA + OAA \rightarrow CIT + CoA$ | | × | | × | | × |
| | $CIT + NAD^+ \rightarrow AKG + CO_2 + NADH$ | | × | | × | | × |
| | $AKG + CoA + NAD^+ \rightarrow SCoA + CO_2 + NADH$ | | × | | × | | × |
| | $SCoA + ADP + Pi \rightarrow Suc + CoA + ATP$ | | × | | × | | × |
| | $Suc + NAD^+ \rightarrow Fum + NADH$ | | × | | × | | × |
| | $Fum \rightarrow Mal$ | | × | | × | | × |
| | $Mal + NAD^+ \rightarrow OAA + NADH$ | | × | | × | | × |
| GABA-T | $GABA + AKG \rightarrow SSA + Glu$ | | × | | × | | × |
| SSADH | $SSA + NAD^+ \rightarrow Suc + NADH$ | | × | | × | | × |
| BCAT | $Leu + AKG \rightleftharpoons KIC + Glu$ | | × | × | | × | |
| BCKDH | $KIC + CoA \rightarrow ACoA + CO_2$ | | × | | × | | × |
| OxPhos | $O_2 + 6ADP + 6Pi + 2NADH \rightarrow 6ATP + 2NAD^+$ | | × | | × | | × |
| | **Virtual reactions with energetic cost** | | | | | | |
| | $Glu + 25ATP \rightarrow Glu_{vesicular} + 25ADP + 25Pi$ | | | × | | | |
| | $GABA + 8ATP \rightarrow GABA_{vesicular} + 8ADP + 8Pi$ | | | | | × | |

*The symbol "→ " is used for irreversible reactions, while "⇌ " is used for reversible ones. The relevant abbreviated enzyme acronyms referring to the corresponding reactions are indicated in the first column. The columns refer to compartments that are the locus of the enzyme: cA/mA, cytosol/mitochondria of astrocyte; cGlt/mGlt, cytosol/mitochondria of glutamatergic neuron; cGA/mGA, cytosol/mitochondria of GABAergic neuron. The virtual reactions, transporting glutamate and GABA to a virtual vesicular compartment for neurotransmission are included to attach an undifferentiated energetic cost for the total neurotransmitter cycling, see text for justification.*

(39). A well-known problem in FBA is the lack of a unique solution, i.e., the stoichiometry and bounds alone are not sufficient to identify a unique steady state. It is this non-uniqueness which gives the system the flexibility to adjust to changing physiological conditions. Rather than forcing the uniqueness of the solution by adding extra conditions, a task which may require constructing artificial objectives for the system, we seek to explore computationally the full set of feasible steady state configurations. The methodology to achieve this, which is based on a probabilistic description of the problem, has been developed in a series of papers by the authors, see, e.g., Heino et al. (7); Calvetti and Somersalo (8); Calvetti et al. (9), and references therein. We give a concise overview of the approach below.

In this probabilistic setting, feasible flux-transport vectors are assumed to be distributed in the space of all vectors so as to approximately satisfy both the equilibrium condition equation (3) while strictly respecting the bound constraint inequality (4). This is achieved by defining a truncated Gaussian probability density

$$\pi(u) \propto H(Cu - c) \exp\left(-\frac{1}{2}(Au - r)^{\mathrm{T}} \Sigma^{-1} (Au - r)\right). \quad (5)$$

Here, "$\propto$" stands for "proportional to," $H$ is the multidimensional Heaviside function that vanishes except when all the components of the vector $Cu - c$ are positive, in which case it assumes the value one, and $\Sigma$ is a covariance matrix, which we assume to be

**Table 2 | The transports implemented in the model.**

| | Blood | | ECS | | Cytosol | | Mitochondria |
|---|---|---|---|---|---|---|---|
| Glc | • | → | • | → | • | | • |
| Lac | • | ↔ | • | ↔ | • | | • |
| Pyr | • | | • | | • | → | • |
| $O_2$ | • | → | • | → | • | → | • |
| $CO_2$ | • | ← | • | ← | • | ← | • |
| $NH_4^+$ | • | → | • | ↔ | • | ↔ | • |
| Leu | • | → | • | ↔ | • | ↔ | • |
| KIC | • | | • | ↔ | • | ↔ | • |
| Ala | • | ↔ | • | ↔ | • | | • |
| ATP | • | | • | | • | ↔ | • |
| ADP | • | | • | | • | ↔ | • |
| Pi | • | | • | | • | ↔ | • |
| Glu | • | | • | ↔ | • | ↔ | • |
| GABA | • | | • | ↔ | • | ↔ | • |
| Gln | • | ← | • | ↔ | • | ↔ | • |

| | Malate-aspartate shuttle (MAS) |
|---|---|
| OGC | Mal (cyto) + AKG (mito) → Mal (mito) + AKG (cyto) |
| AGC1 | Glu (cyto) + Asp (mito) → Glu (mito) + Asp (cyto) |

*In the columns, "→" and "←" mean that the transport has been implemented as unidirectional in the direction indicated by the arrow, while "↔" means that a priori no preferred direction is specified. Observe that in the numerical simulations, we may implement bound constraints that limit the direction of some of the reactions and transports, as indicated in the text.*

**Table 3 | Input values defining the non-vanishing components of the right hand side *r* in equation (3).**

| $r_j = Q/F(C_j^a - C_j^v)$ | $Q = 0.55$ | $F = 1$ | |
|---|---|---|---|
| | $C_j^a - C_j^v$ | $C_j^a$ | $C_j^v$ |
| Glc | $0.54 \pm 0.05$ | – | – |
| Lac | $-0.18 \pm 0.02$ | – | – |
| $O_2$ (free and bound) | – | $9.15 \pm 0.5$ | Estimated |
| $CO_2$ (free and bound) | – | $23 \pm 1$ | Estimated |
| | $0 \pm 0.2$ | – | – |
| Leu | $0 \pm 0.1$ | – | – |
| Gln | $0 \pm 0.1$ | – | – |

*The system is scaled so that it corresponds to 1 g tissue. The units of the blood flow Q are milliliter per minute, the mixing ratio F is unit free, and the concentrations are given in micromoles per milliliter. The flux values are expressed in micromoles per minute. In the table, "estimated" means that the quantity is included in the list of unknowns and estimated together with the flux values. Observe that the formula for $r_j$ above for oxygen and carbon dioxide refers to concentrations of free gases, and a conversion from bound to free needs to be performed [see, e.g., Ref. (51)].*

diagonal. The diagonal entries of $\Sigma$ express how tightly each one of the steady state equations is enforced. We refer to the square roots of these entries as standard deviations of the model equations. A rather stringent condition is required in our calculations: the standard deviation is set to $\sigma = 0.005\ \mu$mol/min for every model equation, except for equations that define the influx/efflux from blood to ECS. The latter standard deviations are given in **Table 3**.

To investigate feasible steady state configurations with some of the fluxes close to a desired target value, the distribution can be modified accordingly in a simple way: the belief that a given flux or transport $u_\ell$, such as the efflux of a given neurotransmitter, should be close to a target value $u_\ell^{\text{target}}$, can be implemented by augmenting the probability density equation (5) with an extra factor,

$$\pi(u) \to \pi(u) \times \exp\left(-\frac{1}{2w^2}\left(u_\ell - u_\ell^{\text{target}}\right)^2\right), \qquad (6)$$

where $w > 0$ is the standard deviation controlling how stringently the target value is pursued. We specify the values of these parameters later on. To explore the resulting probability density, and thus the distribution of all feasible flux-transport configurations, we use Markov chain Monte Carlo (MCMC) techniques to generate a representative sample of those vectors,

$$S = \{u^1, u^2, \ldots, u^N\}, \qquad (7)$$

such that for $N$ large, the vectors $u^j$ are, at least asymptotically, distributed according to the probability density $\pi(u)$. For details, see Heino et al. (7). Based on this sample, we obtain a summary statistics of the distribution, e.g., by computing the sample mean and variance. In the sequel, we shall use almost exclusively the sample mean as a representative flux-transport configuration for a given stoichiometric model with bounds and target controls defined in equations (4) and (6).

The values for the input parameters used in the computations are given in **Table 3**.

## 4. QUANTITATIVE ANALYSIS OF AMMONIUM SHUTTLING

It is commonly accepted that the glutamate and GABA neurotransmitter cycling between neurons and astrocyte is completed by the glutamine transport, following the glutamate/glutamine cycle for excitatory transmission between the glutamatergic neuron and astrocyte, or the GABA/glutamine cycle for the inhibitory transmission between GABAergic neuron and astrocyte. Both cycles require the ammonium fixation by GS in astrocytic cytosol, and metabolizing glutamine into glutamate in neuron by PAG. In both transmitter cycles, a stoichiometric shortage of one $NH_4^+$ in the astrocytic cytosol, and excess of one $NH_4^+$ in the neuronal cytosol ensues, see **Figure 1**.

As pointed out in Rothman et al. (2), the stoichiometric imbalance requires one of the following alternatives to take place:

1. the excess $NH_4^+$ in the neuronal cytosol diffuses via ECS to the astrocyte, probably in the form of $NH_3$, or
2. the excess $NH_4^+$ in the neuronal cytosol diffuses in the mitochondria and is fixed to $\alpha$-ketoglutarate by GDH to form glutamate.

The former alternative readily resumes the stoichiometric equilibrium of ammonia, while the latter requires a further shuttling mechanism to restore the balance. In the literature, different mechanisms have been proposed.

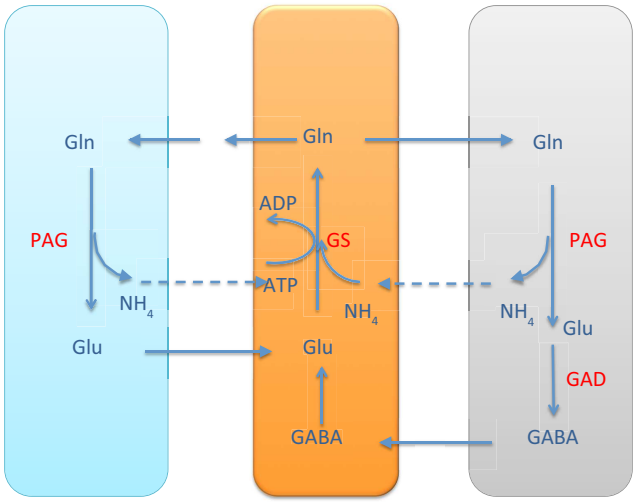


**FIGURE 1 | The glutamate-glutamine cycle and GABA-glutamine cycles**. The excess ammonium freed by PAG in the neurons needs to be transported back to astrocyte, where a shortage is created by the GS activity. In the pure glutamate/GABA-glutamine cycle paradigm, the ammonium diffusion or surrogate shuttle mechanisms need to go at the flux rate of the glutamine efflux from astrocyte. The quantitative analysis indicates that the full picture may be more complex.

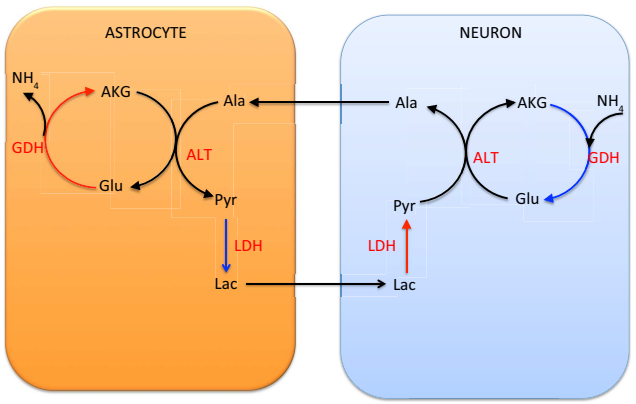


**FIGURE 2 | The lactate-alanine cycle between astrocyte and neuron**. Observe that in order to complete the cycle, lactate needs to move from astrocyte to neuron. The shuttle is in redox equilibrium, since in both cells the oxidative (red arrows) and reductive (blue arrows) reactions compensate each other. However, in order for this shuttle to be efficient at transferring ammonium, it requires reductive activity of the GDH in the neuronal mitochondria, a scenario that has been questioned in the literature both for theoretical reasons and in the light of experimental evidence (1).

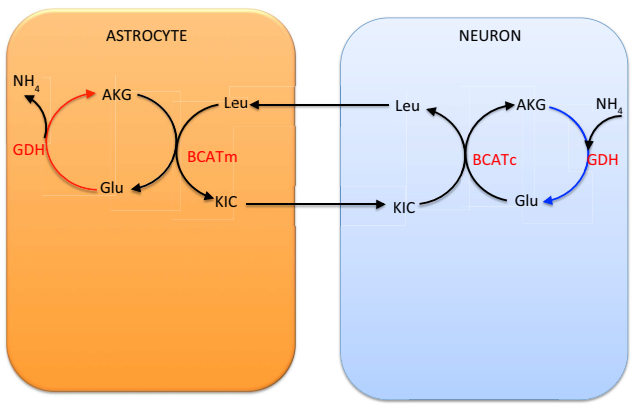


**FIGURE 3 | The branched chain amino acid cycle**. In order to effectively move the $NH_4^+$ from neuron to astrocyte, the latter has to be fixed to $\alpha$-ketoglutarate by mitochondrial GDH, thus requiring this reaction to go in the reductive direction, hence running into the same criticism as the lactate-alanine shuttle (see **Figure 2**).

In the alanine-lactate shuttle (22), the mitochondrial glutamate in neuron enters cytosol either by the AGC1 exchanger or the GC carrier, and consequently is transaminated to $\alpha$-ketoglutarate by ALT, while concurrently forming alanine from pyruvate. In this scenario, alanine is then shuttled to astrocyte, where it is transaminated to pyruvate by ALT, concomitantly forming glutamate from $\alpha$-ketoglutarate. To attain the carbon balance, pyruvate is shuttled from astrocyte to neuron in the form of lactate, produced by LDH, which converts lactate to pyruvate in a reverse reaction in neuron. We refer to **Figure 2** for an illustration.

An alternative shuttle mechanism uses the branched chain amino acids, leucine in particular in our model, as carriers of the amino group. In this scenario, glutamate is transaminated to $\alpha$-ketoglutarate by BCATc in the cytosol of the neuron, while $\alpha$-ketoisocaproate is converted into leucine. The branched chain amino acids and corresponding keto acids are then exchanged between the neuron and the astrocyte, and the reverse reaction, facilitated by BCATm, produces glutamate and $\alpha$-ketoisocaproate in the mitochondria of the astrocyte. This shuttle mechanism is illustrated schematically in **Figure 3**.

Several factors complicate the analysis of the interplay between the various shuttling mechanism, including the following:

1. The different mechanisms are probably completing each other, none of them alone compensating the ammonium imbalance;
2. Glutamate has a complex role, being not only a neurotransmitter but also a metabolite;
3. The two proposed shuttling mechanisms require that GDH in neuron runs in the reductive direction with a significant flux, which has been experimentally and theoretically been questioned as a possible option;
4. both $NH_4^+$ and leucine are assumed to be replenished from the blood, while glutamine may leak out, making the shuttling mechanisms stoichiometrically leaky.

These are some of the considerations which make a flexible computational tool for following the fluxes particularly attractive.

## 5. NUMERICAL SIMULATIONS

In Calvetti and Somersalo (8), three different simulated activation levels were considered: Excitation state, characterized by high glutamate efflux from glutamatergic neuron, awake state, defined by moderate glutamate efflux, and inhibition state, in which the GABA efflux from GABAergic neuron is set at a specified level.

These states are not intended to model the whole brain, which is known to be predominantly glutamatergic (40, 41), but rather a small portion of it that is assumed to correspond to the prescribed activity. Without specifying the volume of the domain modeled, we scale the fluxes to correspond a volume of one gram tissue. Our *in silico* analysis focuses on inhibitory activity, which is achieved by defining a target value for the GABA efflux from the GABAergic neuron (nGABA). In equation (6), we choose

$$u_\ell = j^{\rm GABA}_{\rm nGABA\to ECS}, \quad u_\ell^{\rm target} = 0.13\ \mu{\rm mol/min}.$$

This is the level of inhibitory activity state used in Calvetti and Somersalo (8), based on the experimental analysis in Shulman et al. (42) and Hyder et al. (34). Here, only the inhibitory activation state is considered as a representative of complex interplay between the astrocytes and the two neuron types.

The parameter $w$ in equation (6) defining the standard deviation of the GABA flux from the target value is set to $w = 0.005\ \mu$mol/min. While it is understood here that the GABA efflux should be a response to some excitatory activity, we do not explicitly specify the level of excitatory activity in the model, but rather let it be determined by the stoichiometry. By the way the model is set up, the uptake of nutrients, oxygen, and glutamine of the glutamatergic neuron settle to some levels that satisfy the equilibrium conditions. In the various simulations with the current model discussed later, the mean glutamate efflux from glutamatergic neurons to ECS is 0.08–0.09 $\mu$mol/min. The energy demand of the astrocytes, measured in terms of mean ATP turnover, in all our simulations is approximately 45% of the total ATP turnover. This indicates a high oxidative activity of astrocyte during inhibition, which is in line with the earlier computational results (8) as well as with experimental findings (37, 43).

Using the software package Metabolica, we generate a sample of $N = 200{,}000$ sample vectors $u^n$, each of them representing a possible equilibrium or near-equilibrium flux configuration. In the following analysis, we report for the most part the mean transport-flux vector that is obtained by averaging over the sample, that is, we define the mean $\overline{u}$ by

$$\overline{u} = \frac{1}{N}\sum_{n=1}^{N} u^n.$$

We run three different tests, differing from each other by the imposed positivity constraints specified below, designed to put the focus on ammonium traffic and GABA formation.

### 5.1. OXIDATIVE GDH AND AMMONIA DIFFUSION

In the first computational simulation, we constrain the directions of some key important fluxes. To analyze the ammonium uptake pattern, we first assume that GDH in all cell types goes in the oxidative rather than reductive direction, that is,

$$\text{GDH}: \text{Glu} + \text{NAD}^+ \to \text{AKG} + \text{NH}_4^+ + \text{NADH}, \quad \varphi_{\rm GDH} \geq 0. \tag{8}$$

These constraints constitute three rows in the system equation (4), one corresponding to each cell type.

In addition, we prescribe the direction of the branched chain amino acid transport from ECS to astrocyte (ast), by implementing the positivity constraint

$$j^{\rm Leu}_{\rm ECS\to ast} \geq 0, \tag{9}$$

constituting an additional row in the system equation (4). We point out that this constraint is an *a priori* bound, included to make the model conform with the proposed shuttle mechanism as well as with the observation that leucine is predominantly taken up by astrocytes rather than neurons (44, 45). The effect of removing the constraint is also investigated later on.

#### 5.1.1. Ammonium traffic through ECS

As expected, the bound constraint equation (8) disables the ammonium fixation in the neuron, and therefore makes both the alanine-lactate shuttle and the BCAA shuttle ineffective as possible pathways for trafficking $\text{NH}_4^+$ from neuron to astrocyte. **Figure 4** summarizes quantitatively the ammonium trafficking between the compartments under the constraint equation (8).

