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Arne Schousboe
Ursula Sonnewald *Editors*

The Glutamate/GABA-Glutamine Cycle

Amino Acid Neurotransmitter Homeostasis

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Chapter 1
Introduction to the Glutamate–Glutamine Cycle

Ursula Sonnewald and Arne Schousboe

Abstract The term 'glutamate–glutamine cycle' was coined several decades ago based on the observation that using certain ^{14}C-labeled precursors for studies of brain metabolism the specific radioactivity of glutamine generated from glutamate was higher than that of glutamate, its immediate precursor. This is metabolically impossible unless it is assumed that at least two distinct pools of these amino acids exist. This combined with the finding that the enzyme synthesizing glutamine from glutamate was expressed in astrocytes but not in neurons formed the basis of the notion that a cycle must exist in which glutamate released from neurons is transported into astrocytes, converted to glutamine which is subsequently returned to neurons and converted to glutamate by an enzyme the activity of which is much higher in neurons than in astrocytes. Originally this cycle was supposed to function in a stoichiometric fashion but more recent research has seriously questioned this.

This volume of Advances in Neurobiology is intended to provide a detailed discussion of recent developments in research aimed at delineating the functional roles of the cycle taking into account that in order for this system to work there must be a tight coupling between metabolism of glutamate in astrocytes, transfer of glutamine to neurons and de novo synthesis of glutamine in astrocytes. To understand this, knowledge about the activity and regulation of the enzymes and transporters involved in these processes is required and as can be seen from the table of contents these issues will be dealt with in detail in the individual chapters of the book.

Keywords Glutamate • Glutamine • Transport • Synthesis metabolism

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1.1 Introduction

The concept of a mechanism in the brain which could handle the large amounts of glutamate being cycled as a neurotransmitter was fostered by biochemical investigations of glutamate and glutamine metabolism in different brain tissue preparations using radioactively labelled ([^{14}C]) precursors. Depending on the precursor used, the specific radioactivity of these two amino acids was found to follow the predicted substrate–product relationship, i.e. the precursor (glutamate) exhibiting the highest specific radioactivity or the opposite, i.e. the highest specific radioactivity was seen in the product, glutamine (for references, see Berl and Clarke (1969) and Schousboe (2012)). The latter result could not be easily explained unless the assumption was made that at least two compartmentalized pools of the precursor glutamate must exist (Garfinkel 1966; Berl et al. 1968; van den Berg and Garfinkel 1971). This combined with the seminal observation that the enzyme responsible for glutamine synthesis, glutamine synthetase (GS) is selectively expressed in astrocytes not neurons (Norenberg and Martinez-Hernandez 1979) led to the concept of the glutamate–glutamine cycle in which glutamate from glutamatergic neurons is taken up into astrocytes and subsequently returned to the neurons in the form of glutamine.

1.2 Release and Uptake of Glutamate

Due to its high concentration in the brain it was not easy to accept that glutamate could function as an excitatory neurotransmitter. Even though it was demonstrated that glutamate exerts a depolarizing action on spinal cord neurons, its legitimate status as a neurotransmitter was not immediately accepted since the depolarizing action could be mimicked by other amino acids as well (Curtis et al. 1959). However, the discovery of specific agonists and antagonists of glutamate receptors finally allowed the firm conclusion that glutamate is a *bona fide* neurotransmitter (Watkins and Evans 1981). Moreover, clearly the release of glutamate from synaptic terminals and glutamatergic neurons in culture fulfils the criteria for a neurotransmitter (Nicholls 1989; Ehrhart-Bornstein et al. 1991). In addition, in keeping with the status as a neurotransmitter there is a specific mechanism operating for inactivation of the receptor-mediated excitatory action of glutamate since extremely efficient high affinity transporters for glutamate have been identified (Schousboe 1981; Danbolt 2001), transporters which have been shown to be preferentially expressed in astrocytes (Gegelashvili and Schousboe 1997; Danbolt 2001).

The purification of a glutamate transporter from a rat brain synaptosomal preparation (Danbolt et al. 1990) paved the way for cloning of altogether five different high affinity glutamate transporters, GLAST/EAAT-1, GLT1/EAAT-2, EAAC-1/EAAT-3, EAAT-4 and EAAT-5 (Storck et al. 1992; Pines et al. 1992; Kanai and Hediger 1992; Fairman et al. 1995; Arriza et al. 1997) of which the former two, that were found to be localized primarily in astrocytes, account for the majority of the

glutamate transport capacity in the brain (Danbolt 2001). As these transporters clearly are of fundamental functional importance not only as direct partners in the regulation of excitatory neurotransmission but also in prevention of the excitotoxic actions of elevated extracellular concentrations of glutamate in the brain (Choi 1988), it is not surprising that their expression level is tightly regulated. This was first demonstrated by the observation that neuronally secreted factors were able to increase the expression of the high affinity glutamate transporter activity in cultured astrocytes (Drejer et al. 1983). This finding was subsequently strengthened by the demonstration that exposure of cultured astrocytes to either conditioned medium from neuronal cultures or by direct contact with neurons resulted in a significant increase in the expression of GLT-1, a transporter not normally expressed to any significant extent in cultured astrocytes (Gegelashvili et al. 1997; Swanson et al. 1997; Schlag et al. 1998). It may also be of interest that other factors such as arachidonic acid, pH and the redox state seem to affect the expression level and activity of astrocytic glutamate transporters (Levy 2002).

The functional activity of the glutamate transporters depends on an intact cellular sodium gradient and hence, the operation of the Na^+–K^+–ATPase (Danbolt 2001). It is therefore not surprising that the transporters have been shown to be tightly coupled to the ATPase in the plasma membrane (Cholet et al. 2002; Rose et al. 2009; Bauer et al. 2012; Matos et al. 2013; Roberts et al. 2014; Illarionova et al. 2014). As the sodium-dependent transport obviously requires chemical energy in the form of ATP it is of considerable interest that the astrocytic glutamate transporters have been shown to be closely associated with both glycolytic enzymes and mitochondria (Genda et al. 2011; Bauer et al. 2012; Jackson et al. 2015). It seems of particular importance that there is a correlation between glutamate transporter activity and the mobility of mitochondria in the fine astrocytic processes (Jackson et al. 2014). Thus, an increase in neuronal activity and glutamate uptake leads to arrest of mitochondrial mobility anchoring the mitochondria in close proximity to the transporters (Jackson et al. 2014).

1.3 Metabolism of Glutamate in Astrocytes

As described earlier, extracellular glutamate is very efficiently taken up into astrocytes via transporters and the glutamate content inside the astrocytes increases with increasing extracellular glutamate concentrations (McKenna et al. 1996). It is well established that astrocytes metabolize glutamate taken up from the extracellular milieu to glutamine (Waniewski and Martin 1986; Zielke et al. 1990; Farinelli and Nicklas 1992) but additionally glutamate can be oxidatively metabolized (Yu et al. 1982; McKenna et al. 1996). This conversion of extracellular glutamate to glutamine is a part of the so-called glutamate–glutamine cycle (Hertz 1979).

There are conflicting results regarding the relative amount of glutamate metabolized via the TCA cycle versus the amount directly converted to glutamine (Yu et al. 1982; Yudkoff et al. 1986; Waniewski and Martin 1986; Zielke et al. 1990;

Sonnewald et al. 1993). McKenna et al. (1996) showed an increase in the % of glutamate metabolized via the TCA cycle as compared to the amount converted to glutamine directly. At extracellular glutamate concentrations of 0.01, 0.05, 0.25 and 0.5 mM it has been shown by several investigators (Waniewski and Martin 1986; Yu et al. 1982; Yudkoff et al. 1986; Sonnewald et al. 1993; McKenna et al. 1996) that a significant amount of glutamate is converted to α-ketoglutarate but at concentrations of 0.01 mM in the presence of 10 % fetal calf serum (Zielke et al. 1990) or 0.05 mM in the presence of 1 mM pyruvate (Farinelli and Nicklas 1992), glutamate appears to be almost exclusively converted to glutamine.

In order for exogenous glutamate to be metabolized in the TCA cycle it must be converted to α-ketoglutarate a process which can occur as a transamination or a deamination. Studies of glutamate metabolism in astrocytes (Farinelli and Nicklas 1992; Sonnewald et al. 1993; Waniewski and Martin 1986; Yu et al. 1982; Yudkoff et al. 1986; Zielke et al. 1990) have yielded somewhat conflicting results regarding the significance and mechanism of oxidation through the TCA cycle. The significance of transamination can be probed by using aminooxyacetic acid (AOAA), a non-specific inhibitor of pyridoxal phosphate-dependent enzymes (Yu et al. 1982). AOAA inhibits the major transaminases involved in glutamate metabolism: aspartate aminotransferase (AAT) and alanine aminotransferase (ALAAT), both in the cytosol and in mitochondria (Kauppinen et al. 1987; Cooper 1988). To establish the significance of transamination for glutamate metabolism in astrocytes, cells were incubated with [U-^{13}C]glutamate in the presence or absence of AOAA. The ^{13}C labelling pattern in compounds present in extracts of the cells and the incubation media were subsequently determined using ^{13}C magnetic resonance spectroscopy. Since glutamate was uniformly labelled, detection of incorporation of label into metabolites is unambiguously due to ^{13}C-^{13}C-spin coupling patterns. Biochemical methods were used to quantify ammonia, lactate, amino acids and citrate. After blocking transamination of glutamate with AOAA, entry into the TCA cycle of the carbon units from glutamate was still taking place since lactate and citrate labelling with ^{13}C were observed. Glutamate and glutamine synthesis from α-ketoglutarate could, however, not be detected under this condition showing the importance of transamination for glutamate formation from α-ketoglutarate.

As described earlier, a considerable proportion of the glutamate taken up by astrocytes is metabolized via the TCA cycle (Yu et al. 1982; Waniewski and Martin 1986; Schousboe et al. 1993a, b; Bachelard et al. 1994; McKenna et al. 1996) and converted to TCA cycle-related products, including lactate. Sonnewald et al. (1993) have demonstrated using ^{13}C magnetic resonance spectroscopy that the carbon skeleton of extracellular [U-^{13}C]glutamate is converted to lactate to a large extent. In order to accomplish conversion of glutamate to lactate, glutamate must obviously enter the TCA cycle for conversion to metabolites like malate, oxaloacetate, citrate, α-ketoglutarate and aspartate, which can subsequently leave the mitochondria. Citrate leaving the mitochondria may give rise to oxaloacetate in the cytoplasm and subsequently to pyruvate *via* the activity of malic enzyme or by the concerted action of phosphoenolpyruvate carboxykinase and pyruvate kinase. Subsequently, pyruvate may be metabolized to lactate, alanine or acetyl-CoA. The release of lactate by

astrocytes has been demonstrated by several groups (Walz and Mukerji 1988; Larrabee 1992; Sonnewald et al. 1993) and considerable evidence exists that lactate is an effective substrate for maintaining energy metabolism in synaptic terminals (Schurr et al. 1988; McKenna et al. 1993, 1994).

As is apparent from the results presented earlier, glutamate metabolism is highly regulated and in addition, there is evidence of the existence of several glutamate pools in astrocytes: one pool, with low capacity, in which glutamine formation and some transamination is taking place and another pool with large capacity, in which detoxification of large amounts of glutamate taken up from the extracellular space might take place. This latter pool allows the use of glutamate as an energy substrate via the TCA cycle. This pool has a low requirement for α-keto acids, since deamination does not require them, a clear advantage under conditions where these substances may not be present in adequate amounts (Yudkoff et al. 1994a, b). The above-mentioned results confirm earlier reports of compartmentation of glutamate metabolism in which it was shown that extracellular, radioactively labelled glutamine gave rise to a higher specific labelling of intracellular glutamate than glutamine and that aspartate formation was different when extracellular glutamate or glutamine was used as precursors (Schousboe et al. 1993a, b).

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Chapter 2
Glucose, Lactate, β-Hydroxybutyrate, Acetate, GABA, and Succinate as Substrates for Synthesis of Glutamate and GABA in the Glutamine–Glutamate/GABA Cycle

Leif Hertz and Douglas L. Rothman

Abstract The glutamine–glutamate/GABA cycle is an astrocytic-neuronal pathway transferring precursors for transmitter glutamate and GABA from astrocytes to neurons. In addition, the cycle carries released transmitter back to astrocytes, where a minor fraction (~25 %) is degraded (requiring a similar amount of resynthesis) and the remainder returned to the neurons for reuse. The flux in the cycle is intense, amounting to the same value as neuronal glucose utilization rate or 75–80 % of total cortical glucose consumption. This glucose:glutamate ratio is reduced when high amounts of β-hydroxybutyrate are present, but β-hydroxybutyrate can at most replace 60 % of glucose during awake brain function. The cycle is initiated by α-ketoglutarate production in astrocytes and its conversion via glutamate to glutamine which is released. A crucial reaction in the cycle is metabolism of glutamine after its accumulation in neurons. In glutamatergic neurons all generated glutamate enters the mitochondria and its exit to the cytosol occurs in a process resembling the malate-aspartate shuttle and therefore requiring concomitant pyruvate metabolism. In GABAergic neurons one half enters the mitochondria, whereas the other one half is released directly from the cytosol. A revised concept is proposed for the synthesis and metabolism of vesicular and nonvesicular GABA. It includes the well-established neuronal GABA reuptake, its metabolism, and use for resynthesis of vesicular GABA. In contrast, mitochondrial glutamate is by transamination to α-ketoglutarate and subsequent retransamination to releasable glutamate essential for the transaminations occurring during metabolism of accumulated GABA and subsequent resynthesis of vesicular GABA.

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Keywords Astrocyte • β-Hydroxybutyrate • Branched chain amino acids • GABAergic neuron • Glucose • Glutamatergic neuron • Glutamate–glutamine cycle • Neuronal GABA uptake • Succinate

Abbreviations

^{13}C MRS	^{13}C magnetic resonance spectroscopy
α-KG	α-ketoglutarate
AAT	Aspartate aminotransferase
Ac.CoA	Acetyl coenzyme A
ADP	Adenosine diphosphate
AGC	Glutamate/aspartate exchanger (in brain AGC1 or aralar)
ANLS	Astrocyte neuron lactate shuttle
AOAA	Alpha-aminooxyacetic acid
ATP	Adenosine triphosphate
AV	Arteriovenous
BCAA	Branched chain amino acid
BCAT	Branched chain amino acid transaminase
BCATc	Cytosolic branched chain amino acid transferase
BCATm	Mitochondrial branched chain amino acid transferase
DNA, RNA	Deoxyribonucleic acid, ribonucleic acid
GABA	γ-aminobutyric acid
GABA-T	GABA transaminase
GAD65, GAD67	Glutamate amino decarboxylase 65 and 67 KD isoforms
GAT1, GAT2, GAT3	GABA transporters 1, 2, 3
GDH	Glutamate dehydrogenase
GLAST	Glutamate aspartate transporter
GLT-1	Glutamate type 1 transporter
GS	Glutamine synthase
KIC	Alpha-ketoisocaproic acid
MAS	Malate-aspartate shuttle
MCT1	Monocarboxylic acid transporter 1
MCT2, MCT3	Monocarboxylic acid transporters 2, 3
MCTs	Monocarboxylic acid transporters
NADH/NAD$^+$	Nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate
OAA	Oxaloacetate
PAG	Phosphate activated glutaminase
PC	Pyruvate carboxylase
PDH	Pyruvate dehydrogenase complex
SAT1, SAT2	System A glutamine transporters 1 and 2
SN1	System N glutamine transporter 1
SSA	Succinic semialdehyde
TCA cycle	Tricarboxylic acid cycle

2.1 Introduction

The brain is almost unique among organs in having an obligate requirement of glucose for function. However, the brain can use a variety of other substrates including lactate, β-hydroxybutyrate, acetate, and medium and long chain fatty acids. Although the brain's relative preference for substrates was well known from the pioneering AV difference studies of Kety and colleagues (Siesjö 1978), the reason for requirement for glucose has remained unexplained. In this paper, we examine evidence ranging from isolated enzymes, to cell culture, to in vivo studies to address this question. Our overall hypothesis is that the obligate requirement for glucose is due to the special needs for supporting the large glutamate and γ-aminobutyric acid (GABA) neurotransmitter cycling fluxes in the awake brain. We describe a model for glutamate–glutamine cycling that can explain the observed glucose dependence, as well as a revised model that explains the reduced dependence on glucose in GABAergic neurons. These models are consistent with the present results over a wide range of systems. Although the definitive in vivo studies remain to be done to test these models they will hopefully be useful in guiding experimentation.

2.2 Brain Metabolism of Glucose, Lactate, and β-Hydroxybutyrate

2.2.1 Glucose

The adult mammalian brain consumes about 20 % of total bodily oxygen and thus calorie utilization (Raichle and Gusnard 2002), and it normally uses glucose as its only or main substrate (Sokoloff et al. 1977). Studies in the rodent and human brain in vivo using ^{13}C-based magnetic resonance spectroscopy (MRS) and labeled glucose or acetate, which is oxidized in astrocytes but not in neurons (Muir et al. 1986; Waniewski and Martin 1998) showed that in the awake human brain astrocytes contribute ~25 % of the oxidative metabolism in brain gray matter (Gruetter et al. 2001; Lebon et al. 2002; Mason et al. 2007). Most of this glucose is metabolized via formation of two molecules of pyruvate in the cytosol from one molecule of glucose (glycolysis) followed by mitochondrial formation of acetyl coenzyme A (ac.CoA) from pyruvate (Fig. 2.1). The latter reaction is catalyzed by the pyruvate dehydrogenase complex (PDH), which is present in all brain cells. In the tricarboxylic acid (TCA) cycle the acetyl part of acetyl CoA (containing two carbon atoms) condenses with a four-carbon TCA intermediate, oxaloacetate (OAA), to form a six-carbon TCA cycle constituent (citrate), which via two decarboxylations regenerates OAA (4 carbon atoms), ready to condense with another molecule of acetyl CoA.

TCA cycle activity followed by oxidative phosphorylation in the respiratory chain creates a large amount of energy in the form of adenosine triphosphate (ATP) in both astrocytes and neurons. Since OAA is consumed at the beginning of the TCA cycle and regenerated at the end of the cycle, this process continues as long as

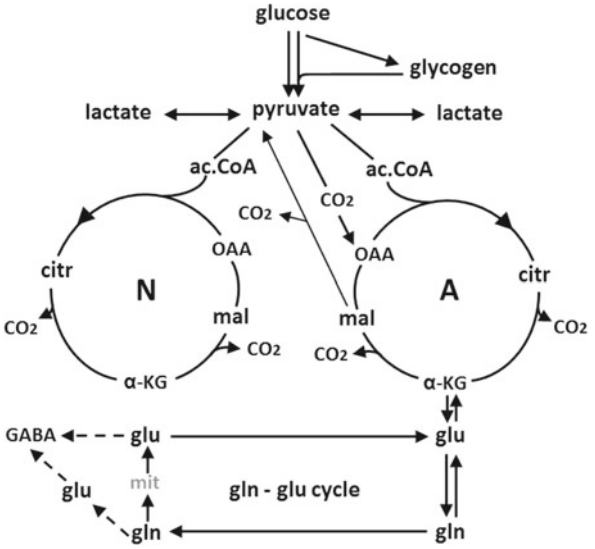

Fig. 2.1 Cartoon of glucose metabolism via pyruvate in neurons (*left*—N) and astrocytes (*right*—A) and of glutamine–glutamate (GABA) cycling. One molecule glucose is metabolized by glycolysis in the cytosol to two molecules of pyruvate in a complex and strictly regulated pathway, where one oxidative process requires transfer of reducing equivalents to the mitochondria (see, e.g., Hertz and Dienel 2002). In both neurons and astrocytes pyruvate metabolism via acetyl Coenzyme A (ac.CoA) leads to formation of citrate by condensation with preexisting 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 reformation of oxaloacetate, ready for another turn of the cycle, and to reduction of NADH, creating large amounts of energy (ATP) via reoxidation in the electron transport chain. Pyruvate carboxylation, which is active in astrocytes, but absent in neurons (reviewed in Hertz 2013), 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. α-Ketoglutarate (α-KG), one of the intermediates of the TCA cycle can leave the cycle to form glutamate (glu), catalyzed by either aspartate aminotransferase or glutamate dehydrogenase. Further metabolism by the cytosolic and astrocyte-specific enzyme glutamine synthetase leads to the formation of glutamine (gln). In glutamatergic neurons all glutamate formed by deamidation of glutamine enters the mitochondria (mit) and is returned to the cytosol in a complex process, which requires simultaneous glucose metabolism. In GABAergic neurons this is only the case for some of the glutamate, whereas the remainder enters the cytosol directly. This mechanism, which is further described in Fig. 2.6, its legend, and associated text may make GABA production less sensitive to replacement of glucose as the substrate with β-hydroxybutyrate in patients receiving very high amounts of ketone bodies to prevent seizures. Released glutamate is almost quantitatively reaccumulated in astrocytes, together with at least part of the released GABA [upper line of the glutamine–glutamate/GABA cycle (glu–gln cycle)] and reaccumulated in the astrocytic cytosol. Here, about 75 % is converted to glutamine and reenters the glutamine–glutamate/GABA cycle. The remaining ~25 % is oxidatively degraded, via one of two partly different pathways. In both α-ketoglutarate is reconverted to malate. In one malate exits to the cytosol, is decarboxylated by cytosolic malic enzyme to pyruvate, which is oxidized in the TCA cycle via acetyl Coenzyme A. In the other malate does not exit the TCA cycle but may be further metabolized to α-ketoglutarate after condensation with acetyl Coenzyme A, allowing resynthesis of another molecule of glutamate from only one molecule pyruvate. In either case the degraded glutamate must in the long term be replaced by a quantitatively similar production of glutamate from glucose, in the first case by complete de novo synthesis from one molecule glucose, in the second from one half of a glucose molecule. However, temporary fluctuations in the content of glutamate occur. The initial part of GABA metabolism is different, as all GABA is metabolized via succinic semialdehyde, succinate, and α-ketoglutarate to glutamate. Modified from Hertz (2013)

ac.CoA is available and energy is consumed, but it cannot give rise to a new molecule of any TCA cycle constituent. Nevertheless, it does lead to labeling of glutamate and its derivatives, a labeling that forms the basis of ^{13}C-NMR spectroscopy. However, this labeling is due to a transamination-mediated bidirectional exchange between α-KG and glutamate (Fitzpatrick et al. 1990).

Recently, experiments by Patel et al. (2005, 2015) and Duarte and Gruetter (2013) have shown a considerable flow of neurotransmitter GABA to astrocytes via the GABA/glutamine cycle. GABA after its release from inhibitory neurons is metabolized in astrocytes via succinic semialdehyde and succinate. It will later be discussed that a similar reaction may occur in GABAergic neurons. Succinate is a TCA cycle intermediate between α-ketoglutarate (α-KG) and malate (Fig. 2.1) and in the TCA cycle it is metabolized via OAA to citrate, requiring uptake of one molecule ac.CoA. Citrate is further metabolized to α-KG and transaminated to glutamate. Subsequently, the generated glutamate can be returned to neurons via astrocytes in the conventional glutamine–glutamate/GABA cycle via glutamine (Fig. 2.1). Duarte and Gruetter (2013) showed using metabolic modeling that the ac.CoA flow needed in the astrocyte to accommodate GABA metabolism constitutes a considerable fraction of total astrocyte ac.CoA production. The formation of ac.CoA needed for metabolism to citrate of OAA derived from GABA is included in the 25 % of total brain glucose consumption assigned to astrocytes; however, from the standpoint of ATP production the flow from succinate also needs to be considered.

Although pyruvate formation from glucose takes place in the cytosol one oxidative process, formation of diphosphoglycerate from glyceraldehyde 3-phosphate is involved. As an oxidation this process is coupled to formation of nicotinamide dinucleotide NADH from NAD^+. Since NADH is unable to cross the mitochondrial membrane for reoxidation in the mitochondria a reducing equivalent must be carried across the mitochondrial membrane for oxidation with return of the corresponding oxidized equivalent. In the brain this transport is mediated by the malate-aspartate shuttle (MAS). In the MAS (Fig. 2.2), cytosolic NADH is reoxidized to NAD^+ by reduction of cytosolic OAA to malate. Malate traverses the mitochondrial membrane but not in direct exchange for OAA (which cannot leave the mitochondria), but in exchange for α-KG, using the malate/α-ketoglutarate carrier (OGC-Slc25a11), and it is reoxidized to OAA in the TCA cycle. In the mitochondria OAA is transaminated by aspartate transaminase (AAT) to the corresponding amino acid, aspartate, which then exits the mitochondrial membrane in exchange for glutamate, using the glutamate/aspartate exchanger (AGC), in brain AGC1 or aralar (Slc25a12). This exchanger is abundantly expressed not only in neurons but also in astrocytes (Lovatt et al. 2007; Li et al. 2012). The glutamate that enters the mitochondria is simultaneously transaminated to α-KG, its corresponding keto acid. α-KG exits the mitochondria in exchange with incoming malate in the process catalyzed by malate/α-ketoglutarate carrier. This carrier is also operating in brain (Passarella et al. 1987), including astrocytes (Pardo et al. 2011). In the cytosol α-KG is transaminated to glutamate with simultaneous conversion of aspartate to OAA, closing the cycle (Fig. 2.2). AAT activity is high in most cells and in retina, a cerebral tissue, it is expressed both in the cytosol and in mitochondria (Ross and Godfrey 1987).

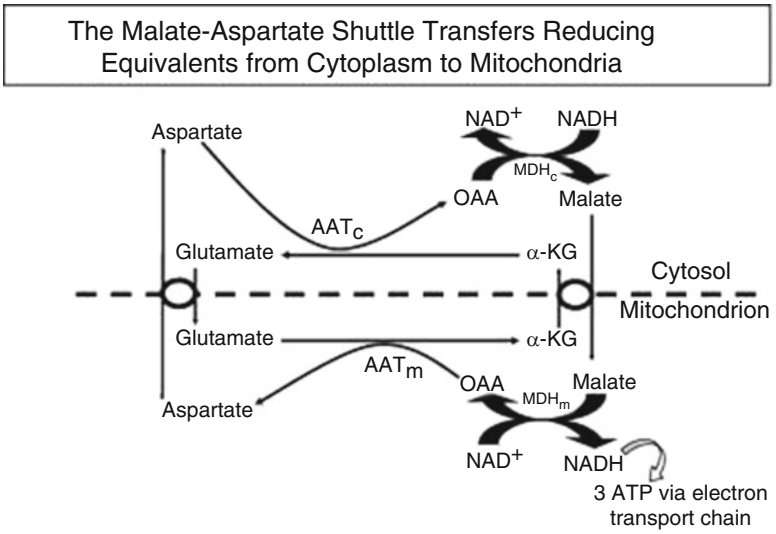

Fig. 2.2 The malate-aspartate shuttle (MAS). In the malate-aspartate shuttle (MAS), which serves to transfer reducing equivalents across the mitochondrial membrane, cytosolic malate dehydrogenase (MDHc) oxidizes NADH and converts oxaloacetate (OAA) to malate (top right of figure), which enters the mitochondria in exchange with α-ketoglutarate (α-KG). The mitochondrial malate dehydrogenase (MDHm) reoxidizes malate to OAA, which is transaminated to aspartate by the mitochondrial aspartate aminotransferase (AATm). Aspartate leaves the mitochondria in exchange with glutamate, requiring aspartate-glutamate exchanger (aralar in brain). 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. From Hertz and Dienel (2002)

Due to the dependence of oxidative glycolysis on MAS activity, complete and direct oxidation of glucose requires not only that pyruvate enters the mitochondria as ac.CoA, but also that not only malate and aspartate, but also glutamate and α-KG are transported across the mitochondrial membrane. Note also that the dependency between these processes is mutual: the MAS would not be able to operate without NAD^+ reduction.

In addition to entering the TCA cycle via ac.CoA, pyruvate can also be carboxylated to OAA, an anaplerotic process, which expands the amount of TCA cycle intermediates and is catalyzed by the astrocyte-specific pyruvate carboxylase (PC). This enzyme is virtually absent in neurons both in the brain in vivo (Shank et al. 1984; Hutson et al. 2008) and in cultured cells (Yu et al. 1983). Condensation of one molecule OAA with one molecule of acetyl-CoA forms a new molecule of citrate. This molecule as well as all molecules derived from it, including α-KG and glutamate will be labeled from [^{13}C]glucose in different positions than corresponding molecules generated by PDH activity alone, which is the key principle used for determination of rate of pyruvate carboxylation in the brain by ^{13}C-MRS (Gruetter et al. 2001; Sibson et al. 2001; Mason et al. 2007). Although pyruvate carboxylation is

necessary to replace lost TCA intermediates, by far its main role in the brain is to produce glutamate which is then transferred to neurons for use as transmitter glutamate and, after decarboxylation, transmitter GABA. A minor part of synthesized glutamate is also used by the astrocytes themselves as gliotransmitter, for synthesis of glutathione or for detoxification of ammonia to glutamine. Since carboxylation represents ~10 % of total pyruvate metabolism and it must be combined with a similar PDH-mediated metabolism, a large fraction of the 25 % of total pyruvate metabolism occurring in astrocytes must serve to produce glutamate. This does not mean that no energy is produced since combined production and oxidative degradation of glutamate, which also mainly occurs in astrocytes, produces almost as much ATP as direct oxidation of glucose (Gruetter et al. 2001; Hertz et al. 2007).

Besides the pathway shown in Fig. 2.1 a small amount of glucose (2–5 % of total glucose utilization in brain) is metabolized via the pentose phosphate pathway (Gaitonde et al. 1987; Hostetler and Landau 1967; Ben-Yoseph et al. 1995). This pathway generates pentoses, necessary for synthesis of DNA and RNA. It also produces NADPH, a reducing equivalent not replaceable by NADH, used for synthesis of lipids and in a few metabolic pathways, including reduction of oxidized glutathione (Vogel et al. 1999). Although this pathway is relatively small it has been recently suggested as responsible for part of the 5–10 % mismatch[1] between total brain glucose uptake and oxidation (Raichle 2015).

2.2.2 Lactate

Pyruvate is rapidly reduced to lactate and vice versa and most normal cells contain considerably higher concentrations of lactate than of pyruvate. Both can also cross the cell membrane in either direction catalyzed by equilibrating monocarboxylate transporters (MCTs). Lactate uptake is followed by its oxidation to pyruvate with concomitant reduction of NAD^+ to NADH. This has the consequences that lactate oxidation competes with glucose oxidation and that metabolism of lactate, like that of glucose activates MAS. MCT transporters have different kinetics in astrocytes (displaying MCT1 and 3) and neurons (displaying MCT2). It is important that these transporters, like the glucose transporters, are equilibrating, not concentrative, because that means that they are unable to accumulate (or release) the unaltered compound to a concentration beyond that from which it is accumulated or released (reviewed by Hertz and Dienel 2005). However, once the native compound is removed by metabolism, uptake or release can continue. It has been suggested that

[1] Considerable work has been done studying this mismatch and the larger mismatches that occur during functional activation. There are several theories to explain apparent uncoupling of glucose uptake and oxidation including the astrocyte neuron lactate shuttle (ANLS), discussed again later, which has received a great deal of attention. As this chapter focuses on oxidative metabolism we refer the reader to two recent reviews on opposite sides of the ANLS controversy (Pellerin and Magistretti 2012; Dienel 2012).

astrocytically released lactate is taken up and metabolized largely by neurons after transfer by the astrocyte neuron lactate shuttle ANLS (Pellerin and Magistretti 2012) and this may be critical for complex functions such as memory formation (Steinman et al. 2016), but considerable evidence is available *against* this hypothesis (e.g., Dienel 2012; Patel et al. 2014). Furthermore, astrocytes have a high ability to transport lactate between themselves through gap junctions which may substantially reduce transport to neurons (Gandhi et al. 2009). Nevertheless a small amount of glycogenderived lactate seems to be transferred from astrocytes to neurons during learning (Steinman et al. 2016). If this lactate is accumulated into the minute dendritic spines which have no mitochondria it may create ATP by oxidation to pyruvate without interfering with glucose metabolism (Hertz and Chen 2016). The established importance of glial cells for memory formation depends upon glycogen but is most likely independent of a major lactate transfer between cell types (Hertz and Chen 2016).

The unlikelihood of astrocytes supplying neurons with glucose-derived lactate does not mean that lactate metabolism is unable to cover a substantial fraction of the brain's energy demand. Thus, Van Hall et al. (2009) showed that lactate at a plasma level of 6.9 mM contributed 27 % of energy demand in human brain; high exercise intensity can decrease brain glucose uptake by almost 40 % (Kemppainen et al. 2005), and a similar decrease occurs in the anesthetized rat when plasma lactate concentration is increased to 5.8 % (Wyss et al. 2011). Boumezbeur et al. (2010) used ^{13}C magnetic resonance spectroscopy (^{13}C MRS) to estimate the relationship between plasma lactate level and cortical lactate metabolism. They found under resting awake conditions that plasma lactate at physiological levels (low mM range) accounted for ~10 % of consumption by the TCA cycle. However, based on the transport kinetics determined it was concluded that it could account for up to 60 % under maximally elevated conditions, a value comparable with those cited earlier. The labeling patterns observed were the same as with glucose, suggesting a similar percentage contribution to neuronal and glial metabolism. In mature cultured cortical astrocytes, glucose utilization can similarly be reduced by 60–70 % in the presence of lactate (Swanson and Benington 1996; Rodrigues et al. 2009), but in very young cultures of cortical neurons lactate can cover even more of total energy consumption (Bouzier-Sore et al. 2003, 2006). In this context it should be remembered that lactate oxidation to pyruvate competes with the oxidation of glyceraldehyde 3-phosphate for cytosolic NADH and thereby may inhibit glucose utilization. Nevertheless, a small increase in lactate is under certain conditions capable of increasing glucose uptake into brain at low glucose concentrations without major effect on lactate contribution to metabolism (De Feyter et al. 2013; Herzog et al. 2013). This effect appears to be more marked in neurons than in astrocytes. It is unknown whether it might be related to the signaling effect of physiological concentrations of lactate on noradrenergic neurons (Tang et al. 2014).