There are two explanations why the $\text{NH}_4^+$ flux from ECS into the astrocyte is not equal to the glutamine efflux from astrocyte to ECS. First, the tissue takes up leucine from the blood, which in turn is taken up by astrocyte, thus providing a source of ammonium. Second, some of the glutamine synthesized in astrocyte is released in the blood and is thus removed from the system. We observe that the $\text{NH}_4^+$ flux out of the neurons is slightly higher than the glutamine flux into the neurons. The difference is due to the oxidation of glutamate in neurons, GDH oxidizing glutamate to $\alpha$-ketoglutarate. The percentage of glutamine-derived glutamate which is oxidized is about 14% in glutamatergic neuron and 22% in GABAergic neuron. Also, there is a slight offset between the ammonium uptake flux of the astrocyte and the glutamine efflux. The difference, or missing ammonium in astrocyte, is compensated by the uptake of leucine from the blood.

#### 5.1.2. Glutamine as precursor of GABA

The simplified picture of the GABA/glutamine cycle is that glutamine is taken up by GABAergic neuron, transformed to glutamate by PAG and further to GABA by GAD. Our analysis indicates that while this core chain is valid, the full picture is much more complicated. The mean fluxes indicate that the GAD activity (0.2 $\mu$mol/min) is more than twice the PAG (0.09 $\mu$mol/min) activity, raising the question of the origin of the extra glutamate in the cytosol of the GABAergic neuron. Mitochondrial glutamate can be replenished via three transaminases (AAT, ALT, and BCAA), and two transport mechanisms (AGC1 and glutamate-hydroxyl carrier GC) from the cytosol. As indicated in **Figure 5**, in a combined action, they exactly account for the excess glutamate. Another noteworthy detail is that the GAD produces not only the neurotransmitter GABA, but also a significant amount of GABA that is oxidized in the mitochondria, corresponding to about 35% of all GABA produced by GAD. The GABA entering mitochondria feeds the GABA shunt, the rate of which is slightly less than 50% of the total TCA cycle flux measured by the succinate dehydrogenase flux. This result is in line with the experimental results in rat brain reported in Hassel et al. (46).

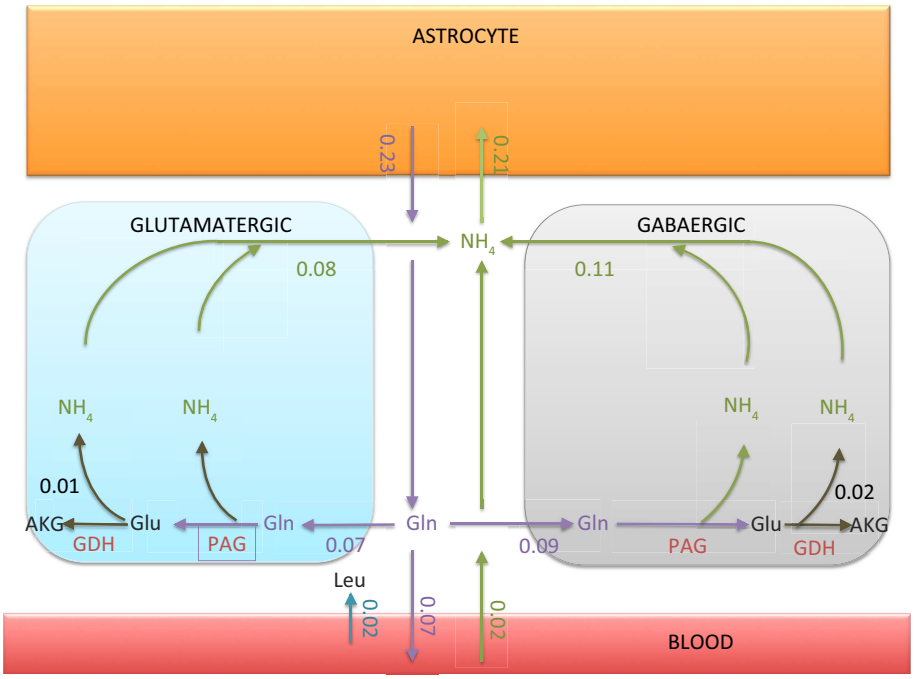


**FIGURE 4 | The transport rates of glutamine and ammonium between ECS and other compartments**. The numbers indicated in the figure are in units micromoles per minute per 1 g tissue. Observe that the ammonium flux into the astrocyte is 0.02 $\mu$mol/min smaller than the glutamine efflux. The imbalance, however, is compensated by the influx of leucine from blood to ECS, as indicated in the figure. The net influx of $NH_4^+$ carried by leucine into astrocyte turns out to be exactly 0.02 $\mu$mol/min.

### 5.2. BIDIRECTIONAL GDH AND THE ROLE OF AMINO GROUP SHUTTLES

The results reported in the previous section demonstrate quite clearly that without neuronal GDH running in the reductive direction, both the lactate-alanine shuttle and the branched chain amino acid shuttle play only a marginal role in the ammonium group traffic. This observation underlines the difficulty of reconciling the proposed shuttle mechanisms with experimental observations that brain tissue is incapable of ammonium fixing even under hyperammonemic conditions (21, 47, 48). To test computationally the viability and mutual equilibrium values of these shuttles, we repeat the sampling, removing the constraint inequality (8) from all cell types, which is tantamount to allowing bidirectional GDH activity. What we anticipate in this case is a rather significant reductive GDH activity in both neuron types. **Figure 6** shows the smoothed histograms of the GDH activity in each cell type, indicating also the sample mean. The histograms indicate that a wide range of GDH activity levels are possible. However, in equilibrium conditions and with no bound constraints for the GDH, the fluxes seek to balance the reductive activity in neurons ($\varphi_{\rm GDH} < 0$) by the oxidative activity in astrocyte ($\varphi_{\rm GDH} > 0$). Because this equilibrium is not possible when the direction of the activity is restricted, the mismatch was balanced with ammonium diffusion between the cells.

When GDH is assumed to be able to operate in both directions, it is possible to reduce, or even reverse the $NH_4^+$ diffusion from neuron to astrocyte by replacing it with the two shuttle mechanisms discussed in Section 4. The schematics in **Figure 7** illustrate the mechanism in astrocyte that removes the ammonium from alanine and leucine via a joint action of GDH and the transaminases ALT and BCATm. The mean flux values in the figure indicate that the two shuttle mechanisms have almost the same level of activity. Moreover, the rate of the $NH_4^+$ production by this process is almost twice as large as the flux of GS depleting it: the residual $NH_4^+$ is released to ECS, from where it is taken up by the neurons. Therefore, the combined action of the two shuttle mechanisms, in the mean flux configuration, not only replaces the need for ammonium diffusion, but in fact, overwhelms it. This finding suggests that the mean flux configuration with freely reversible GDH in the neurons may not represent a physiologically meaningful steady state.

One of the attractive features of the sampling-based approach to metabolic networks is that from the full sample of steady state configurations, it is possible to select subsamples of states that satisfy physiologically more meaningful bounds. In the present case, we select only those reaction-transport vectors $w^j$ for which the net ammonium flux from ECS into the cytosol of astrocyte is positive. Restricting the analysis to this subsample, we recompute the mean fluxes. **Figure 8** shows the smoothed histograms of the subsampled GDH fluxes in each cell type. Interestingly, the mean GDH activity in the GABAergic neurons vanishes, while in the astrocyte and in the glutamatergic neurons GDH runs, in the mean, at the same low rate but in opposite direction. The counterpart of **Figure 7** with mean fluxes calculated from the subsample are shown in **Figure 9**. The numbers indicate that about one fifth of the ammonium required by the GS originates from the GDH, while the rest enters the astrocyte by diffusion from the ECS.

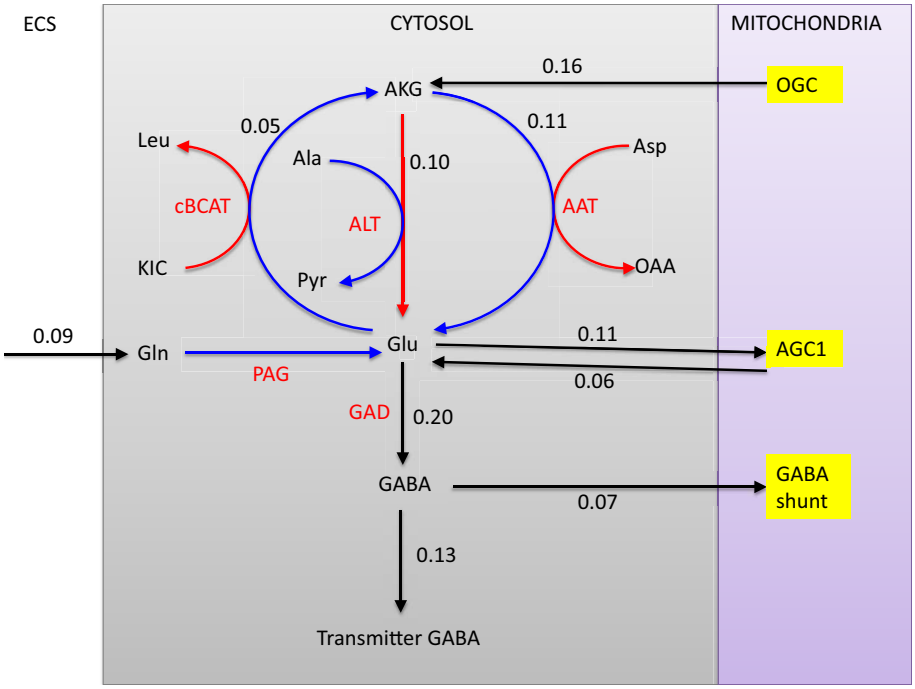


**FIGURE 5 | The fates of glutamine in the GABAergic neuron**. All glutamine is transformed into glutamate in the cytosol by PAG, after which the pathway branches. The three transaminases with respective flux rates (cBCAT, ALT, and AAT) are marked in red. Observe that the rate of GAD is more than twice the rate of glutamine uptake and PAG. Of all GABA produced, 35% enters the mitochondria where it is transaminated by GABA-T and further oxidized. The glutamine influx comprises about 70% of the transmitter GABA efflux, although the complex pathway pattern complicates the tracking of the precursors.

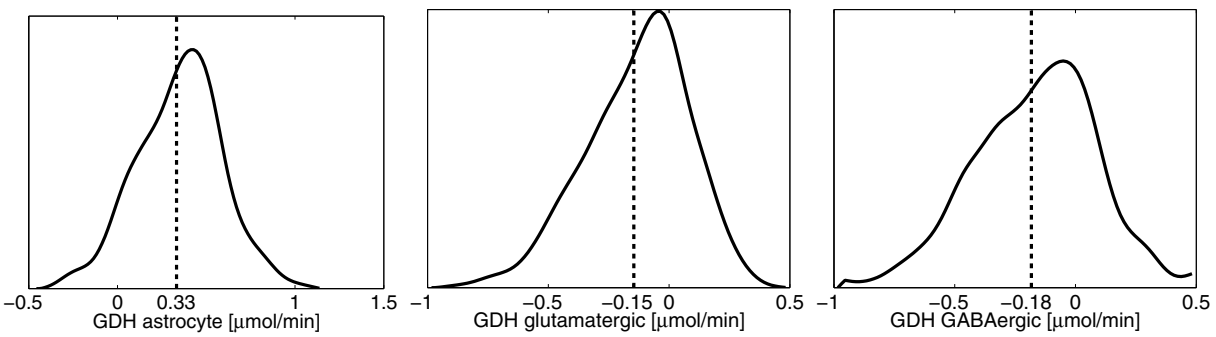


**FIGURE 6 | The smoothed histograms of the GDH fluxes in each of the three cell types, calculated from the sample of 200,000 realizations**. The sample mean in each cell is indicated by the vertical line. Observe that the sum of the mean activities in the neurons equal the negative of the astrocytic activity.

### 5.3. BRANCHED CHAIN AMINO ACID SHUTTLE: AN ALTERNATIVE FOR GLUTAMINE CYCLE?

The BCAA shuttle, which has been suggested as a vehicle for returning the ammonia freed by PAG in neuron into the astrocyte for fixation by GS, requires the questionable reductive action of GDH in neuron. When we remove the bound constraint inequality (9) on the direction of the transport of leucine, the pathway analysis tool Metabolica suggests an alternative function for BCAA that cannot be excluded by stoichiometric considerations alone. A stoichiometric equilibrium can be found in which BCAA is running in the reverse direction, transaminating astrocytic $\alpha$-ketoisocaproate to leucine, which is transported to neuron, where the reverse reaction (Leu + AKG → KIC + Glu) replenishes the glutamate pool. In other words, leucine assumes the role of glutamine as a precursor of transmitter glutamate and GABA. This shuttle does not completely replace the glutamine as a precursor of glutamate in the mean equilibrium state, but acts as an additional source. The analysis suggests also that, when leucine can be taken up by neuron, the alanine-lactate shuttle runs in the direction suggested in Waagepetersen et al. (22), as shown in **Figure 10**. The

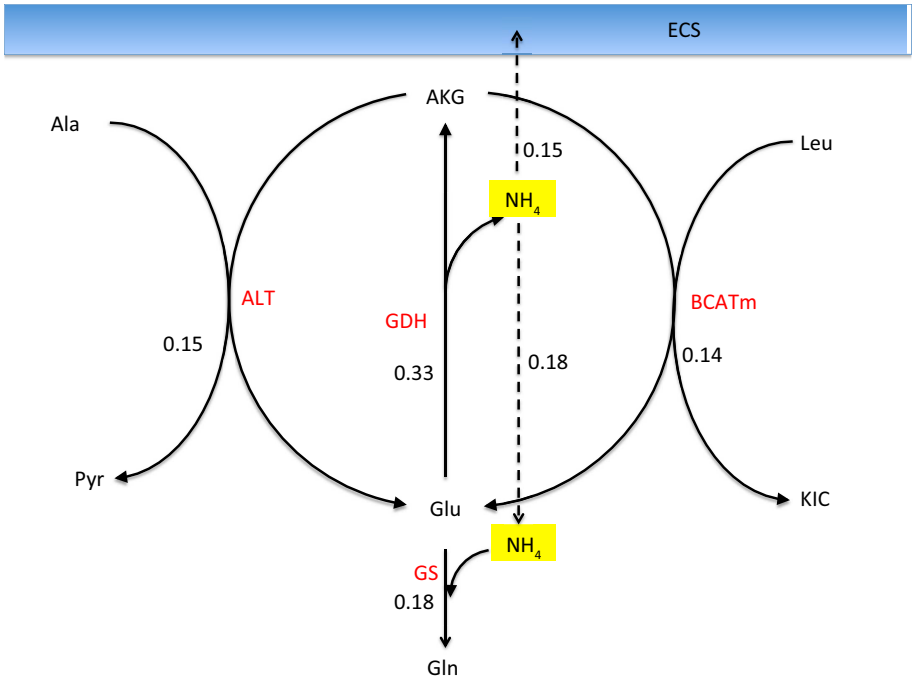


**FIGURE 7 | Reactions in the astrocyte that liberate ammonium from alanine and leucine transported into astrocyte from the neurons when allowing a bidirectional GDH activity**. The values refer to the mean fluxes over a 200,000 sample. The oxidative GDH activity is higher than the GS flux, leading to an excess of $NH_4^+$ which is transported to the ECS and further taken up by neurons. This mean flux configuration may not be physiologically meaningful because of the unexpected efflux of $NH_4^+$ from astrocyte.

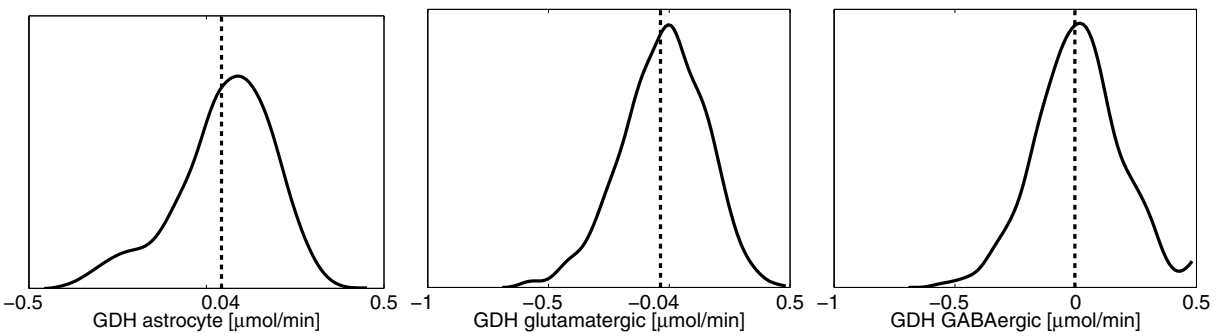


**FIGURE 8 | The smoothed histograms of the GDH fluxes in each of the three cell types, calculated from the subsample of the flux vectors in which the $NH_4^+$ flux goes in the direction ECS → astrocyte**. The sample mean in each cell is again indicated by the vertical line.

situation is similar for the astrocyte and GABAergic neuron pair, in which the action of GAD from glutamate to GABA needs to be incorporated.