As a substrate lactate is able to sustain all the functions of glucose shown in Fig. 2.1 except glycolysis. It can support both PDH and PC activity and lactate and pyruvate can even give rise to glycogen synthesis (Dringen et al. 1993; Huang et al. 1994; Dringen et al. 2005). Lactate interacts with MAS. It cannot be used as

a substrate for the pentose phosphate shunt; however, cytosolic NADPH can be produced from lactate conversion by pyruvate carboxylase into OAA and by malic enzyme into pyruvate with the conversion of an $NADP^+$ to an NADPH.

2.2.3 *Acetate, Fatty Acids, and Ketone Bodies*

Most dietary lipids are triglycerides. After lipolysis glycerol is converted via glyceraldehyde 3-phosphate to pyruvate (Zabłocki and Bryła 1988), and fatty acids are β-oxidized leading to the formation of ac.CoA (Lynen 1953). The formed ac.CoA can either be metabolized as such by condensation with OAA (e.g., in the heart), or two molecules of ac.CoA can be converted to the ketone bodies acetoacetate and its reduced metabolite β-hydroxybutyrate, a reaction occurring in the liver (McPherson and McEneny 2012). After release from liver cells circulating ketone bodies are transported by MCTs (Vannucci and Simpson 2003) and utilized as metabolic substrates in several organs, but normally not to an major degree in adult brain. This is in contrast to brain energy metabolism in young animals where MCT activity in the blood–brain barrier is high (Cremer et al. 1976) and ketone bodies plentiful in blood. However, ketone bodies, mainly in the reduced form of β-hydroxybutyrate, accumulate when glucose metabolism is failing, e.g., in diabetes. Ketone bodies also accumulate during fasting (due to metabolism of fatty acids), and Owen et al. (1967) showed (1) a considerable arteriovenous difference for ketone bodies across the brain of obese patients treated with starvation and (2) that ketone bodies accounted for ~60 % of brain oxygen consumption in these patients. An increased ketone body concentration in the systemic circulation and upregulation of blood–brain barrier permeability may have contributed to the increase (Robinson and Williamson 1980), but an elevation in D-β-hydroxybutyrate dehydrogenase activity has also been described in the brains of fasting rats (Smith et al. 1969).

That ketone body oxidation to approximately 50–60 % can replace glucose oxidation in intact brain has repeatedly been confirmed (Zhang et al. 2013). Chowdhury et al. (2014) compared rate of β-hydroxybutyrate metabolism in awake, mildly anaesthetized (halothane), and in deeply anaesthetized (pentobarbital) rats with isoelectric brain activity. In the awake animals ketone body oxidation increased with its plasma concentration and at a saturating plasma β-hydroxybutyrate concentration of 17 mM reached a plateau of ~60 % of total substrate oxidation in brain cortex, a similar value as that observed by Owen et al. (1967). However, in the deeply anaesthetized rats the much less active neuronal TCA cycle was fully supported by ketone bodies at a β-hydroxybutyrate plasma level of ~12 mM, in spite of the fact that the animals were euglycemic. Accordingly, ketone bodies can provide complete energetic support of basal (nonsignaling) processes in brain when available in sufficient concentration, but they are unable to fully support signaling processes even at the highest concentrations. By subtracting the nonsignaling dependent component Chowdhury et al. (2014) concluded that in the awake rat cerebral cortex approximately 50 % of neuronal energetics required for signaling cannot be replaced by β-hydroxybutyrate and has to derive from glucose oxidation (Fig. 2.4).

The functional capabilities of ketone bodies compared to glucose are likely to be related to the different metabolic pathways followed in their oxidative degradation. β-Hydroxybutyrate is oxidized to acetoacetate intramitochondrially, and acetoacetate is converted to two molecules of ac.CoA which then condense with OAA to form citrate. Since the oxidation of β-hydroxybutyrate is intramitochondrial it does not compete with oxidation of glyceraldehyde 3-phosphate. This is in contrast to lactate, and metabolism of β-hydroxybutyrate does also not share the ability of glucose and lactate to interact with MAS. In contrast to lactate, it can only support PDH-mediated activity, not pyruvate carboxylation. It supports not only neuronal (Amaral 2013; Chowdhury et al. 2014) but also astrocytic PDH activity (Melø et al. 2006; Gibbs et al. 2009; Jiang et al. 2011; McKenna 2012). Similarly, Pan et al. (2002) showed in awake human subjects that the labeling pattern from β-hydroxybutyrate was similar to that of glucose and calculated a similar relative percentage contribution to neuronal and glial oxidative metabolism as has been found with glucose. This is in contrast to acetate, which in cell culture is almost exclusively metabolized (although only via ac.CoA) by astrocytes (Waniewski and Martin 1998), confirming previous findings in brain and retina (Muir et al. 1986). In ^{13}C MRS studies on humans and rodents the labeling kinetics have also shown glial localization of acetate metabolism (Lebon et al. 2002; Jiang et al. 2013). Although acetate is normally found in low levels in the blood stream it can be substantially elevated by alcohol consumption to levels over 1 mM. At these levels it has been measured using ^{13}C MRS to account for as much as 50 % of glial oxidative ATP needs, particularly in subject groups exposed to hypoglycemia such as intensively insulin-treated type 1 diabetes patients (Gulanski et al. 2013). Acetate has even been reported to have protective effect against hypoglycemic seizures (Urion et al. 1979), which would suggest a major contribution by astrocytes to the seizures. Consumers of large amounts of alcohol also show increased acetate metabolism, reflecting that alcohol is metabolized to acetate (Jiang et al. 2013; Volkow et al. 2013, 2015).

2.3 The Glutamine–Glutamate/GABA Cycle

2.3.1 Synthesis and Degradation of Glutamate and GABA

In the adult brain perfusion techniques have shown very limited transport of glutamate and its precursor glutamine from blood to the brain, and changes in plasma concentration of glutamine have only little influence on brain glutamate content (Smith 2000; Patel et al. 2015). Accordingly, neurotransmitter glutamate and its decarboxylation product GABA must be synthesized within the brain. As discussed in Sect. 2.2.2 formation of glutamate requires synthesis of a new molecule of a TCA cycle intermediate and both PDH and PC activities. It therefore occurs in astrocytes but not in neurons. Transport to neurons is mediated by the glutamine–glutamate/GABA cycle where formation of neuronal glutamate accounts for about 75–80 % of

the flux (measured from neurons toward astrocytes) and the remaining 20–25 % is made up by GABA (Patel et al. 2005, 2015; Duarte and Gruetter 2013). A recent study of glutamate amino decarboxylase 67 KD isoform GAD67 knock out mice suggested lower contributions by GABA, but the possibility of an underestimate was mentioned (Walls et al. 2015).

The cycle matures slowly, i.e. after 1 month in rats (reviewed by Chowdhury et al. 2007; Hertz 2013; Brekke and Morken 2015) and in the adult brain it has a flux equal to rate of glucose utilization in neurons (Sibson et al. 1998; Chowdhury et al. 2007; Hyder et al. 2013a, b) or ~75 % of the total rate of glucose utilization in brain. However, only a minor part (25 % or less) of the glutamate flux from astrocytes to neurons represents newly synthesized glutamate, whereas the remainder is made up of glutamate that has already been released as transmitter but subsequently is accumulated by astrocytes and returned to neurons in the cycle (Rothman et al. 2011). Almost all released transmitter glutamate is treated in this manner, whereas only little is reaccumulated directly into glutamatergic neurons (Danbolt 2001; Zhou and Danbolt 2013). Some neuronal reuptake has been described in cultured hippocampal neurons (Coco et al. 1997) but might be due to the young age of the cultures (see Hertz 2013).

In the long term the glutamate concentration in adult healthy brain must stay unaltered (Lebon et al. 2002; Sonnewald 2014), meaning that a fraction similar to that synthesized de novo (~25 %) must be oxidatively degraded in astrocytes, or to a much lesser extent lost from the brain as glutamine, a process mainly occurring during ammonia detoxification (Zielińska et al. 2014). For oxidative degradation glutamate is converted to α-KG, which after circling in the TCA cycle to malate can exit into the cytosol and be converted by the astrocyte-enriched (Kurz et al. 1993) cytosolic malic enzyme to pyruvate. Pyruvate can then enter the TCA cycle and be oxidatively degraded (Fig. 2.1). However, Sonnewald (2014) hypothesized that a relatively low degree of pyruvate recycling (turnover of pyruvate or one of its metabolites in the TCA cycle to regenerate pyruvate) suggested that instead pyruvate was released from the cells as lactate, which would constitute a similarly effective cataplerosis. This hypothesis is supported by her previous observation that lactate generated from TCA cycle intermediates is found in the medium (Sonnewald et al. 1993), but these experiments were performed in cultured cells known to release very large amounts of lactate. On the other hand, labeled ac.CoA was derived from TCA cycle intermediates in brain mitochondria, demonstrating malic enzyme activity and pyruvate recycling by Bakken et al. (1997) from the same group. Also, release from brain of specifically glutamate-derived lactate would cause a very considerable decrease in the amount of ATP produced by astrocytes. A complication in determining pyruvate recycling using labeling methods is also that the astrocyte-derived pattern of labeling it produces in lactate will be substantially diluted by the large amount of lactate from neuronal metabolism. Furthermore, similar scrambling occurs in glucose released by the liver which can make determination ambiguous.

A third possibility is that some of the ~25 % of returning glutamate which is converted to α-KG is only partly degraded. This would happen if malate did not leave the astrocytic TCA cycle but was converted to OAA, condensed with a new molecule of ac.CoA and further metabolized via α-KG to glutamate. Such a process

would allow formation of another molecule of glutamate and glutamine using only one pyruvate molecule and requiring no pyruvate carboxylation. Accordingly it would not require glucose or lactate utilization but could also use β-hydroxybutyrate as the source. Maciejewski and Rothman (2008) described several alternate pathways of glutamate neurotransmitter cycling that involved glutamate oxidation but did not require pyruvate carboxylation, but at present there is no evidence of their importance for the glutamine–glutamate/GABA cycle in the brain in vivo, at least under normal conditions (Rothman et al. 2012).

In the short term glutamate content in brain can transiently increase, e.g., during learning (Gibbs et al. 2007), in some cases of epileptic seizures and during visual stimulation (Bjørnsen et al. 2007; Peca et al. 2010; Mangia et al. 2010, 2012; Perez et al. 2012). The involvement of astrocytic metabolism during anaplerosis/cataplerosis provides huge possibilities for astrocytic regulation. However, even astrocytic involvement only during return of previously released transmitter without conversion of glutamate to α-KG enables some astrocytic regulation, because glutamine can move within the astrocytic syncytium (Cruz et al. 2007). Perhaps glutamate released from neurons might even be redirected to GABA-ergic neurons and vice versa. Formation of a new molecule of glutamate by conversion of glutamate-derived malate to α-KG and glutamate in the TCA cycle and incorporation of ac. CoA during this process would increase the astrocytic influence.

2.3.2 Individual Steps of Glutamate Formation and Degradation

An important, debated question is whether the initial conversion of α-KG to glutamate in astrocytes is catalyzed by glutamate dehydrogenase (GDH) or by aspartate aminotransferase (AAT). Westergaard et al. (1996) showed that in cultured astrocytes conversion of a-KG (Greek alpha) to glutamate is catalyzed by AAT wheres glutamate oxidation is mediated by GDH. The equilibrium constant for AAT is close to unity (Krebs 1953), making the reaction easily reversible. In contrast, in the GDH reaction the reductive amination to glutamate is thermodynamically favored (Engel and Dalziel 1967). However, a high K_m for ammonia together with a high NAD^+:NADH ratio in brain may enable oxidative deamination of glutamate (Zaganas et al. 2001, 2009). GDH is highly regulated by allosteric activators and inhibitors (Schousboe et al. 2013), whereas AAT is dependent upon the presence of suitable transactivation partners, for example, conversion of aspartate to OAA concomitant with glutamate formation from α-KG.

One of us (L.H) bears some responsibility for the concept of a large involvement of GDH in glutamate oxidation, since this process was first suggested to depend on GDH in his laboratory, based on findings in cultured astrocytes (Yu et al. 1982). This finding has repeatedly been confirmed (e.g., McKenna et al. 1996), and GDH is also the enzyme involved in glutamate degradation in astrocytes obtained from brain tissue (Whitelaw and Robinson 2013). However, in both of these situations

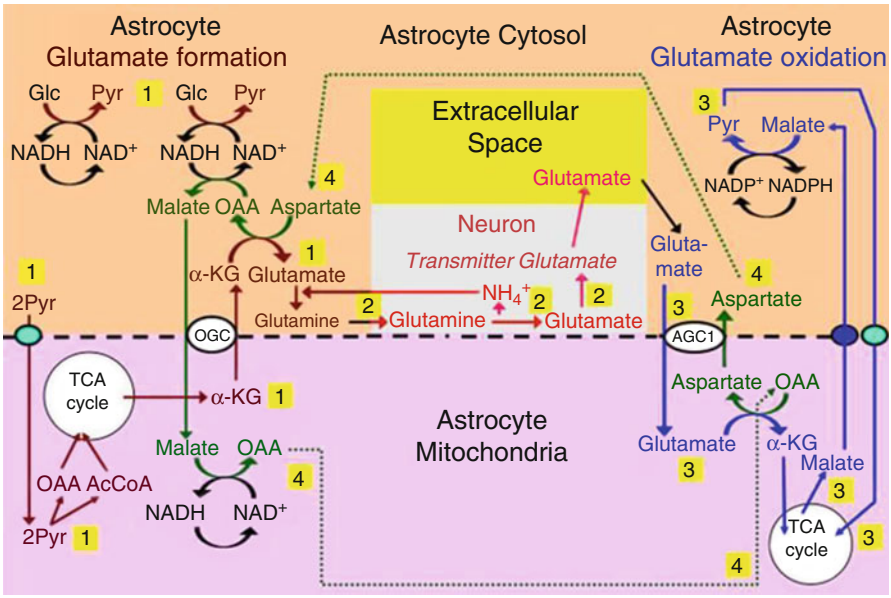

Fig. 2.3 Proposed pathway for coupled production and metabolism of transmitter glutamate using aspartate transamination for exchange between α-ketoglutarate and glutamate. Joint pyruvate carboxylase and pyruvate dehydrogenase activation generates a "new" molecule of citrate (*lower left corner*) as detailed in Fig. 2.1. 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 oxaloacetate formed from aspartate (Pardo et al. 2011). Glutamate is amidated to glutamine (pathway 1), which is transferred to glutamatergic neurons (without indication of any extracellular space in the figure) and extracellular release as transmitter glutamate (pathway 2), and subsequent reuptake of glutamate and oxidative metabolism in astrocytes (pathway 3). During oxidative metabolism transamination of glutamate to α-ketoglutarate generates aspartate that can be used in the transamination of α-ketoglutarate to glutamate in pathway 1 after transfer via pathway 4. Metabolism of α-ketoglutarate via malate is only shown to pyruvate. 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 circle* shows pyruvate carrier into mitochondria and *small purple circle* malate carrier out from mitochondria. *AGC1* aspartate/glutamate exchanger, aralar; *α-KG* α-ketoglutarate; *Glc* glucose; *Pyr* pyruvate; *OGC* malate/α-ketoglutarate exchanger (From Hertz 2013)

the astrocytes are isolated without the possibility of functional interactions with neurons and a complete glutamine–glutamate/GABA cycle. A suggested, but not proven, functional interaction between glutamate anaplerosis and cataplerosis (Hertz 2011a, 2013) is therefore not able to operate. The suggestion of such a correlation between glutamate formation and degradation (Fig. 2.3) was triggered by a large stimulation of glutamate/glutamine formation in astrocytes in intact brain in

the presence of aspartate found by Pardo et al. (2011). As previously mentioned, a high concentration of aspartate facilitates glutamate formation from α-KG by AAT but it will not affect the corresponding GDH-mediated reaction. The model proposed in Fig. 2.3 represents an attempt to find an endogenous source of aspartate capable of exerting this important stimulation. As seen in this figure synthesis and degradation along the suggested pathways, which are described in detail in the legend of this Fig and in Hertz (2011a, b, 2013), would be able to supply aspartate for glutamate synthesis. Moreover, both cytosolic and mitochondrial aspartate aminotransferase activity are very high in brain (Cooper 2013), allowing rapid nitrogen exchange between glutamate and aspartate. Furthermore, in brain mitochondria glutamate oxidation depends mainly on AAT (Balazs 1965; Dennis et al. 1977), and in GDH knockout mice, most functions remain unchanged except for a reduced glutamate oxidation in cultured, and thus isolated astrocytes (Frigerio et al. 2012).

Nevertheless, a recent paper (Karaca et al. 2015) shows abnormalities in glutamate oxidation in the knockout mice. Those found in astrocytes and mitochondria may be related to the fact that no concomitant synthesis and degradation of glutamate can occur in these isolated preparations, but an increase in ADP/ATP ratio in brain cannot. However, GDH1, the enzyme studied in the mice, shows a reduced K0.5 for ammonia when ADP is lowered from 1 to 0.1 mM (Zaganas et al. 2013), which may enhance glutamate oxidation when GDH is operating. Elimination of this mechanism in the knockout animals might possibly explain the increased ADP/ATP ratio. However, the findings do suggest that also in the brain in vivo GDH does contribute to glutamate formation/oxidation although the contribution might be minor. This would be consistent with a ten times higher activity of AAT than of GDH in mitochondria (compare Zaganas et al. 2001 with McKenna et al. 2006), and it would not disagree with the observations by Balazs (1965) and Dennis et al. (1977). They also confirm that glutamate is an important metabolic substrate (McKenna 2012, 2013) perhaps more because of its direct access to the TCA cycle than of the amount oxidized which must be much smaller than that of glucose, since brain glutamate exclusively originates from its astrocytic production from glucose and astrocytes only account for about one quarter of brain volume and metabolism (reviewed by Hertz 2011b). Finally, although the model suggested in Fig. 2.3 requires that glutamate formation from α-KG and glutamate degradation to α-KG are catalyzed by the same enzyme that enzyme may not catalyze 100 % of the interconversion.

An alternative mechanism for anaplerotic resynthesis of glutamate from α-KG was originally proposed by Yudkoff et al. (1996), who found a rapid uptake of the branched chain amino acid (BCAA) leucine into cultured astrocytes (V_{max} 54 nmol/mg of protein/min and K_m 450 μM). Astrocytic accumulation of leucine was three times greater in the presence of alpha-aminooxyacetic acid (AOAA), a transamination inhibitor, suggesting that these isolated astrocytes rapidly transaminate leucine to alpha-ketoisocaproic acid (KIC), which they then release into the extracellular fluid. This finding was linked to glutamate oxidation by Hutson and coworkers (Hutson et al. 1998, 2001), who suggested that mitochondrial branched chain amino acid transferase (BCATm) donates an amino nitrogen group to α-KG, forming glutamate which is then converted by glutamine synthase to glutamine. In their original model the amino nitrogen was fixed in the neuron by GDH and transferred by cyto-

solic neuronal BCAT (BCATn) to a branched chain keto acid, which then shuttled from the neuron to the astrocyte as a BCAA. However, it is now believed that this is more likely an internal glial reaction, based on lack of evidence that neuronal GDH can mediate glutamate synthesis. Rothman et al. (2012) determined the flux via BCAT in brain tissue vivo to ~0.1 μmol/nmol/mg, which would just be enough to catalyze transamination of the 20 % of glutamate synthesis that represents anaplerosis. Although it is unusual that a transaminase activity is similar to the net rate of the actual process (usually being several to many times greater) BCAT activity might therefore catalyze anaplerotic synthesis of glutamate. An advantage of using BCAT is that BCAAs are continuously supplied to the brain from the blood, and the nitrogen all four BCAAs together bring is almost stoichiometrically equivalent to the amount that leaves the brain as glutamine (Rothman et al. 2012 and see later). This means that the reaction described by Yudkoff et al. (1996) would suffice to generate glutamate from α-KG. The rate of α-KG synthesis (20–25 % of ~0.6 nmol/mg of protein/min) is at least as high, and in the pathways suggested in Fig. 2.3 the amount of aspartate supplied equals the glutamate uptake and metabolism in astrocytes (Hertz et al. 1978; McKenna 2012), which is much more intense than those of the BCAAs. Important evidence against participation of BCAT in glutamine–glutamate/GABA cycle glutamate synthesis is that the 50 % stimulation of glutamate synthesis caused by administration of aspartate could not be replicated by administration of the branched chain amino acid leucine (Pardo et al. 2011). Thus, use of BCAT for glutamate synthesis related to neurotransmitter cycling, as opposed to nitrogen balance, in intact tissue under normal conditions is not likely. However, in the situations where synthesis of glutamate exceeds its metabolic degradation (and thus rate of aspartate formation) BCATs could play a role in glutamate synthesis by converting BCAAs to branched chain keto acids. The latter could be reconverted to BCAAs when cataplerosis catches up with the increased anaplerosis. GDH maight also play a role. Glutamate is converted to glutamine by the astrocyte-specific (Martinez-Hernandez et al. 1977; Norenberg and Martinez-Hernandez 1979; Hutson et al. 2008; Anlauf and Derouiche 2013) enzyme glutamine synthetase (GS), which is localized in the cytosol. Inhibition of this enzyme in retinal Müller cells (an astrocyte-like cell) almost immediately interrupts glutamatergic activity in the retina (Barnett et al. 2000). The inhibition also increases retinal cell death (Gorovits et al. 1997; Bringmann et al. 2013), whereas a hormonally induced increase in Müller cell glutamine synthase GS protects against neuronal injury (Ola et al. 2011). Similarly acute administration of MSO to inhibit GS in vivo leads to rapid elevation of extracellular glutamate and can result in seizure and chronic administration will lead to the development of epilepsy (Eid et al. 2008).

Enhanced metabolism of BCAAs also counteract ammonia toxicity (Watanabe et al. 1986; Muto et al. 2005), and a nonnegligible oxidative metabolism (following transamination) and incorporation of BCAAs into protein in cultured astrocytes is strongly inhibited by a toxic concentration of ammonia (Murthy and Hertz 1987). At one time ammonia detoxification was believed to be the major pathway of glutamine synthesis but it was shown to be extremely slow compared to that in the glutamate–glutamine cycle, even during hepatic encephalopathy (Shen et al. 1998; Sibson et al. 2001; Keiding et al. 2006; Cudalbu et al. 2012). Under hyperammonemic

conditions the glutamate formed for detoxification is probably formed by glutamate dehydrogenase GDH working in the glutamate synthesis direction due to the elevated NH_4^+ concentration (Shen et al. 1998; Cooper 2012; Cudalbu et al. 2012; Kanamori and Ross 1995), but the ammonia interactions with BCAAs may suggest that they are also involved.

Glutamine exit from astrocytes and entry intro neurons are equally important as glutamine synthesis for regulation of de novo synthesis of glutamate and GABA and it is essential even during the return of released transmitter glutamate to neurons after its initial uptake in astrocytes. The amino acid transporter SN1 mediates electroneutral and bidirectional glutamine transport in astrocytes (Nissen-Meyer and Chaudhry 2013). Its activity is regulated by many factors, e.g., by extracellular pH, because protons compete with Na^+, which is required for its transport activity. There are also consistent observations that SN1 is down-regulated by protein kinase C phosphorylation, probably by internalization (Nissen-Meyer and Chaudhry 2013). After its release to the extracellular space glutamine is accumulated into glutamatergic neurons by a different transporter which is coupled to Na^+, SAT1, or at some places SAT2. It is subsequently converted to glutamate by phosphate-activated glutaminase (PAG).

As discussed in more detail by Hertz (2013) and Schousboe et al. (2013), the conversion of glutamine to glutamate in glutamatergic neurons is remarkably complex. After PAG-mediated deamidation within the mitochondrial membrane glutamate enters the mitochondrial matrix and is returned from here to the cytoplasm in a "pseudomalate-aspartate shuttle." This has been demonstrated both in cultured astrocytes (Palaiologos et al. 1988, 1989) and in mitochondria (Ziemińska et al. 2004; Bak et al. 2008). The "pseudomalate-aspartate shuttle" is identical to the 'real' MAS (Fig. 2.2) with the exception that glutamate on the cytosolic side is generated by deamidation of glutamine by PAG, abolishing the need for reaccumulation into mitochondria of cytosolic glutamate formed from α-KG by transamination. This glutamate thus becomes freely available in the cytosol for use as transmitter glutamate. Since cytosolic malate formation is an indispensable part of MAS (Fig. 2.2) and thus also of pseudo-MAS, formation of transmitter glutamate must also coincide with cytosolic reduction of NAD^+ to NADH. This will in general mean formation of one molecule of pyruvate from glucose, i.e., utilization of half a molecule of glucose. The energy obtained from this process and subsequent oxidation of glucose does not need to be used in the glutamine–glutamate cycle, which only requires little ATP to function (for glutamate and glutamine uptake and glutamine synthesis). However, it contributes substantially (1/2 molecule glucose per molecule glutamate formed) to the ratio between glucose (i.e., 2 pyruvate) utilization and glutamate flux, which as shown in Fig. 2.4 equals approximately 85 % of cerebral-cortical neuronal glucose oxidation in the resting awake state and 60–70 % of total cerebralcortical glucose oxidation (with the remainder due to glia and GABAergic neurons primarily) (Sibson et al. 1998; Rothman et al. 2011). Since the "pseudo-MAS" only operates in glutamate formation which accounts for 80 % of the total flux the contribution must be about 40 %. Anaplerotic astrocytic glutamate formation from glucose which accounts for about one quarter of total flux must account for another 20 %.

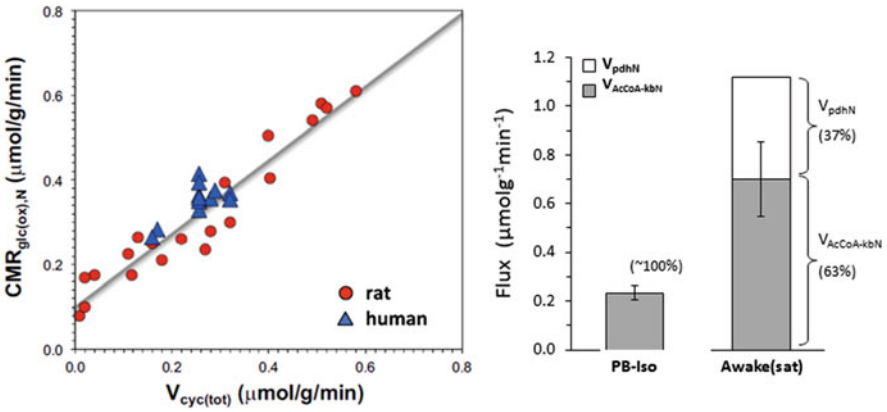

Fig. 2.4 Rate of the glutamate/glutamine cycle versus glucose oxidation and ability of ketone oxidation to displace glucose. *Left*: Measured relation between the rate of the glutamate/glutamine cycle in rat cerebral cortex versus neuronal glucose oxidation based on 12 published studies in rodents and 9 in humans (adapted from Hyder et al. 2013b). The relation is close to linear throughout the range with a slope of close to 1:1. *Right*: Saturating ketones dark part of columns can displace all of the glucose oxidation under isoelectric conditions but only approximately 50 % of the glucose oxidation needed to support neuronal signaling in glutamatergic neurons (adapted from Chowdhury et al. 2014). This percentage is in agreement with the prediction of the pseudo malate asparate shuttle model which is discussed in the text (and see Figs. 2.1, 2.2, and 2.3) VAcCoA-kbN, rate of neuronal acetyl-CoA utilization from ketone bodies; VpdhN, neuronal pyruvate dehydrogenase flux (i.e., rate of acetyl-CoA utilization from glucose)

After glutamate has been released from neurons as a transmitter most glutamate is accumulated in astrocytes by the powerful glutamate transporters Glut-1 and GLAST (Danbolt 2001; Zhou and Danbolt 2013). Although some glutamate may also be taken up by axonal terminals in glutamatergic neurons this is not sufficient to maintain glutamate homeostasis (Bjørnsen et al. 2014). In astrocytes a part (20–25 %) is oxidized as already discussed, whereas the remaining 75–80 % is again converted to glutamine and returned to neurons for reuse as transmitter (Rothman et al. 2011). However, total flux in the glutamine–glutamate/GABA cycle is so large and the association between glutamate uptake and metabolism so close that the fraction of glutamate which is oxidized in astrocytes is more than high enough to support its own energy-consuming uptake (McKenna 2012). Metabolic utilization of glutamate is strongly supported by a close association between astrocytic glutamate transporters and metabolic enzymes demonstrated by the Robinson group (Genda et al. 2011; Bauer et al. 2012; Whitelaw and Robinson 2013; Jackson et al. 2014). A close functional interaction between formation and degradation of glutamate (Fig. 2.3) is also consistent with the quantitative similarity between anaplerosis and cataplerosis and the evidence that the latter also mainly occurs in astrocytes. However, the conclusion that glutamate is a metabolizable substrate is in distinct contrast to a view expressed in a recent "Neuron" review by Magistretti and Allaman (2015), despite the studies discussed earlier and findings that human, cat, and guinea pig brain slices show at

least as high rates of oxygen uptake with glutamate alone as the substrate as with glucose alone (McIlwain 1953; Kratzing 1953; Takagaki and Tsukada 1957).

The presumed pathway for oxidative metabolism in intact tissue (Fig. 2.3) is relatively simple: Glutamate enters the mitochondria in exchange with aspartate, formed from OAA generated during glutamate synthesis. It is subsequently transaminated to α-KG in the same transamination process that formed aspartate from OAA, and α-KG enters the TCA cycle. One problem with this hypothesis is that glutamate may not necessarily be synthesized and degraded in the same astrocyte. One potential solution to this problem may be that lactate, which is rapidly transported between astrocytes (Gandhi et al. 2009), may be metabolized and facilitate glutamate synthesis when return of aspartate is deficient as a result of reduced glutamate oxidation (Hertz et al. 2014). For example, lactate might support production of OAA and thus of aspartate in pathway 4 in Fig. 2.3 if insufficient glutamate is being oxidized in a specific astrocyte. As already mentioned BCAA transamination or GDH activity may also alleviate this problem.

2.3.3 Individual Steps of GABA Formation and Degradation

It has been mentioned that GABA-derived glutamate accounts for ~20 % of the total glutamine–glutamate/GABA cycle flux from neurons toward astrocytes in the brain in vivo (Patel et al. 2005), and an approximately similar magnitude was found by Duarte and Gruetter (2013) and Patel et al. (2015). This means that in spite of a considerable rate of GABA reuptake into GABA-ergic neurons (the functional importance of which will be discussed later) GABA synthesis ultimately requires operation of the glutamine–glutamate/GABA cycle. It is likely that glutamine production and release in astrocytes for GABA production proceeds along similar pathways as those for glutamate production. It is therefore of interest to establish whether astrocytic formation and degradation of GABA might be interconnected in a similar manner as suggested for glutamate. Figure 2.5 shows that this might well be the case, although the needed conversion of GABA to glutamate before it can be returned to astrocytes in the glutamine–glutamate/GABA cycle makes the pathway for astrocytic return of GABA as glutamate more complex than that of transmitter glutamate.

GABA uptake has repeatedly been demonstrated in cultured astrocytes (Schousboe et al. 1977b; Hertz et al. 1978), and high GABA transporter currents have been measured in striatal astrocytes by patch-clamp intracellular recording (Goubard et al. 2011). Initially released GABA must after its cytosolic uptake be transferred to the mitochondria, since the conversion of GABA to succinic semialdehyde (SSA) is initiated by GABA transaminase (GABA-T) (Wong et al. 1974), which is a mitochondrial enzyme (Schousboe et al. 1977a). Figure 2.5 suggests a glutamate/GABA antiporter at the mitochondrial membrane, although such a transporter has presently not been demonstrated in mitochondria. It has, however, been shown in the bacterial membrane (Sa et al. 2015).

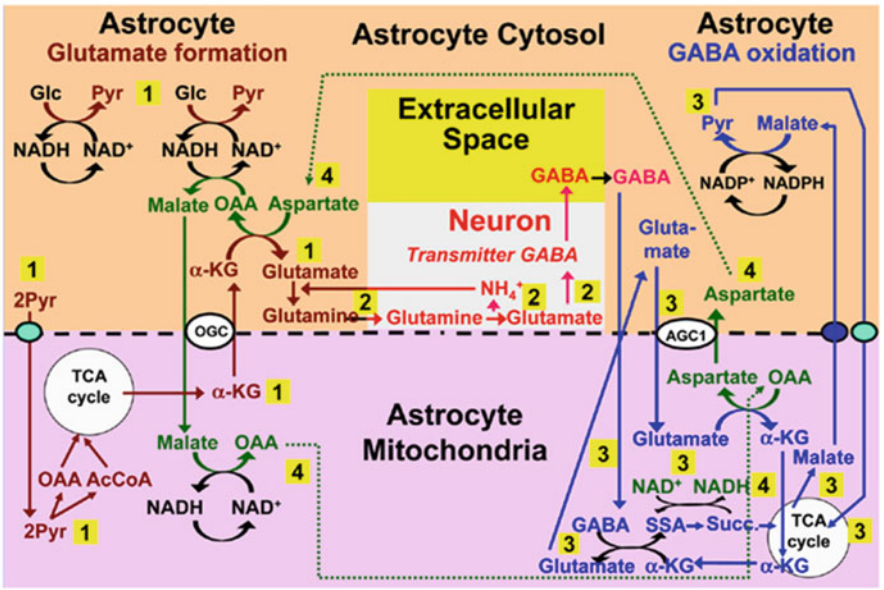

Fig. 2.5 Proposed pathway for coupled production and metabolism of transmitter GABA using aspartate transamination for exchange between α-ketoglutarate and glutamate. Pathways, color coding, and most abbreviations are as in Fig. 2.3. Most differences are in the lower right corner of the figure. The suggested glutamate-GABA exchange at the mitochondrial membrane has not been described in mammalian brain, but metabolism of GABA via succinic semialdehyde (SSA) to succinate (Succ.), a TCA cycle constituent is a well-established pathway. Metabolism of succinate via malate is only shown to pyruvate. As in Fig. 2.3 glutamate metabolism provides aspartate that can be used in the transamination of α-ketoglutarate to glutamate in pathway 1. It also generates α-ketoglutarate needed for the transamination of GABA to SSA

SSA is further oxidized to succinate, which enters the mitochondria. Figure 2.5 also suggests that glutamate reenters the mitochondria through the glutamate-aspartate exchanger (requiring aralar) and that the simultaneous exit of aspartate from mitochondria to cytosol as in Fig. 2.3 may secure the AAT substrate needed for transamination-mediated glutamate production at the initial step of GABA production. As in the metabolism of accumulated glutamate this aspartate is formed from OAA generated during the initial formation of precursor glutamate (Pathway 4) in the transamination process converting glutamate to α-KG. Instead of being oxidized α-KG retransamination to glutamate enables concomitant GABA transamination to SSA and at the same time creates the glutamate suggested to exit the mitochondria in exchange with GABA. McKenna and Sonnewald (2005) have provided some experimental evidence for these suggestions. In a study of metabolism in cultured astrocytes they found increased aspartate content and larger formation of [U-^{13}C]aspartate from added [U-^{13}C]glutamate when the cells were incubated with nonlabeled GABA. This was tentatively interpreted as caused by increased entry of [U-^{13}C] glutamate into the TCA cycle to allow for the transamination of GABA. This

explanation is strikingly similar to what is shown in Fig. 2.5 for GABA degradation, but in intact tissue, where GABA production and degradation may be linked, extracellular glutamate is not needed for this purpose, since cytosolic glutamate can be derived from mitochondrial glutamate formed as described earlier.

Duarte and Gruetter (2013) described a complete metabolic scheme for the return of GABA taken up by the astrocyte in the glutamine–glutamate/GABA cycle, where malate stayed in the TCA cycle to ultimately give rise to α-KG and glutamate. The GABA-derived carbon could also undergo complete oxidation by conversion to pyruvate by malic enzyme, which may be reintroduced and completely metabolized in the TCA cycle (Fig. 2.5). The possibility of GABA oxidation, which would then require anaplerosis for replacement is supported by the observation by Zhang et al. (1995) that small amounts of CO_2 are formed from methyl esters of succinate in rat brain cells. It is possible that glutamate and succinate are accumulated in different types of mitochondria, since astrocytes show mitochondrial heterogeneity (Sonnewald et al. 1993; Collins et al. 2002; Waagepetersen et al. 2006).

In spite of the assumed similarities between the GABA/glutamine and glutamate/glutamine cycles in astrocytic formation of glutamate and glutamine and the transfer of the latter to neurons, deamidation of glutamine to glutamate in neurons may occur in a very different manner. Part of the glutamate produced by PAG in the intermembrane space of the mitochondria is released directly to the cytosol (the direct pathway) whereas another fraction is transaminated to α-KG in the mitochondria (the indirect pathway) (Waagepetersen et al. 2001; Leke et al. 2011). The contribution of each pathway to GABA synthesis is about the same. Both pathways were concluded to contribute to vesicular GABA release and to be involved in determining GABAergic tone resulting from exit of cytosolic GABA via reversal of uptake (Waagepetersen et al. 2001; Walls et al. 2011; Leke et al. 2011), for example, by exposure to elevated extracellular K^+ (Romei et al. 2014). The glutamate decarboxylase GAD 67 is functioning in both pools, whereas GAD 65 operates only in the vesicular pool (Waagepetersen et al. 2001) and is crucial for biosynthesis of synaptic GABA (Kaufman et al. 1991; Tian et al. 1999; Walls et al. 2011) and is the primary isoform in vivo responsible for activity-dependent GABA metabolism (Patel et al. 2006).

Waagepetersen et al. (1999, 2001) and Schousboe et al. (2013) have described that the α-KG entering the mitochondria undergoes a full turn of TCA cycle flux, regenerating α-KG. This conclusion was based on the finding of incorporation of isotope from 0.5 mM [U-^{13}C]glucose into GABA during a 4 h incubation of GABAergic cerebrocortical neurons. An important consequence of this complete turn in the TCA cycle is introduction of ac.CoA between OAA and citrate. If this ac.CoA is derived from glucose or lactate it can enable exit of α-KG from the mitochondria to the cytosol and its conversion to glutamate similar to what was described for astrocytes (pathway 1 in Figs. 2.3 and 2.5). This will, however, not be the case if the ac.CoA is derived from β-hydroxybutyrate, which enters the mitochondria directly. Cytosolic glutamate can then be decarboxylated by glutamate decarboxylase (GAD), a cytosolic enzyme (Balazs et al. 1966) to GABA. Thus, cycling of α-KG within the mitochondria during GABA synthesis via the indirect pathway

may enable exit of glutamate from mitochondria, but it requires that the ac.CoA required to metabolize OAA to citrate is formed from glucose or lactate.

In contrast to the low rate of reaccumulation of glutamate in glutamatergic neurons, GABA is reaccumulated into GABA-ergic neurons in brain slices (Iversen and Neal 1968). Cell culture experiments have indicated that neuronal uptake exceeds that into astrocytes (Larsson et al. 1983; Yu et al. 1984; Schousboe et al. 2013). Müller cells in intact retina also show a high rate of GABA uptake, and in addition GABA-T is activated and NAD(P)H fluorescence increased at the mitochondrial location after GABA uptake (Biedermann et al. 2002). By aid of immunocytochemistry it has been shown that the transporter 3 (GAT 3) is expressed in astrocytes and in some animals also oligodendrocytes, whereas GAT 1 is expressed in neurons with very little astrocytic expression (Scimemi 2014a). In the mouse GABA from vesicular sources is accumulated by GAT1, while that from nonvesicular sources is taken up by the astrocytic GAT3 and 4 (Song et al. 2013). Blockade of uptake by the GAT1-specific inhibitor tiagabine during continued release of GABA should therefore be expected to reduce the neuronal content of GABA. Nevertheless, tiagabine has no effect on tissue amino acid levels and on ^{13}C enrichments from [2-^{13}C]acetate in lightly anesthetized rats (Patel et al. 2015). This is the case although similar concentrations of tiagabine cause a large increase in extracellular GABA (Fink-Jensen et al. 1992; Ipponi et al. 1999). Rather than enhancing neurotransmitter cycling tiagabine tended to reduce rates of neurotransmitter cycling in both glutamatergic and GABAergic neurons slightly without any change in the ratio between the two (Patel et al. 2015). These observations suggest that GABA taken up into GABAergic neurons from the extracellular space is either metabolized or released. In this context it is relevant that Romei et al. (2015) concluded that high-affinity uptake of GABA into nerve terminals seems to have functions other than recapture of the transmitter. The small impact of tiagabine on the astrocytic GABA/glutamine cycle may also reflect different K_m's of the transporters with much higher transient levels of GABA anticipated from vesicular release than back transport of GABA during depolarization. The high affinity of the neuronal GABA transporter is consistent with this possibility.

Romei et al. (2015) used synaptosomes from mouse cerebellum prelabeled with [^{3}H]GABA and superfused with GABA. Influx of GABA through GAT1 transporters stimulated efflux of [^{3}H]GABA. The authors suggested that this was partly by homoexchange, although such a process had not been found for GABA in cultured neurons by Hertz et al. (1978). Similarly Scimemi (2014a, b) presented evidence that not only GABA biosynthesis but also GABA uptake is essential to sustain GABAergic synaptic transmission. Based on these results, those by Duarte and Gruetter (2013) and Chowdhury et al. (2014) and the observation by Biedermann et al. (2002) that Müller cells show enhanced GABA-T expression and stimulation of mitochondrial function after GABA uptake we hypothesize that metabolism of accumulated GABA in neurons may facilitate the transport between mitochondrial and cytosolic compartments needed for production of GABA via the indirect pathway. This suggestion is not in disagreement with the experimental findings by Waagepetersen et al. (1999, 2001), since the 4-h incubation period used by these authors provides ample time for GABA release, reuptake, and metabolism.

In accordance with the observations by Waagepetersen et al. (2001) the suggested pathway assumes that for every two glutamine molecule one is converted in the cytosol to glutamate (light blue pathway in Fig. 2.6) from which GABA is formed by decarboxylation and released by arrow 1. The other glutamine molecule enters the mitochondria where it is deamidated to glutamate and converted by transamination to α-KG, contingent upon the presence of OAA for simultaneous conversion to aspartate (sea-blue pathway). This OAA becomes available when released GABA, which at the beginning originates exclusively from the cytosol, is reaccumulated (gray arrow and arrow 2) and metabolized. As in astrocytes, GABA is assumed to enter the mitochondria in exchange with glutamate. Here it is transaminated to SSA (in contrast to the pathways shown in Figs. 2.3 and 2.5 this process has to be a transamination), with concomitant transamination of the α-KG, formed from the glutamine that initially entered the mitochondria, to glutamate. This glutamate exits to the cytosol via a glutamate carrier (sea-blue pathway) and from there the extracellular space, from where it is accumulated by astrocytes for return in the glutamine–glutamate/GABA cycle or for metabolic degradation. Accordingly, the glutamine accumulated into the mitochondria does not give rise to any formation of GABA, but it is essential for the transamination of incoming GABA to SSA, and it also delivers aspartate for the subsequent conversion of succinate-derived alpha-KG to glutamate and glutamate for return to astrocytes. SSA is oxidized to succinate which is metabolized via OAA, condensing with ac.CoA to alpha-KG which is transaminated to glutamate, and conversion of aspartate to OAA. This glutamate molecule exits to the cytosol (in exchange with GABA) and is again decarboxylated to a molecule of GABA which is released as synaptic GABA (arrow 3). Once the vesicular release is established, it is accordingly this GABA which is reaccumulated (arrow 4 and the pathway shown in red in Fig. 2.6), since Song et al. (2013) have shown that it is vesicularly released GABA which is accumulated by the neuronal GAT1. Thus, although the released and reaccumulated GABA does not give rise to any increase in GABA concentration it is used as the source for production of another glutamate molecule which can enter the cytosol and form releasable GABA. The whole process (direct and indirect pathways together) produces two molecules of GABA from two molecules of glutamine, but they are both formed from the glutamine molecule that entered the cytosol by the direct pathway. The indirect pathway is not used for direct production of GABA, but it facilitates

Fig. 2.6 (continued) from there to the extracellular fluid and probably accumulated into astrocytes (*Italics*) in the glutamine–glutamate/GABA cycle. Exchange of OAA, α-KG, and Asp between the two pathways is indicated in the figure, and there is stoichiometric balance between synthesis and utilization. Thus for each 2 Gln molecules the neurons receive from the astrocytes they return 1 Glu and 1 GABA molecule in the glutamine/glutamate/GABA cycle. These pathways are more complex than that originally suggested by Waagepetersen et al. (1999, 2001) but fully compatible with the experimental data obtained by these authors since the 4-h. incubation period used allows time for GABA uptake and metabolism and release via *arrow 3*. In vivo data by other authors supporting the proposed pathways are cited in the text. To prove or disprove this pathway it will be necessary to study the effect of extracellular GABA on GABA content in GABAergic neurons in the presence of extracellular glutamine

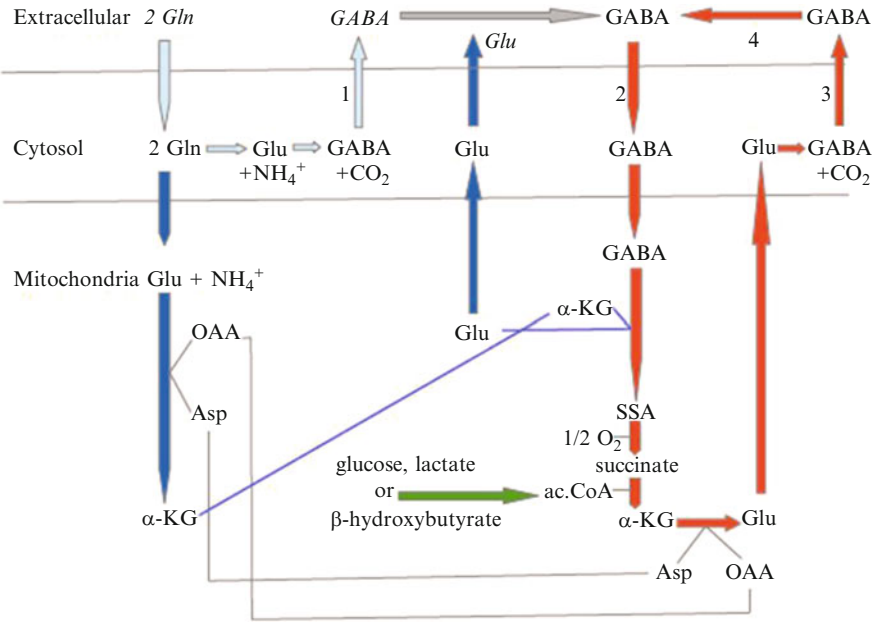

Fig. 2.6 Proposed pathways for production of cytosolically and vesicularly released GABA in GABAergic neurons from glutamine (Gln) released from astrocytes and accumulated into the astrocytic cytosol. Since initially one half of accumulated Gln deamidated to glutamate by glutaminase (PAG) located in the intramembranaceous space enters directly in the cytosol (*light blue*), whereas another one half enters the mitochondria in a similar manner as in glutamatergic neurons (Waagepetersen et al. 1999, 2001) the figure depicts the metabolic fate of two molecules of glutamine (*Italics*), whereas each of the two pathways follows the metabolism of one molecule Glu. Glu released directly to the cytosol is decarboxylated to GABA and released to the extracellular space (*light blue and arrow 1*). When GABAergic signaling is initiated it is reaccumulated into the cytosol of the GABAergic neuron (*red and arrow 2*) and from there into the mitochondria, probably in exchange with glutamate. Here it is metabolized via succinic semialdehyde (SSA) to succinate which is accumulated into the TCA cycle, where it is metabolized to α-ketoglutarate (α-KG), probably explaining the incorporation of radioactivity from the TCA cycle in released GABA shown by Waagepetersen et al. (1999, 2001). This cycling requires condensation of oxaloacetate (OAA) with acetyl coenzyme A (co.A), which may be derived from either glucose, lactate, or β-hydroxybutyrate (green arrow). α-KG is transaminated to Glu, which exits from the mitochondria (probably in exchange with incoming GABA), is decarboxylated to GABA, which is released to the extracellular space (*red and arrow 3*). Results by Song et al. (2013) show that during established GABAergic signaling in vivo it is probably mainly or exclusively the GABA formed by this mechanism (released via *arrow 3*) that becomes reaccumulated into the GABAergic neuron (*arrow 4*). Thus during established GABAergic signaling formation of vesicularly released GABA sustains itself by the neuronal uptake of GABA and its metabolism to "new" releasable GABA, whereas neuronal reuptake of GABA formed from glutamate released directly to the cytosol (unlabelled gray arrow) ceases. This allows uptake of this GABA (*Italics*) into astrocytes in the glutamine–glutamate/GABA cycle. The Gln initially released into the mitochondria is converted to α-KG (as also concluded by Waagepetersen et al. 1999, 2001), and this α-KG is reconverted to Glu in the transamination of GABA to SSA. This has the consequence that the cycle of vesicular release and neuronal reuptake and production of "new" GABA only is possible when another molecule of Gln simultaneously is converted to mitochondrial Glu and α-KG. Glu is released to the cytosol and

resynthesis of GABA from accumulated and metabolized GABA. According to the proposed mechanism it is thus not the mitochondrial α-KG formed from astrocytically delivered glutamine which is circled a full turn in the TCA cycle but succinate (which is formed in the TCA cycle by decarboxylation of α-KG) which is converted via almost a complete turn of the cycle to α-KG. An important difference from the original concept by Waagepetersen et al. (1999, 2001) is that the exogenous ac.CoA which is needed to convert mitochondrial OAA to citrate can equally well be supplied by glucose and β-hydroxybutyrate. This would make GABA synthesis much less sensitive to replacement of most glucose with a ketogenic diet than glutamate synthesis. This conclusion is in agreement with a better metabolic efficiency of β-hydroxybutyrate of rats on ketogenic diet found by Roy et al. (2015). The same authors found a 44 % increase in brain content of the BCAAs leucine and isoleucine associated with the presence of free leucine and isoleucine in the liver that was never seen in control rats. Along similar lines Chowdhury et al. (2014, see description earlier and Fig. 2.4) had found a very drastic reduction of glucose:glutamate ratio in the animals treated with high doses of β-hydroxybutyrate (from 1:1 in controls to 0.4:1). Together, the findings by these two groups suggest more drastic changes in glutamine–glutamate/GABA cycling than only replacement of glucose with β-hydroxybutyrate in the processes indicated in this review.

The proposed pathway might explain how the neuron disposes of excess TCA cycle constituents although it shows no pyruvate recycling, indicating lack of formation of pyruvate from TCA cycle constituents (Waagepetersen et al. 2002) and express little if any malic enzyme (Kurz et al. 1993). Of the two glutamine molecules followed in Fig. 2.6 one is returned to astrocytes as released glutamate, the other by the GABA released by arrow 1 after it is no longer reaccumulated by the neurons (cessation of the process shown by the gray arrow) after the reaccumulation of synaptically released GABA has been established. This GABA is converted to glutamate as described by Duarte and Gruetter (2013), but if the pathway scheduled in Fig. 2.6 is followed only one half of this conversion (one GABA molecule) occurs in astrocytes, the other (the GABA molecule metabolized in the pathway shown in red) in the GABAergic neurons. The mechanism seems to be consistent with the conclusion by Van den Berg et al. (1974) 'that there are two metabolic spaces involved in the degradation of GABA…. One of the GABA degradation metabolic spaces leads to a high labeling of glutamine, the other not.' The space leading to the high labeling of glutamine is astrocytic. The other neuronal, where it can be seen that no glutamine is formed in the pathway shown in red. It can probably also be concluded that GAD 67, which participates in the formation of both cytosolic and vesicular GABA catalyzes the formation of the GABA released by nonvesicular means (arrow 1). GAD 65, which selectively leads to formation of vesicular GABA the release of which is shown in arrow 3.

GABA production and degradation also contribute to the 1:1 ratio between glutamate flux and neuronal glucose utilization in individuals on a normal diet. It was concluded earlier that the requirement for pyruvate formation during conversion of glutamine to glutamate in glutamatergic neurons and pyruvate utilization for anaplerosis together could account for 60 % of the glucose consumption. De novo GABA

production depends also on glucose metabolism because newly synthesized astrocytic glutamate normally will be derived from glucose, but this is already included in the 60 %. However, an additional 10–12 % (one half of the 20–25 % of the glutamine–glutamate/GABA cycle used for GABA synthesis) can be explained by the complex processes occurring both in astrocytes (Duarte and Gruetter 2013) and according to Fig. 2.6 also in GABAergic neurons, when GABA is converted to glutamate for recycling (see green arrow in Fig. 2.6). In addition, the rates of GABAergic neuronal glucose oxidation and glutamate/glutamine cycling increase proportionate to glutamatergic neuron glucose oxidation and glutamate/glutamine cycling indicating that the proportionality will be maintained across changes in activity. Thus, under normal conditions with glucose as the only important substrate the combined turnover of glutamate and GABA can account for 70 % of the 1:1 ratio between glutamate cycling and neuronal glucose metabolism.

2.4 Concluding Remarks

The glutamine–glutamate/GABA cycle is an astrocytic-neuronal pathway which is essential for production of transmitter glutamate and GABA and thus for normal brain function. Glucose or lactate is essential precursor for some processes involved in the cycle, whereas they can be replaced by ketone bodies, a BCAA, or acetate at other steps. Formation of glutamate from glutamine is complex in both glutamatergic and GABAergic neurons. It is completely dependent upon concomitant glucose metabolism in glutamatergic neurons, whereas β-hydroxybutyrate can replace glucose in GABAergic neurons. Accordingly, formation of GABA may be less sensitive than that of glutamate to inhibition by diets highly enriched in fats. Energetically glutamate and GABA production via the glutamine–glutamate/GABA cycle is much more expensive than neuronal production, reuptake, and oxidation of glutamate (and GABA) would have been. However, a higher neuronal reuptake capacity would mean that the levels of glutamate and GABA in the extracellular space would be influenced to a greater extent by membrane depolarization as opposed to vesicular release, which could interfere with neuronal communication especially in the case of glutamate. Such a process seems to occur in the very immature brain and the development of the glutamine–glutamate/GABA cycle may be a key factor explaining the difference between the functional capability of the immature and mature brain. It also provides astrocytes with the possibility to coregulate glutamatergic and GABAergic transmission. The modified pathway for synthesis of GABA shown in Fig. 2.6 seems to be able to explain several previous findings of association of specific GAD isozymes and transporters with cytosolic and vesicular released GABA and it may also be relevant for tonic and phasic GABA signaling.

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Chapter 3
Anaplerosis for Glutamate Synthesis in the Neonate and in Adulthood

Eva Brekke, Tora Sund Morken, Anne B. Walls, Helle Waagepetersen, Arne Schousboe, and Ursula Sonnewald

Abstract A central task of the tricarboxylic acid (TCA, Krebs, citric acid) cycle in brain is to provide precursors for biosynthesis of glutamate, GABA, aspartate and glutamine. Three of these amino acids are the partners in the intricate interaction between astrocytes and neurons and form the so-called glutamine–glutamate (GABA) cycle. The ketoacids α-ketoglutarate and oxaloacetate are removed from the cycle for this process. When something is removed from the TCA cycle it must be replaced to permit the continued function of this essential pathway, a process termed anaplerosis. This anaplerotic process in the brain is mainly carried out by pyruvate carboxylation performed by pyruvate carboxylase. The present book chapter gives an introduction and overview into this carboxylation and additionally anaplerosis mediated by propionyl-CoA carboxylase under physiological conditions in the adult and in the developing rodent brain. Furthermore, examples are given about pathological conditions in which anaplerosis is disturbed.

Keywords Pyruvate carboxylation • Neonate • Adult • Anaplerosis • Pyruvate • Glutamate

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Abbreviations

AD	Alzheimer's disease
AMPA	α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
AMPK	AMP activated protein kinase
CBZ	Carbamazepine
GS	Glutamine synthetase
ME	Malic enzyme
MRS	Magnetic resonance spectroscopy
P	Postnatal day
PAG	Phosphate activated glutaminase
PC	Pyruvate carboxylase
PCC	Propionyl-CoA carboxylase
PDH	Pyruvate dehydrogenase
PEPCK	Phosphoenolpyruvate carboxykinase
PPP	Pentose phosphate pathway
PTZ	Pentylenetetrazole
TCA	Tricarboxylic acid

3.1 Introduction

The major fuel for the brain is glucose and relevant metabolic pathways are shown in Fig. 3.1. Glucose metabolism is necessary for glutamate synthesis which is essential for brain function since the blood–brain barrier prevents glutamate from entering the brain. Synthesis of this excitatory amino acid neurotransmitter in neurons is particularly important since 90 % of synapses in the forebrain are glutamatergic (Attwell and Laughlin 2001) and glutamate is the precursor of the other major amino acid neurotransmitter, GABA. The synthesis of the carbon skeleton of these neurotransmitters is dependent on the production of the anaplerotic substrate oxaloacetate from glucose (Fig. 3.2). Sir Hans Kornberg coined the expression: *anaplerotic sequences* to describe a series of enzymatic reactions or pathways that replenish the pools of metabolic intermediates in the tricarboxylic acid (TCA) cycle. Since both glutamate and GABA are derived from the TCA cycle intermediate α-ketoglutarate the anaplerotic reactions securing the synthesis of α-ketoglutarate in brain are important. However, because of the coupling of this process with cataplerosis, i.e. the exit of intermediates from the cycle, both pathways are equally important for the regulation of amino acid, glucose and fatty acid homeostasis (Sonnewald 2014).

One role of this cycle in all mammalian cells is the oxidation of acetyl-CoA to carbon dioxide and energy production. However, in brain synthesis of precursors for the neurotransmitters glutamate, GABA and aspartate are also an essential part of its function. Pyruvate carboxylase (PC) is the enzyme with major responsibility for oxaloacetate production from glucose and thus anaplerosis in

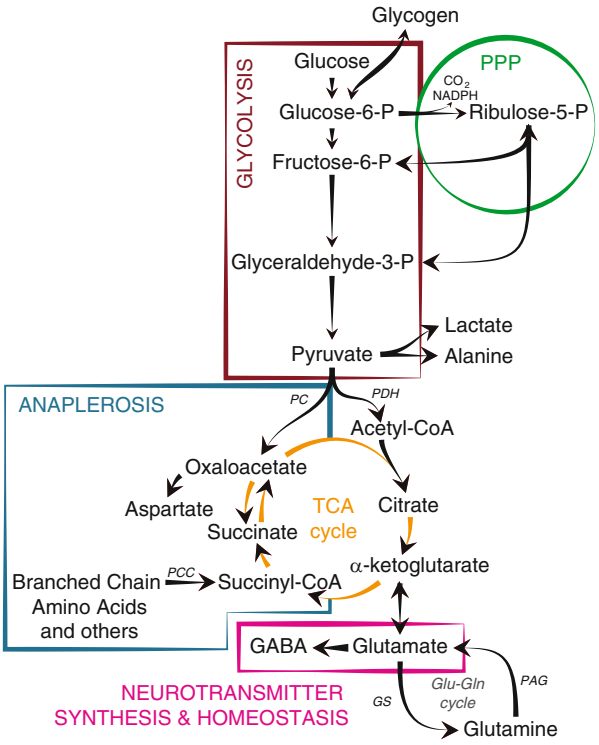

Fig. 3.1 Pathways for glucose metabolism. *Gln* glutamine, *Glu* glutamate, *KG* ketoglutarate, *ME* malic enzyme, *GS* glutamine synthetase, *NADPH* nicotinamide adenine dinucleotide phosphate, *PAG* phosphate-activated glutaminase, *PC* pyruvate carboxylase, *PCC* propionyl-CoA carboxylase, *PDH* pyruvate dehydrogenase, *PPP* pentose phosphate pathway, *TCA* tricarboxylic acid

brain (Mohler et al. 1974; Patel 1974b; Kanamatsu and Tsukada 1999). However, pyruvate carboxylation and neurotransmitter synthesis and release do not occur in the same compartment (Fig. 3.3). In the brain only astrocytes express PC (Yu et al. 1983; Shank et al. 1985; Cesar and Hamprecht 1995) but the two amino acid neurotransmitters are produced in the neurons. This dependence of neurons on astrocytes has been investigated extensively in the adult brain of animals and humans (McKenna et al. 2012) and is defined by the details of the glutamate–glutamine cycle (Fig. 3.3) which can also encompass GABA (Berl and Clarke 1983). Using ^{13}C magnetic resonance spectroscopy (MRS) and ^{13}C-labelled glucose it has been shown that pyruvate carboxylation occurs in humans, rats and mice in vivo as well as in vitro in neural tissue and cell preparations (Badar-Goffer et al. 1990; Shank et al. 1993; Hassel and Sonnewald 1995; Griffin et al. 1999; Waagepetersen et al. 2001a; Rae et al. 2005; Mason et al. 2007). An excellent review article gives an overview over the different MRS studies performed to evaluate the relationship between pyruvate carboxylation and dehydrogenation (Hertz 2011). In order to perform de novo synthesis of glutamate, an

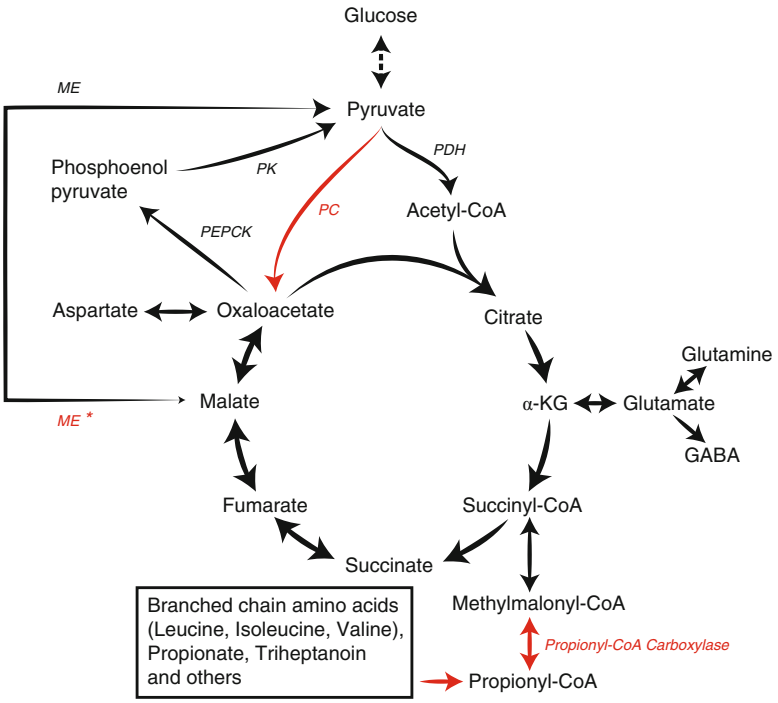

Fig. 3.2 Schematic presentation of reactions pertinent to pyruvate carboxylation. *In the brain ME appear to operate primarily in the decarboxylation direction, see Section 3.2. *KG* ketoglutarate, *ME* malic enzyme, *PC* pyruvate carboxylase, *PDH* pyruvate dehydrogenase, *PEPCK* phosphoenolpyruvate carboxykinase, *PK* pyruvate kinase

anaplerotic substrate must be transferred from astrocytes to neurons, since the latter cell type does not contain PC. In contrast to glutamate, glutamine can safely be released to the extracellular milieu without a disturbing interaction with receptors for the two mentioned amino acid neurotransmitters. When glutamine is released from astrocytes into the synapse it is taken up by specific transporters into neurons (Varoqui et al. 2000) where it can be converted to glutamate by phosphate activated glutaminase (PAG) (Hogstad et al. 1988). As can be seen in Fig. 3.3, after release in the neurotransmission process, glutamate is taken up via specialized, high affinity transporters located primarily in astrocytes (Danbolt et al. 1992; Danbolt 2001). In astrocytes, glutamate is either rapidly converted to glutamine via the enzyme glutamine synthetase (GS), localized only in astrocytes (Norenberg and Martinez-Hernandez 1979), or converted to α-ketoglutarate, thereby entering the TCA cycle (McKenna et al. 1996; Schousboe et al. 2014). This sequence of processes is termed the glutamate–glutamine cycle (Fig. 3.3) (references in (McKenna et al. 2012)), which is essential in order to ensure precise neural signalling, preserve carbon atoms for neuronal glutamate synthesis and limit excitotoxicity which may result from excessive glutamate receptor stimulation (Danbolt 2001). Only limited information is

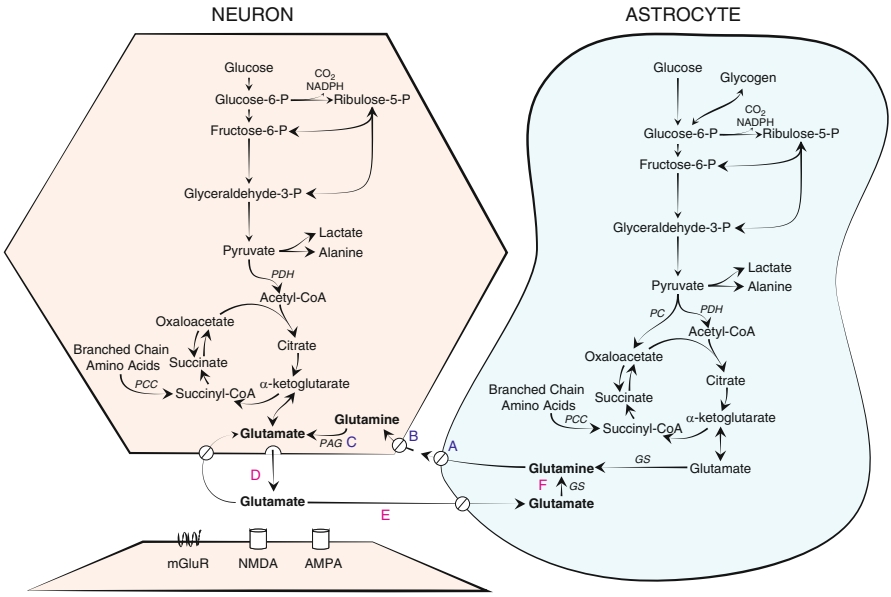

Fig. 3.3 The glutamine–glutamate cycle. *AMPA* α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor, *A,B* glutamine transport, *C,D* glutamate transport, *E* conversion of glutamate to glutamine, *GS* glutamine synthetase, *NADPH* nicotinamide adenine dinucleotide phosphate, *NMDA N*-methyl-D-aspartate receptor, *PAG* phosphate activated glutaminase, *PC* pyruvate carboxylase, *PCC* propionyl-CoA carboxylase, *PDH* pyruvate dehydrogenase, *mGluR* metabotropic glutamate receptors

available on the above-mentioned processes in the neonatal brain. However, there are indications that the glutamate–glutamine cycle at this stage does not operate as a cycle, but rather as a delivery system of glutamine from astrocytes to neurons, since transport of glutamate from neurons to astrocytes is negligible while that of glutamine from astrocytes to neurons is higher than in the adult brain (Morken et al. 2013; Brekke et al. 2014).