## 6. CONCLUSION AND PERSPECTIVES

In this work, we investigate the neurotransmitter cycling in a steady state multi-compartment model using the computational tool Metabolica, with a particular emphasis on the traditional glutamate/GABA-glutamine cycling scenario between astrocytes and neurons. The basic brain metabolism multi-compartment model is enriched with other cycling mechanisms, such as branched chain amino acid shuttle and alanine-pyruvate shuttle. The focus in this work is on the stoichiometric implications of selected bound constraints on some of the key reaction fluxes and transport rates. This work is bridging the earlier computational works on brain energy metabolism by the authors with recently published works on neurotransmitter synthesis and cycling, [see Ref. (2, 49)]. As pointed out in Sibson et al. (33) and several other studies later on, the neurotransmitter cycling activity and the energy metabolism are tightly coupled, and therefore any model for one has implications for the other. However, the modeling paradigm adopted here which is based on modeling the reaction and

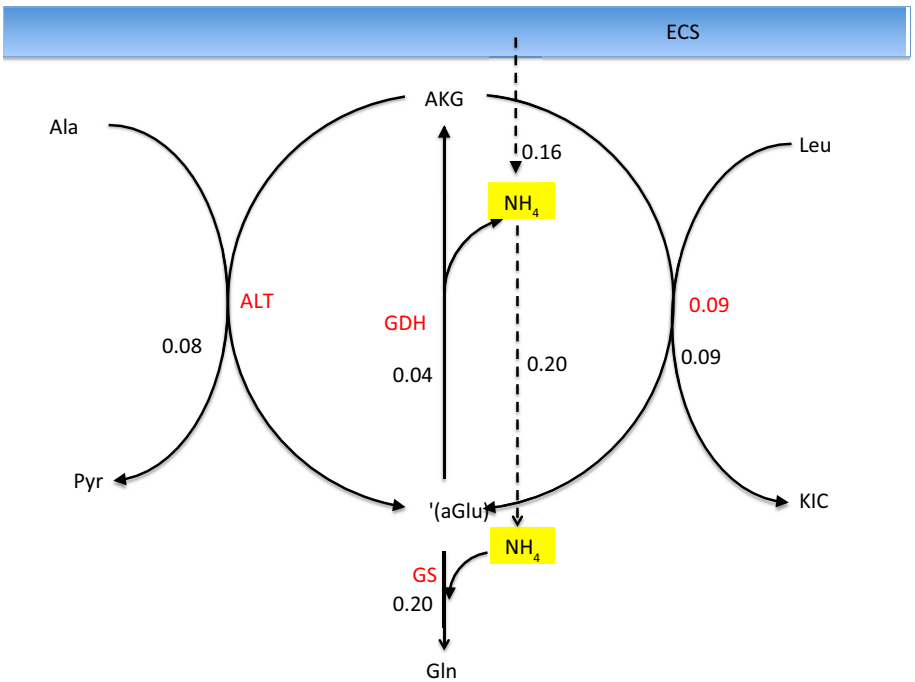


**FIGURE 9 | The corresponding fluxes as in Figure 7, calculated as a mean over a subsample of those sample vectors for which the $NH_4^+$ flux is from ECS into the astrocyte**. The ammonia influx and the GDH flux add up to equal the GS flux. Observe that the GDH runs at a much lower rate than the two transaminases, and the corresponding lactate-alanine shuttle and the branched chain amino acid shuttle, demonstrating that considering the isolated shuttle mechanisms may be misleading.

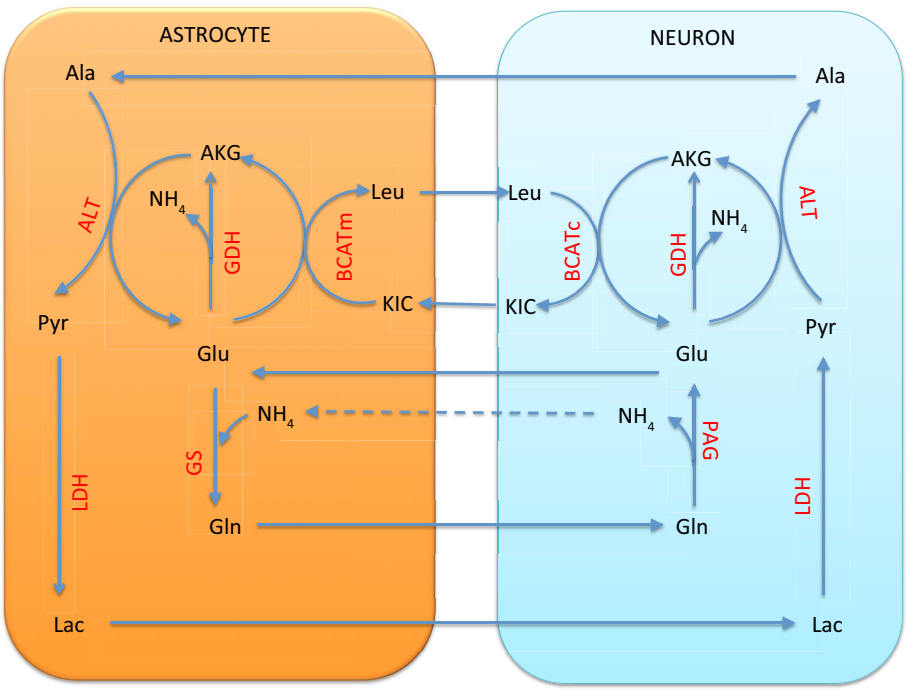


**FIGURE 10 | The branched chain amino acid cycle can provide a stoichiometrically viable alternative for the traditional glutamate-glutamine cycle**. Leucine is transported from astrocyte to neuron, where it is transaminated to $\alpha$-ketoisocaproate, forming glutamate from $\alpha$-ketoglutarate. Unlike the glutamate-glutamine cycle, this cycle requires no return traffic of ammonia. A stoichiometric equilibrium can be found in which the GDH runs in oxidative direction in all cells, and the combined action of alanine-lactate shuttle and GDH replenishes the $NH_4^+$ pool in astrocyte. A flux of $NH_4^+$ from neuron to astrocyte is still needed for equilibrium.

transport fluxes in terms of distributions indicate that within the same energetic level, measured in terms of tissue glucose uptake, it is possible to find a range of stoichiometrically viable scenarios that differ in the details about the shuttling of the amino groups between the various cell types. As demonstrated in the article, the sample based approach makes it possible to narrow down the distributions by means of subsampling, thus excluding realizations that represent steady state configurations that lead to model predictions which are in conflict with observations.

The main findings of the article may be summarized in few points: first, if the GDH is only allowed to run in the oxidative direction in the neurons, as the experimental data suggest, a significant diffusion of $NH_4^+$ from neuron to astrocyte is needed to compensate the ammonium imbalance due to the GS-PAG activity. If, however, the reductive action of GDH is allowed with no further restrictions, the model seeks an equilibrium in which the GDH overcompensates, and the diffusion of $NH_4^+$ is reversed, going from astrocytes to neurons. Since this scenario does not seem plausible in the light of what is known experimentally, we selected from the sample of possible equilibrium states those flux vectors in which the ammonium traffic goes from ECS into the astrocyte, leading to an equilibrium in which the GDH activity is significantly reduced, and the equilibrium state is more in line with experimental observations.

Finally, we point out that if the branched chain amino acid shuttle is not restricted to run in the direction suggested in the literature, with leucine entering astrocyte, a viable equilibrium can be found, in which leucine plays a role of glutamine, enriching the glutamate pool in neuron without the need of compensating $NH_4^+$ diffusion from neuron to astrocyte. In our model, this glutamate/GABA-leucine cycle does not replace the traditional glutamate/GABA-glutamine cycle, but completes it. Although experimental evidence supports the uptake of leucine predominantly by astrocytes rather than neurons, the stoichiometric viability of this shuttle may be of interest under some atypical conditions.

The role that advanced statistical computational models may have in the brain research is to point out scenarios and alternative patterns in the complex metabolic network that may not be evident, and may be hard to find by simple flux balance analysis. In this way, the analysis may turn out to be useful as a guideline for designing new experiments. As pointed out in Rowley et al. (50), the details of neurotransmitter and amino group cycling are particularly important since they provide potential targets for drug discovery to control, e.g., epileptic seizures, hepatic encephalopathy, various mental disorders, or progression of neurodegenerative diseases.

## ACKNOWLEDGMENTS

This work was partially supported by grant from the Simons Foundation (# 246665 to Daniela Calvetti). The work of Erkki Somersalo was supported in part by NSF grant DMS 1016183.

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**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

*Received: 05 June 2013; paper pending published: 02 July 2013; accepted: 16 September 2013; published online: 08 October 2013.*
*Citation: Calvetti D and Somersalo E (2013) Quantitative in silico analysis of neurotransmitter pathways under steady state conditions. Front. Endocrinol. **4**:137. doi: 10.3389/fendo.2013.00137*
*This article was submitted to Cellular Endocrinology, a section of the journal Frontiers in Endocrinology.*

frontiers in
ENDOCRINOLOGY

REVIEW ARTICLE
published: 11 November 2013
doi: 10.3389/fendo.2013.00165

# GABA and glutamate transporters in brain

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The mammalian genome contains four genes encoding GABA transporters (GAT1, slc6a1; GAT2, slc6a13; GAT3, slc6a11; BGT1, slc6a12) and five glutamate transporter genes (EAAT1, slc1a3; EAAT2, slc1a2; EAAT3, slc1a1; EAAT4, slc1a6; EAAT5, slc1a7). These transporters keep the extracellular levels of GABA and excitatory amino acids low and provide amino acids for metabolic purposes. The various transporters have different properties both with respect to their transport functions and with respect to their ability to act as ion channels. Further, they are differentially regulated. To understand the physiological roles of the individual transporter subtypes, it is necessary to obtain information on their distributions and expression levels. Quantitative data are important as the functional capacity is limited by the number of transporter molecules. The most important and most abundant transporters for removal of transmitter glutamate in the brain are EAAT2 (GLT-1) and EAAT1 (GLAST), while GAT1 and GAT3 are the major GABA transporters in the brain. EAAT3 (EAAC1) does not appear to play a role in signal transduction, but plays other roles. Due to their high uncoupled anion conductance, EAAT4 and EAAT5 seem to be acting more like inhibitory glutamate receptors than as glutamate transporters. GAT2 and BGT1 are primarily expressed in the liver and kidney, but are also found in the leptomeninges, while the levels in brain tissue proper are too low to have any impact on GABA removal, at least in normal young adult mice. The present review will provide summary of what is currently known and will also discuss some methodological pitfalls.

**Keywords: GABA uptake, glutamate uptake, BGT1, GAT1, GAT3, GAT2, EAAT2, EAAT1**

## GLUTAMATE AND GABA AS NEUROTRANSMITTERS

Gamma-aminobutyric acid (GABA) and glutamate are, respectively, the major inhibitory and the major excitatory neurotransmitters in the mammalian central nervous system (1–3), and are thereby involved directly or indirectly in most aspects of normal brain function including cognition, memory, and learning. They are exocytosed from nerve terminals, and it is currently debated whether they are also exocytosed from astrocytes [e.g., Ref. (4–6)]. When interpreting data in the literature, it is important to keep in mind that astrocytic preparations differ greatly depending on the source of the cells and the culturing conditions, and cultured astrocytes may differ substantially from mature brain astrocytes (5).

## THE IMPORTANCE OF CELLULAR UPTAKE OF GABA AND GLUTAMATE

Both GABA and glutamate exert their signaling roles by acting on receptors located on the surface of the cells expressing them [e.g., Ref. (7–13)]. Therefore it is the transmitter concentration in the surrounding extracellular fluid that determines the extent of receptor stimulation. It is of critical importance that the extracellular concentrations are kept low [e.g., Ref. (3, 14–16)]. This is required for a high signal to noise (background) ratio in synaptic as well as in extrasynaptic transmission.

Low extracellular levels can only be maintained by cellular uptake because there is no extracellular metabolism of GABA and glutamate [e.g., Ref. (17–24)].

For glutamate, there is another reason to keep the extracellular levels low. Excessive activation of glutamate receptors is harmful, and glutamate is thereby toxic in high concentrations [for review and references, see Ref. (3)].

## EARLY CHARACTERIZATION OF GABA AND GLUTAMATE UPTAKE

It was soon realized that the mechanisms responsible for the uptake of GABA and glutamate are independent of each other (21, 25, 26) and that there is heterogeneity both within GABA uptake (27) and glutamate uptake (28–33). Uptake activity of both GABA and glutamate uptake was found to be present both in glial cells and in neurons [for review, see Ref. (1, 24, 34)]. The uptake processes are electrogenic and in the case of glutamate uptake it is driven by the ion gradients of $K^+$ and $Na^+$ [for review, see Ref. (35)] while the uptake of GABA is driven by the gradients of $Na^+$ and $Cl^-$ (35–39). The dependency of the transport process on the electrochemical gradients across the plasma membranes further implies that the uptake can reverse if the gradients are sufficiently weakened. In fact, during cerebral ischemia massive amounts of glutamate are released (40) and transporter reversal may be one

**Abbreviations:** BGT1, betaine-GABA transporter (slc6a12); DTT, dithiothreitol; EAAC1, excitatory amino acid carrier (EAAT3; slc1a1); EAAT, excitatory amino acid transporter; GAT1, GABA transporter 1 (slc6a1); GAT2, GABA transporter 2 (slc6a13); GAT3, GABA transporter 3 (slc6a11); GABA, gamma-aminobutyric acid; GLAST, glutamate–aspartate transporter (EAAT1; slc1a3); GLT-1, glutamate transporter 2 (EAAT2; slc1a2).

of the mechanisms [e.g., Ref. (41–45)] albeit not the only one (3). Further, the transporters can operate as exchangers. The latter phenomenon complicated early attempts to study binding of glutamate to the uptake sites in membrane preparations (46–48), and it took some time before it was realized that transportable uptake inhibitors induce release of internal endogenous substrates by enabling heteroexchange [e.g., Ref. (3, 49)].

## IDENTIFICATION OF GABA AND GLUTAMATE TRANSPORTERS

The first neurotransmitter transporter to be molecularly identified was the GABA transporter (**Table 1**) now known as GAT1 (slc6a1). This was accomplished by purifying the protein in active form using reconstitution of transport activity as the assay to monitor the purification process (50). Based on peptide sequences derived from the pure protein, probes were synthesized and cDNA was successfully isolated from a rat brain library (51). This turned out to be the first member of a new family of transporters. Another three GABA transporters (GAT2, slc6a13; GAT3, slc6a11; BGT1, slc6a12) were subsequently identified (52, 53). The first cloning of BGT1 resulted from screening of a Madin–Darby canine kidney (MDCK) cell cDNA library for expression of a betaine transporter in *Xenopus* oocytes (54). BGT1 homologs were subsequently cloned from mouse brain (53), and from human brain (55) and kidney (56). In fact, the mammalian genome contains about 20 transporters with structural similarities to GAT1 (37, 38, 57). Interestingly, none of these were glutamate transporters.

The first glutamate transporter (**Table 2**) to be isolated in active form (58) and localized (59, 60) was the one now known as EAAT2 [GLT-1; slc1a2; Ref. (61)]. Simultaneously, but independently of each other, three other research teams succeeded in cloning another two glutamate transporters using completely different approaches (62–64). The three human counterparts were quickly identified and named Excitatory Amino Acid Transporter (EAAT) 1–3 (65). Another two glutamate transporters were found later: EAAT4 (66) and EAAT5 (67). All the EAATs catalyze $Na^+$- and $K^+$-coupled transport of L-glutamate as well as L- and D-aspartate, but not D-glutamate. Further, down-regulation of glial glutamate transporters after glutamatergic denervation suggested complex regulation (68). Glutamate transporter expression turned out to be regulated via several different pathways and neurons were found to influence astroglial expression levels [e.g., Ref. (69–72)]. In fact, there is regulation on apparently all levels from transcription to posttranslational modification and trafficking (73, 74). This degree of complexity suggested that the transporters might have more roles than simply representing drainage and re-cycling systems [for review, see Ref. (3, 73, 75–79)]. Pharmacological manipulation of transporter expression or function would be highly interesting from a therapeutic point of view (80). A spider toxin has been found to enhance EAAT2 transport activity (81), but the compound responsible has not yet been identified. Recently, it was discovered EAAT2 expression can be increased by β-lactam antibiotics (82, 83), and that finding has got considerable attention.

## FUNCTIONAL PROPERTIES OF GABA TRANSPORTERS

The molecular functioning of GAT1 has been extensively studied (84–92), but there are also data on the other three GABA transporters (93–100). GAT1 and GAT3 are coupled to both sodium and chloride. Like for the glutamate transporters (see below), there is also uncoupled transport (101–103). The affinities for GABA vary greatly. The reported $K_m$ values for the mouse isoforms are 0.8, 8, 18, and 80 μM, respectively, for GAT3, GAT1, GAT2, and BGT1 (52, 53, 104). Nipecotic acid and β-guanidinopropionate inhibit the GAT1–3, but not the taurine transporter (105). GAT2 (slc6a13) transports β-alanine and also taurine with $K_m$ of 28 and 540 μM, respectively (52, 106). Mouse BGT1 (slc6a12) transports betaine with a $K_m$ of about 200 μM,

**Table 1 | Overview of the nomenclature of plasma membrane GABA transporters.**

| Name adopted by HUGO (www.genenames.org) | Other names |
|---|---|
| GABA transporter 1 (GAT1; slc6a1) | Rat GAT1, human GAT1, mouse GAT1 (51, 52) |
| GABA transporter 2 (GAT2; slc6a13) | Rat GAT2, human GAT2, mouse GAT3 (52, 277) |
| GABA transporter 3 (GAT3; slc6a11) | Rat GAT3, hGAT-3, mGAT4, GAT-B (52, 277, 278) |
| Betaine-GABA transporter (BGT1; slc6a12) | Rat BGT1, rat GAT-4, rat NTBE, human GAT-4, mouse GAT2 (52–56, 180) |
| Taurine transporter (TAUT; slc6a6) | (279), (280) |
| Proton-coupled amino acid transporter 1 (PAT1; slc36a1) | Imino acid carrier, LYAAT-1, tramdorin 3 (281) |

*The neurotransmitter transporter family (SLC6) comprises four GABA transporters as well as the taurine transporter (280). Both the taurine transporter and the proton-coupled amino acid transporter 1 (282) are able to transport GABA, but with low affinity ($K_m$ > 1 mM) and are therefore usually not classified as "GABA transporters." The nomenclature used here is the one adopted by the HUGO Gene Nomenclature Committee (283).*

**Table 2 | Overview of the nomenclature of plasma membrane glutamate transporters.**

| HUGO name | Other names |
|---|---|
| Excitatory amino acid transporter 1 (EAAT1; slc1a3) | GLAST (63–65) |
| Excitatory amino acid transporter 2 (EAAT2; slc1a2) | GLT-1 (61, 65) |
| Excitatory amino acid transporter 3 (EAAT3; slc1a1) | EAAC1 (62, 65, 211) |
| Excitatory amino acid transporter 4 (EAAT4; slc1a6) | (66) |
| Excitatory amino acid transporter 5 (EAAT5; slc1a7) | (67) |

*Glutamate transporters do not belong to the slc6-family, but to the slc1-family (high-affinity glutamate and neutral amino acid transporter family). Although there are several proteins with ability to transport glutamate, the term "glutamate transporter" is usually used to describe the five "High-Affinity Glutamate Transporters" also called "Excitatory Amino Acid Transporters (EAATs)." The actual meanings of the acronyms (GLAST, glutamate–aspartate transporter; GLT-1, glutamate transporter; EAAC, excitatory amino acid carrier; EAAT, excitatory amino acid transporter) are not important, as they do not reflect functional differences among the transporters. The nomenclature used here is the one adopted by the HUGO Gene Nomenclature Committee (283).*

but no significant transport of β-alanine could be detected (53). Mouse GAT2 and GAT3 are more potently inhibited by isoserine, β-alanine, and hypotaurine than GAT1 and BGT1 (107). Tiagabine is highly selective for GAT1 (24, 108, 109). Recently, new functional assays have been developed for compound screening (110, 111) leading to development of several new compounds (112–116).

## FUNCTIONAL PROPERTIES OF GLUTAMATE TRANSPORTERS

Most of the reported $K_m$ values of EAAT2 for glutamate are at around 20 μM and the affinities of EAAT1 and EAAT3 differ from EAAT2 with a factor of <2 (3), while the affinities of EAAT4 and EAAT5 are, respectively, one order of magnitude higher and lower (3, 65–67). Stoichiometry of the transport mediated by EAAT1–3 is exchange of one internal potassium ion with the following external substrates: one glutamate, three sodium ions, and one hydrogen ion (117–119). The coupling to three sodium ions makes these transporters less prone to reversal than the GABA transporters which are coupled to two sodium ions. In addition to the coupled (stoichiometric) transport, there are uncoupled fluxes. Thus, the transporters also function as chloride channels (66, 117, 120–122). EAAT4 and EAAT5 have the largest chloride conductance, and may function more as inhibitory glutamate receptors than as transporters (123, 124). In addition, a general feature of sodium coupled transport appears to be transport of water (125, 126). Obviously, these transporters are complex molecules, and it is important to determine their exact structure. Although the mammalian transporters have not yet been crystallized, a detailed picture is emerging (127, 128). The mammalian EAAT2 and EAAT3 proteins are believed to be homotrimers where the subunits are non-covalently connected (129). This is supported by recent evidence including crystallization of a bacterial glutamate transporter (130, 131). However, crosslinking studies indicate that there may be differences between the EAAT subtypes (123). These proteins are integral membrane proteins and they depend on the lipid environment. For instance, the GABA transporters, at least GAT1, need cholesterol to be fully active (132). EAAT2 is more robust, but is influenced by fatty acids such as arachidonic acid (133–135) and oxidation (136, 137). Arachidonic acid elicits a substrate-gated proton current associated with the glutamate transporter EAAT4 (138, 139).

All the five EAATs transport L-glutamate and DL-aspartate with high affinities (3, 140). There are some important differences, however. One of them is that EAAT3 transports cysteine (141). Another is that EAAT2 is blocked by kainate and dihydrokainate (65). Importantly, kainate analogs block both net uptake and exchange [for the importance of this, see Figure 5 in Ref. (3)] while most other inhibitors are substrates. Recently, a pan-EAAT blocker was developed by Shimamoto and co-workers. They synthesized a series of compounds (TBOA and variants) based on aspartate (142). The only known biological effect of these compounds is to bind to EAATs with higher affinity than glutamate (143, 144) and they do not interact with ASCT2 (145). An EAAT1 selective inhibitor has also been developed (146).

## LOCALIZATION AND FUNCTION – NUMBERS MATTER

Still, most of the data on protein distribution relies on immunohistochemistry. Unfortunately, validation of the specificity of immunochemical labeling is difficult and the literature reflects that [for detailed discussion, see Ref. (147–149)]. The most difficult part is to obtain good negative controls. When studying human samples, post-mortem proteolysis may complicate interpretation because the termini of EAAT1 and EAAT2 are rapidly proteolyzed (150–152). Post-mortem changes affect GAT3 more than GAT1 (153). It is a good idea to use additional methods, including *in situ* hybridization and Western blotting. For instance, Western blotting can be used to validate regional and temporal differences in labeling intensity. However, there are pitfalls here too. One of them is that non-transporter proteins may interfere with the binding of transporters to the blotting membranes causing underestimation of expression levels (106, 154).

The presence of a protein is one thing. But to be physiologically relevant, sufficient numbers of molecules must be present. The number of molecules needed to accomplish a given task depends on what that task is. This consideration is particularly relevant for neurotransmitter transporters as the transport process is fairly slow. The cycling time of EAAT2 and EAAT3 are about 30 glutamate molecules per second at $V_{max}$ (14, 155, 156). EAAT5 is even slower and is reported to behave as a slow-gated anion channel with little glutamate transport activity being more than an order of magnitude slower than EAAT2 (157). The cycling time for the GABA transporters has not been determined, but is believed to be similar to that of EAAT2.

The TBOA glutamate uptake blocker (143, 158) showed that there is a rapid extracellular turnover of glutamate (159). Despite that, the resting levels of extracellular glutamate in normal brains are low, possibly as low as 25 nM (160) which is 0.1–0.2% of the reported $K_m$ values for glutamate uptake (see above). At this concentration <0.1% of the glutamate transporter molecules are expected to be actively transporting. Consequently, if ambient concentrations of 25 nM shall be maintained, then there must be so many EAAT molecules that 0.1% of them is sufficient to keep up with the release. In fact, this is what has been determined experimentally (123, 155, 161, 162). Buffering synaptically released glutamate on a submillisecond time scale is just as demanding (163).

Also the ambient GABA levels around synapses are low; probably well below 1 μM (164–166) and thereby below the $K_m$ of GAT3. The low levels mean that GABA is removed efficiently and down to a level where BGT1 (and GAT2) is much less efficient that GAT1 and GAT3. This illustrates the point that it is not interesting whether a few BGT1 molecules can be found or not, but whether there are enough of them to make a difference. Because BGT1 has lower affinity (see above) and is expressed in the brain at much lower levels than that GAT1, it cannot contribute to GABA inactivation and does not affect seizure thresholds (167).

## DISTRIBUTION OF GABA TRANSPORTERS

The purified rat GAT1 protein (50) was used to generate the first antibodies to a GABA transporter and these were used to localize GAT1 in young adult rat brain tissue (168). These antibodies were probably selective for GAT1, but reactivity toward the other GATs were never tested. Nevertheless this antibody did not label cell bodies and the strongest labeling was found in GABAergic terminals. Basket cell terminals around the base of the Purkinje

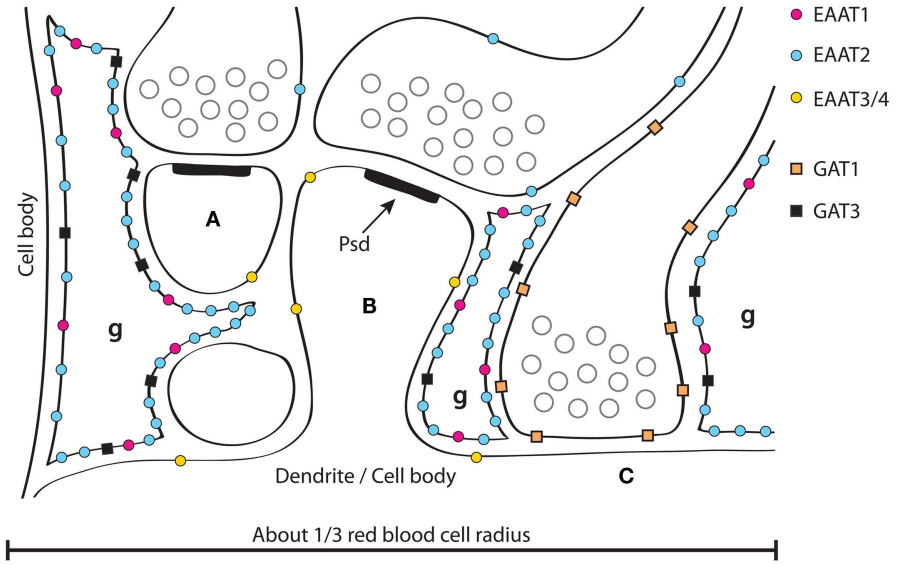


**FIGURE 1 | A schematic illustration of GABA and glutamate transporter distributions around synapses in the hippocampus**. Two glutamatergic synapses **(A,B)** are shown forming synapses asymmetric specializations with prominent post synaptic densities (PSD, one of which is labeled). GABAergic synapses **(C)** are often onto dendritic trunks rather than spines, and the synaptic specializations are typically symmetric. Three fine astrocyte branches are indicated (g). Note that the synapses in the hippocampus are usually not surrounded by astrocytes, but rather contacted by an astrocyte (like a finger pointing to it, and typically from the postsynaptic side). Also note that there are no astrocytes between synapse **(A,B)**. About 1/3 of neighboring synapses in the hippocampus have no astrocytes between them in contrast to the molecular layer of the cerebellum where most synapses onto spines are typically completely surrounded by astrocytes (**Figure 2**) and thereby isolated from their neighbors (162, 275). Glutamate and GABA transporters are indicated. EAAT1 (184, 185) and GAT3 (153, 172, 175–177) are selective for astrocytes, while EAAT2 is predominantly expressed in astrocytes (59), but there is also some (about 10%) in hippocampal nerve terminals (229). This has some resemblance to GAT1 as GAT1 is mostly neuronal (170–173), but with some expression in astrocytes; particularly in the thalamus (172). There is more EAAT2 than EAAT1 in the hippocampus and the other way around in the cerebellum (162, 184). EAAT3 is selective for neurons, but is expressed at levels two orders of magnitude lower than EAAT2 and is targeted to dendrites and cell bodies (193) (Copyright: Neurotransporter.org; Reproduced with permission).

cells, for instance, were strongly labeled. Most GABAergic terminals were labeled, but with two notable exceptions: striatonigral and Purkinje cell axon terminals. There was also some labeling of astrocytes. These findings were confirmed by antibodies produced to synthetic peptides (169, 170) and thereby to sequences known to differ between GABA transporter subtypes (170–173). In thalamus, however, GAT1 was not found in terminals. All the immunolabeling was in astrocytes together with GAT3 (172). GAT1 is present in neuronal cell bodies for a short time during development (174). In contrast to GAT1, GAT3 (**Figures 1–3**) is considered to be selectively expressed in astrocytes throughout the CNS [e.g., Ref. (153, 172, 175–177)]. The highest GAT1 levels are in the cerebral cortex while the highest GAT3 levels are in the brainstem (178, 179).

In contrast, GAT2 and BGT1 are predominantly expressed in hepatocytes in the liver and kidney (52, 53, 106, 180, 181). Within the skull (**Figure 4A**), GAT2 is found only in the leptomeninges and some large blood vessels (169, 181, 182) while BGT1 (**Figure 4B**) is expressed in the leptomeninges (106, 178).

## DISTRIBUTION OF GLUTAMATE TRANSPORTERS

When the first polyclonal and monoclonal antibodies were raised against the purified EAAT2 protein (58), it was immediately clear that EAAT2 is highly expressed in astrocytes in all parts of the brain and spinal cord. The highest levels were found in the hippocampus and the neocortex (59, 60). Soon thereafter antibodies were raised to synthetic peptides representing parts of the various subtypes. This made it easier to ensure that the antibodies were subtype specific. The conclusions on the distribution of EAAT2 were confirmed (183, 184), while EAAT1 was localized for the first time (184) and also this protein was found in astrocytes throughout the central nervous system (184–188). With immunogold and electron microscopy, it was shown that both EAAT1 and EAAT2 are preferentially targeted to the plasma membranes, and that plasma membranes facing neuropil have higher densities than those facing cell bodies, other astrocytes, and pia mater (189). Quantitative immunoblotting of brain tissue extracts compared with known amounts of purified glutamate transporters showed that EAAT2 protein represents about 1% of the total forebrain protein and that it is about four times more abundant than EAAT1 in the hippocampus and six times less abundant than EAAT1 in the cerebellum (162). The high expression levels are part of the reason why the first post-embedding immunogold electron micrographs of EAAT1 and EAAT2 (189) as well as EAAT4 (123) were so clear. Of course, good antibodies and good tissue preparation are key factors, but to get good immunogold images, there must also be a large number of molecules in the plane of the section.

Immunoadsorption of transport activity revealed that EAAT2 represent about 95% of the total glutamate uptake activity in young adult forebrain tissue (59, 129). Deletion of the EAAT2 gene

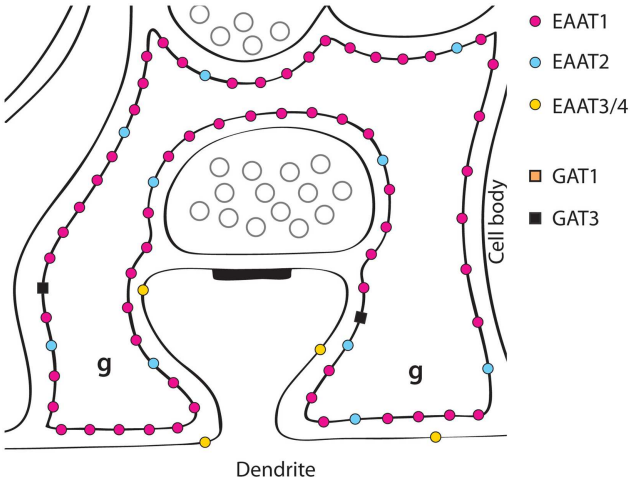


**FIGURE 2 | A schematic illustration of glutamate transporter distributions around parallel fiber synapses on Purkinje cell spines in the molecular layer of the cerebellum**. EAAT1 is expressed in the astrocytes (Bergmann glial cells) at very high levels, and about six times higher than EAAT2 (162, 184). Most of the EAAT4 protein is found in cerebellar Purkinje cells (the glia-covered parts of the membranes of Purkinje cell dendrites), but low levels are also found in scattered neurons in the neocortex (123). EAAT3 is also found in the Purkinje cell dendrites, as well as in the other neuron types present, but at low levels (193) (Copyright: Neurotransporter.org; Reproduced with permission).

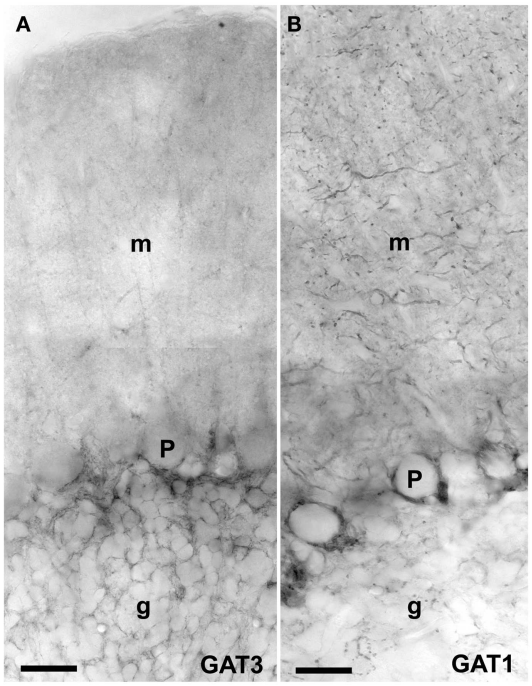


**FIGURE 3 | Peroxidase labeling of GAT3 (A) and GAT1 (B) in the cerebellum [as described Ref.** (175, 276)**]**. GAT1 (slc6a1) and GAT3 (slc6a11) are the two GABA transporters that are functionally relevant for brain function, and these transporters are not expressed in the liver and kidney (181). GAT1 is mostly localized in axonal terminals in molecular layer (m) and GAT3 is in astroglial processes in granular layer (g). Note very high levels of GAT1 in basket cell terminals around the Purkinje cells (P). Scale bar: 20 μm (Copyright: Neurotransporter.org; Reproduced with permission).

in mice confirmed this conclusion as the glutamate uptake activity was reduced to 5% compared to wildtype mice (190–193) without changing the expression of other glutamate transporters, glutamine synthetase (GS), and glutamate GluR1 receptors (194). Further, electrophysiological recordings of glutamate transporter currents from hippocampal astrocytes and from human embryonic kidney 293 cells expressing human EAAT2 are statistically indistinguishable suggesting that the transporter currents in astrocytes result from EAAT2 or a functionally identical isoform (155).

EAAT1 (**Figures 1** and **2**) is the predominant glutamate transporter in the cerebellum (162, 195), the inner ear (196, 197), the circumventricular organs (198), and in the retina [Ref. (199–204); for review, see Ref. (205)].

EAAT2 and EAAT1 are the only glutamate transporters expressed in brain astrocytes as both EAAT3 (193, 206) and EAAT4 (123, 207, 208) are neuronal. Within the CNS, EAAT5 is preferentially expressed in the retina and expression in the brain is very low. It is interesting to note that also in insects (at least in the cabbage looper *Trichoplusia ni*) glial cells have high densities of glutamate transporters in their plasma membranes (209, 210).

EAAT3 is a neuronal transporter as originally suggested (62, 183, 211) and is not expressed in glial cells (193, 206). It appears to be expressed in the majority if not all neurons throughout the CNS, but is selectively targeted to somata and dendrites avoiding axon terminals (193, 206). Within the CNS, it is found in the highest concentration in the hippocampus, but the total tissue content in young adult rat brains is about 100 times lower than that of EAAT2 (193).

EAAT4 is predominantly found in the cerebellar Purkinje cells (66, 123) where it is targeted to the dendrites, the spines in particular (123), but there is also some EAAT4 in a subset of forebrain neurons (123, 207, 208).

Outside the CNS, EAAT2 is primarily expressed in glandular tissue, including mammary gland, lacrimal gland, and ducts and acini in salivary glands (212) and by perivenous hepatocytes (212, 213). It is not present in the heart (214). Thus, the main roles of EAAT2 are in the brain [for review, see Ref. (3, 15, 16)]. EAAT1 is found in several non-neuronal tissues (212) including, the heart, fat cells, and taste buds (212, 214, 215), but does not appear to be important in controlling bone growth (216). EAAT3 is present in the kidney (62, 193). The heart expresses EAAT1, EAAT3, EAAT4, and EAAT5, but not EAAT2 (214).

## NEURONAL EXPRESSION OF THE EAAT2 PROTEIN

EAAT2 mRNA is detected in astrocytes, but is also found in some neurons: pyramidal cells in CA3 hippocampus and in layer VI of the parietal neocortex (217–219). In fact, EAAT2 mRNA is reported in the majority of neurons in the neocortex and in parts of the olfactory bulb, thalamus, and inferior olive (188). That neurons have the potential to express EAAT2 protein is clear. Cultured neurons from hippocampus and cortex can express EAAT2 protein (220–222). Further, EAAT2 is transiently localized on growing axons of the mouse spinal cord before establishing astrocytic expression (223). Finally, in the normal and mature mammalian retina, EAAT2 protein is not expressed in retinal glial

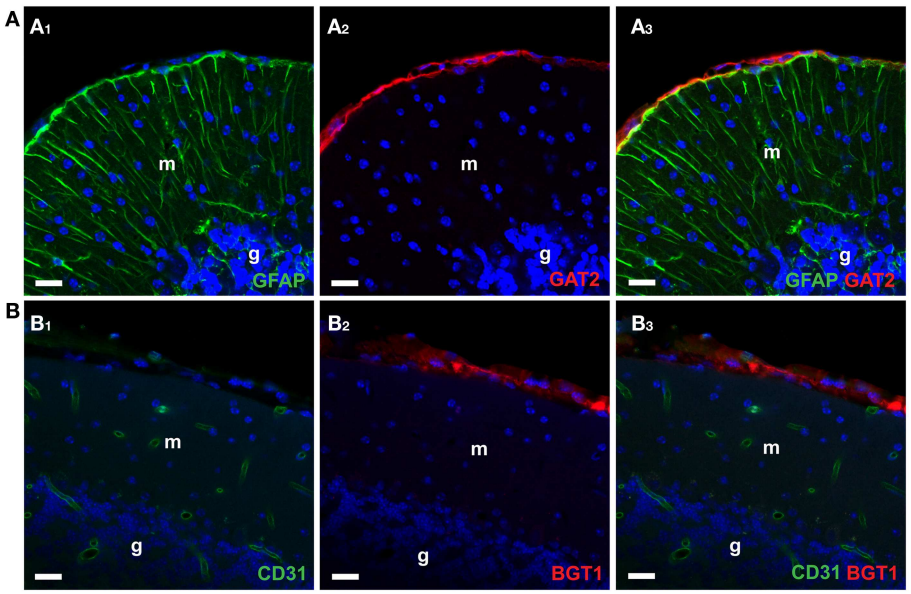


**FIGURE 4 | Confocal imaging of GAT2 and BGT1 protein in the cerebellum [as described Ref.** (106, 181)**].** (**A1–A3)** Show double labeling for GAT2 (red) and the astroglial marker GFAP (green). (**B1–B3)** Show double labeling for BGT1 (red) and the endothelial marker CD31 (green). The nuclei were visualized in blue by DAPI. Note that both GAT2 and BGT1 are found at the leptomeninges external to glia limitans. Also note that there is no co-localization of GAT2 with GFAP, and no co-localization of BGT1 with endothelial marker. In fact, BGT1 (106, 167) and GAT2 (181) are hardly expressed in the brain at all, but are highly expressed in the liver and kidney. Abbreviations: m, molecular layer; g, granular layer. Scale bar: 20 μm (Copyright: Neurotransporter.org; Reproduced with permission).

cells (neither in the Müller cells nor the astrocytes), but is exclusively expressed in neurons (cone photoreceptors and bipolar cells) (201, 205, 224–226).