3.2 Carboxylation in the Brain

There are several enzymes that theoretically would have the potential for catalysing carboxylation processes in the brain (Fig. 3.2) such as pyruvate carboxylase (PC), propionyl-CoA carboxylase, phosphoenolpyruvate carboxykinase (PEPCK) and malic enzyme (ME). The latter three enzymes are present both in astrocytes and neurons (Kurz et al. 1993; Alves et al. 1995; McKenna et al. 1995, 2000; Vogel et al. 1998a, b). However, in the brain PEPCK and ME appear to operate primarily if not exclusively in the decarboxylation direction (Kurz et al. 1993; Alves et al. 1995; McKenna et al. 1995, 2000; Vogel et al. 1998a, b).

3.3 Propionyl CoA Carboxylation

The anaplerotic pathway from propionyl-CoA via methylmalonyl-CoA to succinyl-CoA (Fig. 3.2) is known to operate in peripheral tissues. Anaplerotic molecules metabolized to propionyl-CoA and the propionyl-CoA carboxylase pathway include the branched chain amino acids, isoleucine and valine (Bak et al. 2009), propionate and molecules containing fatty acids with an uneven number of carbon atoms, such as triheptanoin (Borges and Sonnewald 2012). This pathway has been shown to be active in brain and supplementation of diet with triheptanoin has been attempted as a treatment against epilepsy (Willis et al. 2010; Borges and Sonnewald 2012; Kim et al. 2013; Marin-Valencia et al. 2013; Smeland et al. 2013).

3.4 Pyruvate Carboxylation

3.4.1 Pyruvate Carboxylation in the Adult

Pyruvate carboxylase is an ATP-dependent mitochondrial enzyme catalysing the carboxylation of pyruvate to form oxaloacetate. It is a nuclear encoded homotetramer found in most tissue from eukaryotes and in many prokaryotes and is a member of the family of biotin-dependent carboxylases (Wallace et al. 1998). In the brain it is involved in various anabolic pathways such as lipogenesis and the synthesis of neurotransmitter substances (Wallace et al. 1998; Schousboe et al. 2015). A defect in the expression or biotinylation of pyruvate is a rare autosomal recessively inherited disorder in humans resulting in developmental delays and failure to thrive starting in the neonatal or early infantile period, early death, or severe debilitating psychomotor retardation (Wallace et al. 1998; Schiff et al. 2006).

While the cellular location of PC in astrocytes but not neurons (Yu et al. 1983) is not disputed, pyruvate carboxylation has been suggested to take place also in neurons (Hassel 2001; Merle et al. 2002). However, a whole range of experiments, using different preparations, in different laboratories have essentially unequivocally confined this carboxylation to glia. Primary mono-cultures of neurons and astrocytes were used to obtain information about the cellular location and magnitude of pyruvate carboxylation using ^{13}C labelled precursors. However, the interpretation of results obtained in these studies may be ambiguous since oxaloacetate can be converted not only to citrate but also via several steps to the symmetrical molecule fumarate (back cycling) and subsequently from oxaloacetate and citrate (cycling). The latter pathway will lead to scrambling of label resulting in misinterpretation of some labelling results. Merle et al. (2002) suggested incomplete back cycling for astrocytes and complete back cycling for cerebellar neurons. If back cycling in neurons is indeed complete, the study mentioned in the previous sentence, utilizing [1-^{13}C]glucose, was not capable of showing carboxylation in neurons. In order to

clarify this, Waagepetersen et al. (2001a) have performed a study using [U-^{13}C]glucose or [U-^{13}C]lactate and 3-nitropropionic acid to specifically block the TCA cycle at the succinate dehydrogenase step (Alston et al. 1977). In this way multiple cycling of TCA cycle constituents was avoided and carboxylation could be clearly detected in labelling of the C-3 position, regardless of back cycling. Analysing the ^{13}C labelling patterns in amino acids, pyruvate carboxylation was detected in astrocytes but not in neurons (Waagepetersen et al. 2001a). Furthermore, also in studies of brain tissue slices and cell cultures carboxylation was detected in astrocytes (Griffin et al. 1998, 2003; Waagepetersen et al. 2001a). In a very elegant study using unrestrained awake rats which were continuously infused with a combination of $H^{14}CO_3^-$ and [1-^{13}C] glucose in over 50 infusions ranging from 5 to 60 min it was shown that synthesis of glutamine from $H^{14}CO_3^-$ was substantial, amounting to 32 % of the glutamate/glutamine cycle (Xu et al. 2004).

3.4.2 Pyruvate Carboxylation in the Neonate

Also in the neonatal brain pyruvate carboxylation is predominantly localized in glia since the level of [2,3-^{13}C]glutamine derived from [1,2-^{13}C]glucose is higher than that of [2,3-^{13}C]glutamate (Morken et al. 2013; Brekke et al. 2014). As pointed out earlier, synthesis of the neurotransmitters glutamate and GABA is coupled to pyruvate carboxylation and this is particularly important in development when neurotransmitter levels increase. GABA levels are only slightly lower at postnatal day 7 (P7) compared to later in life, but glutamate has a sharp increase early in brain development (Chowdhury et al. 2007; Morken et al. 2013). In humans, glutamate levels double from gestational week 32 until term and continue to increase during the first year of life (Kreis et al. 2002). In rats, glutamate levels double between P7 and adulthood. This increase in de novo synthesis of glutamate must happen via anaplerosis catalysed by PC.

PC activity in the rat brain is very low at birth, but increases sharply during the first weeks of life (Larsson et al. 1985), coinciding with the major period of gliogenesis (Schousboe 1972; Bandeira et al. 2009). During the first days of life, the activity of the TCA cycle-related enzymes is also low (Wilbur and Patel 1974; Larsson et al. 1985). Thus, at the time of birth, astrocytes may not have the same capacity as in the adult brain for metabolic support of neurons but the relative contribution of PC to pyruvate metabolism might still be significant. Indeed, a larger part of the available glucose is channelled into this pathway in the P7 rat than in the adult (Morken et al. 2013; Brekke et al. 2014). In fact, for every glucose molecule metabolized through glycolysis to become pyruvate, half is metabolized via pyruvate dehydrogenase, and the other half is used for anaplerosis in the P7 rat (Morken et al. 2013; Brekke et al. 2014).

Since pyruvate carboxylation occurs in astrocytes, it is possible to calculate the relative glucose utilization in astrocytes compared to neurons at the level of pyruvate using results from experiments with [1,2-^{13}C]glucose injection. This was calculated to be 34 ± 3 % of glucose metabolism at the level of pyruvate in the P7 rat

brain (Morken et al. 2013; Brekke et al. 2014). This is in surprisingly good accordance with results from the adult brain based on a corresponding equation using [U-^{13}C]glucose. Qu et al. (2000) found that astrocytes metabolize maximally 34 % of glucose at the level of pyruvate in the adult brain, and similar results have been reported by Hassel et al. (1995). This could indicate that in rats both astrocytes and neurons have lower glucose metabolism at P7 compared to adult animals. However, the distribution between nonneuronal cells and neurons changes considerably from P7 until adult age, since the number of astrocytes increases dramatically in this period to outnumber neuronal cells at adult age (Baburamani et al. 2013; Bandeira et al. 2009). This may suggest that each astrocyte has a higher metabolic rate relative to each neuron in the neonatal brain compared to later in life.

The exchange of glutamate and glutamine between neurons and astrocytes in rats is radically different in the neonatal brain compared to the adult brain (Morken et al. 2013; Brekke et al. 2014). The necessary transporters for glutamine to enter neurons are present from late gestation (Weiss et al. 2003) (Fig. 3.3B) and the number of glutamine transporters on astrocytes actually reaches higher levels than the adult brain on postnatal day 14 (Boulland et al. 2003) (Fig. 3.3A). However, the expression of astrocytic glutamate transporters (GLT-1 and GLAST) is low early in brain development (Danbolt 2001) (Fig. 3.3E), indicating that the ability of astrocytes to take up glutamate from the synapse may be limited in the neonatal brain. Indeed, the transport of newly synthesized glutamine from astrocytes to glutamatergic neurons is higher in the P7 rat brain than the adult rat brain (Morken et al. 2013, 2014) while the reciprocal transfer of glutamate from neurons to astrocytes was very low at P7 compared to adults. The latter may also be due to low release of glutamate from neurons (Fig. 3.3D) at this stage and/or low rate of conversion into glutamine in the astrocytes (Fig. 3.3F). In the neonatal rat brain the action potentials are smaller in amplitude and the firing rate is lower (McCormick and Prince 1987) and the EEG does not develop an adult pattern until P12 (Snead and Stephens 1983). Moreover, glutamine synthetase activity, just like PC activity, is very low at birth, but increases sharply during the first weeks of life (Wilbur and Patel 1974; Hertz et al. 1978; Juurlink et al. 1981; Larsson et al. 1985). Thus, several factors may lead to this difference regarding astrocyte–neuron interactions in the neonatal and the adult brain.

3.5 What Factors Affect Pyruvate Carboxylation?

3.5.1 Increased Pyruvate Carboxylation

Cerebral hyperammonemia is believed to play a pivotal role in the development of hepatic encephalopathy (HE), a debilitating condition arising due to acute or chronic liver disease (Butterworth 2003). In the brain, ammonia is thought to be detoxified via the activity of the astrocytic enzyme glutamine synthetase. However, glutamine cannot be synthesized without increased activity of PC as pointed out earlier. Zwingmann

(2007) showed that an elevated ammonia concentration increased pyruvate carboxylation both in astrocytes in culture and in animal models. This carboxylation might be coupled to the increased glutamine formation which is a hallmark of ammonia detoxification (Zwingmann 2007). Glutamine formation is the only efficient method of ammonia fixation in brain and also in other cell culture studies increased production of glutamine was detected (Leke et al. 2011). It may be of interest that upon inhibition of the glutamine synthesizing enzyme glutamine synthetase ammonia fixation is brought about by the concerted action of glutamate dehydrogenase and alanine aminotransferase leading to net synthesis of alanine, a process stimulating glycolysis to provide pyruvate for alanine production (Dadsetan et al. 2011, 2013).

The ketogenic diet is a high-fat, adequate-protein, low-carbohydrate diet that is used primarily to treat refractory epilepsy in children (Yudkoff et al. 2004). The diet causes an increase in the metabolism of fatty acids rather than carbohydrates. The effect of ketone bodies on pyruvate carboxylation by rat brain mitochondria has been investigated (Patel 1974a) and it was shown that pyruvate carboxylation increased subsequent to feeding rats a ketogenic diet (Melø et al. 2006). This increase in pyruvate carboxylation was coupled to an elevated pyruvate recycling (Melø et al. 2006), i.e. conversion of oxaloacetate to pyruvate which re-enters the TCA cycle via pyruvate dehydrogenase, as expected since synthesis of a metabolite has to be equalled by its degradation.

3.5.1.1 Neurotransmission

Activation of the glutamatergic α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor induces large post-synaptic action potentials (Rao and Finkbeiner 2007) and should therefore stimulate release of neurotransmitter glutamate. In experiments with brain tissue slices metabolizing [1-^{13}C]glucose it was possible to show evidence of pyruvate carboxylation in aspartate which is obtained by transamination of oxaloacetate, the product of pyruvate carboxylation by pyruvate carboxylase (Rae et al. 2009). Increased pyruvate carboxylation was detected when the slices were incubated with AMPA (Rae et al. 2009) suggesting that increased neuronal activity increases carboxylation of pyruvate, a finding compatible with the notion that glutamate biosynthesis requires anaplerosis coupled to pyruvate carboxylation. Furthermore, it was shown that pyruvate carboxylation in the awake rat brain was several fold higher than under deep pentobarbital anaesthesia (Oz et al. 2004).

3.5.2 Decreased Pyruvate Carboxylation

3.5.2.1 Anxiety and Epilepsy

Decreased pyruvate carboxylation has been reported after pentylenetetrazole (PTZ) injection in rats (Eloqayli et al. 2004). PTZ administration is a generally accepted model for epileptic seizures but when given in low doses PTZ can cause anxiety

(Eloqayli et al. 2004). Interestingly, the antiepileptic drug carbamazepine (CBZ) decreased the biotin concentration in both humans and rats and decreased pyruvate carboxylase activity in rat brain (Rathman et al. 2003). These results support the use of biotin supplementation as a concurrent strategy during CBZ administration to help maintain pyruvate carboxylation.

3.5.2.2 Ischaemia

Not surprisingly, ischaemia has a profound effect on pyruvate carboxylation, a pathway dependent on adenosine triphosphate availability. In adult rats subjected to 120 min of middle cerebral artery occlusion followed by 120 min of reperfusion it could be shown that the utilization of precursors in astrocytic metabolism originating from the pyruvate carboxylase pathway was markedly reduced compared to that of precursors originating from the pyruvate dehydrogenase pathway. Hence, glutamate synthesis was reduced and that of GABA completely stopped in the ischaemic core area (Håberg et al. 2006). Furthermore, in the re-perfused penumbra, glutamatergic and GABAergic neurons used relatively more astrocytic metabolites derived from the pyruvate carboxylase pathway than in the ischaemic core (Håberg et al. 2006). In the canine cardiac arrest model of global ischaemia, pyruvate carboxylation was decreased in the hippocampus in animals that had undergone hyperoxic resuscitation (Scafidi et al. 2009).

Interestingly, in the Rice–Vannucci model of neonatal hypoxia–ischaemia in postnatal day 7 rats, PC activity is relatively preserved compared to that of the pyruvate dehydrogenase pathway. Labelling via pyruvate dehydrogenase was reduced by 44 % while labelling via pyruvate carboxylase was reduced by 30 % in the hypoxic–ischaemic hemisphere in the Rice–Vannucci model (Brekke et al. 2014) while, as pointed out, in adult models of middle cerebral artery occlusion, PC activity is more profoundly reduced than pyruvate dehydrogenase activity (Haberg, Qu et al. 1998, 2006). This illustrates how the neonatal and adult brain can respond differently to similar insults and may need different treatments.

The consequences of relatively preserved PC activity in the neonatal brain following hypoxia–ischaemia may be ambiguous. As mentioned earlier there is negligible transport of glutamate from neurons to astrocytes during normal conditions in the neonatal brain (Morken et al. 2014). During hypoxia–ischaemia, there is a large release of glutamate that may originate from neurons as well as astrocytes through reversal of glutamate transporters (Puka-Sundvall, Sandberg et al. 1997; Kusaka et al. 2004). However, no increase in transfer of glutamate from neurons to astrocytes was found following neonatal hypoxia–ischaemia at postnatal day 7 in rats. Both glutamate uptake and glutamine synthesis are energy demanding processes, which may explain the lacking augmentation in transfer following hypoxia–ischaemia. Contradicting this, transfer of glutamine from astrocytes to neurons as well as glutamine synthesis and PC activity, which are ATP demanding processes were preserved at this time point following injury suggesting that astrocytes have at least partly maintained or re-established their energy balance (Morken et al. 2014). It is conceivable that preservation of anaplerosis and transfer of newly synthesized glutamine to neurons in the immediate recovery phase following hypoxia–ischaemia

may have harmful effects, but this remains to be investigated. On the other hand, following the massive release of glutamate into the extracellular space during hypoxia–ischaemia, neurons might be depleted of TCA cycle intermediates (such as α-ketoglutarate). Under these conditions anaplerosis might be beneficial since de novo synthesized glutamine transferred from astrocytes may be necessary for neurons to re-establish oxidative metabolism.

Surprisingly, at 6 h after hypoxia–ischaemia the transfer of glutamate from neurons to astrocytes was transiently decreased, even though at this point, the energy levels are almost back to normal (Yager et al. 1992). The exact mechanism of this has yet to be explored.

3.5.2.3 Glutamate Exposure

Qu et al. (2001) showed that exogenous glutamate had an inhibitory effect on pyruvate carboxylation in astrocytes, presumably by formation of oxaloacetate from α-ketoglutarate derived from glutamate metabolism. Inhibition of pyruvate carboxylation was also observed when glutamine was added to astrocytes (Hassel and Sonnewald 2002). When metabolism of ^{13}C-labelled glucose was investigated in cultured mouse cerebellar astrocytes, it could be shown that carboxylation of pyruvate was more important for biosynthesis of releasable glutamine and citrate, compared with their intracellular pools (Waagepetersen et al. 2001b). Activation of the cellular energy sensor, AMP-activated protein kinase (AMPK), has been shown to reduce the metabolism of glutamate in the TCA cycle of astrocytes. Specifically, the synthesis of citrate from glutamate involving malic enzyme and pyruvate carboxylase was reduced. Since AMPK is known to upregulate catabolic processes, this may suggest that glutamate has an anabolic function in astrocytes (Voss et al. 2015).

3.5.2.4 Alzheimer's Disease

Regional hypometabolism of glucose in the brain is a hallmark of Alzheimer's disease (AD) (Gibson et al. 1998, 1999; Huang et al. 2003; Klivenyi et al. 2004). However, little is known about the specific alterations of neuronal and astrocytic metabolism involved in homeostasis of glutamate and GABA in AD. In a recent study, the effects of amyloid β pathology on energy and neurotransmitter metabolism were investigated in the transgenic McGill-R-Thy1-APP rat model of AD, at age 15 months (Nilsen et al. 2014). Rats were injected with [1-^{13}C]glucose and [1,2-^{13}C]acetate, and brain extracts of the hippocampal formation as well as several cortical regions were analysed using ^{1}H- and ^{13}C nuclear magnetic resonance spectroscopy and high performance liquid chromatography. A decrease of pyruvate carboxylation in the hippocampal formation and retrosplenial/cingulate cortex was observed in the McGill-R-Thy1-APP rat and this decrease might have affected the level of glutamine in the hippocampal formation and levels of glutamate, glutamine, GABA and aspartate in the retrosplenial/cingulate cortex (Nilsen et al. 2014). Future treatment of AD patients might be targeted at increasing anaplerosis in the brain.

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Chapter 4
Enzyme Complexes Important for the Glutamate–Glutamine Cycle

Mary C. McKenna and Gustavo C. Ferreira

Abstract Transient multienzyme and/or multiprotein complexes (metabolons) direct substrates toward specific pathways and can significantly influence the metabolism of glutamate and glutamine in the brain. Glutamate is the primary excitatory neurotransmitter in brain. This neurotransmitter has essential roles in normal brain function including learning and memory. Metabolism of glutamate involves the coordinated activity of astrocytes and neurons and high affinity transporter proteins that are selectively distributed on these cells. This chapter describes known and possible metabolons that affect the metabolism of glutamate and related compounds in the brain, as well as some factors that can modulate the association and dissociation of such complexes, including protein modifications by acylation reactions (e.g., acetylation, palmitoylation, succinylation, SUMOylation, etc.) of specific residues. Development of strategies to modulate transient multienzyme and/or enzyme–protein interactions may represent a novel and promising therapeutic approach for treatment of diseases involving dysregulation of glutamate metabolism.

Keywords Transient multienzyme complexes • Metabolon • Glutamate • Brain • Transporters • Acylation

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Abbreviations

α-KG	α-Ketoglutarate
AAT	Aspartate aminotransferase
AMPA	α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
BCAA	Branched-chain amino acid
BCAT	Branched-chain aminotransferase
BCATc	Cytosolic branched-chain aminotransferase
BCATm	Mitochondrial branched-chain aminotransferase
BCKD	Branched-chain ketoacid dehydrogenase
BCKDC	Branched-chain ketoacid dehydrogenase complex
GABA	Gamma-aminobutyric acid
GAD	Glutamic acid decarboxylase
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GDH	Glutamate dehydrogenase
GLAST	EAAT1 or excitatory amino acid transporter 1
GLT-1	EAAT2 or excitatory amino acid transporter 2
hBCAT	Human branched-chain aminotransferase
hBCATm	Human mitochondrial branched-chain aminotransferase
ICDH	Isocitrate dehydrogenase
KGDH	α-Ketoglutarate dehydrogenase
MDH	Malate dehydrogenase
ME	Malic enzyme
mGluR	Metabotropic glutamate receptor
mMDH	Mitochondrial malate dehydrogenase
mTOR	Mammalian target of rapamycin
NCX	Na^+/Ca^{2+} exchanger
nNOS	Neuronal nitric oxide synthase
OAA	Oxaloacetate
PAG	Phosphate-activated glutaminase
PC	Pyruvate carboxylase
PDH	Pyruvate dehydrogenase
SCHAD	Short-chain 3-hydroxyacyl-CoA dehydrogenase
SIRTs or sirtuins	Silent information regulator proteins
SUMO	Small ubiquitin-like modifiers
TCA	Tricarboxylic acid
VDAC	Voltage-dependent anion channel
vGLUTs	Vesicular glutamate transporters

4.1 Introduction

There is increasing evidence that the formation of transient multienzyme complexes (metabolons), which can rapidly associate and dissociate, can greatly influence metabolism. The association and dissociation of these transiently formed

heteroenzyme complexes is governed by fluctuations in the concentration of key metabolites in mitochondria and to some extent by protein modifications (Fahien et al. 1977, 1985, 1988; Teller et al. 1990; Fahien and Teller 1992; Malik et al. 1993; McKenna et al. 2000a, 2006b; McKenna 2007, 2011; Gibson et al. 2015; McKenna and Rae 2015; Yang et al. 2015; Robinson and Jackson 2016). Formation of these transient enzyme and/or enzyme–protein complexes occurs dynamically in response to localized changes in the concentration of metabolites and thus has the ability to modulate metabolism in "real time." Biochemical modifications of enzyme molecules can modulate activity and/or the ability of these proteins to associate into enzyme complexes. Given the importance of glutamate and glutamine metabolism in brain for neurotransmission and bioenergetics, an understanding of the intricate regulation of participating enzymes and associated proteins is crucial.

This chapter includes information about the enzymes involved in glutamate metabolism and summarizes reports on the formation and modulation of transient multienzyme complexes that could potentially influence glutamate and glutamine metabolism and cycling in astrocytes and neurons. We discuss the regulation of glutamate dehydrogenase as this large homohexameric enzyme may have a role in organizing multienzyme enzyme complexes (Fahien et al. 1989). Common post-translational modifications that influence the activity and/or properties of proteins directly or indirectly involved in glutamate metabolism are also discussed.

4.2 Overview of Glutamate and Glutamine Metabolism and Compartmentation in Brain

Glutamate is the primary excitatory neurotransmitter in brain and has essential roles in normal brain function including learning and memory (Schweigert et al. 2005; McKenna 2007, 2013; Schousboe et al. 2014; Connor and Wang 2015; Kumar 2015; Kennedy 2016). Astrocytes are key partners in brain function, and glutamatergic neurotransmission requires a continuous, dynamic interaction between astrocytes and neurons (McKenna 2013). After depolarization of glutamatergic neurons the concentration of glutamate in the synaptic cleft increases from a low resting concentration of ~0.1–1 μM to ~100 μM −1 mM (Bergles et al. 1999; Matsui et al. 2005). The high affinity glutamate transporters in astrocytes rapidly and efficiently remove glutamate from the synaptic cleft, which allows glutamatergic neurotransmission to continue and prevents excitotoxicity (Danbolt 2001; McKenna 2013). Glutamate, taken up by astrocytes is primarily oxidized for energy (McKenna 2007, 2013; Schousboe et al. 2014) or converted to glutamine, which can be released by astrocytes and taken up by neurons to replenish TCA cycle intermediates and neuronal glutamate (Leke and Schouboe 2016). Glutamate can also participate in the purine nucleotide cycle (Yudkoff et al. 1986) or be used for synthesis of proteins or the tripeptide glutathione (Dringen 2000).

Glutamine, which is formed primarily, if not exclusively in astrocytes due to the selective localization of glutamine synthetase in these cells (Norenberg and Martinez-Hernandez 1979), is taken up from the extracellular milieu by neurons and converted

back to neurotransmitter glutamate, oxidized for energy via the tricarboxylic acid (TCA) cycle (Yudkoff et al. 1988), or decarboxylated to form gamma-aminobutyric acid (GABA), the main inhibitory neurotransmitter in brain (Schousboe 1981; Sonnewald et al. 1993b; Westergaard et al. 1995a; McKenna et al. 2011). De novo synthesis of glutamine occurs in astrocytes due to the compartmentation of the enzyme pyruvate carboxylase (PC) in these cells (Yu et al. 1983; Shank et al. 1985). By adding a carbon to pyruvate to form the 4-carbon TCA cycle intermediate oxaloacetate, metabolism via PC leads to formation of glutamine that is released by astrocytes and ultimately serves to replenish the glutamate and TCA cycle intermediates in neurons that would otherwise be depleted via neurotransmission (McKenna 2007, 2013; Schousboe et al. 2014; Sonnewald 2014 and refs therein).

Numerous transporters and enzymes are needed for glutamate, glutamine, and GABA metabolism, and a number of these proteins are differentially distributed between astrocytes and neurons (McKenna 2007, 2013; Schousboe et al. 2014). Such differential distribution or compartmentation of transporters, enzymes, and metabolic pathways is needed so that glutamatergic and GABAergic neurons and the astrocytes surrounding synapses can perform their highly specialized functions in brain (McKenna 2007, 2013; Schousboe et al. 2014) (Fig. 4.1 and Table 4.1). The phenomenon of compartmentation requires the trafficking or exchange of metabolites between different cell types in brain (Westergaard et al. 1995a; Schousboe et al. 1997; McKenna et al. 2000a). Key factors contributing to compartmentation are the heterogeneity in the localization of transporters and metabolic enzymes, *within* populations of astrocytes and neurons (McKenna et al. 1990, 2000a; Oh et al. 1991; Cesar and Hamprecht 1995), and differences *between* glia and neurons that lead to the unique metabolic capabilities of each cell type (Kvamme et al. 1985; Lai et al. 1989; Schousboe et al. 1997; Sonnewald et al. 1997, 1998; Hanu et al. 2000; McKenna et al. 2000b; Yudkoff et al. 2000; McKenna 2007). A number of the enzymes important in metabolism are regulated differently in astrocytes and neurons (Malik et al. 1993; McKenna et al. 2000a, 2006b; McKenna 2007, 2011 and references therein). *Intracellular* compartmentation of metabolism also occurs in both astrocytes and neurons (McKenna et al. 1990, 1995, 1996a, b, 2000a; Schousboe et al. 1993; Sonnewald et al. 1993a, b; Bakken et al. 1997; Waagepetersen et al. 1998, 1999 and references therein). In addition to compartmentation among different cell types and organelles, indication of cytoplasmic compartmentation of metabolites in neural cells has been reported (McKenna et al. 1994, 1995, 1996a, b, 2006a; Bakken et al. 1997; Waagepetersen et al. 1998). Indeed, the gel-like composition of the cytoplasm contains proteins and extensive membranous surfaces and that can restrict the diffusion of small molecules (Jones 1986; Pollack 2001). Interestingly, the metabolic state of the cell may regulate mobility of glycolytic enzymes (Pagliaro and Taylor 1992).

Since compartmentation leads to differences in neuronal and glial metabolism, recognizing the importance of this phenomenon is essential for understanding regulation of metabolism in brain (McKenna et al. 2000a; McKenna 2007). A knowledge of the enzymes involved in the formation and disposal of glutamate, glutamine, and related metabolites is essential for understanding glutamate metabolism in brain. Enzymes with a role in glutamate and glutamine metabolism in astrocytes and neurons are shown in Fig. 4.1 and Table 4.1.

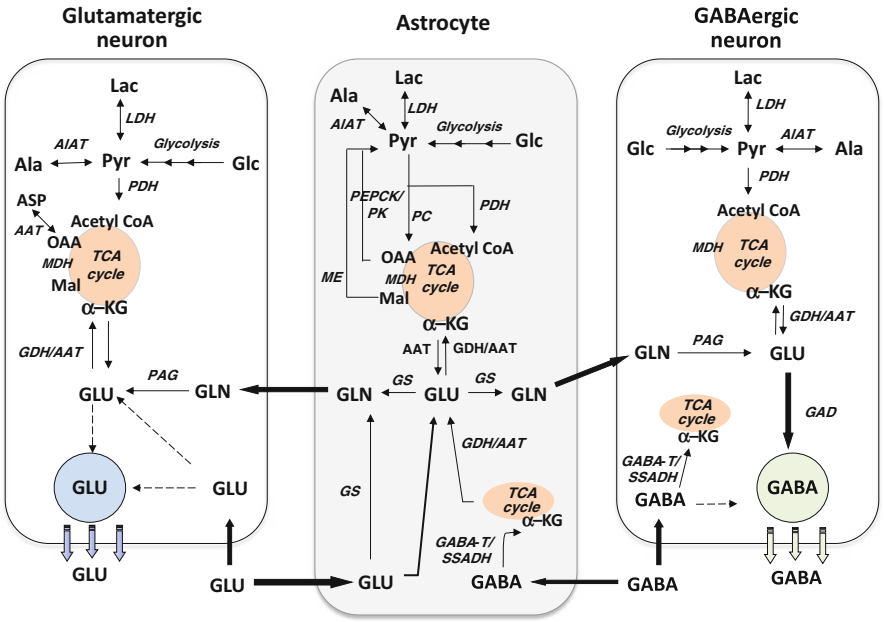

Fig. 4.1 Neurotransmitter glutamate (GLU) interacts with receptors and is rapidly taken up by the high affinity transporters on astrocytes surrounding the synaptic cleft. In the astrocyte, glutamate is either metabolized in the TCA cycle for energy or converted to glutamine (GLN) via the enzyme glutamine synthetase (GS), which forms part of an open glutamate–glutamine cycle. Glutamine is released by astrocytes and taken up by glutamatergic neurons to be used for energy or converted to glutamate via phosphate-activated glutaminase (PAG). Glutamate is converted to the TCA cycle intermediate α-ketoglutarate by the activity of GDH or via AAT or other aminotransferase enzymes. The carbon skeleton of glutamate can replenish the pool of TCA cycle intermediates in neurons and astrocytes. In GABAergic neurons, GABA is synthesized from GLU via the activity of glutamate decarboxylase (GAD). The carbon skeleton of glutamate can also be partially or fully oxidized via the pyruvate recycling pathway which involves malic enzyme (ME), or phosphoenolpyruvate carboxykinase (PEPCK) and pyruvate kinase (PK). The de novo synthesis of glutamate and glutamine from glucose occurs via the combined action of PDH and pyruvate carboxylase (PC) in astrocytes leading to net synthesis of TCA cycle intermediates. Some of the enzymes discussed in this chapter are not shown in this figure. It should be noted that aspartate formation from OAA occurs in all of three cell types but is shown only in one cell type; and pyruvate recycling, shown only in astrocytes may also occur in neurons. *Ala* alanine, *ASP* aspartate, *Glc* glucose, *GLN* glutamine, *GLU* glutamate, *α-KG* α-ketoglutarate, *Lac* lactate, *Mal* malate, *OAA* oxaloacetate, *Pyr* pyruvate, *AlAT* alanine aminotransferase, *AAT* aspartate aminotransferase, *GDH* glutamate dehydrogenase, *GAD* glutamate decarboxylase, *GABA-T* GABA transaminase, *LDH* lactate dehydrogenase, *MDH* malate dehydrogenase, *ME* malic enzyme, *PC* pyruvate carboxylase, *PDH* pyruvate dehydrogenase, *SSADH* succinic semialdehyde dehydrogenase (Figure adapted from Schousboe et al. 2014). See references therein and this chapter for more details

Table 4.1 Compartmentation of a number of enzymes and proteins involved in glutamate metabolism in brain

Localization	Enzymes/transporters/proteins
Located in neurons (not in astrocytes)	cBCAT, cytosolic branched-chain aminotransferase
	BCKDC-E1α, branched-chain α-ketoacid dehydrogenase complex subunit E1α
Located only in GABAergic neurons	GAD, glutamic acid decarboxylase
Highly enriched in neurons	PDH, pyruvate dehydrogenase
	PAG, phosphate-activated glutaminase
	mME, mitochondrial malic enzyme
Neuronal glutamate transporters	EAAC1/EAAT3, EAAT4, EAAT5
Located in astrocytes (not in neurons)	GS, glutamine synthetase
	PC, pyruvate carboxylase
	hBCATm, mitochondrial branched-chain aminotransferase (in rodents, but not in humans)
Highly enriched in astrocytes	cME, cytosolic malic enzyme
Astrocytic glutamate transporters	GLAST/EAAT1, GLT1/EAAT2
Located in both astrocytes and neurons	AAT, aspartate aminotransferase (both mitochondrial and cytosolic isoforms)
	GDH, glutamate dehydrogenase
	Binding protein on the inner mitochondrial membrane that interacts with AAT, MDH, and mutienzyme complexes (unidentified but expected to be present); see section 4.3.3
Located in endothelial cells	BCKDC-E1α, branched-chain α-ketoacid dehydrogenase complex E1α subunit (in humans)
	hBCATm, mitochondrial branched-chain aminotransferase (in humans) (Hull et al. 2012)

Adapted from McKenna (2007)

4.3 Glutamate Dehydrogenase (GDH) and Aminotransferase Enzymes Have Essential Roles in Linking Glutamate Metabolism to Energy Metabolism

4.3.1 Metabolism of Glutamate to α-Ketoglutarate

As noted earlier glutamate taken up by astrocytes can be oxidatively metabolized via the TCA cycle. The first step in this process is conversion to the TCA cycle intermediate α-ketoglutarate either by deamination by the enzyme glutamate dehydrogenase (GDH) or transamination by one of the aminotransferase enzymes, aspartate aminotransferase, branched-chain amino acid transaminase, or alanine aminotransferase (McKenna 2011; Schousboe et al. 2014; McKenna et al. 2016 and refs therein).