Nevertheless, this was controversial for a long time and remains to be fully resolved. What is clear is that there is a significant glutamate uptake into glutamatergic nerve terminals, at least in the hippocampus CA1 [Ref. (227) for a review, see section 4.2 in Ref. (3)]. Glutamatergic axon terminals in hippocampus CA1 express EAAT2 protein, albeit at low levels (154, 228–231) and this transporter is responsible for the glutamate uptake activity in hippocampal terminals because it is absent in EAAT2 knockout mice and is sensitive to inhibition by dihydrokainate (193, 229). Because these terminals originate from the CA3 pyramidal cells, it makes sense that these cells have high levels of EAAT2 mRNA. This is further confirmed by the recent observation from EAAT2 eGFP BAC reporter mice (232). There is now consensus up to here, but at least two questions remain:

(a) Is nerve terminal glutamate uptake functionally relevant? Why was about half of all D-aspartate taken up by hippocampus slices found to in axon terminals when terminals only contain around 10% of the EAAT2 protein (229)? This uptake cannot simply be disregarded as an *in vitro* artifact due to a higher rate of heteroexchange than net uptake (233). Preliminary data from selective deletion of EAAT2 in axon terminals indicate disturbances in synaptic transmission (234), and thereby may suggest that EAAT2 in terminals is functionally relevant.
(b) Do CA3 pyramidal neurons represent special cases or is most of the so called synaptosomal uptake measured in other brain regions also due to nerve terminal EAAT2?

Data obtained with EAAT2 eGFP BAC reporter mice (232) tend to favor a "yes" to this question, while *in situ* hybridization data argue for a "no" [e.g., Ref. (154, 235)].

## LESSONS FROM GABA TRANSPORTER KNOCKOUTS

GAT1-deficient mice were generated as an intermediate in the construction of the mGAT1-GFP strain (236). As GAT1 is the major GABA transporter, one might expect that deletion would lead to increased extracellular GABA levels and inhibition. Reduced aggression (237), hypoalgesia (238), reduced anxiety, and depression-like behaviors (239) and altered behavioral responses to ethanol (240) may be largely as expected. However, things are more complicated. One complicating factor is that the brain still expresses GAT3 in astrocytes. Another point is that GAT1 is mostly in terminals where it recycles GABA, and GAT1 deletion leads to decreased quantal GABA release, and a differential tonic activation of GABA(A) versus GABA(B) receptors in the hippocampus (241), as well as to tremor, ataxia, nervousness, and increased GABA-induced tonic conductance in cerebellum (242). This phenotype resembles adverse effects of tiagabine treatment. Tiagabine is highly selective for GAT1 (115). It has effects on seizure control and behavior, but side effects are fatigue, dizziness, psychomotor slowing, ataxia, gastrointestinal upset, weight change, and insomnia (243). In human populations there is genetic variation within the GAT1 gene (slc6a1) and these may be associated with anxiety disorders with panic symptoms (244).

Deletion of BGT1 in mice does not affect seizure thresholds (corneal kindling; minimal clonic and tonic extension threshold test; 6 Hz seizure threshold test; pentamethylenetetrazole-induced seizure) of adult mice (167) in agreement with the fact that it is

predominantly expressed in the liver and at lower levels in the kidneys and at the brain surface (106). Deletion of GAT2 in mice leads to changes in liver and brain taurine contents (181), but also does not appear to give any symptoms from the nervous system under non-challenging rearing conditions (181). Obviously, it would be interesting to study the consequences of the deletion of GAT3. These studies are under way as GAT3 knockout mice have been made by Yun Zhou, C. Guo, and Niels Christian Danbolt.

## LESSONS FROM GLUTAMATE TRANSPORTER KNOCKOUTS

The possibility of connections between malfunctioning glutamate transporters and disease has got considerable attention [e.g., for review, see Ref. (3, 74, 245, 246)]. Observations of the EAAT2 knockout mice illustrate why (190, 191). Deletion of the EAAT2 gene causes, in agreement with biochemical data (59, 129), a reduction in glutamate uptake activity by about 95% (155, 190, 192, 193) and increased extracellular glutamate levels (247, 248). This has dramatic consequences as they grow up. Mice deficient in EAAT2 are not conspicuous at birth, but at 3 weeks of age they can readily be identified because they are hyperactive, epileptic, and smaller than their wildtype littermates. About half of the mice die from spontaneous seizures before they reach 4 weeks of age (190). The gradual increase in severity parallels the postnatal increase in EAAT2 expression in wildtype animals (249, 250), and in production of transmitter glutamate via the glutamate-glutamine cycle [reviewed by Ref. (251)]. The heterozygote EAAT2 knockout mice (±) exhibit a 59% decrease in EAAT2 protein levels in the brain, but do not show any apparent morphological brain abnormalities and have a similar life-span as their wildtype littermates (192). There are only moderate behavioral alterations (mild sensorimotor impairment, hyperlocomotion lower anxiety, better learning of cue-based fear conditioning), but worse context-based fear conditioning (192). However, the histological outcomes following traumatic spinal cord injury is worse in agreement with the notion that they are less protected (252). No humans have been identified at being EAAT2 deficient, but there are some reports on mutations. One patient with amyotrophic lateral sclerosis was found to harbor a mutated EAAT2 (253, 254) and associations of mutations with alcoholism (255), schizophrenia (256), smoking behavior (257), essential tremor (258), and bipolar disorder (259) have been reported, but it is too early to make firm conclusions.

Mice lacking EAAT1 (260) develop normally, but show symptoms of insufficient glutamate uptake in regions where EAAT1 is the major glutamate transporter. Thus, cerebellar function is affected resulting in reduced motor coordination and increased susceptibility to cerebellar injury (260), disturbance of the inner ear with exacerbation of noise-induced hearing loss (261) and disturbed retinal function (262). The EAAT1 knockout mice also display poor nesting behavior; abnormal sociability, reduced alcohol intake, and reward (260, 263–265). Lack of GLAST does not lead to spontaneous seizures like those seen in connection with EAAT2-deficiency, but when seizures are initiated, then lack of GLAST increases seizure duration and severity (266). In humans, mutations in EAAT1 are associated with episodic ataxia (246, 267, 268).

Mice lacking EAAT3 (269) develop dicarboxylic aminoaciduria, and possibly a somewhat reduced spontaneous locomotor activity (open field). They do not show signs of neurodegeneration at young age and do not have epilepsy (269–271), but may age prematurely (270). Humans lacking EAAT3 develop dicarboxylic aminoaciduria as expected from the mice data (272). Further, human EAAT3 polymorphisms have been reported to be associated with obsessive–compulsive disorders (273, 274).

## CONCLUDING REMARKS

The various GABA and glutamate transporters have select expression patterns and distributions. The literature, however, has become confusing in part due to poorly controlled immunocytochemistry. A major reason for the latter is the reliance on the pre-absorption test which easily gives a misleading impression of specificity [for discussion, see Ref. (149)]. Post-mortem proteolysis has also contributed to confusion concerning distributions in humans (152). To sum up (for references, see above): GAT3 and EAAT1 (GLAST) are both selectively expressed in astrocytes throughout the CNS, while EAAT3 (EAAC1) and EAAT4 are selective for neurons. EAAT3 is expressed by most if not all neurons, while EAAT4 is only expressed in subpopulations. GAT1 and EAAT2 (GLT-1) are both in terminals (GABAergic and glutamatergic, respectively) and in astrocytes, but differ in that EAAT2 is predominantly in astrocytes throughout the CNS except in the retina, while GAT1 is only predominantly astrocytic at some locations (e.g., thalamus). EAAT2 is the only one of these transporters that is required for survival under non-challenging conditions. GAT2 and BGT1 are both expressed in the leptomeninges, but are not significantly expressed not around synapses (in neuropil). GAT2 is also found in some blood vessels. All these transporters are highly conserved between mammals, and they play different roles, some of which remains to be fully understood.

## ACKNOWLEDGMENTS

The authors thank Gunnar Lothe for help with **Figures 1** and **2**. This work was supported by the Norwegian Research Council (FUGE II-183727-S10).

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**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

*Received: 05 October 2013; paper pending published: 16 October 2013; accepted: 17 October 2013; published online: 11 November 2013.*
*Citation: Zhou Y and Danbolt NC (2013) GABA and glutamate transporters in brain. Front. Endocrinol. **4**:165. doi: 10.3389/fendo.2013.00165*
*This article was submitted to Cellular Endocrinology, a section of the journal Frontiers in Endocrinology.*

frontiers in
ENDOCRINOLOGY

ORIGINAL RESEARCH ARTICLE
published: 17 September 2013
doi: 10.3389/fendo.2013.00123

# Inhibitors of glutamate dehydrogenase block sodium-dependent glutamate uptake in rat brain membranes

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We recently found evidence for anatomic and physical linkages between the astroglial $Na^+$-dependent glutamate transporters (GLT-1/EAAT2 and GLAST/EAAT1) and mitochondria. In these same studies, we found that the glutamate dehydrogenase (GDH) inhibitor, epigallocatechin-monogallate (EGCG), inhibits both glutamate oxidation and $Na^+$-dependent glutamate uptake in astrocytes. In the present study, we extend this finding by exploring the effects of EGCG on $Na^+$-dependent L-[$^3$H]-glutamate (Glu) uptake in crude membranes (P2) prepared from rat brain cortex. In this preparation, uptake is almost exclusively mediated by GLT-1. EGCG inhibited L-[$^3$H]-Glu uptake in cortical membranes with an $IC_{50}$ value of 230 μM. We also studied the effects of two additional inhibitors of GDH, hexachlorophene (HCP) and bithionol (BTH). Both of these compounds also caused concentration-dependent inhibition of glutamate uptake in cortical membranes. Pre-incubating with HCP for up to 15 min had no greater effect than that observed with no pre-incubation, showing that the effects occur rapidly. HCP decreased the $V_{max}$ for glutamate uptake without changing the $K_m$, consistent with a non-competitive mechanism of action. EGCG, HCP, and BTH also inhibited $Na^+$-dependent transport of D-[$^3$H]-aspartate (Asp), a non-metabolizable transporter substrate, and [$^3$H]-γ-aminobutyric acid (GABA). In contrast to the forebrain, glutamate uptake in crude cerebellar membranes (P2) is likely mediated by GLAST (EAAT1). Therefore, the effects of these compounds were examined in cerebellar membranes. In this region, none of these compounds had any effect on uptake of either L-[$^3$H]-Glu or D-[$^3$H]-Asp, but they all inhibited [$^3$H]-GABA uptake. Together these studies suggest that GDH is preferentially required for glutamate uptake in forebrain as compared to cerebellum, and GDH may be required for GABA uptake as well. They also provide further evidence for a functional linkage between glutamate transport and mitochondria.

**Keywords: glutamate, GLT-1, EAAT2, GLAST, GABA, glutamate dehydrogenase, sodium-dependent uptake, epigallocatechin-monogallate**

## INTRODUCTION

Glutamate is the predominant excitatory neurotransmitter in the mammalian CNS and mediates the vast majority of cell-to-cell communication in the brain [for review, see (1)]. In addition to being required for millisecond cell-to-cell communication, plasticity of excitatory synaptic transmission likely underlies learning and memory [for reviews, see (1–3)]. Aberrant glutamatergic transmission has been implicated in a wide variety of neurodevelopmental, neurologic, and psychiatric conditions [for review, see (3)]. For example, an accumulation of glutamate in the extracellular space and the consequent excessive activation of glutamate receptors likely contributes to the cell death that is observed after acute insults to the nervous system, such as stroke and head trauma [for review, see (2)].

Extracellular glutamate is cleared by $Na^+$-dependent glutamate transport systems [for reviews, see (4–6)]. In mammals, there are five $Na^+$-dependent glutamate transporter gene products; these are called excitatory amino acid transporters (EAAT1-5). EAAT1 (also called GLAST) is found on glia; expression is enriched in cerebellum but also found throughout forebrain (7). EAAT2 (also called GLT-1) is essentially restricted to astroglia with modest expression by a subset of neurons in hippocampus (7, 8). Results from several different types of studies strongly suggest that GLT-1 and GLAST mediate the bulk of glutamate uptake in the mammalian brain [(9); for review, see (10)]. This clearance into astroglia differentiates glutamate from most of the other classical neurotransmitters that are directly recycled back into the presynaptic nerve terminal [for reviews, see (11, 12)]. These transporters co-transport three $Na^+$ ions and one $H^+$ with glutamate in the inward direction; the cycle is completed with the counter-transport one $K^+$ ion (13). With this stoichiometry, these transporters are capable of generating up to a one million-fold concentration gradient of glutamate across the plasma membrane [for review, see (14)].

The astroglial transporters, GLT-1 and GLAST, are enriched on fine astroglial processes near synapses *in vivo* (15, 16). As might be expected, recent studies suggest that these transporters co-compartmentalize with the enzymes/organelles that would be required to efficiently fuel transport in these spatially restricted domains (17). For example, GLT-1 or GLAST co-localize with and physically/functionally interact with the $Na^+/K^+$ ATPase (18). Recently we showed that GLT-1 is part of a co-immunoprecipitable complex with the $Na^+/K^+$-ATPase, most of the glycolytic enzymes, and a subset of mitochondrial proteins (17). We also demonstrated significant co-localization of GLT-1 with a mitochondrial protein *in vivo* and anatomic overlap of mitochondria with GLT-1 in individual astrocytes in organotypic slice cultures. In a subsequent study, we documented similar interactions and anatomic overlap between GLAST and mitochondrial proteins (19). In this later study, we measured the percentage of glutamate that is oxidized in astrocytes. We also examined the effect of an inhibitor of glutamate dehydrogenase (GDH), a mitochondrial enzyme that could contribute to glutamate oxidation, on glutamate uptake and found that it inhibited uptake in astrocytes (19). This effect was not characterized beyond testing of a single concentration of one inhibitor in astrocytes that only express GLAST. In the present study, we characterized the potential effects of inhibitors of GDH on uptake in crude rat brain membranes (P2).

## MATERIALS AND METHODS

### MATERIALS

Adult male Sprague-Dawley rats were obtained from Charles River (Wilmington, MA, USA). All protocols were reviewed and approved by the Institutional Animal Care and Use Committee of the Children's Hospital of Philadelphia (Philadelphia, PA, USA). L-[$^3$H]Glu (40–80 Ci/mmol), D-[$^3$H]Asp (10–25 Ci/mmol), and γ-amino[$^3$H]butyric acid ([$^3$H]GABA; 70–100 Ci/mmol) were obtained from PerkinElmer (Waltham, MA, USA). The specific activity of all ligands was diluted with non-radioactive L-Glu, D-Asp, or GABA, respectively (Sigma-Aldrich Co., St. Louis, MO, USA). (−)-Epigallocatechin-monogallate (EGCG; ≥95%, from green tea), hexachlorophene (HCP), bithionol (BTH), Hepes, KCl, $CaCl_2$, and $K_2HPO_4$ were obtained from Sigma-Aldrich Co. (St. Louis, MO, USA). Tris base, Tris HCl, NaCl, $MgCl_2$, dextrose, and sucrose were obtained from Fisher Scientific (Pittsburgh, PA, USA). Tween-20 was obtained from Bio-Rad (Hercules, CA, USA).

### MEMBRANE PREPARATIONS

Crude membranes (P2) were prepared from cortex and cerebellum as previously described (20). The preparation is commonly referred to as "crude synaptosomal membranes." In the current paper, we refer to this preparation as crude membranes (P2) to avoid giving the impression that it contains strictly neuronal elements. Cortex or cerebellum was dissected on a metal plate cooled to 4°C. All subsequent steps were performed at 4°C. The tissue was homogenized in 20 volumes (wet weight of tissue) of ice-cold 0.32 M sucrose using a Dounce Teflon/glass homogenizer at 400 rpm for seven strokes (tissue homogenate) and centrifuged at 800 × *g* for 10 min. The supernatant (S1) was then centrifuged at 20,000 × *g* for 20 min. In a subset of experiments, the resultant supernatant was collected (S2). The resultant pellet (P2) was resuspended in 40 vols. of sucrose (0.32 M) by vortexing and centrifuged at 20,000 × *g* for 20 min. This washed crude membrane pellet (P2) was resuspended by vortexing in 50 vols. of sucrose (0.32 M) and homogenized (two strokes at 400 rpm). This resulted in a suspension of approximately 30 μg of protein per 50 μl as determined by the Pierce BCA (bicinchoninic acid) protein assay (Thermo Scientific, Rockford, IL, USA).