The mitochondrial enzyme GDH functions in brain primarily to catalyze the deamination of glutamate to the TCA cycle intermediate α-ketoglutarate, releasing NH_3 and forming NAD(P)H (Cooper and Meister 1985; Yudkoff et al. 1990, 1991; Wysmyk-Cybula et al. 1991; McKenna et al. 1993, 1996a, 2000a; Westergaard et al. 1996; Zaganas et al. 2009; Cooper 2011). Although the thermodynamic equilibrium of GDH favors the energy requiring reductive amination of α-ketoglutarate to glutamate, the NAD^+/NADH ratio in the mitochondria and the high Km for ammonia favors the oxidative deamination in brain (Cooper and Meister 1985). Two forms of GDH are found in human brain, GDH1 which is widely expressed (Stanley 2004; Raizen et al. 2005; Spanaki et al. 2010), and GDH2 which is particularly high in astrocyte mitochondria and is also labeled in neuronal mitochondria (Spanaki et al. 2010). The structure and regulation of GDH1 and GDH2 have been reported (Plaitakis et al. 2000; Plaitakis and Zaganas 2001; Smith et al. 2001; Mastorodemos et al. 2005, 2009, 2015; Zaganas et al. 2009; Li et al. 2011; Stanley 2011; Botman et al. 2014; Karaca et al. 2015; Rasgado et al. 2015). Changes in GDH activity have been reported in several conditions leading to neurodegeneration (Plaitakis et al. 1982, 1984, 2003; Plaitakis and Zaganas 2001; Zaganas et al. 2009; Shashidharan and Plaitakis 2014).

Aminotransferase enzymes mediate the transfer of amino nitrogen in brain. These enzymes, which require the cofactor pyridoxal-5′-phosphate, catalyze the reversible transfer of amino groups between glutamate and α-ketoglutarate, in conjunction with another amino acids and their cognate keto acids (Cooper 1988; McKenna et al. 2000a). Aspartate aminotransferase (AAT) which has the highest transaminase activity in brain catalyzes the transamination of oxaloacetate to aspartate in conjunction with the interconversion of glutamate and α-ketoglutarate (Cooper and Meister 1985; Cooper 2011). GDH and AAT act together in metabolism in most tissues since GDH either adds an amino group to α-ketoglutarate or removes an amino group from glutamate (Yudkoff et al. 1990, 1991; Wysmyk-Cybula et al. 1991; McKenna et al. 1993, 1996a, 2000a; Westergaard et al. 1996), whereas aminotransferase enzymes, including AAT, transfer the amino groups to or from glutamate to convert keto acids into amino acids (Cooper and Meister 1985; Yudkoff et al. 1990, 1991; Wysmyk-Cybula et al. 1991; McKenna et al. 1993, 1996a, 2000a; Westergaard et al. 1996). The extent to which GDH and AAT or other aminotransferase enzymes work together in brain cells is not known. However, work from the Hutson lab recently reported that GDH and enzymes involved in branched-chain amino acid (BCAA) metabolism can form a functional complex in some tissues (Islam et al. 2010; Conway and Hutson 2016).

GDH and AAT have specific roles in astrocytes and neurons in brain (Moran and Rivera-Gaxiola 1992; Westergaard et al. 1996; Moran et al. 1999; McKenna et al. 2000a; Yudkoff et al. 2005). Formation of α-ketoglutarate from exogenous glutamate can be catalyzed by both GDH and AAT or other transaminases; however, many studies support a more predominant role for GDH in astrocytes (McKenna et al. 1996b, 2000a; Schousboe et al. 2014). The association of glutamate transporters, GDH and mitochondria into complexes in astrocytes would favor metabolism via GDH and subsequent oxidation for energy (Genda et al. 2011; Bauer et al. 2012;

Robinson and Jackson 2016). In contrast, the resynthesis of glutamate from α-ketoglutarate occurs primarily via AAT in astrocytes (Westergaard et al. 1996). It is well established that *exogenous* glutamate taken up from the extracellular milieu, and glutamate formed endogenously from glutamine via the action of glutaminase are metabolized differently in brain cells (McKenna et al. 1996b; Sonnewald and McKenna 2002; McKenna 2007; Schousboe et al. 2014). This is likely due to compartmentation and the close interaction of enzymes that can essentially channel metabolites through sequential reactions (McKenna et al. 2000a, 2006b; McKenna 2011 and references therein).

Interestingly, the entry of endogenous glutamate (formed from glutamine via glutaminase) into the TCA cycle is mediated only via GDH in synaptosomes, as the oxidation of glutamine by synaptosomes is not inhibited by aminooxyacetate which inhibits AAT and all aminotransferase enzymes (McKenna et al. 1993). The compartmentation and roles of AAT and GDH in brain have been reviewed by our group and others (McKenna et al. 1993, 1996b, 2000a, 2006b; Westergaard et al. 1996; McKenna 2007, 2011; Zaganas et al. 2012; Schousboe et al. 2014; Spanaki et al. 2014, 2015). Protein modifications such as acylation and palmitoylation can modify the kinetic parameters and binding properties of enzymes, including those involved in glutamate and glutamine metabolism (McKenna et al. 2006b; Zhao et al. 2010; McKenna 2011; Gibson et al. 2015; McKenna and Rae 2015).

The enzymes alanine aminotransferase (AlAT) and branched-chain amino transferase (BCAT) can also form α-ketoglutarate from glutamate via transamination with pyruvate or branched-chain keto acids, respectively (Yudkoff et al. 1989, 1991, 1993, 1996; Westergaard et al. 1995b; Yudkoff 1997; Waagepetersen et al. 2000; Hutson 2001; Hutson et al. 2001; Sweatt et al. 2004a; Bak et al. 2005, 2007; Islam et al. 2010). It has been proposed that both enzymes, particularly BCAT, function to transfer nitrogen between neurons and astrocytes to provide nitrogen for glutamate formation in brain (Cooper 2011; Hutson et al. 2011; Schousboe et al. 2014; Hull et al. 2015b; Conway and Hutson 2016). Studies by the Hutson lab have reported the compartmentation of BCAT in brain and interaction with other brain enzymes (Bixel et al. 2001; Garcia-Espinosa et al. 2007; Islam et al. 2010; Hutson et al. 2011; Cole et al. 2012; Hull et al. 2015b; Conway and Hutson 2016).

4.3.2 Regulation of GDH in Brain

Regulation of GDH is discussed as this enzyme may have a role in organizing heteroenzyme complexes (Fahien et al. 1993). Regulation of GDH has been extensively studied since this enzyme is so important in nitrogen metabolism in brain and other tissues (Cooper and Plum 1987; Plaitakis and Zaganas 2001; Zaganas et al. 2009, 2012; Cooper 2011; Plaitakis et al. 2011; Spanaki et al. 2012, 2014). GDH1 and GDH2 associate into homohexamers but are regulated differently (Stanley 2004; Raizen et al. 2005; Spanaki et al. 2010). GDH1 activity is controlled by the

energy needs of cells and is allosterically inhibited by GTP (Stanley 2004; Raizen et al. 2005; Spanaki et al. 2010). GDH2 from human brain is not inhibited by GTP, and therefore can metabolize glutamate in the presence of an active TCA cycle and high energy levels (Smith and Stanley 2008; Spanaki et al. 2010; Li et al. 2011; Plaitakis et al. 2011; Stanley 2011).

The lipid modifications acetylation and palmitoylation can regulate GDH by altering activity and binding properties of the protein (Schlicker et al. 2008). Activity of GDH can also be modulated by mitochondrial silent information regulator proteins (sirtuins or SIRTs). Human SIRT3 binds and deacetylates GDH leading to activation (Schlicker et al. 2008); whereas SIRT4 inactivates GDH by ADP-ribosylation of the enzyme in liver and likely in brain (Haigis et al. 2006). Modulation of GDH activity by sirtuins is consistent with the roles of SIRT3 and SIRT4 in controlling many important mitochondrial processes including metabolism, intracellular signaling, energy production, and apoptosis (Verdin et al. 2010).

Overexpression of GDH1 in cultured neurons from mice led to altered synaptic plasticity, increased release of glutamate, and selective vulnerability of CA1 neurons (Bao et al. 2009). Interestingly, both decreased activity and gain-of-function mutations of GDH led to seizures and hyperexcitability of brain (Raizen et al. 2005; Malthankar-Phatak et al. 2006; McKenna 2011; Stanley 2011) and discussed by McKenna (2011 and references therein). Studies inducing decreased expression of GDH in astrocytes by siRNA and knockout mice with no GDH activity showed that reduction/loss of GDH activity impaired oxidation of glutamate in the TCA cycle (Skytt et al. 2012).

4.3.3 Aminotransferase Enzymes Can Be Subject to Regulation

Even though aminotransferase catalyzed reactions are considered freely reversible as the direction of the reaction is driven by the concentration of substrates and products, there is evidence that these enzymes may be subject to other levels of regulation (McKenna et al. 2000a, 2006b; Hull et al. 2015b; Yang et al. 2015). For example, Teller et al. (1990) reported that full activation of AAT requires the enzyme to associate with a binding protein that transfers it to lipids on the inner mitochondrial membrane. Palmitoylation enhances the interaction between GDH and AAT (Fahien and Kmiotek 1983). A report from McKenna et al. (2006b) demonstrating that addition of palmitate and other small molecules including malate, citrate, glutamate, bovine serum albumin, and Mg^{2+} modulated AAT activity in synaptic and nonsynaptic mitochondria from brain is consistent with regulation of this enzyme. Furthermore, it has recently been shown that acetylation of three lysines on mitochondrial AAT enhances the association between mAAT and mitochondrial malate dehydrogenase (mMDH; also known as MDH2) and promotes the transfer of the reducing equivalents from NADH into mitochondria via the malate-aspartate shuttle (Yang et al. 2015). Taken together, these studies demonstrate that AAT activity, and

thus the metabolism of glutamate, may be regulated in part by protein modifications and metabolites that influence the formation of multienzyme complexes and/or binding of the enzyme to mitochondrial membranes and proteins. Interestingly, the association of AAT with its mitochondrial binding protein may facilitate the transfer of endogenously formed glutamate to AAT rather than to GDH in astrocytes, consistent with our demonstration that conversion of the glutamate formed endogenously from glutamine to α-ketoglutarate occurs primarily via AAT, as it can be blocked by the transaminase inhibitor aminooxyacetic acid (AOAA) (McKenna et al. 1993, 1996b, 2000a, 2006b). Our group recently reported that exogenous glutamate is oxidized in astrocytes at a rate several fold higher than endogenously formed glutamate (McKenna 2012). Constraints in mitochondrial binding of enzymes reported by Teller et al. (1990) can shed light on the compartmentation and differential metabolism of exogenous and endogenously formed glutamate. As discussed in depth by McKenna et al. (2000a), Teller showed that either AAT or MDH (alone or MDH associated with TCA cycle enzymes) can bind to the inner mitochondrial membrane (Teller et al. 1990). Although the binding of MDH to Complex I on the inner membrane has higher affinity than the association of AAT with its binding protein, the binding of AAT prevents the binding of MDH (Teller et al. 1990 and references therein). Given these constraints, it is logical that the endogenous glutamate formed from glutamine is not as rapidly oxidized as exogenous glutamate taken up from the extracellular milieu, since the AAT used to metabolize *endogenous* glutamate would have to dissociate from the binding protein and/or lipids, permitting MDH to bind, for oxidation via the TCA cycle to occur (McKenna et al. 1996b, 2000a).

In contrast to astrocytes, studies of metabolism in synaptosomes isolated from rat brain are consistent with the channeling of endogenous glutamate, formed from glutamine through a complex between GDH and phosphate-activated glutaminase (PAG) that facilitates neuronal oxidation of glutamine (McKenna et al. 1993). ^{13}C-NMR studies showing differential compartmentation of the metabolism of exogenous and endogenously formed glutamate also support the possibility of a PAG–GDH complex that provides substrate to a neuronal TCA cycle associated with GABA formation via glutamic acid decarboxylase (GAD) (Sonnewald and McKenna 2002). GDH has also been shown to associate with a complex between AAT and PC; MDH also associated with this ternary complex (Fahien et al. 1993). It is not known if this complex occurs in brain.

It should be emphasized that an essential, but sometimes overlooked, role of glutamate in both neurons and astrocytes is its role in the malate-aspartate shuttle that transfers reducing equivalents produced during glycolysis into the mitochondria so the energy can be harvested via the electron transport chain (McKenna et al. 2006c). This transfer of reducing equivalents is essential to maintain the NAD^+/NADH ratio required for continued glycolysis. Proteins that participate in this shuttle include both cytosolic and mitochondrial forms of AAT and MDH, the mitochondrial aspartate-glutamate carrier Aralar, and the malate-α-ketoglutarate carrier. This shuttle has a crucial role in brain, thus the formation of any complexes that facilitate either entry of glutamate into mitochondria or the activity of the malate-aspartate shuttle could have a major influence on metabolism.

The sections earlier provided an overview of glutamate and glutamine metabolism and compartmentation. In the section below we address some known and possible multienzyme/multiprotein complexes that can potentially affect metabolism of glutamate and related compounds, as well as factors that can modulate the association and dissociation of these complexes. The formation, dynamics, and metabolic consequences of these transient multiprotein complexes are likely to represent a further level of regulation of the metabolism of glutamate in brain.

4.4 Multienzyme Complexes in Metabolism

4.4.1 Evidence for and Consequences of Multienzyme Complex Formation

The metabolic outcome of the transient formation of these enzyme complexes, together with mitochondrial binding proteins that interact with enzymes and transporters (Teller et al. 1990; McKenna et al. 2000a), is variable. The activity of some enzymes and/or metabolism via certain pathways can be decreased by such interactions; whereas, metabolism through other pathways may be enhanced as the high affinity of participating enzymes for substrates can lead to 'channeling' of metabolites into specific pathways (Fahien et al. 1977, 1988, 1989; Beeckmans and Kanarek 1981; Beeckmans et al. 1990; Stanley 2011). Intrinsic mitochondrial properties, including the high density of enzymes, proteins, and lipids, restrict diffusion of both metabolites and proteins and increase the likelihood of multienzyme complex formation (Lopez-Beltran et al. 1996; McKenna et al. 2000a; Pollack 2001). Evidence supporting the formation of multienzyme complexes is from a wide variety of experimental tools, from simple studies assessing the in vitro binding capabilities of isolated/purified enzymes or purified mitochondrial membranes to other proteins and/or membranes (Fahien et al. 1977, 1985, 1988, 1989, 1990; Beeckmans and Kanarek 1981, 1987; Beeckmans et al. 1990, 1993; Teller et al. 1990; Fahien and Teller 1992; McKenna et al. 2000a) (and references therein), to more sophisticated approaches, using photobleaching (Haggie and Brindle 1999; Haggie and Verkman 2002) and proteomics using mass spectrometry (Rose et al. 2009; Li et al. 2010a; Genda et al. 2011). To date a number of transient multienzyme complexes that may directly or indirectly affect the metabolism of glutamate and glutamine have been described (Islam et al. 2007; Rose et al. 2009; McKenna 2011; Yang et al. 2015; Conway and Hutson 2016; Robinson and Jackson 2016; and references in these papers) (see Table 4.2). The dynamic assembly/disassembly of multienzyme complexes provides unique kinetic advantages that can coordinately impact multiple metabolic pathways, and the knowledge in this field will undoubtedly increase as novel technologies are developed and utilized to identify expected and unexpected interactions in living cells. Several studies reported evidence of heteroenzyme complex formation in vivo (Haigis et al. 2006; Li et al. 2011; Stanley 2011; Regenold et al. 2012; Shan et al. 2014).

Table 4.2 Known and likely enzyme complexes that may occur in brain

Enzymes interacting	Effect/predicted effect	References
CS + MDH	Increases TCA cycle activity	Fahien and Kmiotek (1983) and Morgunov and Srere (1998)
ICDH + KGDH + Complex I	Increases TCA cycle activity	Porpaczy et al. (1987)
CS + MDH + KGDH	Increases TCA cycle activity	Fahien et al. (1988)
KGDH + MDH + AAT	Facilitates metabolism via malate-aspartate shuttle	Fahien et al. (1988)
KGDH + MDH + AAT + GDH	Facilitates metabolism via malate-aspartate shuttle	Fahien et al. (1988)
AAT + GDH + ME	Transfers substrate and cofactor; increases pyruvate recycling pathway	McKenna et al. (1995)
AAT + inner mitochondrial binding protein + lipids	Activates mitochondrial AAT	Teller et al. (1990)
PAG + GDH	Facilitates entry of glutamine into TCA cycle for oxidation only via GDH in synaptosomes	McKenna et al. (1993)
PAG + GAD (glutamate decarboxylase)	Colocalized in thalamus; likely facilitates conversion of glutamine to GABA	Fisher (2007)
AAT + GDH	GDH adds or removes NH_3; AAT transfers amino group to oxaloacetate or from aspartate; increases TCA cycle activity	Fahien and Kmiotek (1983)
AAT + CS	Increases TCA cycle activity	Fahien and Kmiotek (1983)
MDH + GDH	Increases GDH activity (by removing NADH from the catalytic site)	Fahien and Kmiotek (1983)
GDH + AlAT	GDH adds or removes NH_3; AlAT transfers amino group to pyruvate or from alanine	Fahien et al. (1977)
GDH + BCAT	GDH adds or removes NH_3; BCAT transfers amino group to branched-chain ketoacids or from branched-chain amino acids (BCAA)	Islam et al. (2010)

GDH + BCAT + BCKD	Facilitates NH_3 transfer and oxidation of BCAA	Islam et al. (2010) and Hutson et al. (2011)
GDH + GLT1 (glutamate transporter)	Facilitates delivery of exogenous glutamate to mitochondria and oxidation in astrocytes	Genda et al. (2011)
GLT1 + hexokinase + mitochondria	Facilitates glutamate stimulation of glucose metabolism in astrocytes	Genda et al. (2011)
GLT1 + mitochondria	Facilitates delivery of exogenous glutamate to mitochondria and oxidation in astrocytes	Genda et al. (2011)
GLT1 + Na^+/K^+-ATPase	Drives Na^+-dependent glutamate uptake against an unfavorable gradient	Genda et al. (2011)
GLAST + mitochondria	Facilitates delivery of exogenous glutamate to mitochondria and oxidation in astrocytes	Bauer et al. (2012)
GLAST + Na^+/K^+-ATPase + HK + GAPDH + mitochondrial enzymes	Provides energy and drives Na^+-dependent glutamate uptake against an unfavorable gradient—supports local glutamate uptake	Bauer et al. (2012)
GLT1 + GDH	GDH inhibition leads to decreased Na^+-dependent L-glutamate uptake	Whitelaw and Robinson (2013)
KGDHC + PDH	KGDHC succinylates PDH which increases PDH activity; KGDHC can also acylate PDH which decreases activity	Gibson et al. (2015)
KGDHC + fumarase	KGDHC succinylates fumarase which increases activity	Gibson et al. (2015)
SCHAD + GDH	Inhibitory regulation of GDH by SCHAD—lost in SCHAD deficiency, leading to hyperinsulinism	Li et al. (2010a)

4.4.2 *Many Factors Influence the Association and Dissociation of Transiently Formed Enzyme Complexes in Mitochondria*

A number of different transient multienzyme complexes may be formed between mitochondrial enzymes, transporters, and proteins in brain. There are many effectors that influence the kinetics and binding of binary, ternary, and quaternary enzyme complexes between proteins (Fahien et al. 1977, 1985, 1988, 1989, 1990; Halper and Srere 1977; Beeckmans and Kanarek 1981; Moore et al. 1984; Sumegi and Srere 1984; Srere 1987; Beeckmans et al. 1990; Teller et al. 1990; Fahien and Teller 1992; McKenna et al. 2000a) (and references therein). As noted by McKenna (2011) "specific complexes are formed depending on (a) the binding affinities of enzymes for other enzymes, (b) the relative concentration of each enzyme in the mitochondrial matrix, (c) the affinity of binding to the mitochondrial membranes, (d) or to specific binding proteins in the mitochondrial membrane, (e) the order in which binding occurs, (f) the concentration of metabolites (e.g. citrate, oxaloacetate, α-ketoglutarate, malate, aspartate, fumarate, etc.) and ions (e.g. Mg^{2+}) that influence binding of specific enzymes (Fahien et al. 1977, 1985, 1988; Teller et al. 1990; Fahien and Teller 1992; Malik et al. 1993; McKenna et al. 2000a, 2006b; McKenna 2007), (g) the form in which enzymes are found e.g., AAT and MDH are dimers, GDH forms homohexamers when the concentration is sufficient, (h) the stoichiometry of enzyme binding, [e.g. α-ketoglutarate dehydrogenase (KGDH) complex can associate with AAT and MDH with a ratio of 10 total AAT or MDH bound per KGDH (Teller et al. 1990)], and (i) palmitoylation which alters protein conformation and allows proteins to associate and dissociate from specific subcellular microdomains (Fukata and Fukata 2010), e.g. palmitoyl-CoA has been shown to enhance the interactions between GDH and AAT (Fahien and Kmiotek 1983)." In addition, attachment of other types of acyl-moieties to proteins including acetylation (Zhao et al. 2010) and succinylation (Zhang et al. 2011; Gibson et al. 2015; McKenna and Rae 2015) can significantly alter the physicochemical properties and binding affinity as discussed later.

4.4.3 *Fluctuations in the Concentration of Small Molecules Can Modulate Enzyme Activity and Complex Formation*

Our group and others have reported that many small molecules can modulate the activity of key enzymes including MDH, mitochondrial AAT, and malic enzyme (ME) in nonsynaptic and synaptic mitochondria (McKenna et al. 1990, 1994, 1996a, b, 2000a, b, 2006b; Malik et al. 1993; McKenna and Sonnewald 2005; McKenna 2007; Gibson et al. 2015). A recent report from Gibson's group demonstrated KGDH-mediated succinylation modulates the activity of several brain enzymes including KGDH, fumarase, and pyruvate dehydrogenase (PDH) (Gibson et al. 2015;

McKenna and Rae 2015). The concentration of small molecule effectors fluctuates during normal brain metabolism and can change dramatically during pathological conditions (McKenna et al. 2006b; McKenna 2007). Compounds known to influence complex formation and/or enzyme activity include citrate, oxaloacetate, α-ketoglutarate, malate, aspartate, fumarate, glutamate, Ca^{2+}, and Mg^{2+} (McKenna et al. 2006b and references therein); Ca^{2+} also stimulates the activity of the mitochondrial aspartate-glutamate carrier (Contreras et al. 2007; Bak et al. 2012; Llorente-Folch et al. 2013). Small metabolite effectors are important since they can modulate the association and dissociation of transient multienzyme complexes that can rapidly influence metabolism (McKenna et al. 2006b; McKenna 2007).

Due to the viscous mitochondrial matrix, diffusion of enzymes away from other enzymes or proteins or away from membranes would be slow even when multienzyme complexes are dissociated (Srere 1987; Srere et al. 1987). With such close physical proximity, complexes could associate when the concentration of key effectors favors association, and dissociate when the concentration of effectors fluctuates under physiological or pathological conditions.

4.5 Transient Multienzyme Complexes That Affect Metabolism of Glutamate and Related Compounds

4.5.1 Possible Role of GDH and Other Large Enzymes in Organizing Complexes

Relatively little is known about the localization, regulation, and/or roles of transiently formed enzyme complexes that can influence metabolism in brain. Interestingly, many of the complexes formed contain GDH, leading Fahien and coworkers to propose that GDH "could play an organizational role" "in the quaternary complex between GDH, the aminotransferases, MDH, and pyruvate carboxylase" similar to the role performed by the KGDH complex in some tissues (Fahien et al. 1993) [see related discussion in (McKenna 2011)]. Such an organizational role for GDH would be extremely important as it would provide a mechanism to position enzymes that do not bind directly to each other (e.g., AAT and MDH) so that they are physically close enough to transfer substrates and cofactors (Fahien et al. 1993). The high concentration of GDH (up to 10 % of mitochondrial matrix protein in astrocyte processes) would allow for such an organizational role (Fahien and Teller 1992; Fahien et al. 1993; Rothe et al. 1994, 1995). In addition, a key role for GDH in organizing other enzymes into multienzyme complexes would help to explain the severe neurological consequences of mutations that cause conformational changes in GDH (Bahi-Buisson et al. 2008a, b).

Fahien and coworkers noted that the rate-limiting step in the GDH reaction is dissociation of NADH, and that binding of MDH to GDH in the quaternary complex (GDH, MDH, AAT, and fumarase) facilitated the removal of NADH from

GDH (Fahien and Kmiotek 1983; Fahien and Teller 1992; Fahien et al. 1993). KGDH, which like GDH is a large stable enzyme complex, can also associate with the AAT–GDH–MDH complex: however, as GDH is found in much higher concentration it would likely perform the organizational role of forming the complex with AAT and MDH in brain (Salganicoff and De Robertis 1965; Faff-Michalak and Albrecht 1991; Fahien et al. 1993; McKenna et al. 2000a, 2006b and references therein). Despite the fact that high GDH activity has been reported in neurons and synaptosomes, immunolabeling of GDH is lower in neurons than in astrocytes (Erecinska and Silver 1990; McKenna et al. 1993, 2000a; Rothe et al. 1994, 1995). Many groups have reported the activity of brain enzymes (Salganicoff and De Robertis 1965; Lai and Clark 1989; Faff-Michalak and Albrecht 1991; McKenna et al. 2000a, 2006b) but there is little information on the relative concentration of the different brain enzymes in different cell types and cellular compartments.

4.5.2 GDH Participates in Heteroenzyme Complexes with AAT and TCA Cycle Enzymes

As reviewed by McKenna et al. (2000a) and McKenna (2011) both AAT and MDH can form complexes with GDH, and the presence of AAT increases the affinity of MDH binding to GDH (Fahien and Kmiotek 1983; Fahien and Teller 1992); however, due to stoichiometry of binding, multiple complexes can be formed. The Fahein group observed that under the conditions used, the molar ratio of enzymes bound in vitro was always >1 AAT per GDH homohexamer (Fahien and Teller 1992). The only complexes formed were ternary complexes of GDH–AAT–MDH and GDH–MDH–(AAT)2 and the binary complex of GDH–AAT (Fahien and Teller 1992). A quaternary complex was formed by binding of fumarase to the ternary complex (Fahien and Teller 1992). It is important to note that even when such a complex is dissociated the physical proximity of the participating enzymes is so close that even very small crosslinker molecules (with only 12 Å span) can link all three of these enzymes or GDH and MDH (Fahien et al. 1985; Fahien and Teller 1992).

Binding of allosteric effectors induces conformational changes that impact enzyme activity (Fahien et al. 1989). Binding of substrate to AAT facilitates the reaction of this enzyme (Hayashi et al. 2003). Even though a complex between MDH and AAT is not actually formed (Fahien and Teller 1992), the ternary complex of AAT–GDH–MDH brings these two enzymes in close enough physical proximity to transfer the oxaloacetate from MDH to AAT (Fahien and Teller 1992). In addition, a binary complex between AAT and GDH can associate with malic enzyme (ME) and facilitates transfer of both substrate and cofactors (Teller et al. 1990; Fahien and Teller 1992) to ME, which has a higher Km for malate than mitochondrial MDH (McKenna et al. 1995, 2000b). This association "would facilitate conversion of TCA cycle metabolites into pyruvate" and thus increase metabolism via the pyruvate recycling pathway in brain. In liver, palmitoyl-CoA enhances the association of AAT and GDH into a complex (Fahien and Kmiotek 1983); whereas

citrate enhances dissociation of AAT from GDH (Fahien et al. 1985), as discussed by McKenna (2011). The concentrations of mitochondrial enzymes in liver are an order of magnitude higher than the dissociation constants of the heteroenzyme complexes (Fahien and Teller 1992); however, such information is not readily available for brain.

4.5.3 Glutamate Transporters Can Form Complexes with Metabolic Enzymes and Mitochondria

The striking difference in glutamate concentration among distinct biological compartments within (e.g., vesicles, mitochondria, cytosol) or outside (e.g., synaptic cleft) neural cells is driven by glutamate transporters. As glutamate cannot be degraded extracellularly, GLAST (Excitatory amino acid transporter 1—EAAT1) and GLT-1 (Excitatory amino acid transporter 2—EAAT2), high affinity transporters on astrocytes have the key role of removing glutamate from the synaptic cleft to prevent excessive stimulation of glutamate receptors on neuronal plasma membranes (Danbolt 2001). Vesicular glutamate transporters (vGLUTs) are responsible for vesicular packing of this excitatory amino acid (Danbolt 2001).

Studies over the past decade, using a variety of preparations, have shown that glutamate transporters interact with a number of crucial proteins from different metabolic pathways of the CNS, including PSD-95 (Gonzalez-Gonzalez et al. 2008, 2009), Na^+/H^+ exchanger regulatory proteins (Lee et al. 2007; Sato et al. 2013), and glutamine transporters (SNAT3) (Martinez-Lozada et al. 2013). There is also evidence that glutamate transporters interact with proteins involved in brain bioenergetics, including Na^+/K^+-ATPase (Rose et al. 2009) and Na^+/Ca^{2+}exchanger (NCX) (Magi et al. 2012, 2013), as well as glycogen metabolizing enzymes, glycolytic enzymes, and mitochondrial proteins (Genda et al. 2011; Bauer et al. 2012). Some of these protein complexes facilitate metabolism, e.g., the macromolecular complex between GLAST and GDH facilitates the oxidative metabolism of transported glutamate in astrocyte mitochondria for energy production (Bauer et al. 2012), which offsets the energy requirements associated with glutamate uptake (McKenna 2013). The colocalization of GLT-1 with glycolytic enzymes may facilitate any glutamate and/or potassium stimulated increase in glucose metabolism that may occur after neurotransmitter release (Pellerin and Magistretti 1994; Peng et al. 1994, 2001), and would provide "a mechanism to spatially match energy and buffering capacity to the demands imposed by transport" as noted by Genda et al. (2011). Although the binding of GLT-1 to mitochondrial proteins may occur through GDH (Bauer et al. 2012), hexokinase was also hypothesized as a putative link between these proteins. However, the presence of membrane-permeant peptides, which are known to disrupt the interaction between hexokinase and the voltage-dependent anion channel (VDAC), did not affect coimmunoprecipitation and interactions between GLT-1 and the mitochondrial proteins adenine nucleotide translocator (ANT) or the ubiquinol–cytochrome-c reductase complex in a brain subfraction, suggesting that the hexokinase is not the protein that anchors GLT-1 to mitochondria (Jackson et al. 2015).

However, displacing hexokinase from VDAC diminished GLT-1-dependent glutamate uptake, implying that "this interaction is either required to fuel uptake or supports co-compartmentalization" as noted by Jackson et al. (2015).

Interactions of glutamate transporters and other proteins appear to have functional relevance. For instance, acute activation of adenosine A2A receptors, which were shown to interact with GLT-1 and the Na^+/K^+-ATPase α2 isoform in astrocytes, leads to decreased glutamate uptake and Na^+/K^+-ATPase activity, while glutamate uptake is enhanced following deletion of astrocytic A2A receptors (Matos et al. 2012a, b, 2013). In addition, it has recently been shown that the glutamate transporter EAAC1 (EAAT3) is found on both neuronal and astrocyte mitochondria, and that a physical and functional interaction of the EAAC1 and NCX1 has a modulatory effect on glutamate-enhanced ATP production in brain mitochondria (Magi et al. 2012, 2013). The functional coupling between these systems may support glutamate uptake, as well as be regulated by glutamate transport (Robinson and Jackson 2016).

Recent studies suggest that synaptic activity promotes the colocalization of astrocyte membrane transporters with mitochondria following synaptic activity, since colocalization between mitochondria and GLT1 was observed in mixed cultures of astrocytes and primary cortical neurons that contained a higher amount of mitochondria in astrocytic processes; whereas, no colocalization was found in pure astrocyte cultures (Ugbode et al. 2014). Interestingly, even though GLAST and GLT-1 are often colocalized on astrocyte membranes, they may take part in distinct multienzyme complexes, as there is no evidence for coimmunoprecipitable interactions between them (Haugeto et al. 1996). Understanding the nature, dynamics, and regulation of these protein complexes will enable us to better unravel their role(s) in the physiological functioning of different brain cells, as well as any role(s) that association/dissociation of macromolecular complexes may have in the pathophysiology of CNS diseases. Glutamate receptors can also form complexes with other proteins, for example glyceraldehyde 3-phosphate dehydrogenase (GAPDH), a crucial enzyme for glycolysis, was shown to interact with the GluR2 subunit of AMPA receptors. The inability to form GluR2/GAPDH interactions protected cells against AMPA receptor-mediated excitotoxicity (Wang et al. 2012; Zhai et al. 2013), and thus may represent an alternative therapeutic target for glutamate toxicity.