### WESTERN BLOT ANALYSES

The subcellular fractions were mixed with equal volumes of Laemmli sample buffer. Dual color molecular weight marker (Bio-Rad) and 3 or 10 μg of protein from each fraction were resolved on 10% SDS-polyacrylamide gels, and transferred to immobilon FL polyvinylidene fluoride (PVDF) membranes (Millipore, Bedford, MA, USA) as described previously (17, 19). The PVDF membranes were blocked in TBS-T (50 mM Tris, pH 8.0, 150 mM NaCl, 0.1% Tween-20) containing 5% non-fat dry milk for 1 h at 25°C. The membranes were then probed with the appropriate antibody overnight at 4°C: rabbit anti-GLT-1 (1:5,000; Dr. Rothstein), mouse anti-GLAST (1:50; Miltenyi Biotec, Auburn, CA, USA), mouse anti-*N*-methyl-D-aspartate (NMDA) receptor subunit 1 (NR1; 1:500; BD Biosciences, San Jose, CA, USA), rat anti-glial fibrillary acidic protein (GFAP; 1:500; Dr. Lee), or goat anti-neurofilament light polypeptide (NF-L; 1:250; Santa Cruz Biotechnology, Santa Cruz, CA, USA). The membranes were then washed with TBS-T containing 1% milk and incubated with the appropriate fluorescently conjugated anti-mouse, anti-rabbit, anti-goat, or anti-rat antibodies (1:10,000; LiCor Biosciences, Lincoln, NE, USA). Blots were scanned using an Odyssey Infrared Imager (LiCor Biosciences). The yield was calculated as the percentage of total immunoreactivity found in a particular fraction divided by the total immunoreactivity found in the tissue homogenate. The enrichment was calculated as the total immunoreactivity found in a particular fraction divided by the total amount of protein found in the fraction; this was normalized to the tissue homogenate. Therefore a number greater than 1 reflects relative enrichment in a fraction compared to homogenate.

### TRANSPORT ASSAYS

Sodium-dependent transport of L-[$^3$H]Glu, D-[$^3$H]Asp, and [$^3$H]GABA was measured as previously described (20). Duplicate assays were performed in a final volume of 0.5 ml containing Tris base (5 mM), HEPES (10 mM), NaCl (140 mM), KCl (2.5 mM), $CaCl_2$ (1.2 mM), $MgCl_2$ (1.2 mM), $K_2HPO_4$ (1.2 mM), dextrose (10 mM), and substrate in the absence or presence of inhibitors (pH = 7.2). In parallel assays, the uptake was measured in the absence of sodium with the substitution of equimolar amounts of choline chloride for NaCl. As HCP and BTH are not readily soluble in aqueous solutions, they were first dissolved in ethanol (HCP) or dimethyl sulfoxide (BTH) as 10 mM stocks. These stocks were diluted such that the same concentration of solvent (0.1% final concentration) was added to each assay; this meant that the highest concentrations of HCP or BTH used in these assays were

10 μM. In all experiments, 0.1% solvent (ethanol or dimethyl sulfoxide) was added to control assays and this concentration had no effect on uptake (data not shown, $n = 3$). EGCG was prepared and diluted in uptake buffer immediately before measuring uptake. All components excluding the crude membranes were combined into 12 mm × 75 mm glass tubes and equilibrated to 37°C. The assay was initiated with the addition of P2 membranes (50 μl) and stopped with 2 ml of ice-cold (4°C) choline-containing buffer after 3 min. For analyses of the effects of pre-incubation with HCP, the assay was initiated with the addition of radioactive substrate to the crude membranes (P2) incubated with HCP. After the addition of cold choline-containing buffer, the assays were filtered onto pre-wetted glass filter paper (FP-100; Brandel, Gaithersburg, MD, USA) using a cell harvester (Brandel, Gaithersburg, MD, USA). Filters were rinsed three times with 2 ml of cold choline-containing buffer. The radioactivity trapped in the membranes was solubilized with 5 ml of Cytoscint ES (MP Biomedicals, Solon, OH, USA) and measured using scintillation spectrometry (Beckman-Coulter Instruments, LS 6500). Sodium-dependent uptake was determined by subtracting the signal in the choline-containing buffer from the signal in the sodium-containing buffer. The total concentration of substrate (radioactive and non-radioactive) was 0.5 μM unless otherwise indicated. The $Na^+$-independent signal observed was less than 5% of the total uptake in the presence of sodium.

### DATA ANALYSIS

All values reported are the mean ± SEM of at least three independent experiments that were performed on different days. Concentration-response curves were fit to one-site and two-site competition curves, and these fits were compared by *F*-test using Prism 5.0 (GraphPad Software Inc., La Jolla, CA, USA). The top of the curves were constrained to 100% (no inhibition), and for all three compounds, the maximal predicted inhibition from the curve fits was essentially 100% (complete inhibition). Kinetic analyses of glutamate transport performed in the absence and presence of HCP were fit by linear regression as Eadie–Hofstee plots. $K_m$ and $V_{max}$ values were compared using one-way ANOVA with a Bonferroni *post hoc* test using InStat (GraphPad Software Inc., La Jolla, CA, USA). The data for L-[$^3$H]Glu, D-[$^3$H]Asp, and [$^3$H]GABA uptake in cortex and cerebellum was analyzed using one-way ANOVA with a Bonferroni *post hoc* test using InStat.

## RESULTS

We recently found that EGCG, a compound extracted from green tea that inhibits GDH (21), blocks $Na^+$-dependent glutamate uptake in astrocytes. EGCG was only tested at a single concentration (1 mM) using a single concentration of glutamate (19). The goal of the present study was to determine if inhibition of GDH might inhibit $Na^+$-dependent glutamate transport in membrane preparations from brain. As astrocytes in culture express GLAST and not GLT-1 (22, 23), we used crude membranes (P2) from cortex to further explore this effect. Genetic deletion of GLT-1 from mice essentially eliminates uptake from crude cortical membrane preparations (P2), and the pharmacology of glutamate uptake in this preparation parallels that observed for GLT-1 (9, 20, 24). Although this is classically considered a subcellular fraction that contains nerve terminals, it also contains substantial amounts of astroglial elements (25, 26). We analyzed the subcellular distributions of two different glial glutamate transporters [GLT-1 and GLAST; (15, 16)], a neuronal receptor [the NR1 subunit of the NMDA receptor; (1)], a glial cytoskeletal protein (GFAP), and a neuronal cytoskeletal protein [NF-L; (27); see **Figure 1**; **Table 1**]. The yield of both glutamate transporters was about 40% in the cortical P2 fraction and about 60% in the cerebellar P2 fraction, while the yield of NR1 was about 75% in the cortical P2 fraction and only about 40% in the cerebellar P2 fraction. The yields of both cytoskeletal proteins were 10% or less in both the cerebellar and cortical P2 fractions. Together these studies show that the P2 fractions contain glial and neuronal membrane proteins consistent with the earlier studies (25, 26).

In the first set of experiments, the effects of increasing concentrations of EGCG on $Na^+$-dependent uptake were examined (**Figure 2A**). The effects of EGCG were concentration-dependent and inhibited uptake with an $IC_{50}$ value of 234 μM. The maximal inhibition observed was 83% at 1 mM; higher concentrations were not tested because of solubility concerns. EGCG inhibits purified GDH with an $IC_{50}$ value of ∼0.5 μM (21, 28), but EGCG is relatively hydrophilic and has limited stability in solution (29).

Several other inhibitors of GDH were recently identified in a high-throughput screen (28). We chose two additional inhibitors of GDH to reduce the likelihood that effects of EGCG on glutamate uptake might be attributed to a non-specific interaction with a target other than GDH. All three of these compounds interact with different sites on GDH (30). The first compound examined, hexachlorophene (HCP), caused a concentration-dependent inhibition of $Na^+$-dependent glutamate uptake (**Figure 2B**). When these data were fit to a single site, the $IC_{50}$ value was 3.9 μM,

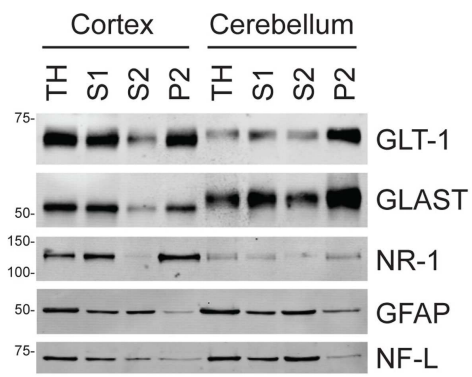


**FIGURE 1 | Analyses of neuronal and astroglial proteins in tissue homogenate (TH) and various subcellular fractions as described in Section "Materials and Methods**." To ensure that signal was in the linear range, 3 μg of protein from each fraction were used for the analyses of GLT-1 or GLAST and 10 μg of protein were for the analyses of the other proteins (NR1 subunit of the NMDA receptor, NR1; GFAP, and NF-L). The western blots shown are all from one animal; these analyses have been reproduced in three additional animals (see **Table 1** for data analyses).

**Table 1 | Analyses of neuronal and astroglial proteins in tissue homogenate (TH) and various subcellular fractionations as described in Section "Materials and Methods."**

| | TH | | S1 | | S2 | | P2 | |
|---|---|---|---|---|---|---|---|---|
| | **Enrichment** | **Yield** | **Enrichment** | **Yield** | **Enrichment** | **Yield** | **Enrichment** | **Yield** |
| **Cortex** | | | | | | | | |
| GLT-1 | 1.0 | 100 | 1.1 ± 0.2 | 93 ± 20 | 0.44 ± 0.06 | 17 ± 2 | 1.2 ± 0.4 | 39 ± 13 |
| GLAST | 1.0 | 100 | 1.4 ± 0.3 | 112 ± 23 | 0.41 ± 0.07 | 15 ± 3 | 1.2 ± 0.4 | 39 ± 12 |
| NR1 | 1.0 | 100 | 1.3 ± 0.2 | 107 ± 20 | 0.14 ± 0.005 | 5.2 ± 0.1 | 2.3 ± 0.4 | 74 ± 14 |
| GFAP | 1.0 | 100 | 0.41 ± 0.08 | 34 ± 7 | 0.5 ± 0.1 | 19 ± 4 | 0.26 ± 0.07 | 8 ± 2 |
| NF-L | 1.0 | 100 | 0.42 ± 0.09 | 34 ± 7 | 0.23 ± 0.04 | 9 ± 1 | 0.3 ± 0.1 | 10 ± 3 |
| **Cerebellum** | | | | | | | | |
| GLT-1 | 1.0 | 100 | 1.3 ± 0.09 | 100 ± 5 | 0.4 ± 0.3 | 13 ± 8 | 2.7 ± 0.7 | 67 ± 20 |
| GLAST | 1.0 | 100 | 1.4 ± 0.05 | 108 ± 2 | 0.7 ± 0.1 | 22 ± 4 | 2.4 ± 0.6 | 59 ± 17 |
| NR1 | 1.0 | 100 | 0.5 ± 0.2 | 36 ± 14 | 0.4 ± 0.3 | 14 ± 10 | 1.2 ± 0.1 | 37 ± 9 |
| GFAP | 1.0 | 100 | 0.37 ± 0.05 | 28 ± 4 | 0.57 ± 0.08 | 19 ± 2 | 0.18 ± 0.02 | 4.4 ± 0.7 |
| NF-L | 1.0 | 100 | 0.36 ± 0.08 | 27 ± 7 | 0.7 ± 0.2 | 24 ± 6 | 0.09 ± 0.02 | 2.1 ± 0.3 |

*Enrichment and yield are normalized to the levels of the tissue homogenate as described in Section "Materials and Methods." Data are the mean ± SEM of at least three independent measurements.*

but the inhibition data were best fit to two sites with $IC_{50}$ values of 30 nM and 14 μM. The maximal inhibition observed was 70% at 10 μM, and higher concentrations were not tested to avoid effects of solvent on uptake. The reported $IC_{50}$ value for inhibition of GDH is 1.7 μM (28). We also examined the effects of bithionol (BTH), which caused a concentration-dependent inhibition of $Na^+$-dependent glutamate transport activity in crude cortical membranes (P2; **Figure 2C**). The $IC_{50}$ value was 4.1 μM when the data were fit to a single site, but the inhibition data were best fit to two sites with $IC_{50}$ values of 84 nM and 26 μM. The maximal inhibition observed was 60% at 10 μM, and higher concentrations were not tested to avoid effects of solvent on uptake. The reported $IC_{50}$ value for inhibition of GDH is 5.5 μM (28). Together, these studies show that three different inhibitors of GDH also inhibit $Na^+$-dependent glutamate uptake in crude cortical membranes (P2).

One might expect that the effects of inhibition of GDH would increase with pre-incubation. To address this possibility, crude cortical membranes (P2) were pre-incubated with 6.0 μM HCP for up to 15 min prior to initiation of uptake by the addition of L-[$^3$H]Glu. Somewhat surprisingly, the amount of inhibition observed with pre-incubations of 1, 3, 5, 10, and 15 min was not significantly different than that observed with no pre-incubation (**Figure 3**). In these experiments, the crude membranes (P2) were warmed to 37°C for the same amount of time, regardless of the length of the pre-incubation with HCP, to ensure the effects seen were independent of the increased time that the crude membranes (P2) were warmed to 37°C. This shows that the effects of HCP on glutamate uptake are very rapid and essentially instantaneous in this experimental paradigm where uptake is measured for 3 min.

To further characterize the mechanism of action, we examined the effects of HCP on the concentration-dependence for L-[$^3$H]-Glu uptake. As was previously observed by us and others [for review, see (31)], the $K_m$ for L-[$^3$H]-Glu uptake was ∼5 μM and the $V_{max}$ was ∼1.2 nmol/mg/min. HCP had no effect on the $K_m$ value and decreased the $V_{max}$ for L-[$^3$H]-glutamate uptake (**Figure 4**). These data are consistent with a non-competitive mechanism of inhibition of glutamate uptake.

$Na^+$-dependent glutamate uptake into crude cerebellar membranes (P2) displays a dramatically different pharmacology from that observed in crude cortical membranes [P2; (20, 32)]. In fact, the pharmacology of transport in crude cerebellar membranes (P2) is consistent with that of GLAST (24). To determine if the effects of inhibition of GDH are selective for these two different transport activities, we compared the effects of HCP, EGCG, and BTH on $Na^+$-dependent glutamate uptake in crude membranes (P2) prepared from cortex and cerebellum. None of the GDH inhibitors had any effect on $Na^+$-dependent uptake in crude membranes (P2) prepared from cerebellum (**Figure 5A**).

D-Aspartate is transported by the $Na^+$-dependent glutamate transporters (5), but is not a substrate for GDH (33). To determine if the effects of the GDH inhibitors might be related to direct metabolism of the glutamate that moves through the transporter during uptake, we examined the effects of the three different GDH inhibitors on D-[$^3$H]-Asp uptake in crude membranes (P2) prepared from cortex and cerebellum. As was observed with L-[$^3$H]-Glu transport, all three compounds inhibited D-[$^3$H]-Asp uptake in crude cortical membranes (P2), and no effects were observed in cerebellar membranes (P2; **Figure 5B**).

The inhibitory neurotransmitter, γ-aminobutyric acid (GABA), is also cleared by $Na^+$-dependent transport systems. The role of GDH in GABA metabolism is unclear, but it may play a role in the deamination of glutamate formed from the transamination of GABA by GABA-transaminase, also found in mitochondria [for discussions, see (34, 35)]. Therefore, the effects of the three different GDH inhibitors on $Na^+$-dependent [$^3$H]-GABA transport were examined in crude membranes (P2) prepared from cortex and cerebellum. As was observed with L-[$^3$H]-Glu or D-[$^3$H]-Asp,

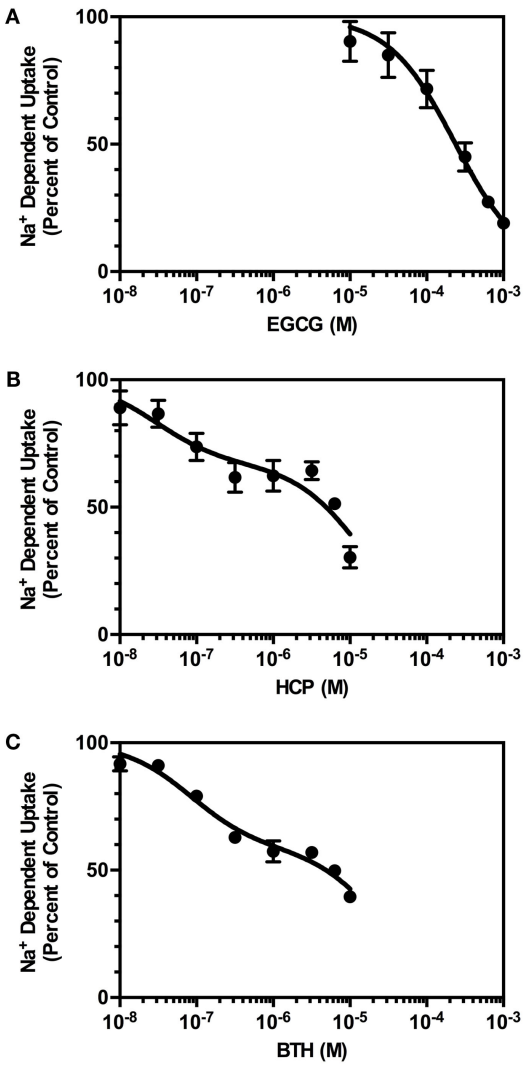


**FIGURE 2 | Concentration-dependence of the effects of GDH inhibitors on of $Na^+$-dependent L-[$^3$H]-glutamate transport into crude membranes (P2) prepared from cortex**. Transport of L-[$^3$H]-glutamate (0.5 μM) was measured in the absence or presence of increasing concentrations of inhibitors, as described in Section "Materials and Methods." The percent of control represents the velocity of transport measured in the presence of inhibitor divided by that observed in the absence of inhibitor multiplied by 100. **(A)** Inhibition by EGCG was fit to a one-site competition model with an $IC_{50}$ value of 234 μM. **(B)** Inhibition by HCP was fit to a two-site competition model. The $IC_{50}$ value for the high affinity component was 30 nM, and the $IC_{50}$ value for the lower affinity component was 14 μM. Based on this fit, 33% of the sites were high affinity and 67% were of lower affinity. **(C)** Inhibition by BTH was fit to a two-site competition model with an $IC_{50}$ for fraction 1 (42%) of 84 nM and an $IC_{50}$ for fraction 2 (58%) of 26 μM. Data are the mean ± SEM of at least three independent measurements (except for incubation of 1 μM BTH, of which there were only two observations).

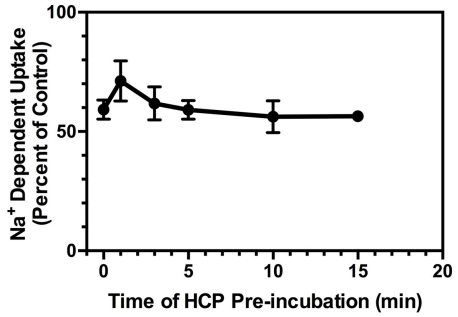


**FIGURE 3 | Effects of pre-incubation with HCP (6 μM) on $Na^+$-dependent L-[$^3$H]-glutamate transport into crude membranes (P2) prepared from cortex**. HCP (or vehicle) was added to crude membranes (P2) at 37°C at 1, 3, 5, 10, or 15 min prior to the initiation of transport with the addition of L-[$^3$H]-glutamate, or simultaneously with L-[$^3$H]-glutamate (time = 0 min). Percent of control represents the velocity of transport in the presence of inhibitor divided by that observed in the absence of inhibitor multiplied by 100. Data are the mean ± SEM of at least three independent measurements.