4.5.4 Branched-Chain Aminotransferase (BCAT) and Branched-Chain Ketoacid Dehydrogenase (BCKD) Participate in a Metabolon with GDH in Some Tissues

Leucine and other branched-chain amino acids (BCAA) may serve as a source of nitrogen in the brain and other tissues (Hutson et al. 2011). Plasma concentrations of BCAA are reflected in peripheral tissues as levels increase proportionately with higher intake of BCAAs (Hutson and Harper 1981; Neishabouri et al. 2015). BCAA levels in brain are moderately elevated following a high protein diet, since

competition with other amino acids limits uptake across the blood–brain barrier (Hutson and Harper 1981; Neishabouri et al. 2015). The two first enzymes in their catabolic pathway, namely, the mitochondrial branched-chain aminotransferase (BCATm) and branched-chain ketoacid dehydrogenase (BCKD), can form a heteroenzyme metabolon that facilitates degradation of BCAA (Islam et al. 2007, 2010). Work from Hutson's group (Islam et al. 2010; Hutson et al. 2011) reported that this metabolon interacts with GDH in rat liver as well as with bovine GDH1. GDH binds to the pyridoxamine-5′-phosphate form of BCATm in vitro (Islam et al. 2010; Hutson et al. 2011). BCAA oxidation was stimulated by the formation of BCAT/BCKD complex and the provision of α-ketoglutarate for the transfer of nitrogen from the BCAT to glutamate was facilitated by the binding of GDH1 to the complex, ultimately enhancing BCKD enzyme activity. It is possible that GDH may have a role in assembling/facilitating this complex.

It was demonstrated that the cytosolic BCAT (BCATc) is the predominant isozyme of BCAT in the CNS (Hall et al. 1993; Sweatt et al. 2004a, b). While BCATc is expressed in "select populations of glutamatergic and GABAergic neurons" (Sweatt et al. 2004a; Garcia-Espinosa et al. 2007), BCATm is enriched in astrocytes of rodents (most often located in the neuropil and myelinated tracts, instead of in gray matter) and the "labeling for BCATm in these astrocytes extends into the cell processes, indicating a wide intracellular distribution of the enzyme in this cell type" (Cole et al. 2012). Labeling for the BCKDC-E1α subunit is present in several neuronal cell types, including glutamatergic, and cholinergic neurons, while astrocytes lack BCKDC labeling (Cole et al. 2012). "The segregation of BCATm to astrocytes and BCKDC to neurons provides further support for the existence of a BCAA-dependent glial-neuronal nitrogen shuttle since the data show that branched-chain keto acids produced by glial BCATm must be exported to neurons" (Cole et al. 2012). Since the complex between GDH and BCAT/BCKD identified by the Hutson group (Islam et al. 2010; Hutson et al. 2011) was restricted to BCATm, one can assume that neurons are devoid of such complexes. However, the expression of BCATm and the GDH-related glutamate disposal in astrocytes, leading to formation of α-ketoglutarate and subsequent oxidation suggest that a complex could exist in astrocytes. It should be noted that the conversion of α-ketoglutarate to glutamate in astrocytes is mediated by AAT, not by GDH, as noted earlier (McKenna et al. 1996a; Sonnewald et al. 1996; Westergaard et al. 1996).

Besides the ability to form a metabolon with BCKD and GDH, a novel functional role for human BCAT (hBCAT) in redox protein folding was recently proposed, particularly when hBCAT was S-glutathionylated (El Hindy et al. 2014). The formation of complexes between hBCAT with unfolded proteins, catalyzing their refolding, suggests that mitochondrial hBCAT (hBCATm) may also function as chaperone, by a thiol disulfide exchange mechanism or as a redox-regulated oxidoreductase (El Hindy et al. 2014).

In distinct contrast to rodent brain, hBCATm is not found in astrocytes in humans, where GDH is predominantly expressed (Hull et al. 2015a). A recent study from Hull and colleagues (2015a) reported an increase of hBCATm in the frontal and temporal cortex in AD subjects, relative to matched controls, suggesting that the expression of this enzyme may be directly related to the severity of the disease.

As initially hypothesized by Helms and colleagues (2012) and later discussed by Hull and colleagues (2012), the blood–brain barrier itself may play a role in the regulation of brain glutamate levels. Due to the high expression of BCAT throughout the endothelial layer in different areas of the human brain (Hull et al. 2012), one can assume that transamination is taking place in these cells, particularly when glutamate exceeds the Km of the Na^+-dependent transporters involved in its uptake (O'Kane et al. 1999), during periods of excitation or excitotoxicity (Dzubay and Jahr 1999; Moussawi et al. 2011). The potential source of branched-chain ketoacids remains uncertain but mRNA levels of the monocarboxylic acid transporters (MCT1 and MCT2), that are able to transport these monocarboxilic acids, were shown in rat brain endothelial cells (RBE4 cell line) (Mac and Nalecz 2003). The direction of BCAT reaction should be driven by the concentration of substrates and redox state of the cell. Therefore, if α-ketoglutarate is sufficiently provided by the TCA cycle and considering that these cells contain L-system transporter, which allow the transport of BCAAs (Smith et al. 1987; Shulkin et al. 1995; O'Kane and Hawkins 2003; O'Kane et al. 2004), it is also feasible that this reaction would occur in the opposite direction. GDH, if present, could provide an alternative source of α-ketoglutarate for this reaction and the association between BCATm and GDH could replenish this TCA intermediate when BCAT acts in the direction of glutamate formation (Hutson et al. 2011). Therefore, a novel role for hBCATm in glutamate homeostasis in the endothelial cells of the human brain vasculature as well as the possible occurrence of a BCAT/BCKD metabolon in these cells has been proposed (Hull et al. 2012, 2015b). The mammalian target of rapamycin (mTOR) complex 1 integrates many intra- and extracellular signals to control major processes in cell metabolism, including protein and lipid synthesis and autophagy (Laplante and Sabatini 2012). Dysregulation of mTOR complex 1 signaling was reported to be involved in the aging process (Gilley et al. 2013) and also in human diseases, including cancer (Menon and Manning 2008, 2013), Fragile X Syndrome (Richter et al. 2015), and depression (Li et al. 2010b). It was also recently reported that BCATc indirectly regulates mTOR complex 1 signaling, by modulating leucine concentration and glycolytic metabolism in immune cells ($CD4^+$ T cells) (Ananieva et al. 2014) and that the chronic mTOR complex 1 activation by BCAAs may result in hypertrophy in peripheral organs, including heart, kidney, and spleen (Neishabouri et al. 2015). However, it is uncertain if these effects also occur in the brain.

4.5.5 Interactions of Short-Chain 3-Hydroxyacyl-CoA Dehydrogenase (SCHAD) and Glutamate/Glutamine Metabolism Enzymes

It has recently been reported that an inherited deficiency of short chain 3-hydroxyacyl-CoA dehydrogenase (SCHAD) can lead to hyperinsulinemia in patients (Li et al. 2010a). Studies in SCHAD knockout mice provide evidence that in SCHAD deficiency, loss of the inhibitory interaction between SCHAD and GDH leads to

activation of GDH in islet cells of the pancreas resulting in increased insulin release (Stanley 2011). This occurs because SCHAD, like many other multifunctional proteins, can act as a "moonlighting protein" which performs a role different than the primary role of the protein (Jeffery 2005). In this role SCHAD serves to inhibit GDH activity (Stanley 2011). The interaction between SCHAD and GDH was also later reported in brain, kidney, and liver of mice (Narayan et al. 2012). Other enzymes involved in glutamate metabolism known to have protein–protein interactions with SCHAD include AAT and glutamine synthetase (Narayan et al. 2012).

4.6 Posttranslational Modifications Can Influence Enzyme Activity and the Ability to Form Transient Multienzyme Complexes

Posttranslational covalent modifications to proteins can affect their stability and function, modulating their biological activity and interfering with major cellular pathways (Fischer 2014; Perluigi et al. 2014). The addition of each specific type of functional group confers unique properties to modified proteins and results in alterations in protein/protein interactions, membrane binding, targeting of proteins, and in intracellular signaling (Resh 1996; Baeza et al. 2016). The next section describes a number of covalent modifications affecting enzymes related to glutamate and glutamine metabolism, including covalent attachment of an acetyl-, palmitoyl-, succinyl-, or small ubiquitin-like modifiers (SUMO) moiety, and indicates the role(s) of sirtuins in these modifications of other proteins (Verdin et al. 2010; Osborne et al. 2014; Baeza et al. 2016) (Table 4.3).

4.6.1 Acetylation Is a Common Posttranslational Modification That Alters Enzyme Activity

Acetylation of lysine residues is a widespread posttranslational modification affecting metabolic enzymes and the underlying mechanism is believed to involve non-enzymatic attachment of an acetyl moiety from acetyl-CoA to reactive lysine residues. Reversible acetylation is one of the most extensively studied posttranslational modifications in mitochondria and is frequently associated to impaired functioning of key metabolic pathways involved in the integrity and redox homeostasis in this organelle (for an extensive review, see Baeza et al. 2016). Acetylation of mitochondrial enzymes is reversed by the NAD^+-dependent deacetylase SIRT3 and mitochondrial sirtuins were recently proposed to play an important role in the modulation of mitochondrial metabolism (Osborne et al. 2014). SIRT3 regulates protein deacetylation and is important for mitochondrial metabolism, cell survival, and longevity (Cimen et al. 2010). Acetylation affects many metabolic enzymes involved

Table 4.3 Common protein covalent modifications affecting glutamate and glutamine metabolism

Protein modification		References
Acetylation	Found on essentially all enzymes in glycolysis, gluconeogenesis, the tricarboxylic acid (TCA) cycle, the urea cycle, fatty acid metabolism, and glycogen metabolism in liver; less studies in brain	Zhao et al. (2010)
	Deacetylation of GDH by SIRT 3 activates its enzyme activity in mitochondrial matrix	Schlicker et al. (2008)
	SIRT 3 deacetylates and increases pyruvate dehydrogenase activity in cancer cells	Ozden et al. (2014)
	Acetylation activates MDH activity in human liver cells	Zhao et al. (2010)
	Acetylation of mitochondrial AAT enhances the association between mAAT and MDH2 and promotes the transfer of reducing equivalents from NADH into mitochondria via the malate-aspartate shuttle. Stimulates NADPH production in mitochondria	Yang et al. (2015)
	Deacetylation of succinate dehydrogenase flavoprotein subunit increases Complex II activity in liver of mice	Cimen et al. (2010)
ADP-Ribosylation	ADP-ribosylation of GDH by SIRT 4 leads to inactivation of GDH activity	Haigis et al. (2006)
Palmitoylation	Palmitoylation enhances interactions between GDH and AAT	Fahien and Kmiotek (1983)
	Palmitoylation of GAD65 is crucial for post-Golgi trafficking of the protein to presynaptic clusters in rat hippocampal neurons	Kanaani et al. (2008)
	Glutamate dehydrogenase isozymes are inhibited by palmitoylation	Fahien and Kmiotek (1981)
	Palmitoylation of GAD65 leads to its anchoring to membranes of small vesicles in rat pancreatic β-cells	Christgau et al. (1992)
Succinylation	Succinylation of isocitrate dehydrogenase decreases its activity in cultured neurons from embryonic C57BL/6 mice	Gibson et al. (2015)
	Succinylation of KGDHC, fumarase, and PDHC increases their activities in cultured neurons from embryonic C57BL/6 mice	Gibson et al. (2015)
	Succinate dehydrogenase is activated by succinylation in human embryonic kidney (HEK) 293T cultured cells	Park et al. (2013)
SUMOylation	SUMOylation of presynaptic proteins increases glutamate release in synaptosomes	Feligioni et al. (2009)
	SUMOylation of metabotropic glutamate receptors (mGluR) results in novel protein interactions and regulates their density, localization, and functional properties	Scheschonka et al. (2007)

in glutamate metabolism in the cell, notably impairing the activities of GDH, PDH (Lombard et al. 2007; Schlicker et al. 2008), and the TCA enzymes isocitrate dehydrogenase (Someya et al. 2010; Yu et al. 2012), the NDUFA9 subunit of Complex I (Ahn et al. 2008), and succinate dehydrogenase flavoprotein (SdhA), one of the Complex II subunits (Cimen et al. 2010; Finley et al. 2011). SIRT3 binds to GDH and leads to its deacylation and subsequent activation (Schlicker et al. 2008). A different sirtuin, SIRT4, uses NAD to inactivate GDH in the brain by ADP-ribosylating it (Haigis et al. 2006). SIRT4 has mitochondrial localization in rodent brain (Komlos et al. 2013), particularly in astrocytes in the postnatal brain and in radial glia during embryogenesis, and GDH inactivation may occur after a modification of a single subunit of an active homohexamer (Herrero-Yraola et al. 2001). In line with this, a repression of transcriptional production of SIRT4 by mTOR complex 1 leads to increased glutamine anaplerosis to the TCA cycle, indicating GDH activation (Csibi et al. 2013).

However, it should be underscored that acetylation does not always impair enzyme activity; for example, acetylation stimulates the activity of aconitase (Fernandes et al. 2015). Interestingly, MDH activity can be stimulated or inhibited depending on the specific lysine residue that is acetylated (Zhao et al. 2010; Hebert et al. 2013). Besides interfering with enzyme activities, acetylation can also modulate binding properties of proteins, as in the case of the triple acetylation of mitochondrial AAT that enhances the formation of a complex between mAAT and MDH2, that increases malate-aspartate shuttle activity, and the transfer of the reducing equivalents from cytosolic NADH into mitochondria (Yang et al. 2015). Production of NADPH to attenuate ROS formation and protect cells from oxidative damage is also enhanced by the formation of this multienzyme complex (Yang et al. 2015). Despite the function of thiamin as a cofactor for enzyme reactions, a novel role for this vitamin as an allosteric regulator of acetyl-CoA metabolism was recently described, ultimately affecting acetylation of proteins (Mkrtchyan et al. 2015). One should not underestimate the importance of this modification since it was recently stated that “reversible acetylation of mitochondrial proteins represents a new layer of protein regulation mediating acute and adaptive changes in mammalian metabolism” (Wagner and Payne 2011).

4.6.2 Palmitoylation of Proteins Can Dynamically Influence Compartmentation, Localization, and Interactions of Proteins and Signaling

Palmitoylation is the attachment of a 16-carbon fatty acid moiety to a protein via a thioester bond (Fukata and Fukata 2010). Unlike some modifications, palmitoylation occurs rapidly and allows proteins to shuttle between different intracellular compartments (e.g., Golgi and plasma membrane), and usually leads to a more rapid turnover of the protein (Fukata and Fukata 2010 and references therein). The

relevance of palmitoylation and other lipid modifications to glutamate and glutamine metabolism was recently discussed and includes modulation of enzyme activities and binding properties (for details, see (McKenna 2011) and references therein). In this scenario, activation of glutamate receptors was shown to modulate palmitoylation of proteins and play a role in brain development and synaptic plasticity (Noritake et al. 2009; Fukata and Fukata 2010). McKenna and colleagues demonstrated that palmitate interfered with AAT activity in synaptic and nonsynaptic mitochondria (McKenna et al. 2006b), which is in line with previous reports indicating that AAT binds to the inner mitochondrial membrane, and must subsequently be transferred to lipids to become fully activated (Teller et al. 1990; Fahien and Teller 1992). Palmitoylation can occur spontaneously (Fukata and Fukata 2010) or by enzymatic reaction of palmitoyl acyltransferase enzymes, while palmitoyl acyl thioesterases mediate depalmitoylation (Fukata and Fukata 2010), and the regulation of these processes under physiological or pathophysiological context is still under debate (Noritake et al. 2009).

4.6.3 Succinylation of Lysine Residues in Enzymes Can Alter Activity

Succinylation of lysine residues in proteins represents a new level of metabolic regulation (Park et al. 2013; Choudhary et al. 2014), affecting key enzymes in glycolysis, the TCA cycle, fatty acid metabolism, and others (Papanicolaou et al. 2014). A recent report demonstrated that succinylation of multiple proteins is reduced when KGDH is inhibited in neural cells, even though KGDH is not the sole mechanism of attachment of this acyl moiety to proteins in the cell (Gibson et al. 2015). Identified targets for protein succinylation by KGDH include KGDH itself and other enzymes involved in brain bioenergetics, such as fumarase, isocitrate dehydrogenase, and pyruvate dehydrogenase (PDH). The catalytic outcome of this modification was varied: in contrast to the decreased isocitrate dehydrogenase activity observed, the activities of KGDH, fumarase, and PDH were found to be increased by succinylation (Gibson et al. 2015), which is in line with a previously reported activation of succinate dehydrogenase by succinylation (Park et al. 2013). Taken together, a putative role for KGDH inducing succinylation includes activation of TCA cycle steps from KGDH to oxaloacetate, and deceleration of the initial steps (from isocitrate dehydrogenase to KGDH), which would facilitate the oxidation of glutamate via a partial TCA cycle (McKenna and Rae 2015). It is worth noting that KGDH activity can also lead to acetylation of proteins in cytosol and mitochondria, including PDH (Gibson et al. 2015). In contrast to the effect of succinylation, glutathionylation (Cooper et al. 2011) and internally generated reactive oxygen species produced by the E3 subunit can inhibit KGDH activity (Bunik and Sievers 2002). In addition to effects on enzyme activity, the attachment of one or more large negatively charged succinyl groups can modify the physicochemical properties of proteins, and thus alter their ability to associate into transient multienzyme complexes (McKenna and Rae 2015), which can substantially influence metabolism

(McKenna et al. 2000a, 2006b; Genda et al. 2011; McKenna 2011; Bauer et al. 2012; Whitelaw and Robinson 2013; Jackson et al. 2014; McKenna and Rae 2015).

There is evidence that SIRT5 participates in the regulation of desuccinylation of proteins, with a weak deacetylation activity (Houtkooper et al. 2012; Newman et al. 2012; Park et al. 2013). Increased levels of protein succinylation are described in SIRT5 knock-out mice, that present with hyperammonemia probably due to the succinylation and inhibition of carbamoyl phosphate synthetase 1 (Nakagawa et al. 2009), but do not display any other evident metabolic abnormalities (Yu et al. 2013). Since succinylation occurs rapidly and dynamically within the cell (Gibson et al. 2015), it is feasible that the modulation elicited by such modification on TCA cycle oxidative capacity might be regulated in response to cellular demands.

4.6.4 SUMOylation of Proteins Can Affect the Glutamate–Glutamine Cycle

Sumoylation of proteins, the covalent addition of small ubiquitin-like modifiers (SUMO), provides an important posttranslational modification for many biological processes in the brain, particularly during development (Kerscher 2007). It may lead to alterations in the localization, activity, and stability of modified proteins (Geiss-Friedlander and Melchior 2007). Feligioni and colleagues demonstrated that SUMOylation of presynaptic proteins increases glutamate release (Feligioni et al. 2009). EAAT2 cleavage by the activation of caspase-3 in a mouse model of ALS results in the formation of a peptide fragment that can be SUMOylated and builds up along with disease course in the nucleus of astrocytes (Gibb et al. 2007), reaching up to 20 % of the $GFAP^+$ cells of the ventral horn of the spinal cord at later stages of disease (Foran et al. 2011). SUMOylation of proteins could also affect protein–protein interactions, by inhibiting, modifying, or enhancing such phenomena (Ulrich 2008). For instance, it was suggested that metabotropic glutamate receptors (mGluR) might be SUMOylated, resulting in specific protein interactions and ultimately regulating their density, localization, and functional properties (Scheschonka et al. 2007).

4.6.5 Protein Modifications Following Oxidative and/or Nitrosative Stress Can Modulate Glutamate Metabolism

Proteins can undergo many oxidative modifications, particularly under pathological conditions, that can alter their intrinsic activity, including carbonylation, nitration, and S-nitrosylation, which are frequently considered deleterious to cell survival (Pedersen et al. 1998; Beal 2002; Johnson and Johnson 2015). However, an interesting case is nitration of a tyrosine residue of glutamine synthetase that leads to the inactivation of the enzyme (Schliess et al. 2002; Haussinger et al. 2005). This

apparent deleterious loss of enzyme function was in fact proposed to be protective during hyperammonemia, since the decrease in glutamine synthetase activity mitigated the metabolic changes contributing to ammonia toxicity including glutamine accumulation, cell swelling, Ca^{2+} influx, and the production of NO and reactive oxygen intermediates in rat astrocytes (Schliess et al. 2002).

A recent report also investigated the biological functions of protein S-nitrosylation in the tripartite glutamatergic synapse (Raju et al. 2015). Proteomic analyses indicated that mice lacking neuronal nitric oxide synthase ($nNOS^{-/-}$) activity had decreased S-nitrosylation of proteins that participate in the glutamate-glutamine cycle, and that brain extracts from $nNOS^{-/-}$ mice converted less glutamate to glutamine and oxidized more glutamate than those from mice of the other genotypes. This most likely is due to increased metabolism of glutamate to α-ketoglutarate via GDH and/or mAAT and subsequent oxidation, as well as a partial loss of glutamine synthetase activity. S-Nitrosylation of GLT-1 at Cys373 and Cys561 residues was absent in $nNOS^{-/-}$ mice, and synaptosomes from these animals showed decreased glutamate uptake sensitive to dihydrokainate (Raju et al. 2015), which inhibits GLT-1 (Robinson 1998), indicating a decrease in GLT-1 glutamate transport capacity. The finding that a functional mutant GLT-1 protein (containing serine instead of cysteine in the equivalent residues) did not have any nitrosative modifications of these residues in the presence of an S-nitrosylating agent further supports the nNOS-dependent S-nitrosylation of this transporter (Raju et al. 2015). These studies indicate that, under certain conditions, glutamatergic neurotransmission may be influenced by nNOS-dependent S-nitrosylation of crucial enzymes involved in glutamate homeostasis in the brain (Raju et al. 2015).

4.7 Clinical Relevance of Transient Multienzyme Complexes Formation

At this point, the clinical relevance of the possible association and dissociation of these multienzyme complexes in vivo is not fully understood. Recent reports provide evidence that the efficiency of brain metabolism may rely on physical interactions involving enzymes or transporters forming complexes with other proteins and/or mitochondria. The disruption of such interactions may be related to brain abnormalities, as demonstrated in patients with psychiatric diseases in which a lack of interaction between hexokinase and the outer mitochondrial membrane has been reported (Regenold et al. 2012), as well as in schizophrenia in which association of hexokinase 1 with GLT1 found in control brain was not found in brain tissue from patients (Shan et al. 2014). Increased expression of hBCATm was identified in specific regions in postmortem brain from patients affected by vascular dementia, dementia with Lewy body (Ashby et al. 2016), and Alzheimer's disease (Hull et al. 2015a). Interestingly, alterations leading to either increased or decreased GDH activity were related to seizures and hyperexcitability of brain (Raizen et al. 2005; Malthankar-Phatak et al. 2006; McKenna 2011; Stanley 2011) as discussed by McKenna (2011 and references therein). GDH insensitivity to

GTP inhibition caused by mutations in the *GLUD1* gene sequence leads to the neurological impairment in patients with hyperinsulinism–hyperammonemia syndrome (Bahi-Buisson et al. 2008b). Impaired GDH activity in the temporal cortex of patients with medial temporal lobe epilepsy was observed (Malthankar-Phatak et al. 2006). Both the expression and activity of GDH are increased in human glioma tissue, and higher GDH level is associated with poor outcome (Zhang et al. 2016).

Since a localized alteration in the amount of an enzyme in a particular cellular and/or mitochondrial domain can alter its binding capacity, which is driven by affinity, it is tempting to speculate that the transient formation and/or disassembly of some multienzyme complexes may play a role in the pathophysiological mechanisms leading to these conditions.

4.8 Concluding Remarks

The occurrence and dynamic control of transient multienzyme and/or multiprotein complexes (metabolons) can facilitate metabolism within cells. As noted in this chapter, there is compelling evidence that the formation of such transient metabolons can markedly influence cell bioenergetics and neurotransmission by channeling substrates into specific pathways including the TCA cycle, malate-asparte shuttle, and oxidative metabolism of glutamate and glutamine for energy in neurons and astrocytes. Although it was not extensively covered in this chapter, the influence of physiological (as well as pathological) fluctuations in the concentration of small molecules on the association/dissociation of complexes that impact glutamate and glutamine metabolism should not be underestimated. There is still much to learn about the extent and role of common protein modifications on the activity of enzymes and transporters involved in these pathways. As noted by Robinson and Jackson (2016), more efforts should be undertaken to determine how transporter/enzyme/mitochondria/proteins complexes are regulated. As novel technologies are developed and employed to identify expected and unexpected interactions in living cells and in vivo, a better understanding of these types of interactions will be reached. Modulation of such transient protein–protein interactions may represent a novel and promising therapeutic approach for treatment of diseases involving dysregulation of glutamate metabolism.

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Chapter 5
BCAA Metabolism and NH_3 Homeostasis

M.E. Conway and S.M. Hutson

Abstract The branched chain amino acids (BCAA) are essential amino acids required not only for growth and development, but also as nutrient signals and as nitrogen donors to neurotransmitter synthesis and glutamate/glutamine cycling. Transamination and oxidative decarboxylation of the BCAAs are catalysed by the branched-chain aminotransferase proteins (BCATm, mitochondrial and BCATc, cytosolic) and the branched-chain α-keto acid dehydrogenase enzyme complex (BCKDC), respectively. These proteins show tissue, cell compartmentation, and protein–protein interactions, which call for substrate shuttling or channelling and nitrogen transfer for oxidation to occur. Efficient regulation of these pathways is mediated through the redox environment and phosphorylation in response to dietary and hormonal stimuli. The wide distribution of these proteins allows for effective BCAA utilisation. We discuss how BCAT, BCKDC, and glutamate dehydrogenase operate in supramolecular complexes, allowing for efficient channelling of substrates. The role of BCAAs in brain metabolism is highlighted in rodent and human brain, where differential expression of BCATm indicates differences in nitrogen metabolism between species. Finally, we introduce a new role for BCAT, where a change in function is triggered by oxidation of its redox-active switch. Our understanding of how BCAA metabolism and nitrogen transfer is regulated is important as many studies now point to BCAA metabolic dysregulation in metabolic and neurodegenerative conditions.

Keywords BCAT • BCAAs • Glutamate • Nitrogen shuttling • Metabolon • Redox-switch • MSUD

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Abbreviations

AGC	Malate/aspartate carrier
ALT	Alanine aminotransferase
BCAA	Branched chain amino acids
BCAT	Branched chain aminotransferase
BCKA	Branched chain α-keto acid
BCKDC	Branched-chain α-keto acid dehydrogenase enzyme complex
E1	Branched chain α-keto acid dehydrogenase
E2	Dihydrolpoyl transacylase subunits
E3	Dihydrolipoyl dehydrogenase
GABA	γ-Amino butyric acid
GDH	Glutamate dehydrogenase
GS	Glutamine synthetase
GSNO	S-nitrosoglutathione
KG	α-Ketoglutarate
KIC	α-Ketoisocaproate
KIV	α-Ketoisovalerate
KMV	α-Keto-β-methylvalerate
MDH	Malate dehydrogenase
ME	Malic enzyme
MSUD	Maple syrup urine disease
mTOR	The mechanistic target of rapamycin
PLP	Pyridoxal phosphate
PMP	Pyridoxamine
TPP	Thiamine pyrophosphate

5.1 Introduction

The branched chain amino acids (BCAA) (leucine, isoleucine, and valine) are three of the nine nutritionally essential amino acids that cannot be synthesized endogenously and must therefore be obtained through a dietary source. Dietary BCAA are required for normal growth and development (Hutson et al. 2005). These amino acids play a key role as nitrogen donors, for the major nitrogen carriers, alanine and glutamine, where nitrogen is shuttled between muscle and liver. In the brain, leucine is an important nitrogen donor for the synthesis of the neurotransmitter glutamate and the inhibitory neurotransmitter γ-amino butyric acid (GABA). Leucine also plays a role as an anabolic nutrient signal regulating insulin secretion and protein synthesis through the mechanistic target of rapamycin (mTOR) pathway in skeletal muscle (Hutson et al. 1980). However, toxic levels of BCAAs and the α-keto acids observed in conditions such as Maple Syrup Urine Disease (MSUD) highlight the importance of regulating their metabolism (Chuang et al. 2006). Control of BCAA transport and oxidation is governed by the cell and tissue expression of the

branched-chain aminotransferase (BCAT) proteins, the branched-chain α-keto acid dehydrogenase enzyme complex (BCKDC), glutamate dehydrogenase (GDH), and the redox state of the cell coupled with phosphorylation/dephosphorylation of branched chain α–keto dehydrogenase enzyme (E1) of BCKDC.

The BCAT proteins catalyze the transamination of the BCAA, leucine, isoleucine, and valine with α-ketoglutarate (α-KG) releasing their respective branched chain α-keto acids (BCKA: α-ketoisocaproate (KIC), α-keto-β-methylvalerate (KMV), and α-ketoisovalerate (KIV)) and glutamate, regenerating the pyridoxal phosphate (PLP) form of the enzyme (Ichihara 1975). The second step in BCAA catabolism is catalyzed by the BCKDC in which irreversible oxidative decarboxylation of the BCKA is the committed step in oxidation of the BCAAs (Harris et al. 2004). Glutamate dehydrogenase, a mitochondrial matrix enzyme, is responsible for the oxidative deamination of glutamate to α-KG and free NH_4^+, using either NAD^+ or $NADP^+$ as a co-factor. Whole body tissue expression and compartmentation of these proteins drives intra- and inter-organ exchange of nitrogen and carbon. In addition to being the major excitatory neurotransmitter, glutamate is also the substrate for Glutamine Synthetase which forms glutamine. These two amino acids are part of the Glutamine/Glutamate Cycle. BCAA are not transaminated in liver hepatocytes and transamination exceeds oxidation in skeletal muscle, which accounts for 40 % of total body weight and is one of the main sites of BCAA uptake. Because skeletal muscle transaminase activity exceeds the oxidative capacity, BCAAs act as nitrogen donors in peripheral metabolism. In the central nervous system, transamination and oxidation also exhibit cell-specific localization and participate in nitrogen and carbon shuttles. In this chapter, we discuss the rodent BCAA-dependent astroglial-neuronal nitrogen shuttle and how the absence of BCATm from astrocytes in human brain indicates distinct differences between species.

BCATm and BCKDC and BCATm and GDH also form metabolons, which facilitate effective substrate channelling (Islam et al. 2010; Hutson et al. 2011). These interactions are regulated through changes in the redox environment, where a reduced environment favours binding and oxidation prevents metabolon formation (Islam et al. 2007). The unique redox-active CXXC motif of the mammalian BCAT becomes oxidised and transamination is terminated. We discuss the potential for this redox switch to behave as a signal for change in function from a metabolic role to operate as a redox chaperone. How this would impact BCAA catabolism and nitrogen transfer remains to be elucidated, but is important to answer, given the role of these amino acids in nutrient cell signalling and protein synthesis.

5.2 Transamination and Oxidative Decarboxylation of the BCAA

The BCAT isozymes are encoded by BCAT1 (located on the short arm of chromosome 12) and BCAT2 (located in chromosome 19) (Hall et al. 1993; Bledsoe et al. 1997). The resulting (predominant) isoforms include a cytosolic isozyme, BCATc, and a mitochondrial isozyme, BCATm, which show both cell and tissue

compartmentalization. These proteins operate as homodimers with a molecular mass of 44,650 Da and 41,730, respectively, and are structurally very similar, where they share 58 % sequence homology (Yennawar et al. 2002, 2006; Goto et al. 2005). However, they possess distinct differences in catalytic efficiency and regulation (Davoodi et al. 1998; Conway and Hutson 2000). The mitochondrial isozyme is ubiquitous and found in most tissues, whereas the cytosolic isozyme is limited to the brain, peripheral nervous system, ovaries, and testes (Suryawan et al. 1998; Hall et al. 1993; Hutson 1988; Hutson et al. 1998; Sweatt et al. 2004a, b; García-Espinosa et al. 2007). Although these two isozymes predominate, two other splice variants have been identified, namely, a novel alternatively spliced PP18b variant found in placental tissue and a novel co-repressor for thyroid hormone nuclear receptors (P3) (Lin et al. 2001; Than et al. 2001). Both spliced variants are homologous to BCATm. Although the function of the PP18b variant is unknown, P3 was reported to act as a co-repressor for thyroid hormone nuclear receptors. The biological significance of these variants remains to be determined, but may have significance under pathological conditions such as Alzheimer's disease (Hull et al. 2015). Therefore, based on existing evidence, with the exception of the brain and peripheral nervous system, BCAA metabolism occurs in the mitochondria.