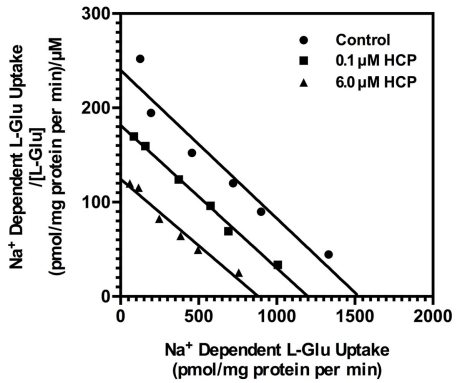


**FIGURE 4 | Eadie–Hofstee plot of the concentration-dependence of L-[$^3$H]-glutamate transport in crude membranes (P2) prepared from cortex with no treatment (control; circle), 0.1 μM HCP (square), or 6.0 μM HCP (triangle)**. Uptake was determined as described in Section "Materials and Methods." Data presented are from a single experiment that has been repeated in four independent experiments with similar results. The $K_m$ values for transport were 5.7 ± 0.3 μM in vehicle control, 5.4 ± 0.4 μM in the presence of 0.1 μM HCP, and 4.2 ± 1.0 μM in the presence of 6.0 μM HCP. The $V_{max}$ values for transport were 1.6 ± 0.4 (nmol/mg protein/min) for vehicle control, 1.2 ± 0.4 in the presence of 0.1 μM HCP, and 0.7 ± 0.1 in the presence of 6.0 μM HCP. The $K_m$ or $V_{max}$ values were normalized to vehicle controls for each experiment (set to 100%) and compared to those observed in the presence of HCP. HCP had no significant effect on $K_m$ value, but significantly reduced the $V_{max}$ value at either 0.1 μM ($p < 0.01$) or 6 μM ($p < 0.001$).

all three GDH inhibitors reduced $Na^+$-dependent [$^3$H]-GABA transport in cortical membranes (P2; **Figure 5C**). However, in contrast to the lack of inhibition observed with L-[$^3$H]-Glu or D-[$^3$H]-Asp, all three compounds also inhibited [$^3$H]-GABA transport in crude cerebellar membranes (P2).

## DISCUSSION

In a recent study, we obtained preliminary evidence that inhibition of GDH might lead to inhibition of $Na^+$-dependent glutamate uptake (19). In the present study, we observed concentration-dependent inhibition of $Na^+$-dependent glutamate uptake in crude membranes (P2) prepared from cortical tissue using three

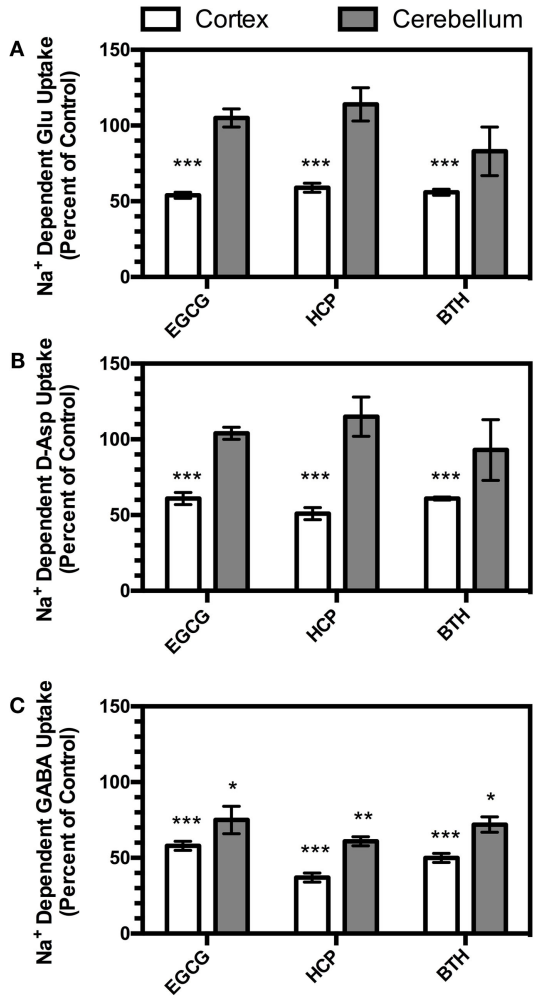


**FIGURE 5 | Effects of EGCG (300 μM), HCP (6 μM), or BTH (3 μM) on $Na^+$-dependent transport of L-glutamate (A), D-aspartate (B), or GABA (C) measured in crude membranes (P2) prepared from cortex (open bars) or cerebellum (shaded bars)**. Transport of all three substrates in both brain regions was measured in parallel experiments with different vehicle controls as described in the Section "Materials and Methods." Data are the mean ± SEM from at least three independent experiments. Data were compared to vehicle controls by ANOVA. *Indicates a $p < 0.05$, ** indicates a $p < 0.01$, and *** indicates a $p < 0.001$.

different inhibitors of GDH. The effects of one of these inhibitors, HCP, were studied further. As pre-incubation did not increase the effects of HCP, inhibition occurs relatively rapidly (within seconds to minutes). HCP had no effect on $K_m$ for glutamate uptake, but reduced $V_{max}$, consistent with a non-competitive mechanism of action. All three compounds also inhibited $Na^+$-dependent D-[$^3$H]-Asp and [$^3$H]-GABA to a similar extent in crude cortical membranes (P2). However, in crude membranes (P2) prepared from cerebellum, these compounds did not affect $Na^+$-dependent L-[$^3$H]-Glu or D-[$^3$H]-Asp, but did inhibit [$^3$H]-GABA transport.

The fact that three different inhibitors of GDH also block $Na^+$-dependent glutamate and GABA uptake suggests that the observed inhibition is likely to be due to an effect on GDH. The potencies of HCP or BTH for inhibition of glutamate uptake are similar to those observed for inhibition of GDH (all in the micromolar range – see Results). One concern is that the concentration of EGCG required to inhibit transport is approximately 500-fold higher than that required to inhibit GDH; one major difference is that the analyses of GDH were conducted with purified enzyme (21). Others have shown that the EGCG is not particularly stable in solution, is subject to active efflux from some cellular systems by ABC cassette proteins, and may be poorly absorbed across membranes (29, 36). Therefore, it is possible that this difference in potency simply reflects the fact that glutamate uptake was measured in an intact system. Based on analyses of crystal structures of GDH, HCP and BTH bind to distinct locations in the interior of the GDH hexamer (30). Based on analyses with epicatechin 3-monogallate (an EGCG analog), it seems that EGCG likely binds to the ADP-activation site, located by the pivot helix of GDH (37). We also considered the possibility that these compounds might block glutamate uptake through a direct interaction with GLT-1, but do not think this is likely for two different reasons. First, the fact that three different inhibitors have the same effect on uptake makes this a less likely explanation. Second, all three inhibitors block sodium-dependent GABA uptake as well, which is mediated by a completely distinct family of transporters that share no homology with the glutamate transporters [for review, see (38)]. Therefore, we suggest that the simplest explanation for the present results is that inhibition of GDH rapidly inhibits $Na^+$-dependent glutamate or GABA uptake.

In the present study, we found that inhibition of GDH had no effect on L-glutamate or D-aspartate transport in crude cerebellar membranes (P2) where GLAST likely mediates uptake (20, 24, 32). Yet, we previously found that EGCG inhibits glutamate uptake in astrocyte cultures (19) where uptake is also mediated by GLAST (22, 23, 39). These measures of transport were conducted in identical buffers; this suggests that the differential effects cannot be attributed to the utilization of different exogenous metabolites to generate ATP. Coupling of GDH to transport may vary depending on the maturation of the system; astrocytes in culture are polygonal, not stellate shaped as is observed *in vivo*, and do not express GLT-1 consistent with an immature phenotype (22, 23). While maturation may explain the difference between astrocytes in culture and crude cerebellar membranes (P2), GDH has been recently deleted from the CNS tissues in mice (40) and also been knocked down in astrocytes in culture (41). Interestingly, the mice with the CNS specific deletion of GDH display no overt behavioral phenotype, and one would predict a seizure phenotype if deletion of GDH resulted in impaired glutamate uptake *in vivo* (42, 43). Therefore, as has been observed with differential metabolism of glutamate in different systems or with increased neuronal activity [for reviews, see (44, 45)], it seems likely that redundant systems may be differentially engaged to support glutamate uptake.

In the present study, we used crude membranes (P2). Previous studies have demonstrated that the pharmacology of glutamate uptake in cortical crude membranes parallels that observed for GLT-1 and is distinct from that observed in cerebellar crude membranes uptake (20, 24, 32). Furthermore, genetic deletion of GLT-1 reduces glutamate uptake in cortical crude membranes to 5% of control (9). These studies provide compelling evidence that uptake

measured in cortical crude membranes is mediated by GLT-1 and that uptake measured in cerebellar crude membranes is mediated by GLAST. We examined the abundance of a neuronal membrane protein, the NR1 subunit of the NMDA receptor, and two glial membrane proteins, GLT-1 and GLAST in this membrane preparation. As was previously observed (25, 26), we found that this fraction contains high levels of neuronal and glial membrane proteins. We also found low levels of the cytoskeletal proteins, GFAP and NF-L. The absence of large amounts of either cytoskeletal protein suggests that the membranes do not uniformly reseal into vesicles and that the resealed vesicles contain only a small fraction of the original cytosol. This conclusion is also supported by the observation that cortical and cerebellar crude membranes contain comparable levels of GLT-1 (**Figure 1**), even though no GLT-1-mediated uptake is detected in cerebellar crude membranes (see above).

GLT-1 expression, when analyzed using immunohistochemical approaches (7, 15, 16), *in situ* hybridization of mRNA [(46, 47), and for reviews, see (4, 5)], or bacterial artificial chromosome GLT-1 promoter reporter mice (48), is essentially restricted to astrocytes. However, there is also evidence that GLT-1 is expressed in some neurons in the CNS (8, 47, 49). The P2 membrane preparation has also been used to examine the relative contributions of the different GABA transporters to uptake. The pharmacology of GABA transport in crude membranes (P2) is consistent with GAT-1 (50, 51), which is thought to be localized predominantly in neurons, but is also found in astrocytes [for review, see (52)]. Although GLT-1 is heavily enriched in astrocytes and GAT-1 is enriched in neurons, it is not possible to determine if the effects of inhibition of GDH are related to inhibition of neuronal or glial pools of this enzyme. This will need to be a topic of further investigation.

Glutamate dehydrogenase is a mitochondrial enzyme that catalyzes the reversible deamination of glutamate to α-ketoglutarate [for reviews, see (53, 54)]. This reaction is accompanied by the production of NADH that can be used to generate ATP; the downstream metabolism of α-ketoglutarate through the tricarboxylic acid cycle will also generate ATP. Cytoplasmic glutamate moves into mitochondria by one of two different families of transporters; neither of these processes transport D-aspartate (55, 56). Furthermore, GDH does not metabolize D-Asp (33). Therefore, although it is theoretically possible that GDH might support inward transport by rapidly metabolizing glutamate and thereby maintaining a concentration gradient, this is unlikely because inhibition of GDH also blocks transport of D-[$^3$H]-Asp. Others have found that synaptosomes enriched from P2 contain relatively high levels of endogenous glutamate (57); therefore, it seems certain that the crude membranes (P2) used in the present study contain endogenous glutamate. Combined with the fact that these inhibitors also block GABA transport, these observations suggest that mitochondrial GDH provides energy for transport using endogenous glutamate to drive the $Na^+$-dependent transport systems.

Several studies have demonstrated that inhibition of mitochondrial function impairs glutamate uptake. For example, the mitochondrial poisons, sodium azide, dinitrophenol, and antimycin A, inhibit glutamate uptake in cultured astrocytes (58). In addition, $MPP^+$ and rotenone, inhibitors of complex 1 of the electron transport chain, inhibit glutamate clearance in both astrocytes and crude cerebral synaptosomes (59, 60). Opening mitochondrial $K_{ATP}$ channels can functionally support glutamate uptake in the presence of $MPP^+$ (61). There is also evidence that glutamate transport couples to ionic changes in mitochondria. In fact, uptake is accompanied by an increase in mitochondrial $Na^+$ (62). Uptake is also accompanied by acidification of mitochondria (63). This may be dependent upon the mitochondrial glutamate–aspartate exchanger, Aralar, which co-transports a $H^+$ with glutamate (55), or the mitochondrial glutamate carrier, which transports glutamate with a $H^+$ or in exchange for a hydroxyl ion (56). The fact that inhibitors of GDH block glutamate uptake provides further support for a functional interaction between transporters and mitochondria.

Epigallocatechin-monogallate is considered the most active of the green tea polyphenols, the likely active ingredients in green tea; the effects of EGCG on biological/pathological processes have been examined in numerous *in vitro* and *in vivo* studies. EGCG is of particular interest in the treatment of Alzheimer's and Parkinson's diseases because it exhibits neuroprotective effects such as radical scavenging, iron chelating, activation of PKC, and anti-apoptotic action [for review, see (64)]. *In vivo* studies have demonstrated the neuroprotective properties of EGCG. For example, these polyphenols improve spatial learning in aged rodents (65), they suppress peripheral nerve degeneration associated with sciatic nerve crush (66), and EGCG protects against hippocampal neuronal damage after global ischemia (67). *In vitro*, EGCG is protective at low concentrations, but at concentrations similar to those used in the present study, EGCG causes cell death in a neuroblastoma cell line (68). EGCG increases the amount of glutamate observed in the effluent upon depolarization of synaptosomes (69). In pancreatic β-cells, EGCG inhibits the increase in insulin secretion and glutamine oxidation caused by the stimulation of GDH (21). In most of these examples, it is unclear if the observed effects are related to inhibition of GDH or to the anti-oxidant activity of EGCG. Inhibition of GDH and glutamate uptake might contribute to some of the observed *in vivo* effects, but this will need to be resolved in future studies.

In summary, we show evidence that inhibition of the mitochondrial enzyme, GDH, can result in inhibition of $Na^+$-dependent L-glutamate or D-aspartate uptake in mammalian cortex. It seems likely that this dependence on GDH is affected by factors that are yet to be defined.

## ACKNOWLEDGMENTS

This work was supported by NIH R01 NS 077774 and P30 HD 06979. We are also grateful to Drs. Thomas Smith, Charlie Stanley, and Marc Yudkoff for their helpful comments and advice throughout this study. We would like to thank Elizabeth Krizman for her help teaching Brendan S. Whitelaw the transport assays and her help with the analyses of the subcellular fractionation. We would like to thank Dr. Virginia Lee and Dr. Jeffery D. Rothstein for their generous provision of the anti-GFAP antibody and the anti-GLT-1 antibody, respectively.

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**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

*Received: 22 May 2013; paper pending published: 05 July 2013; accepted: 30 August 2013; published online: 17 September 2013.*
*Citation: Whitelaw BS and Robinson MB (2013) Inhibitors of glutamate dehydrogenase block sodium-dependent glutamate uptake in rat brain membranes. Front. Endocrinol. **4**:123. doi: 10.3389/fendo.2013.00123*
*This article was submitted to Cellular Endocrinology, a section of the journal Frontiers in Endocrinology.*

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ENDOCRINOLOGY

MINI REVIEW ARTICLE
published: 16 December 2013
doi: 10.3389/fendo.2013.00191

# Glutamate pays its own way in astrocytes

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*In vitro* and *in vivo* studies have shown that glutamate can be oxidized for energy by brain astrocytes. The ability to harvest the energy from glutamate provides astrocytes with a mechanism to offset the high ATP cost of the uptake of glutamate from the synaptic cleft. This brief review focuses on oxidative metabolism of glutamate by astrocytes, the specific pathways involved in the complete oxidation of glutamate and the energy provided by each reaction.

**Keywords: glutamate, astrocytes, oxidative metabolism, glutamate dehydrogenase, pyruvate recycling pathway**

## INTRODUCTION

One of the most essential roles of astrocytes in brain is removal of the neurotransmitter glutamate from the synaptic cleft as it is crucial that a low resting glutamate concentration of ∼1–10 μM be maintained for continued glutamatergic neurotransmission and brain function (1–3). Astrocytes perform this key function by rapidly and efficiently removing glutamate which increases orders of magnitude in concentration to ∼100 μM–1 mM after depolarization of neurons (4, 5). Uptake of glutamate is a very expensive proposition since the astrocyte transporters that mediate glutamate transport 3 $Na^+$ molecules which must be exported by the enzyme $Na^+$, $K^+$-ATPase. Thus the uptake of one molecule of glutamate by an astrocyte requires the expenditure of one molecule of ATP (1). To pay the high cost of removing large amounts of glutamate from glutamatergic synapses, astrocytes must form large amounts of ATP from the metabolism of glucose or other substrates (see **Figure 1A**; **Table 1**). About 30% of the oxidative metabolism in brain *in vivo* takes place in astrocytes (6–9); however, it is not likely that these cells oxidize sufficient glucose to generate the ATP required for the transport of such massive amounts of glutamate (10–16). A number of groups have shown that astrocytes have a sufficiently high rate of glutamate oxidative metabolism to pay the high cost of glutamate uptake (11, 17, 18). This short review summarizes the information on the use of glutamate in astrocytes and recent evidence on the role of protein complexes in facilitating glutamate metabolism (19, 20). The goal of this paper is to provide a short, very concise, and focused review, and to point readers to many excellent more in depth manuscripts recently published (2, 3, 6, 16, 21).

## WHAT IS THE EVIDENCE THAT GLUTAMATE IS METABOLIZED BY ASTROCYTES?

It is well established that astrocytes can oxidize glucose and other substrates for energy including lactate, glutamate, glutamine, fatty acids, and the ketone bodies 3-hydroxybutyrate and acetoacetate (12, 17, 22–30). These substrate are actively oxidized for energy; however, glutamate is oxidized by astrocytes at a rate much higher than the other substrates. The oxidation of glutamate by astrocytes was initially determined with studies using radiolabeled $^{14}$C-glutamate (12, 30–32). However, the more recent use of $^{13}$C-glutamate and $^{13}$C-NMR spectroscopy has provided more complete information about the metabolic fate of glutamate in astrocytes. Sonnewald et al. (18) first reported that more of the label from glutamate metabolism was incorporated into lactate by astrocytes than was converted to glutamine. This key finding was initially considered controversial as it underscored that the glutamate-glutamine cycle is not stoichiometric since only a portion of the glutamate taken up by astrocytes was converted to glutamine. A key study by the McKenna and Sonnewald (29) groups demonstrated that when the exogenous glutamate concentration was increased from 0.1 to 0.5 mM the proportion of glutamate oxidized by the TCA cycle in astrocytes greatly increased and the percent converted to glutamine decreased. Reports from many groups clearly demonstrate (17, 29, 30, 33) that astrocytes have the capability to oxidize the concentrations of glutamate present in the synaptic cleft after depolarization of neurons (100 μM–1 mM) (4). Hertz and Hertz (17) noted that glutamate oxidation by astrocytes is as high as the anaplerotic rate of glutamate production suggesting that synthesis must be balanced by catabolism as glutamate does not readily exit the brain. A recent report by our group (11) showed that glutamate was oxidized by astrocytes at a rate higher than glucose, 3-hydroxybutyrate, glutamine, lactate, or malate, and that none of the other substrates could effectively decrease the oxidative metabolism of glutamate.