The mammalian BCAT proteins are PLP-dependent enzymes that belong to the fold-type IV class of aminotransferase proteins where the proton is abstracted from the C4 atom of the coenzyme-imine or external aldimine on the Re face instead of the Si face of the PLP cofactor (Yoshimura et al. 1996). This is unlike most aminotransferases, which fall into the fold type I family type I (Jansonius 1998; Schneider et al. 2000). Other key aminotransferases include the alanine aminotransferase (ALT) [glutamate pyruvate transaminase or α-keto-glutarate E.C. 2.6.1.2] and the aspartate aminotransferase proteins (AST) [glutamic oxaloacetic transaminase or L-aspartate: α-keto-glutarate aminotransferase, E.C. 2.6.1.1] (Mehta and Christen 2000; Salzmann et al. 2000). These enzymes follow the same basic transamination reaction and exhibit ping-pong kinetic mechanisms that have two half-reactions consisting of three main stages (Fig. 5.1). This reversible transamination step is initiated in the first half-reaction, where the PLP form of BCAT reacts with the α-amino group of the BCAAs. Here, the PLP cofactor is bound to BCAT by an internal aldimine, with a Schiff base between the active site lysine and the PLP cofactor. As the BCAA enters the active site, a transaldimation reaction occurs, where the substrate amino group displaces the amino groups of the active site lysine, forming an external aldimine between the BCAA and the PLP cofactor. This external aldimine is subsequently converted into its ketamine intermediate, where lysine acts as the catalytic base. The first-half reaction is completed with the hydrolysis of the ketamine to give the pyridoxamine phosphate (PMP) form of the enzyme and the respective BCKA. In the second half-reaction, the PMP cofactor binds to α-KG following a reversal of the three main stages. Here, the net result is that the PLP-form of BCAT is regenerated together with the release of glutamate. Transamination in the reverse direction results in the BCKAs receiving the amino group of glutamate to generate BCAAs and α-KG. Although changes in the redox environment do not directly play a role in catalysis, oxidation of the human BCAT proteins inacti-

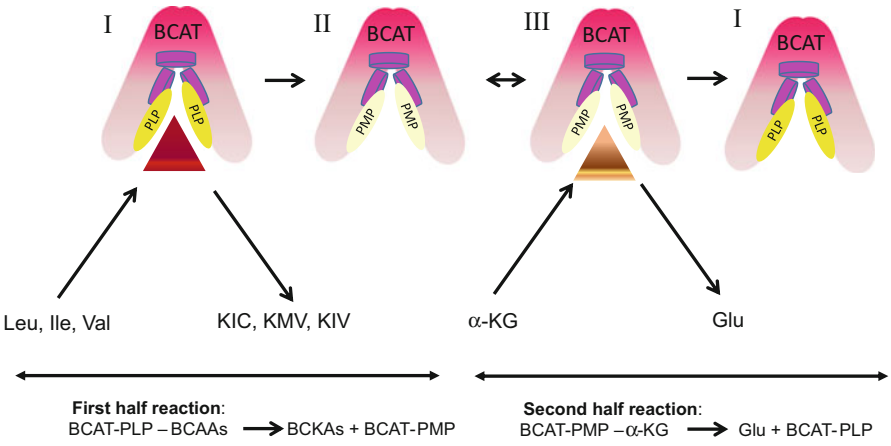

Fig. 5.1 Ping-pong kinetics of the BCAT protein. Each half reaction is divided into three stages. (*I*) Interaction of BCAT-PLP with their respective substrates involves the nucleophillic attack of the α-amino group of amino acid 1 (e.g. isoleucine) with the BCAT-PLP Schiff base carbon atom to form an amino acid-PLP Schiff base (external aldimine) with release of the BCAT. (*II*) After transamination the second step is keto-enol tautomerism which involves the interconversion between the keto-enol form resulting in the formation of an α-keto acid-PMP Schiff base *III*. This is subsequently hydrolysed to PMP and an α-keto acid, the final step in the first half-reaction

vates the enzymes disrupting transamination (discussed later). This redox switch may offer a control point during conditions where oxidative stress prevails.

The BCAT proteins show substrate specificity for the BCAA, with substrate preferences for isoleucine ≥ leucine > valine ⋙ glutamate (Wallin et al. 1990; Hall et al. 1993; Davoodi et al. 1998). Moreover, the kinetic data indicates that the BCKAs and glutamate are the favoured products of the BCAT reaction. In vivo studies using ^{15}N-labeled leucine showed that the majority the ^{15}N was found in glutamate and other amino acids formed through transamination with glutamate, such as alanine and aspartate (Forslund et al. 1998; Kanamori et al. 1998). Cycling of BCAA nitrogen and labelling of the amino acid pool can occur through transamination and reamination. Transfer of nitrogen from the BCAA between tissues happens when BCKAs are released from one tissue, or cells in a tissue and reaminated in other tissues or different cells within a tissue. However, only oxidation and removal of the carbon skeleton will result in a net transfer of BCAA nitrogen to body dispensable amino acids. The widespread expression of BCATs in cells and tissues promotes nitrogen cycling, transfer, and net transfer.

As described, the net transfer of BCAA nitrogen into the nonessential amino acid pool requires oxidation of the BCAA carbon skeleton. Complete oxidation is catalysed by the BCKDC complex (4.5 MDa) located in the mitochondrial matrix and associated with inner mitochondrial membrane (reviewed in Wynn et al. 2004). The activity of the BCKDC determines the rate of oxidation of the BCAA (irreversible loss), which is equivalent to the rate of net nitrogen transfer from BCAAs to glutamate. The BCKDC shares significant functional homology to the pyruvate dehydrogenase

enzyme complex and the α-ketoglutarate dehydrogenase enzyme complex (Reed et al. 1985). It is composed of multiple copies of three enzyme proteins including the branched chain α-keto acid dehydrogenase [E1, heterotetramer of 2E1α and 2E1β subunits] (12 copies) and dihydrolipoyl dehydrogenase [E3] (six copies), which non-covalently surround a core of 24 dihydrolpoyl transacylase enzymes [E2 homodimer]. Also, at the N-terminal end of E2 is a flexible lipoyl moiety, which manoeuvres between the active sites of the enzymes and an inner core domain at the COOH-terminal, important for oligomeric core formation and catalysis of the acyltransferase reaction. For catalysis, several cofactors are required including thiamine pyrophosphate (E1), Coenzyme A (E2), lipoamide, FAD, and NAD (E3) and activity is tightly regulated by phosphorylation and dephosphorylation at Ser 293 of the E1α subunit of the E1 enzyme (Harris et al. 1990; Popov et al. 1992).

Oxidative decarboxylation of the α-keto acid (e.g. KIC) is initiated when the E1β subunit binds thiamine pyrophosphate and catalyses decarboxylation forming 2-methylpropanol-TPP (Wynn et al. 2004). TPP is regenerated when 2-methylpropanol-TPP is oxidised to form an acyl group, while it is simultaneously transferred to the lipoyl cofactor covalently attached to E2. The flexible lipoyl arm carrying the S-acyldihydrolipoamide now swings to E2 where acyl transfer to Coenzyme A forming acyl-CoA occurs. Finally, oxidation of the dihydrolipoyl moiety on E2 occurs via the FAD bound to the E3 enzyme, where NAD^+ acts as the ultimate electron acceptor. The end products of BCKDC-mediated catalysis include isovaleryl-CoA, α-methylbutyryl-CoA, and isobutryl-CoA that undergo further enzymatic reactions and either enter the Krebs cycle or act as precursors for lipogenesis (Ile and Leu). Therefore, the overall end products of BCAA catabolism are acetoacetate and succinyl-CoA (Summarized in Fig. 5.2). Mutations in one or more proteins in the BCKDC result in the inborn error of metabolism, MSUD, characterised by elevated plasma BCAAs and BCKAs (Chuang and Chuang 2000; Chuang et al. 2006).

5.3 Peripheral BCAA Metabolism: Inter-Organ Nitrogen Shuttling

Whole body distribution of the BCAT proteins was first described in rats, followed by human and nonhuman primates (Shinnick and Harper 1976; Hall et al. 1993; Suryawan et al. 1998). In rat, the mitochondrial isoform is expressed in most tissues, but its expression tends to be cell-specific (e.g. in astrocytes in brain, acinar cells of the pancreas, etc.) (Sweatt et al. 2004a, b; Cole et al. 2012). Expression of BCATc is, however, limited to brain (neuronal) and the peripheral nervous system, ovary, and placenta (Hall et al. 1993; García-Espinosa et al. 2007; Sweatt et al. 2004a, b; Hull et al. 2012). Neither BCAT was reported in rat liver, indicating that transamination occurs largely outside the liver and it is not in liver hepatocytes in primates. On the other hand, rat BCKDC shows high activity in the liver, low in skeletal muscle and moderate levels in the kidney and heart, indicating that transamination and oxidative decarboxylation differ between tissues (Goodwin and Harris et al. 1988; Harris et al. 2004). In rat skeletal muscle, however, the ratio of

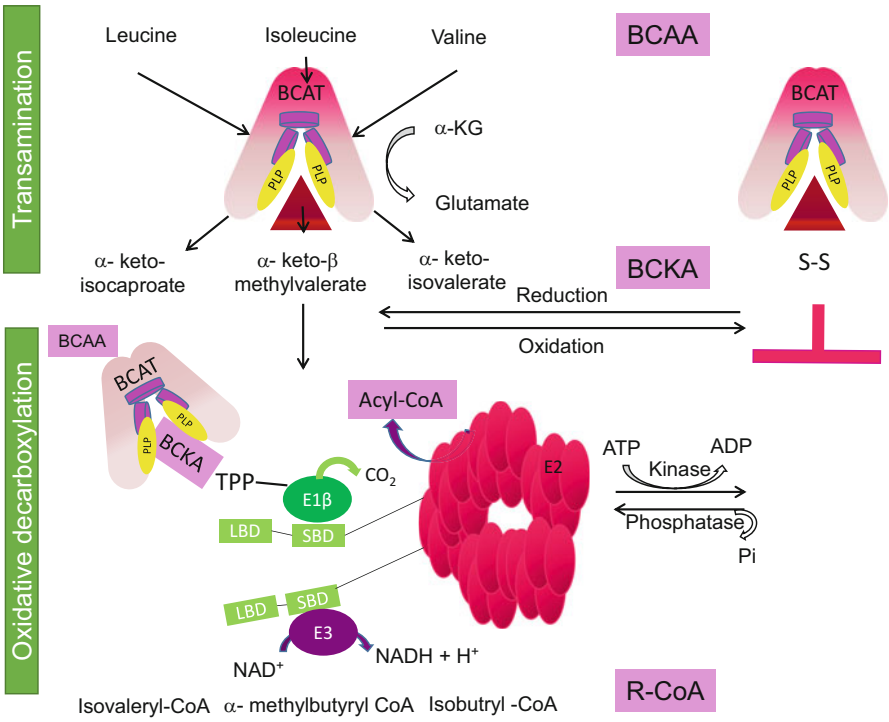

Fig. 5.2 BCAA catabolism. BCAT catalyze the transamination of the BCAA, leucine, isoleucine, and valine to α-ketoglutarate (α-KG) releasing their respective branched chain α-keto acids (BCKAs: α-ketoisocaproate (KIC), α-keto-β-methylvalerate (KMV) and α-ketoisovalerate (KIV)) and glutamate, regenerating the enzyme. The committed step is catalyzed by the second enzyme in the catabolic pathway, the branched-chain α-keto acid dehydrogenase complex (BCKDC) where the respective BCKAs undergo oxidative decarboxylation to generate Isovaleryl-CoA, α-methylbutryl CoA, and Isobutryl-CoA, respectively. Transamination is regulated through a peroxide-sensitive redox switch and oxidation is regulation through phosphorylation/dephosphorylation of the BCKDC

BCAT activity to actual BCKDC activity is ≥90 % and ≥25 % when you take into account the total potential BCKD activity in its unphosphorylated form, i.e. the oxidative capacity. Expression of BCAT and BCKDC in humans showed that although the ratio of BCAT to BCKDC expression in each tissue was comparable with rat, the activity (Table 5.1) and expression pattern showed differences, indicating that transaminase and oxidative capacity is regulated by specific pathways in each species. In humans, overall measured transaminase activity and oxidative capacity, factoring in tissue size, was greatest in muscle; however, high activity reported for brain and kidney indicated that these two organs may also contribute to whole body BCAA metabolism and nitrogen shuttling (Suryawan et al. 1998). Still liver can handle BCAA oxidation in humans (see MSUD section). Although not measured in these studies, based on primate analysis, it is anticipated that the human pancreas will also show high levels of BCAT and BCKDC.

Table 5.1 Distribution of BCAT activity in rat, human, and monkey tissues[a]

Tissue	Rat	Human	Monkey
mU/g wet weight			
Heart	4894±216	387±216	–
Muscle	1599±60	124±216	245±216
Brain	1944±94	510±216	434±216
Iiver	78±5	248±216	250±216
Kidney	3486±142	880±216	1215±216
Pancreas	11,088±1187	–	1790±216
Stomach	5842±415	447±216	559±216
Small intestine	489±22	241±216	383±216
Colon	894±37	254±216	–
Adipose	166±16	84±216	98±216

[a]Data are from Suryawan et al. (1998)

The high ratio of transaminase to oxidative capacity in skeletal muscle of the rat and absence of BCAT and high oxidative capacity in liver led to the prediction of extensive inter-organ shuttling of BCAA metabolites, first postulated by Harper and collaborators (Shinnick and Harper 1976; Hutson et al. 1978; Harper and Benjamin 1984). In rat hepatocytes, the absence of the BCATm isozyme and high activity of BCKDC favour liver oxidation of circulating BCKAs (Suryawan et al. 1998). This would account for the low concentrations of BCKAs found in liver. The liver is the first organ that receives dietary amino acids, where essential amino acids, with exception to the BCAA, undergo irreversible degradation primarily in this organ. As described, in humans, skeletal muscle is the main site for BCAA transamination, generating BCKAs and glutamate. Although skeletal muscle due to its mass also reflected the highest relative value of potential oxidative capacity compared to other sites measured, transaminase activity exceeds the oxidative capacity, indicating that the fate of glutamate and the BCKAs are not coordinated in this tissue. It has been established that BCAA provide nitrogen via glutamate for glutamine and NH_4 production, as well as alanine synthesis (Harper and Benjamin 1984; Elia and Livesey 1983). Glutamine and alanine are major sources of fuel during gluconeogenesis and they are released from muscle and are taken up by the liver, kidney, and small intestine. Alanine and glutamine also are major mechanisms for transferring nitrogen from peripheral amino acid oxidation to liver for urea synthesis. Liver, intestine, and skeletal muscle are the major organs affecting blood glutamine concentrations. Supplementation studies with BCAA have demonstrated that the concentrations of NH_3 and glutamine increase during exercise; however, performance was suggested to be affected, possibly due to a compromised TCA cycle (van Hall et al. 1995a, b). The role of BCAA metabolism in shuttling nitrogen is ultimately linked to the fate of glutamate. The fate of glutamate can be to exit the cell, act as a precursor for alanine or glutamine synthesis, nitrogen donor to other transaminases, or undergo oxidation by GDH. Mitochondrially generated Glu must first exit the mitochondria via the glutamate/hydroxyl carrier, which depletes TCA cycle α-KG. This would need to be

replenished through anaplerosis or amino acid oxidation from protein breakdown. Anaplerosis through pyruvate carboxylase would be a candidate, but it is not abundant in muscle, but is high in brain and liver. Oxidation of glutamate by GDH would generate α-KG and NH_4 and not cause loss of TCA cycle intermediates. GDH1 is found at varying levels in tissues. Coupling of BCAA metabolism to GDH activity would generate the needed NH_4 for glutamine synthesis, without compromising the TCA cycle. Thus, the high ratios of BCAT/BCKDC activities favour (1) release of BCKAs rather than their oxidation and (2) efficient transfer of BCAA nitrogen. This led to the hypothesis that BCAAs play an important role in body nitrogen metabolism, particularly as nitrogen donors for the major nitrogen carriers alanine and glutamine (Odessey and Goldberg 1979; Yudkoff et al. 1994).

Distribution studies in rat were extended to tissues of the digestive tract, which showed BCATc was expressed in peripheral nerves, without the expression of other BCAA catabolic enzymes (Sweatt et al. 2004b). Moreover, the expression of BCATm was pronounced throughout the secretory epithelial cells that secrete acid, enzymes, or enzyme precursors rather than in the absorptive epithelial cells of the intestinal mucosa. This would indicate that during absorption leucine oxidation would be limited, which correlates with previous studies indicating that although transaminase activity was measured in these areas, oxidative capacity was low (Suryawan et al. 1998). The high immunopositive staining of BCATm and BCKDC found in the pancreatic acini rather than the islets was suggested to serve as a regulatory mechanism for leucine signalling in the islets, allowing leucine to activate GDH (Sweatt et al. 2004a, b). Low expression in the islets has been proposed to promote activation of GDH1, related to insulin secretion (Cole et al. 2012). A study by Zhou et al. in 2010 demonstrated that KIC, but not leucine-induced insulin secretion, was abolished in mice deficient in BCATm. The increase in ATP and NADH/NAD^+ ratios normally reported with KIC stimulation was reduced in these deficient mice, and malate concentrations were also attenuated. These studies indicate that transamination in the direction of leucine production is important for insulin release when KIC is the substrate provided to the β-cells, where a role for leucine in stimulating GDH activity, rather than transamination, is important. Recent studies detailing BCATm-induced enhancement of GDH1 metabolism indicate that this reaction involves several metabolic proteins, described in detail later in this chapter (Islam et al. 2010).

The compartmentalization of BCAT isozymes and BCKDC within organs and tissues demonstrates that, for complete oxidation to occur, expression of BCAT with BCKDC is required, but not necessarily in the same tissue or cell. Skeletal muscle clearly has the highest transaminase capacity, which in both rat and human exceeds the oxidative capacity, supporting the role for BCAA as nitrogen donors in peripheral metabolism. Although the expression of BCKDC is high in rat liver, highlighting a role for liver as the main site for BCKA oxidation, the relatively lower levels reported in human does question if the fate of the BCKAs is the same across species. Nevertheless, liver transplantation is being used to treat MSUD, so the liver can serve as the major site of BCAA oxidation in individuals with a defect in BCKDC, hence BCAA oxidation. However, what is certain is that both alanine and glutamine are metabolized in the liver and kidney, in particular during gluconeogenesis.

This concept of nitrogen and BCAA carbon shuttling originally described in peripheral tissues was extended to the Central Nervous System where it has been studied extensively by a number of laboratories including those of Yudkoff and co-workers and Hutson and co-workers. It is generally accepted that BCAAs donate nitrogen to brain glutamate neurotransmitter and are involved in regulating brain glutamate levels.

5.4 Brain Metabolism of the BCAA and the BCAA/BCKA Nitrogen Shuttle (Rodent Model)

In the brain, glutamate is the major excitatory neurotransmitter, a precursor for glutamine as well as the precursor for the synthesis of the major inhibitory neurotransmitter, GABA. Glutamate cannot easily traverse the blood brain barrier, which is important because excess accumulation of glutamate can induce excitotoxicity, leading to neuronal damage (Danbolt 2001). The glutamate/glutamine cycle is the predominant pathway which regulates neuronal glutamate stores (Berl and Clarke 1983; Yudkoff et al. 1993). Replenishment of neuronal glutamate is important because, during excitatory neurotransmission, not all glutamate released from the pre-synaptic neuron is recovered. Under normal excitatory conditions, excess glutamate not involved in transmission is taken up by astrocytes, which express high concentrations of the glutamate-specific transporters (GLAST/EAAT and GLT1/EAAT2) (Chaudhry et al. 1995; Lehre et al. 1995; Rothstein et al. 1994). Astrocytes specifically express the microsomal enzyme glutamine synthetase (GS), which catalyses the conversion of glutamate to glutamine through ATP-dependent amidation. As described for the peripheral generation of glutamine in skeletal muscle, this reaction requires NH_3, supplied from blood or brain metabolism. Glutamine is a non-neuroactive amino acid and can be released to the extracellular fluid for subsequent uptake by the pre-synaptic neuronal cells. Deamidation of glutamine by mitochondrial phosphate-dependent glutaminase regenerates neuronal glutamate, closing the cycle (Yudkoff et al. 2005). Cycling of glutamate between neuronal cells and astrocytes supports the provision of high levels of glutamate in neuronal cells and prevents toxicity in the synaptic space. However, metabolic studies using cultured rat neonatal astrocytes showed that up to 30 % of glutamate removed from the synaptic space by astrocytes was not converted to glutamine (Sonnewald et al. 1993; Hutson et al. 1998). Instead, these studies illustrated that glutamate was transaminated to α-KG (or oxidatively deaminated by GDH) oxidised for energy, especially when glutamate concentrations are high (McKenna et al. 1996a, b). Further metabolism to malate and oxaloacetate and decarboxylation to pyruvate will depend on substrate availability and the distribution and regulation of key metabolic enzymes including Malate dehydrogenase (MDH), Malic enzyme (ME), and pyruvate carboxylase (PC) or whether the source of glutamate is exogenous or endogenous (Shank et al. 1985; Teller et al. 1990; McKenna et al. 1993). For example, when levels of external glutamate are low, the glial glutamate synthetase pathway is favoured, whereas in glutamate excess considerable oxidation occurs (McKenna et al. 1996a). Glutamate is also a source of

carbon for the production of purines and reducing equivalents such as glutathione (Yudkoff 1997). The fate of glutamate other than regeneration through the glutamate/glutamine cycle highlighted a gap in our metabolic understanding of how this 'lost' glutamate carbon could be replaced.

Pyruvate carboxylase, expressed solely in astrocytes, can replenish this lost glutamate, but is dependent on the supply of nitrogen (Gamberino et al. 1997; Hutson et al. 1998). As NH_3 was not considered to contribute to this reaction, other pathways catalysed by the transaminases were suggested, where the BCAA and aspartate serve as potential nitrogen donors (Shank et al. 1985; Brookes 1993; Hutson et al. 1998, 2001; Lieth et al. 2001; Bixel and Hamprecht 1995; Yudkoff et al. 1996a, b, Yudkoff 1997; Kanamori et al. 1998). Several lines of evidence support the role of BCAA as a nitrogen donor in rat brain metabolism. First, they can easily traverse the blood brain barrier, particularly leucine, where a substantial uptake by brain of BCAAs has been reported (Oldendorf 1973; Smith et al. 1987). In rat brain slices, rather than simply providing energy, their metabolism was faster than their incorporation into proteins (Chaplin et al. 1976). As described for skeletal muscle, the overall BCAT activity exceeds that of BCKDC catalysed oxidation in rat brain ($\geq$50). In rat brain, reflected through metabolic studies, transamination of leucine is preferred over complete oxidation. By measuring the incorporation of [^{15}N]leucine into glutamate, these studies illustrated that approximately 30 % of the nitrogen of glutamate/glutamine was derived from leucine alone (Brand 1981; Brand and Hauschildt 1984). In another study, 9 h of continuous intra-gastric feeding in rats estimated that approximately 50 % of brain glutamate was derived from leucine with significant reamination of α-KIC (Sakai et al. 2004). In rat retina models, gabapentin, an inhibitor of BCATc, impacted glutamate synthesis by 30 % in retina cells, also supporting a role for BCAT metabolism in brain glutamate regulation (Lieth et al. 2001; LaNoue et al. 2001). Thus, these studies among others support the flow of metabolites between neuronal and astrocytic cells, complementing the glutamate/glutamine cycle (Yudkoff et al. 1996b; Yudkoff 1997; Daikhin and Yudkoff 2000). As the BCAT and BCKDC activities in human brain are second to muscle, it indicates that this tissue could be an important site of BCAA metabolism.

The role of BCAA metabolism in the CNS was supported further through enzyme distribution studies of rat brain and led to the development of the BCAA/BCKA nitrogen shuttle hypothesis (Fig. 5.3). Early studies showed that BCATc mRNA was expressed in whole rat brain and regionally in the cerebellum and hippocampus (Hutson et al. 1995). In astroglia-rich primary cultures derived from newborn rat brains, BCATc was found expressed in oligodendroglial and O2A progenitor cells. Using immunohistochemistry, BCATc was found only in neuronal cells, i.e. primarily glutamatergic and GABAergic cells of the hippocampus and cerebellum (Sweatt et al. 2004a). No astrocytic staining was observed. Analysis of these images showed that intense staining was observed in the processes rather than the cell bodies of glutamatergic neurons (e.g. granule cells of the cerebellum and dentate gyrus), indicating a role for BCAA metabolism in glutamate production during excitation. Conversely, in GABAergic neurons, the cell body was heavily stained (e.g. cerebellar Purkinje cells and pyramidal basket cells in the dentate gyrus), suggesting a role

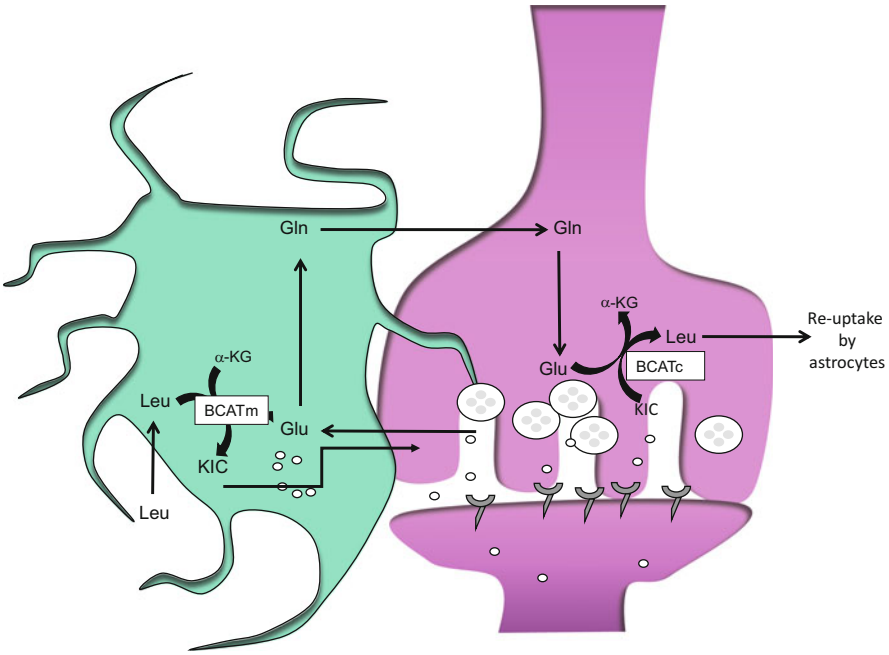

Fig. 5.3 Nitrogen transfer in rodent brain. Leucine is actively taken up by astrocyes where it undergoes transamination via BCATm forming glutamate and α-ketoisocaproate (KIC). The glutamate formed can enter the glutamate-glutamine cycle, whereas KIC, which is poorly metabolised by astrocytes, is metabolized further in neurons cells. Subsequently, the α-keto acid is transaminated with glutamate to regenerate leucine, which can be returned to the astrocyte to complete the cycle. These cycles also operate with the TCA cycle and GDH metabolism (Yudkoff et al. 1990; Hutson et al. 2001)

in maintaining the glutamate storage pool rather than contributing to excitation. In the cerebellar cortex, there were regional differences in the pattern of BCATc immunoreactivity, where intense staining was reported in the glutamatergic inputs to most of the cortex but limited in the vestibule cerebellum, which showed preferential expression in GABAergic cells. The varied expression and intensity of BCATc in these regions suggested differential regulatory controls mechanisms between subtypes of neuronal cells. The pattern of staining in the hippocampus was more consistent, where again staining in glutamatergic and GABAergic cells was reported (Sweatt et al. 2004a). However, more extensive distribution studies of rat brain showed that intracellular staining was not particular to neuronal transmitter phenotype (for example, sertonergic neurons of brainstem contained BCATc), where cell body staining throughout glutamatergic neurons was reported. BCATc was found to be expressed across all regions of rat brain in neurons of different phenotypes, where proposed roles range from generating glutamate and GABA to regulating feeding behaviour through the mTOR pathway (García-Espinosa et al. 2007). In a separate study, BCATc, considered to be expressed solely in neuronal cells, were found in the

peripheral nerves of the salivary gland, the pancreas, and in the Auerbach's and Meissner's plexuses of the gut (Sweatt et al. 2004b). The principal neurotransmitters in this region are acetylcholine, the catecholamines, and neuroactive peptides. Although the role of the BCAT proteins in this region is not known, the authors speculate that leucine may act as an anabolic signal. As BCKDC was not found in this region, oxidation cannot occur, and therefore, the BCKAs would be released.

BCATm expression was initially reported to be highly expressed in astro-glial primary cultures derived from the brains of newborn Wistar rats and also in microglia (Bixel et al. 1997, 2001). However, it was not until recently that BCATm was mapped to specific regions of rat brain (Cole et al. 2012). Consistent with these cellular studies, expression of BCATm was found throughout astrocytes in rat brain. Immunopositive staining of astrocytes was reported in the white matter tracts and in the distal processes of the Bergmann glia of the cerebellar cortex. In the hippocampal region, positive staining was reported in the molecular, granule cell, and polymorphic layers of the dentate gyrus. Labelling of the astrocytic processes surrounding blood vessels along the hippocampal fissure and in the subpial region also showed concentrated BCATm expression. Thus, in rat brain the clear labelling of BCATm to astrocytic processes and cell bodies in the spinal cord and the neuronal specific expression of BCATc supported the proposed BCAT-dependent leucine/glutamine cycle (Yudkoff et al. 1996a, b; Yudkoff 1997) and the BCAA/BCKA nitrogen shuttle (Hutson et al. 2001; Lieth et al. 2001). As described above, for glutamate to be replenished in neuronal cells, additional support networks are needed, in particular a supply of nitrogen. The BCAAs were proposed as a major source of nitrogen required for this pathway, since transfer of BCAA nitrogen to glutamate and glutamine was shown to occur in primary cultures of rat astroglia and in preparations of synaptosomes (Yudkoff et al. 1996a, b; Hutson et al. 1998). Further support using ^{13}C-NMR demonstrated that leucine contributes 25–50 %, of glutamate nitrogen, respectively in rat brain (Kanamori et al. 1998; Sakai et al. 2004). In the BCAA/BCKA nitrogen shuttle model, it was proposed that leucine is taken up by astrocytes, which undergo transamination with α-KG forming glutamate and the respective BCKAs. Glutamate enters the glutamate/glutamine cycle and the BCKAs, which are not oxidized by astrocytes due to lack of BCKDC, are shuttled to neuronal cells. In neuronal cells, BCATc catalyses the transamination of these BCKAs with glutamate, producing the respective BCAAs and α-KG. Leucine is released by neuronal cells for re-uptake by astrocytes, completing the BCAA/BCKA nitrogen cycle (Fig. 5.3). α-KG can enter the TCA cycle or undergo reductive amination to glutamate by GDH. However, as the flux in the direction of reductive amination is low, this is a less likely outcome (Hutson et al. 2001; Yudkoff et al. 1990). Therefore, the role of the BCAT proteins in brain metabolism does not only facilitate a role in energy metabolism, but also in providing essential nitrogen for the anaplerotic regeneration of glutamate.

In rodent brain, BCATm and BCKDC do not co-localize. Immunolocalization of BCATm and BCKDC in rats revealed that BCATm is present in astrocytes in white matter and in neuropil, while BCKDC is essentially found in neuronal populations (Cole et al. 2012). In the rat, BCATm appears uniformly distributed in astrocyte cell bodies throughout the brain. The segregation of BCATm to astrocytes and BCKDC

to neurons provides further support for the existence of a BCAA-dependent astroglial-neuronal nitrogen shuttle and support the hypothesis that BCATm association with GDH1 in astrocytes could provide ammonia for glutamine synthesis (Hutson et al. 2011). In rat brain, GDH1 has been found expressed in many nerve cells, including neurons, astrocytes, the Bergman glia, oligodendrocytes, and satellite cells, but immunoreactivity is predominantly associated with astrocytes (Zaganas et al. 2009). Because BCKDC is also localized in neurons, though BCKDC and BCATc are not necessarily found in the same neurons, net nitrogen transfer occurs when the BCKA product is oxidized. The localization of these key enzymes provides the potential for significant shuttling of BCAA metabolites between different cell types in the rodent brain.

5.5 Branched Chain Amino Acid Metabolon: Substrate Channeling

To further explore the integrated metabolism of the BCAAs and nitrogen transfer, the physical interaction of BCAT with other key metabolic enzymes involved in BCAA oxidation was pursued. Here, overexpressed human BCATm (hBCATm) was reported to bind to the E1α subunit of the E1 enzyme of BCKDC forming a BCAA metabolon (Islam et al. 2007). The association of hBCATm with E1 was governed by several factors including, (1) Only hBCATm in its PLP form could bind (2) Open structure of both proteins during catalysis was required (3) The reduced form of the reactive cysteine residues of hBCATm was necessary. Binding of the PLP-form of hBCAT to E1 increased the kinetic rate of decarboxylation of the BCKAs, whereas no binding occurred when hBCATm was in the PMP form. The K_m values for BCAAs for both enzymes are at (Leu and Ile) or above (Val) the physiological range of plasma and tissue BCAAs with BCATc having lower K_m values and higher k_{cat} than BCATm (Table 5.2). For both enzymes, transamination is responsive to changes in plasma and tissue BCAAs. The K_m for Glu is above tissue concentrations, with the exception of neurons where Glu is concentrated in the nerve terminals in the mM range. In addition, the rate of transamination exceeds that of oxidative decarboxylation by BCKDC. For BCATm, metabolon formation with BCKDC not only increases the k_{cat} for oxidative decarboxylation (DCPIP assay), but also the overall reaction of the complex (Overall assay), but lowers the K_m values for BCAA compared to BCATm alone. Thus, K_m values for BCAA are now within the range observed after a meal. This is especially true for Val. Metabolon formation would also limit BCKA release and facilitate oxidation. Lack of colocalization of hBCATm and BCKDC in the CNS actually promotes metabolite shuttling. Metabolon formation may occur in the capillary endothelium facilitating oxidation.