Data from several different types of studies provide evidence that suggests or demonstrates that glutamate oxidation occurs in astrocytes *in vivo*. These include *in vivo* microdialysis studies demonstrating oxidation of glutamate in the hippocampus of

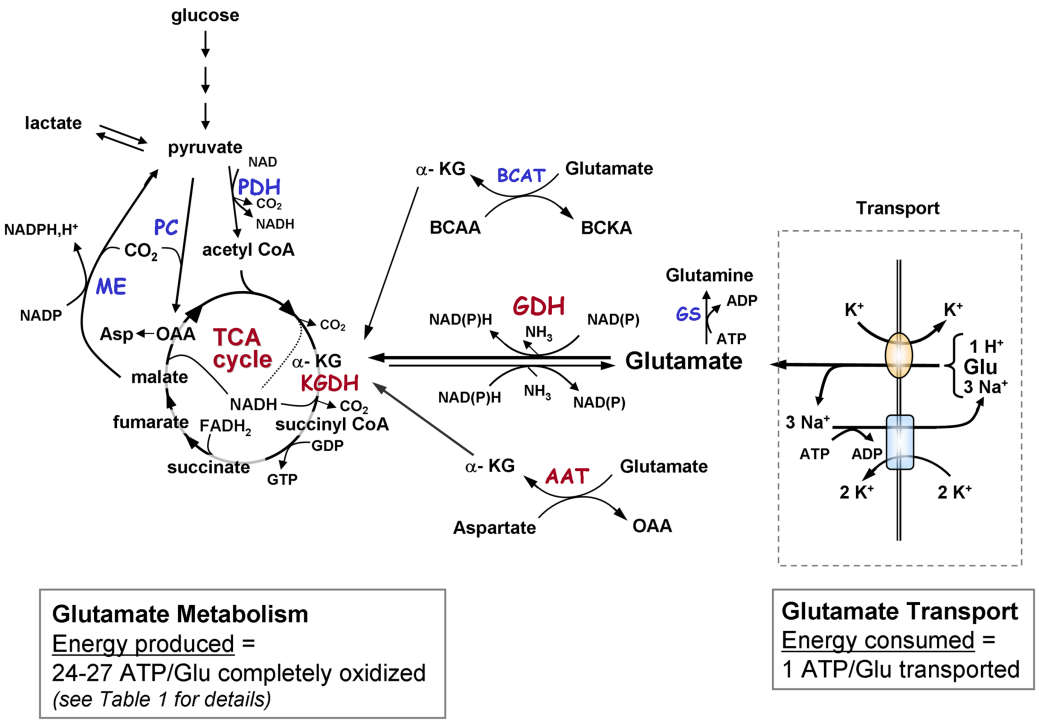


**FIGURE 1A | Schematic diagram indicating the pathways of metabolism of *exogenous* glutamate taken up by astrocytes**. The portion of the figure outlined with a dashed line shows transport of glutamate from the extracellular milieu. The oval depicts the astrocytic glutamate transporter GLT1/EAAT2 or GLAST; rectangle depicts $Na^+$, $K^+$-ATPase. Astrocytic glutamate can be converted to α-ketoglutarate by the energy producing enzyme glutamate dehydrogenase or via one of the transaminase enzymes (primarily via aspartate aminotransferase but also by BCAT and ALAT). α-Ketoglutarate formed from glutamate is metabolized via a partial TCA cycle to either malate or oxaloacetate. For the *complete oxidation* of glutamate to occur malate (or OAA) must leave the TCA cycle, be converted to pyruvate by malic enzyme, and then converted to acetyl CoA by pyruvate dehydrogenase. The acetyl CoA formed by these pyruvate recycling reactions re-enters the TCA cycle and is subsequently oxidized for energy. The dotted in the TCA cycle indicates that the NADH generated from the isocitrate dehydrogenase reaction would only be formed when the acetyl CoA formed from the pyruvate recycling pathway is metabolized via the TCA cycle because the initial entry of carbons α-KG at is subsequent to this step. The complete oxidation of one glutamate molecule can yield 24–27 ATP depending on whether the initial step in metabolism is via GDH or AAT. The ATP is estimated at ∼20 since the maximum theoretical yield of ATP is never recovered due to mitochondrial proton leak. [See **Table 1** for reaction details and **(B)** for labeling pattern of metabolites labeled from the initial metabolism of [U-$^{13}$C]glutamate and the labeling pattern from subsequent metabolism via the pyruvate recycling pathway and re-entry into the TCA cycle]. Abbreviations: Glu, glutamate; α-KG, α-ketoglutarate OAA, oxaloacetate; Asp, aspartate; GDH, glutamate dehydrogenase; AAT aspartate aminotransferase; BCAT, branched-chain aminotransferase; ALAT, alanine aminotransferase; KGDH, α-ketoglutarate dehydrogenase; ME, malic enzyme; PDH, pyruvate dehydrogenase; GS, glutamine synthetase. BCAA, branched chain amino acid; BCKA, branched chain ketoacid.

freely moving rats (34, 35), evidence from several groups documenting that the fine processes of astrocytes enveloping synaptic terminals contain abundant mitochondria (6) (and Tibor Kristian, unpublished), and transcriptome studies on astrocytes isolated from brain of adult rodents that document very high levels of transcripts for glutamate dehydrogenase (GDH) and for enzymes of the TCA cycle (6).

## OXIDATION OF THE CARBON SKELETON OF GLUTAMATE OFFSETS THE COST OF GLUTAMATE UPTAKE

Glutamate taken up by astrocytes can be converted to α-ketoglutarate by two reactions, either by transamination reactions or by the energy producing reaction of the enzyme GDH which is enriched in astrocytes (3, 6, 36, 37). Transamination occurs primarily by aspartate aminotransferase (AAT), but also readily takes place via either branched-chain amino acid aminotransferase (BCAT) or alanine aminotransferase (ALAT) (3, 38–40). Studies from our group and others demonstrate that the oxidative metabolism of *exogenous* glutamate taken up from the extracellular milieu proceeds *primarily* via GDH in astrocytes from rat brain [since it is relatively unaffected by the transaminase inhibitor aminooxyacetic acid, AOAA] (30, 41). The α-ketoglutarate formed from glutamate is metabolized for energy in the sequential reactions of the TCA cycle to the four carbon compound oxaloacetate (**Figures 1A,B**) and yielding the equivalent of nine ATP molecules in this process.

## THE COMPLETE OXIDATION OF GLUTAMATE REQUIRES METABOLISM OF PART OF THE CARBON SKELETON VIA THE PYRUVATE RECYCLING PATHWAY

Studies using $^{13}$C-NMR spectroscopy have provided key insights into the metabolic fate of glutamate in astrocytes and information

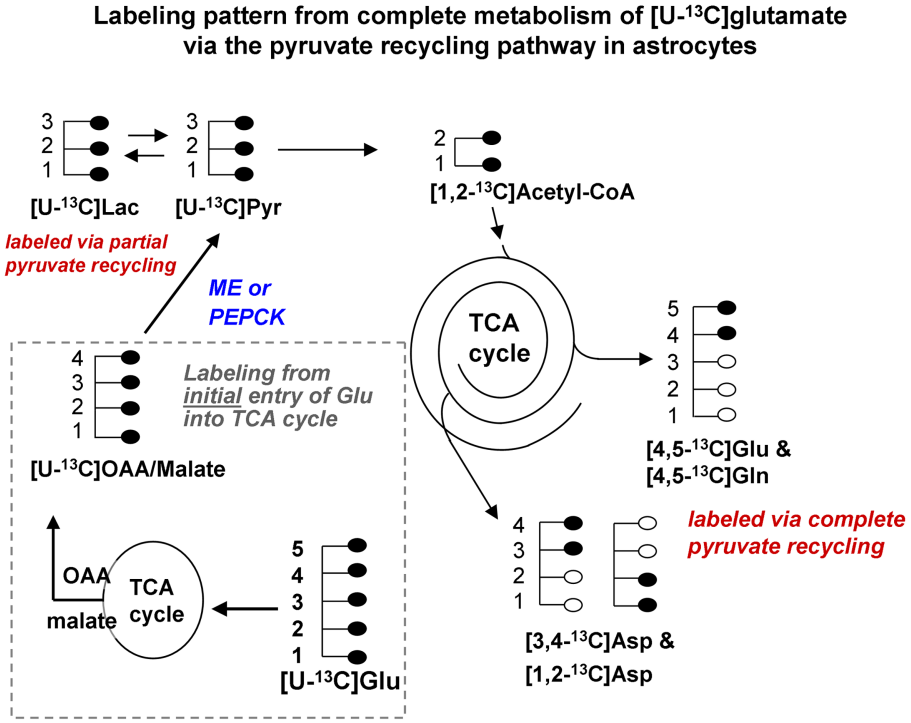


**FIGURE 1B | Labeling pattern from the initial metabolism of [U-$^{13}$C]glutamate and the labeling pattern from the complete oxidation of these glutamate carbons via the pyruvate recycling pathway and re-entry into the TCA cycle.** Labeling from the initial entry of [U-$^{13}$C]glutamate into the TCA cycle is shown inside the dotted line. Note that metabolites generated are also uniformly labeled in all carbons. [Note that any glutamine formed in the cytosol directly from [U-$^{13}$C]glutamate would also be uniformly labeled [U-$^{13}$C]glutamine; *reaction not shown in this figure*]. [U-$^{13}$C]malate or OAA leaving the TCA cycle would be converted to [U-$^{13}$C]pyruvate by malic enzyme or the combined action of PEPCK and pyruvate kinase, and would also give rise to [U-$^{13}$C]lactate. The [U-$^{13}$C]pyruvate can be converted to [1,2-$^{13}$C]acetyl CoA which enters the TCA cycle for further oxidation. Any citrate formed from the condensation of the [1,2-$^{13}$C]acetyl CoA with *unlabeled* oxaloacetate in the cycle would give rise to [4,5-$^{13}$C]glutamate and glutamine, and also to both [1,2-$^{13}$C]aspartate and [3,4-$^{13}$C]aspartate. These partially labeled glutamate, glutamine, and aspartate molecules can be readily distinguished from the [U-$^{13}$C]glutamate, glutamine, and aspartate by $^{13}$C-NMR spectroscopy. It is very likely that studies of pyruvate recycling from [U-$^{13}$C]glutamate underestimate the amount of recycling taking place since any citrate formed from the condensation of [1,2-$^{13}$C]acetyl CoA with [U-$^{13}$C]OAA formed from the initial entry and metabolism of the glutamate into the TCA cycle give rise to [U-$^{13}$C]glutamate which can not be distinguished from the precursor. Abbreviations: Glu, glutamate; Gln, glutamine; OAA, oxaloacetate; Asp, aspartate; Lac, lactate; Pyr, pyruvate; ME, malic enzyme; PEPCK, phosphoenolpyruvate carboxykinase.

about the compartmentation of glutamate metabolism (28, 29, 42–44). Several groups have shown that the carbon skeleton of glutamate can enter the TCA cycle leading to labeling in aspartate and lactate (28, 29, 42–44). The incorporation of label from [U-$^{13}$C]glutamate into [U-$^{13}$C]lactate (see **Figure 1B**) and also into [1,2-$^{13}$C]glutamate and glutamine and specifically labeled molecules of aspartate confirms that the carbon skeleton of glutamate can be metabolized via the pyruvate recycling pathway in astrocytes and reenter the TCA cycle. Thus, all carbons of the glutamate molecule can be completely oxidized for energy via the TCA cycle and pyruvate recycling pathway (45) (see **Figure 1B**).

## OTHER SUBSTRATES CAN FACILITATE THE UPTAKE AND OXIDATIVE METABOLISM OF GLUTAMATE BY ASTROCYTES

The high rate of glutamate oxidation reported by several groups is consistent with the earlier findings of Hertz and Hertz (17) demonstrating that 100 μM glutamate supported $O_2$ uptake by astrocytes as effectively as 7.5 mM glucose. Hertz also demonstrated that $O_2$ uptake and respiration was significantly higher with the combination of glutamate + glucose than with either substrate alone (17). Data from several studies suggests that glucose may facilitate the uptake and oxidation of glutamate by astrocytes. McKenna et al. (30) showed that the presence of 1 mM pyruvate increased the rate glutamate oxidation by astrocytes, possibly by increasing transamination to α-ketoglutarate and metabolism via the TCA cycle in the presence of pyruvate. However, we did not find any effect of glucose or lactate on rate of $^{14}CO_2$ production from [U-$^{14}$C]glutamate in a recent study (11). In contrast, studies by some groups showed that glutamate uptake stimulated glycolysis in astrocytes; however, this has not been found by all groups (46, 47).

## OXIDATION OF GLUTAMATE FOR ENERGY SPARES GLUCOSE AND OTHER SUBSTRATES

Substrate competition studies recently reported by our group demonstrated the robustness of glutamate use by astrocytes as none of the other substrates added, including glucose, had the

**Table 1 | Energy produced in astrocytes from oxidation of one glutamate molecule in the TCA cycle and oxidation via the pyruvate recycling pathway.**

| | | | |
|---|---|---|---|
| **Energy required for uptake of one molecule of glutamate** | | | |
| **Uptake by EAAT2 (GLT1) or GLAST** | | **Rebalancing ions** | **ATP used** |
| 1 Glutamate + 3 $Na^+$ + 2 $K^+$ + 1 $H^+$ taken up | → | 3 $Na^+$ extruded | 1 |
| **Energy provided by complete oxidation of one molecule of glutamate** | | | |
| **Reaction** | | **ATP equivalents** | **(theoretical maximum)** |
| **GDH (glutamate dehydrogenase) reaction** | | | |
| Glutamate → α-ketoglutarate | | NADPH | 3 |
| **ATP equivalents from one Glu metabolized via GDH rxn** | | | **3** |
| *or* ATP equivalents from AAT rxn | | | 0 |
| **TCA cycle reactions** | | | |
| α-Ketoglutarate → succinyl CoA | | NADH | 3 |
| Succinyl CoA → succinate | | GTP | 1 |
| Succinate → fumarate | | FADH2 | 2 |
| Fumarate → malate | | | 0 |
| **ATP equivalents produced from one Glu metabolized via partial TCA cycle to malate** | | | **6** |
| **Malate can stay in the TCA cycle or be metabolized via pyruvate recycling pathway:** | | | |
| (calculations below assume that carbons from one Glu is metabolized via pyruvate recycling) | | | |
| Carbons from one Glu staying in TCA cycle (rather than pyruvate recycling) | | | |
| Malate → oxaloacetate | | NADH | **3**** |
| **Carbons from one Glu metabolized via the pyruvate recycling pathway** | | | |
| **Pyruvate recycling pathway** | | | |
| Malate → pyruvate | | NADPH | 3 |
| Pyruvate → acetyl CoA | | NADH | 3 |
| *From acetyl moiety derived from one glutamate re-entering TCA cycle* | | | |
| Acetyl CoA → oxaloacetate (one complete turn of TCA cycle) | | 3 NADH | 9 |
| | | 1 $FADH_2$ | 2 |
| | | 1 GTP | 1 |
| **ATP equivalents from pyruvate recycling and oxidation of acetyl moiety** | | | **18**** |
| **Total ATP from complete oxidation of one molecule of exogenous glutamate via the TCA cycle and pyruvate recycling pathway** | | | **24–27*** |
| **NET energy yield from uptake and oxidation of one glutamate molecules** | | | **23–26***** |

**Values with asterisks includes the ATP generated from the reoxidation of NADPH formed during conversion of glutamate to α-ketoglutarate in the reaction catalyzed by glutamate dehydrogenase (GDH).*

***Note that when the carbon skeleton from metabolism of glutamate leaves the TCA cycle as malate to proceed through the pyruvate recycling pathway, then NADH will not be formed by malate dehydrogenase (MDH) which converts malate → OAA. However, with multiple molecules of glutamate entering oxidative metabolic pathways in astrocytes some of the glutamate would be converted to OAA and be used for formation of citrate and producing NADH at the MDH step. Note that if a glutamate molecule stays in the TCA cycle 9–12 molecules of ATP would be produced which is less than when it is metabolized via the pyruvate recycling pathway but still considerably more than the ATP required for glutamate transport.*

****The total ATP generated would be 27 if glutamate → α-ketoglutarate proceeds via the GDH reaction, and only 24 if it proceeds via AAT. The ATP values are estimates as noted in **Figure 1A** since the maximum theoretical yield of ATP is never recovered due to the mitochondrial proton leak.*

ability to decrease the oxidation of glutamate (11). Hertz and Hertz (17) found higher respiration and $O_2$ consumption when astrocytes were incubated in the presence of glutamate plus glucose and that the addition of glutamate spared glucose consumption. Earlier studies by Peng et al. (48) showed that added glutamate decreased the rate of glucose oxidation in astrocytes by 75%.

## ASTROCYTE GLUTAMATE TRANSPORTERS AND MITOCHONDRIAL PROTEINS FORM COMPLEXES THAT FACILITATE OXIDATION OF GLUTAMATE FOR ENERGY IN ASTROCYTES

Recent reports from the Robinson lab (19, 20) demonstrate that glutamate uptake by astrocytes is tightly associated with a multi

protein complex which includes the glial glutamate transporters, hexokinase and mitochondrial proteins suggesting that there is a mechanism in astrocytes that insures that a portion of the glutamate taken up is selectively delivered to mitochondria for oxidative energy metabolism. They demonstrated that the astrocyte glutamate transporter GLT1 can co-compartmentalize with hexokinase, other glycolytic enzymes, GDH, and mitochondria (20, 49). A report from the Robinson group in this special issue (49) suggesting that the enzyme GDH associates with the astrocyte glutamate transporters strengthens the evidence for the formation of a protein complex to facilitate the mitochondrial oxidation of glutamate.

Overall, there is compelling data from both *in vitro* and *in vivo* studies that oxidative metabolism of glutamate occurs in astrocytes and provides sufficient energy to pay for the cost of glutamate uptake from the synaptic cleft.

## ACKNOWLEDGMENTS

This work was supported in part by NIH grant HD016596. I thank Dr. Leif Hertz and Dr. Tiago Rodrigues for encouraging me to submit this manuscript and Dr. Susanna Scafidi for her advice regarding **Table 1**.

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**Conflict of Interest Statement:** The author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

*Received: 16 October 2013; accepted: 27 November 2013; published online: 16 December 2013.*
*Citation: McKenna MC (2013) Glutamate pays its own way in astrocytes. Front. Endocrinol. **4**:191. doi: 10.3389/fendo.2013.00191*
*This article was submitted to Cellular Endocrinology, a section of the journal Frontiers in Endocrinology.*