During transamination, the BCAA substrate binds to the PLP cofactor at the bottom of the active site and donates its nitrogen to PLP-hBCATm. This is stabilised by the hydrophobic core, where unlike other transaminases, domain closure does not occur (Yennawar et al. 2002). This open structure has been proposed to facilitate the

Table 5.2 Kinetic constants for branched chain amino acid substrates of hBCATm and hBCATc

	Isoleucine		Leucine		Valine		Glutamate	
Enzyme	k_{cat}/K_m ($\times 10^3$ M^{-1} s^{-1})	K_m (mM)	k_{cat}/K_m ($\times 10^3$ M^{-1} s^{-1})	K_m (mM)	k_{cat}/K_m ($\times 10^3$ M^{-1} s^{-1})	K_m (mM)	k_{cat}/K_m ($\times 10^3$ M^{-1} s^{-1})	K_m (mM)
hBCATm	372±2	0.62±0.03	242±1	0.80±0.02	39±0	4.80±0.25	11±0	12±0.42
hBCATc	3359±2	0.32±0.04	2494±1	0.36±0.02	618±0	2.0±0.45	108±0	9.9±0.42

substrate channelling of the α-keto acid to the E1 subunit of the BCKDC complex, where the active site also stays exposed during catalysis. Also important to this interaction is the redox-active –CXXC– motif, located about 10 Å from the active site (Conway et al. 2002, 2004; Yennawar et al. 2006). The redox sensitivity of the hBCAT proteins is unique to these aminotransferase proteins (Conway et al. 2002). This motif acts as a peroxide-mediated redox-switch, which regulates hBCAT activity, where the oxidised and reduced forms represent the inactive and active enzyme, respectively (Conway et al. 2002). When the hBCATm oxidised or mutant form of the –CXXC– motif was used, complex formation with BCKDC did not occur (Islam et al.). This is because disulphide bond formation increases the net dipole moment of the β-turn and β-sheet residues, which destabilises the substrate and PLP conformation (Yennawar et al. 2006). These changes subsequently interfere with the next step in BCKDC association. Therefore, the thiols in their reduced form and the net dipole charge are essential for association of hBCATm with BCKDC. Once transamination has proceeded, the BCKAs are channelled to the E1 subunit and conversion of PLP-hBCATm to PMP-hBCATm signals release from the E1 subunit. E1 catalysed decarboxylation and reductive acylation. This reaction was enhanced with the addition of α-KG, considered to promote the conversion of PMP-hBCATm to PLP-hBCATm, which can then initiate another cycle (Islam et al. 2007). The interaction was regulated not only by redox, but signalling pathways governed by phosphorylation. When E1 was phosphorylated, hBCATm could not bind. This is interesting because phosphorylation of the E1 subunit renders it inactive and therefore cannot bind BCKAs for decarboxylation. As demonstrated from the distribution studies, skeletal muscle has high expression of the BCKDC kinase, enhancing exit of the BCKAs (Suryawan et al. 1998). Moreover, high ratios of NADH/NAD also prevent BCATm/BCKDC binding and BCKA release or recycling is favoured. Therefore, metabolon formation and its regulation are key events that regulate nitrogen transfer and BCAA oxidation. This switch from a reduced to an oxidised BCATm may also signal a change in function, i.e. from the role of hBCAT in metabolism to its redox-chaperone role, discussed later.

Not only was the BCKDC found to associate with hBCATm, but so too were other key metabolic proteins including GDH, pyruvate carboxylase, 4-nitrophenylphosphatase domain, and non-neuronal SNAP25-like protein homolog 1 (Islam et al. 2010). Pyruvate carboxylase catalyses the formation of oxaloacetate from pyruvate and bicarbonate. Although this protein is important for de novo synthesis of glutamate, its role in this metabolon is unclear. On the other hand, binding of hBCATm with GDH was found to occur in a BCKDC-dependent or BCKDC-independent manner. GDH catalyses the oxidative deamination of glutamate to α-KG, using NAD^+ or NADH as a coenzyme. Following the first half-reaction of transamination and after dissociation with the E1 subunit, the PMP-hBCATm form requires conversion to its PLP form. Binding occurs between PMP-hBCATm and GDH, whereupon the α-KG produced by oxidative deamination of glutamate is channelled to the BCATm active site to generate glutamate and re-establish the PLP form of hBCATm and release GDH. Oxidation of the BCKAs by BCKDC generates the NADH which is a cofactor in the GDH reaction.

Thus, wherever BCATm and GDH are expressed, both are mitochondrial enzymes; α-KG formed from the GDH reaction (preferred direction) is reaminated by metabolon formation, resulting in no net change in glutamate concentration. If BCKDC is also found in the same cell as GDH and BCATm, formation of the metabolon would facilitate BCKA oxidation. However, the ultimate products of the metabolons are the branched chain acyl-CoAs and NH_3. If BCKDC is not present, as is found in rodent astrocytes, then BCKAs would be released and could be reaminated or oxidised in another cell type in brain such as neurons or released into the blood. The ammonia produced could be used for glutamine synthesis in astrocytes, otherwise the ammonia would be released and used in other cells. Another factor other than expression and relative activities of GDH1 and hBCATm (PLP-form v/s PMP-form) is the redox state of the cells (NAD+/NADH). If only GDH and BCKDC are present, the kinetics of both reactions are not affected, because α-KG is not a substrate for BCKDC and GDH does not bind to BCKDC (Hutson et al. 2011). However, although GDH1 alone did not have a significant effect on BCKDC activity, the inclusion of hBCATm and glutamate enhanced the k_{cat} by 150 % when compared to BCKDC and BCKAs alone and by about 40 % compared to the addition of hBCATm alone. Equally important, binding of hBCATm to GDH enhances leucine-induced activation of GDH, where the interaction with hBCATm and leucine promotes nitrogen shuttling and ammonia formation. However, addition of ADP to the above reaction did not enhance oxidative deamination by GDH (Islam et al. 2010). Interestingly, although GTP is an allosteric inhibitor of GDH, addition of PMP-hBCATm protected GDH from inhibition. Conversely, addition of GDH to PMP-hBCATm enhanced the k_{cat} of PMP-hBCATm reamination of α-KG by 15-fold and reduced the k_m by 4.5 fold (Hutson et al. 2011). Therefore, actual net transfer of BCAA nitrogen occurs when BCKDC is active (BCAAs are oxidised) and this is initiated by PLP-hBCATm/E1 complex formation. Together, these metabolons offer a complex yet efficient mechanism for the transfer of nitrogen between enzyme systems providing a possible role for regulating NH_3. However, metabolon formation and the functional significance of the metabolon are ultimately dependent on the expression of these enzymes in tissues and cell types within tissues.

Thus, knowledge of the distribution and relative ratios of these enzymes in cells offers insight into the direction of metabolism, but also the fate of the BCKAs. In rodents, where BCKDC is not co-expressed with BCATm, in tissues such as the liver and brain, this metabolon does not function. In this forum, compartmentation of BCATm to astrocytes (or endothelial cells in humans) and BCKDC to neuronal cells facilitates the shuttling of metabolites between cells, where transamination is favoured in astrocytes or endothelial cells and oxidation in neuronal cells. Also, in skeletal muscle, although BCKDC is expressed, the high transaminase activity and high BCKDC kinase activity will favour BCKA production, preserving the carbon skeleton, which can be reaminated or oxidised in other tissues. The role of these metabolons is also likely to expand to other perhaps to be described functions, where insight into their role in hormone secretion has already been described (Zhou et al. 2010).

5.6 Patterns of BCAA Metabolic Enzyme Expression in Human Brain

BCAT expression studies in the human brain were expected to reflect that of the rodent model, where the BCAT cycle model would support the glutamate/glutamine cycle. Indeed, for hBCATc, consistency between models was reported where immunopositive labelling was predominant in neuronal cells (Fig. 5.4) (Hull et al. 2012). Similar to reports from the rodent models, the majority of hBCATc-labelled neurons in the human brain were either GABAergic or glutamatergic, with GABAergic neurons shown to be more strongly immunopositive than glutamatergic neurons (Table 5.3). However, neurons that were neither glutamatergic nor GABAergic also labelled for hBCATc. Each of the rat distribution studies reported staining that was more intense in some areas over others, and whereas cell body staining was clear in all neuronal cells labelled, staining of processes was more cell-type-specific. For example, in the hippocampus neuronal staining was more intense than the CA1 region. This supports earlier findings, where differential mRNA expression of hBCATc was reported in postnatal and adult brain of mice (Castellano et al. 2007). Compared with axonal staining in the areas of the supraoptic tract, it was more intense in the cell bodies of neuronal cells within the temporal and hippocampus relative to the dendrite regions that had more pronounced staining. Thus, it appears that depending on the neuronal sub-type, hBCATc may have a role in either contributing to the glutamate metabolic pool or the glutamate pool used during excitation (Table 5.2). Therefore, in these neuronal sub-types it was proposed that the role of

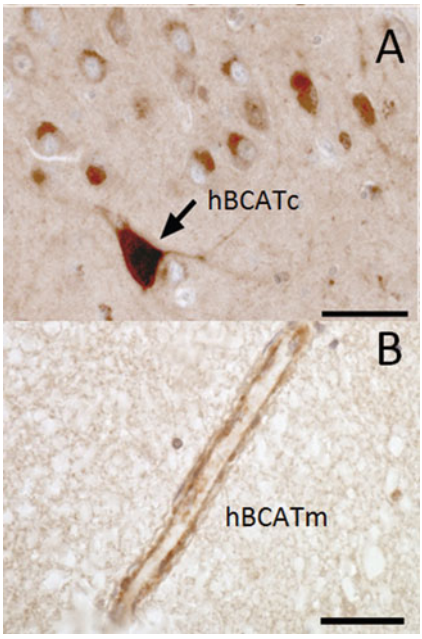

Fig. 5.4 Immunostaining of hBCATc and hBCATm in the temporal lobe and cerebellum. (**a**) The temporal neocortex (inferior temporal gyrus) showing hBCATc immunopositive neurons. (**b**) hBCATm staining of the vasculature surrounding the hippocampus (Hull et al. 2012)

Table 5.3 An overview of hBCATc immunoreactivity throughout the human brain[a]

Area	Immunopositive neuronal cells	Intensity of staining
Temporal lobe		
• *Hippocampus*		
– GABAergic interneurons	+++	+++
– Pyramidal neurons	+	++++
– Dentate gyrus (neurons)	+	+
• *Cortex*		
– Neurons	+++	++
– Subiculum sub-population of neurons	+	++
– Lamina II neurons	++	++
Cortex and white matter		
• Cortical pyramidal cells	+	+++
• Cortical neurons	++	++
• Axonal staining	–/+	++
Putamen and Basal ganglia		
• Large neurons	+	+++++
• Small neurons	+++	+
• Insular cortex (small neurons)	+	++
• Thalamus (neurons)	+	++
• Caudate nucleus (neurons)	+	+
• Lateral geniculate nucleus (neurons)	+	+
Midbrain		
• Widespread neuronal staining	++++	+++
• Periaqueductal grey matter (neurons)	++	++
• Neuropil staining	++	+
• Inferior colliculus (nerve cells)	+	+++
• Supraoptic nucleus of the hypothalamus	+	++++
• Paraventricular neurons of the hypothalamus	+	++++
Cerebellum		
• Oligodendrocyte staining in the white matter.	+	++
• Purkinje cells	+	+
• Swollen axon terminals	++	++
• Interneurons	++	+
• Neurons in the dentate nucleus	++	+
Pons		
• Pontine nuclei (neurons)	+	++
• Tegmental neurons	+	++
• Neuronal processes (nigro striatal processes)	++	+
• Raphe nuclei (neurons)	+	++
• Nucleus basalis of Meynert (cholinergic neurons)	+	++++
Medulla		
• Hypoglossal nucleus (neurons)	+	++
• Dorsal motor nucleus (neurons)	+	++
• Nucleus ambiguus (neurons)	++	+++
• Gracile nucleus	++	++
• Inferior olivary nuclei (neurons)	++	++
• Inferior olivary nuclei neuropil	++	++

[a]Date from Hull et al. (2012)

BCAAs was to produce glutamate, which is corroborated by historical studies that show BCAAs can label a significant proportion of the glutamate pool in neuronal cell culture, which has also been shown in vivo in rats and ex vivo rat retina models (Yudkoff et al. 1983, 1994; Kanamori et al. 1998; LaNoue et al. 2001).

Conversely, unlike the rodent model, which showed expression of BCATm in astrocytes, there is extensive immunopositive staining for hBCATm in the vasculature of the brain with no evidence of staining within astrocytes, suggesting a different role for hBCATm in glutamate regulation in the human brain (Fig. 5.5). Cerebral endothelial cells have a high density of mitochondria (Oldendorf et al. 1977), giving greater capacity for energy production than other capillary endothelial cells and explaining the punctuate staining of mitochondrial hBCAT in these cells. This result was surprising; however, discrepancies between the rat model and humans have previously been described, in particular with respect to transaminase activity and oxidative capacity (Suryawan et al. 1998). If hBCATm is not expressed in human astrocytes, transamination cannot occur indicating that the BCAA/BCKA shuttle is particular to rodents and that in human brain its clear staining in the endothelial layer of the vasculature supports a role in metabolite channelling across the blood

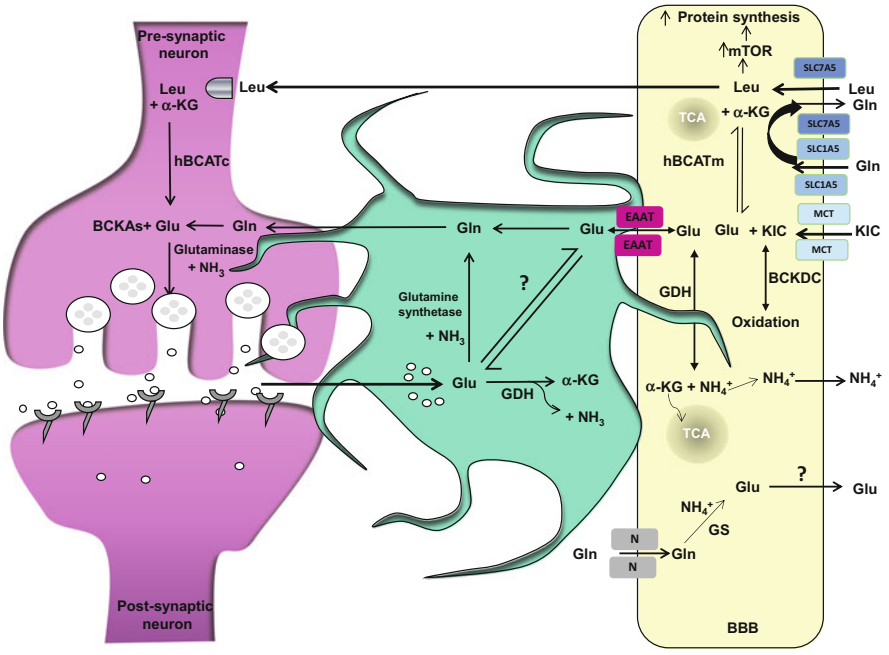

Fig. 5.5 Nitrogen transfer in human brain. Leucine, readily taken up by the blood brain barrier by the L1 system in exchange for glutamine, where it can be taken up by neuronal cells and transaminated by hBCATc or be used as a nutrient signal in endothelial cells. Excess glutamate generated during excitation is taken up by astrocytes, where glutamate is regenerated through the glutamate/glutamine cycle. Glutamate may also be removed by endothelial cells of the vasculature. Here, the fate of glutamate could be (1) exit into blood; (2) oxidative deamination by GDH; and (3) reamination by hBCATm generating leucine for reuptake by neuronal cells (Hull et al. 2012)

brain barrier. The reason behind these differences is not clear. However, hBCATm expression is induced under conditions that promote cell proliferation, which may explain why in cell models hBCATm expression is increased (Pérez-Villaseñor et al. 2005) or that the pattern of expression may be altered by placing cells in culture outside the normal milieu. It may also be due to the age of the rat brain compared with the brains used in the human study, reflecting a younger model relative to older brains used in the human study. Nevertheless, it is most likely the difference in BCAT expression between species.

So, what is the role of hBCATm in the endothelial layer of the vasculature? Based on structural and metabolic studies, we would anticipate that its role will be dependent on the expression of other key metabolic proteins, such as the GDH and BCKDC and the inter-relationships of metabolism to transport key amino acids. Moreover, we anticipate that the redox environment will also feature as it is known to regulate and potentially redirect the function of hBCATs. In humans, hGDH is expressed in two isoforms, hGDH1, that is widely expressed but high in the liver, whereas hGDH2 is expressed in the brain, testis, and kidney (Lai et al. 1986; Schmitt and Kugler 1999; Zaganas et al. 2009, 2010; Spanaki et al. 2010). In human brain, the GDH-specific activity is fourfold less than that described for liver (Spanaki et al. 2010). Similar to reports from rat, in human brain clear astrocyte staining is evident for GDH1 and GDH2, though GDH2 is highly expressed in astrocytes (Spanaki et al. 2014); however, punctuate staining throughout the vasculature was also observed. Here, the authors proposed that this was due to the astrocytic end-feet surrounding the vasculature. However, unpublished work from our lab shows vasculature staining, but indicates that it is the endothelial layer of the vasculature rather than just the astrocytic end-feet. As GDH is found together with hBCATm, then it is possible that the hBCATm/GDH1 metabolon can form and hBCATm does not bind to GDH2 (Hutson et al. 2011). Excess glutamate taken up by EAAT transporters (EAAT1, 2 and 3), the rate of which is thought to be influenced by blood glutamate, could be regulated by the hBCATm/GDH metabolon (Chaudhry et al. 1995; O'Kane et al. 2004; Hosoya et al. 1999; Gottlieb et al. 2003). Glutamate in the ECF must be maintained at low concentrations to avoid excitotoxicity. Glutamine is also transported, primarily by the N transporter, and can be metabolised by glutaminase generating glutamate and ammonium adding to the glutamate pool in endothelial cells (Lee et al. 1998). It is expected that when levels of intra-cellular glutamate rise, net transport across the luminal membrane occurs. Removal of glutamine is also important, given the absence of the urea cycle. Here, an opportunity for the BBB to regulate ammonia levels is important. Accumulation of glutamine in the brain would result in an increased osmolarity and swelling (Hawkins et al. 2006). Oxidation of glutamate by GDH would release α-KG, forming ammonia and NAD^+. If the ammonia produced in the endothelial cell could enter the astrocyte end-feet, it could provide ammonia for glutamine synthesis or would be free to diffuse across the luminal membrane into blood. In the absence of metabolon formation, transamination of glutamate with BCKA would form α-KG that could enter the TCA cycle, generating leucine for neuronal uptake (dependent on the availability of α-keto-acids). Brain transport of α-keto-acids is facilitated by the monocarboxylate transporter expressed in the BBB and neuronal cells (Mac et al. 2000). Provision of blood α-keto-acids

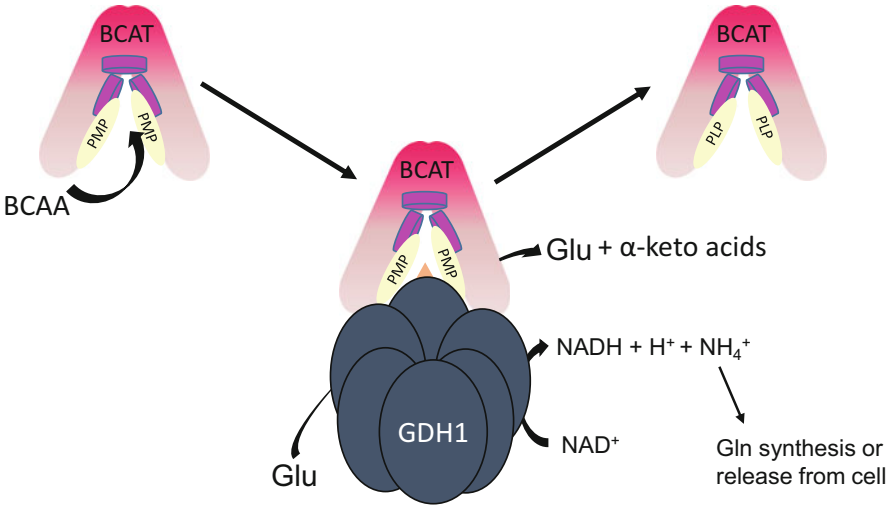

Fig. 5.6 BCATm and GDH1 form a metabolon. The PMP form of BCATm binds to GDH1. The product of oxidative deamination, α-KG, is channeled from GDH1 to PMP BCATm followed by formation of glutamate (Glu) and release of the PLP form of BCATm, which is then ready to transaminate with a BCAA. There is no net change in cellular glutamate. The products of the reaction are NADH and ammonia (NH_4^+). Thus, the metabolon facilitates formation of ammonia and NADH

could drive metabolism in the direction of leucine synthesis. Metabolism of brain glutamate by hBCATm or hBCATm/GDH could therefore serve as an auxillary part to the glutamate/glutamine cycle, the predominant system that regulates brain glutamate between neurons and astrocytes (Fig. 5.6).

Because leucine is easily taken up by specific transporters on the BBB, and as hBCATm is expressed in these endothelial cells, transamination can occur. These amino acid transporters play a key role in the maintenance of free amino acid concentrations in cells and also regulating brain nitrogen. It is the coupling of these amino acid transporters with metabolism that enables the cell to respond to nutrient signals through activation of increased protein translation and growth. Conversely, cell survival through autophagy is stimulated when amino acids are limiting (Chen et al. 2014). Entry of large neutral amino acids such as leucine and tryptophan is facilitated by the membrane transport L1 system, found both on the luminal and abluminal side of the BBB (Oldendorf 1973; Smith et al. 1987; O'Kane et al. 2004; O'Kane and Hawkins 2003). The plasma concentration of tryptophan and competition with other large neutral amino acids (LNAA) has been shown to reflect serotonin levels in the brain and will thus dictate metabolic signalling in the cell (Fernstrom and Wurtman 1972). Recent studies have indicated that in order for leucine to be imported by the solute carrier family 7 member 5 (SLC7A5), glutamine exchange must occur (Nicklin et al. 2009). So, when glutamine is taken up by the solute carrier family 1 member 5 (SLC1A5), it is concomitantly exchanged for leucine (within 1–2 min). This bidirectional transport has been proven to be important for leucine-mediated mTOR activation. Studies indicate that p70S6K was not

activated until glutamine was exchanged for leucine and reactivation of starved cells was dependent on glutamine uptake (Chen et al. 2014). Moreover, knockdown of the SLC1A5 transporter impaired mTor activation, further supporting the synergistic role of glutamine with leucine in regulating the anabolic response (Nicklin et al. 2009).

In contrast to the facilitative transporters on the luminal face, Na^+-dependent transport systems are required on the abluminal side, which allows amino acid removal from the brain via the Na^+ gradient that exists between the ECF and the endothelial cells of brain capillaries (reviewed in Hawkins et al. 2006). In theory, amino acids that both pass the endothelial cell membrane and enter the basement membrane space could be actively and selectively pumped back across the abluminal membrane. It is this gradient system and abluminal expression of the Na^+-dependent carriers that favours removal of nonessential amino acids and toxic amino acids such as glutamate. As L1 transporters also exist on the abluminal side, this indicates that transport can occur in either direction, the impact and kinetics of which remains to be determined (O'Kane and Hawkins 2003). BCKA produced by BCATm could be oxidized by endothelial cell BCKDC (Conway unpublished observations) and enter the blood or the brain. However, further knowledge of the relative expression/activities of these enzyme systems is required, which would inform the BCAA metabolic capacity of these cells. Moreover, we anticipate that as neuronal stores of glutamate require constant replacement, leucine uptake by endothelial cells will be preferentially for this role and mTOR regulation rather than oxidation. Thus, the specific expression of transporters on the respective membranes, together with the metabolic complexes expressed in the BBB, orchestrates the fine tuning of amino acid homeostasis and nitrogen balance, which aims to protect the brain from excitotoxicity. If as we have proposed that in human brain the direction of transamination in neuronal cells is towards glutamate production catalysed by the cytosolic BCATc isozyme, transamination functions in both contributing to the neuronal pool and also excitation, dependent on the neuronal sub-type. On the other hand, if glutamate concentrations are high and BCKAs are available, BCATc could attenuate excitation. As BCKDC is widely expressed in neuronal cells (Cole et al. 2012), oxidation of the BCKAs can occur, but again this will depend on the relative activities of these two enzymes and transport of BCKA either into the mitochondria or release from the cell. As shown in CD4(+) T cells, activation of the T cell receptor induces expression of BCATc and the primary fate of BCKA is released from the T cells (Ananieva et al. 2014). Conversely, in the endothelial layer of the vasculature although the role of hBCATm may work with GDH to metabolise excess glutamate released at the synaptic region, it is also possible that BCATm is detrimental, particularly in conditions such as Alzheimer's disease, where glutamate excitotoxicity prevails. hBCATm was found to be increased by 160 % in the brain of patients with AD relative to matched control (Hull et al. 2015). This was correlated with increased Braak stage, indicating a link with disease severity. On the other hand, multiple forms of BCATm between 41 and 52 kDa were also observed. Redox regulation on BCATm, which inhibits BCAT activity, may alter the function of these proteins and their role in glutamate metabolism.

5.7 The Redox-Active hBCAT Proteins: The Nano-switch

When discussing the role of the hBCAT proteins in metabolon structures, we mentioned the importance of the redox-active –CXXC– motif. Each isoform is differentially regulated through changes in the redox environment, where the mitochondrial isoform can undergo complete reversible oxidation, losing all activity (Conway et al. 2002). The cytosolic isoform is also susceptible to oxidation, where the formation of a disulphide bond between its reactive cysteine residues results in the loss of 50 % hBCATc activity. Interestingly, unlike hBCATm, further oxidation did not affect activity (Conway et al. 2008). The N-terminal cysteine residue of each –CXXC– motif was the most reactive, forming a cysteine sulphenic acid intermediate that quickly undergoes further oxidation to produce a disulphide bond (Conway et al. 2002, 2004). Loss in activity occurs due to the re-orientation of the substrate-binding channel compromising stability, which impacts the second half reaction rather than the first half reaction (Conway et al. 2003; Yennawar et al. 2006). The reactive cysteines of both isoforms are also targets for the S-nitrosylating agent, S-nitroso glutathione (GSNO), where a transition between S-nitrosation and S-glutathionylation was reported dependent on the level of RNS exposure (Coles et al. 2009). The glutaredoxin/glutathione system reversed this inactive form, supporting a role for hBCAT in cellular redox control. We therefore had characterised a reversible redox-sensitive switch, which controlled transaminase activity.

We have already seen that oxidation of hBCATm influences its role in forming a metabolon with the E1 subunit of BCKD, where oxidation prevents metabolite channeling. We propose that this nano-switch has wider implications, such that during periods of oxidative stress the role of hBCAT switches from BCAA metabolism to a redox chaperone (Conway et al. 2008; Conway and Lee 2015). Evidence in support of this stems from recent work where a novel functional role for hBCAT in redox protein folding was reported (El Hindy et al. 2014). Here, these proteins were found to catalyse the refolding of reduced and denatured RNase, the activity of which was regulated by the oxidising environment. When treated with GSNO, high molecular weight (HMW) structures were observed and the rate of refolding increased. Generation of HMW structures during chaperone activity has been reported for other chaperones such as human peroxyredoxin and also Hsp33 (when overoxidised), which are important to their role in protein folding (reviewed in Conway and Lee 2015). We therefore speculate that hBCAT can form oligomers that prevent aggregation and promote correct protein folding. Moreover, as hBCATm also forms a redox complex with protein disulphide isomerase (PDI), influencing the rate of refolding, it is likely that hBCATm is a novel redox chaperone for PDI, operating through a thiol-disulphide exchange mechanism. Or these transaminases may operate independently as an oxidoreductase regulated through the redox environment. In summary, we propose that, under reduced conditions, the role of hBCAT favours transamination and metabolon formation with the BCKDC and/or GDH, metabolic role in brain neurotransmitter metabolism. In an oxidizing environment such as increased ROS or RNS, oxidation changes the structural conformation opening the active site to novel substrates to promote protein folding and chaperone

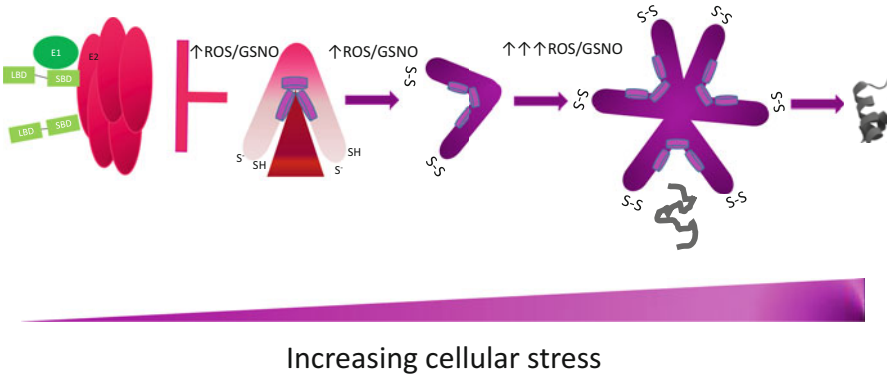

Fig. 5.7 Thiol redox sensor–dual role for BCAT. Oxidation results in the inactivation of BCAT and the prevention of BCAT/BCKDC metabolon formation. With increased oxidation, the BCAT protein can form multimers and function as oxidoreductases, where a role in protein folding has been implicated (El Hindy et al. 2014; Conway and Lee 2015)

activity, supported by the three hydrophobic pockets in the active site. This change in function will be further accelerated as the levels of ATP decrease and the NADH/NAD+ ratios are compromised, impacting the functional state of the metabolons. The physiological role of this interaction has not yet been elucidated, but new data has isolated a protein-folding metabolon, offering exciting new roles for these metabolic proteins as redox chaperones (unpublished observations) (Fig. 5.7).

5.8 Inborn Errors of BCAA Metabolism

A recent case involving mutations in BCATm (BCAT2), which resulted in reduced activity of this enzyme, has been reported (Wang et al. 2015). In this patient, the observed hypervalinemia and hyperleucinemia were associated with brain white matter lesions. This case provides evidence that blocking BCAA catabolism has consequences on brain structure and function that are independent of other BCAA metabolites. Nevertheless, by far the most common and deleterious inborn error of BCAA metabolism is Maple Syrup Urine disease (MSUD; OMIM 248600) caused by deficiency of the mitochondrial BCKDC, the second step in the catabolic pathway. This disease results in the accumulation of BCAA and their corresponding BCKA in tissues and plasma. The disorder typically manifests with potentially lethal episodes of intoxication presenting with acute neurological deterioration, feeding problems, weight loss, and a maple syrup odor to the urine (Morton et al. 2002). These episodes usually occur during states where the body is catabolic such as fasting or intercurrent illnesses. Toxicity is associated with the increase in plasma concentrations of leucine and its α-keto acid KIC. There is little apparent toxicity associated with increases in isoleucine or valine (Korein et al. 1994). Based on its

severity, MSUD has been classified into five clinical subtypes: a “classic” neonatal severe form, an “intermediate” form, an “intermittent” form, a “thiamine-responsive” form, and an “E3-deficient with lactic acidosis” form. Although the correlation between clinical severity and degree of residual enzymatic activity is often inconsistent, the intermediate and intermittent forms are usually associated with some degree of residual activity with later clinical onset, while the classic form usually exhibits extremely low activity (Dancis et al. 1967; Schulman et al. 1970). Current treatment is based on dietary manipulations with protein restriction and a synthetic formula with reduced BCAA content (Morton et al. 2002). More recently, total liver transplant has become a popular alternative for MSUD patients, and they appear to be able to tolerate a normal diet after transplantation. From a recent review that used our published data and another study, it was concluded that the effectiveness of liver transplantation in MSUD indicates that providing 9–13 % of the bodies’ BCKDC activity is sufficient to restore BCAA homeostasis (Suryawan et al. 1998; Burrage et al. 2014). However, even with early detection and dietary restriction or liver transplantation, some mental and social impairments are still present in the majority of these individuals (Simon et al. 2007; Muelly et al. 2013).

Accumulation of leucine and, particularly its α-keto acid KIC, is likely responsible for the brain pathology (Funchal et al. 2005; Ribeiro et al. 2008). Indeed, the BCATm knockout mouse, which exhibits elevated BCAAs without subsequent elevation of BCKAs, does not exhibit an MSUD phenotype (She et al. 2007), although the human clinical case indicated white matter abnormality (Wang et al. 2015a, b). The BCATm KO mouse with concentrations of BCAA seen only in patients undergoing metabolic decompensation exhibited increased energy expenditure due in part to increased protein turnover supporting the hypothesis that the toxic metabolites are the BCKA. It has been reported that BCKA treatment triggers elevation of reactive oxygen species in cells (Funchal et al. 2005), rising BCKA and loss of BCKDC activity increases oxidative stress in MSUD patients (Guerreiro et al. 2015; Sitta et al. 2014). Because oxidative stress can trigger apoptosis in neurons, this could be one mechanism for neuronal cell damage. Neurons are characterized by lower glycolytic rate compared to astrocytes and so are thought to be less resistant to cellular stresses (Bolaños and Almeida 2010). A study by Klee et al. (2013) reported changes in white matter microstructural organization in MSUD patients and interpreted the data as evidence of structural abnormality and reduction in amounts of fibre tracts in these patients. It is known that both mTORC1 and mTORC2 are involved in cytoskeletal polymerization and reorganization (Jacinto et al. 2004; Gulhati et al. 2011; Li et al. 2014). Our unpublished data showed significant reduction of β3-tubulin as well as F-actin in the brain of newborn E1α (E1 enzyme protein) knockout mouse brain and in cultured neurons, when compared to the WT animals. These data suggest either neuronal death or a major cytoskeletal alteration. There was increased depolymerization of actin and β3-tubulin and increased β3 tubulin cleavage in BCKA plus BCAA-treated neurons.

Finally, there are consequences to the brain when BCAA are limiting. Regulation of BCKDC enzymatic activity depends on the phosphorylation status of the E1α

subunit of the E1 enzyme that is specified by a kinase (BDK), which inactivates the E1α subunit, and by a mitochondrial matrix resident type 2C phosphatase gene, *PP2Cm*, that activates it. When phosphorylated, the E1 enzyme is inactive preventing decarboxylation of the BCKA. Mutations in the BCKDC kinase have now been reported (García-Cazorla et al. 2014; Novarino et al. 2012). Impaired kinase activity results in uncontrolled oxidation of BCAA. Therefore, BCAA can become limiting for protein synthesis, which may also alter mitochondrial metabolism (energy metabolism). These mutations have been associated with mild autism, epilepsy, and intellectual disability (Novarino et al. 2012) and developmental delay, microcephaly, and neurobehavioral abnormalities (García-Cazorla et al. 2014). Fortunately, protein-rich diets or oral BCAA supplementation can be used to treat BDK disorders.

5.9 Conclusions

Nitrogen transfer within and between cells and tissues illustrates a fascinating interplay of metabolic pathways that play key roles in protein metabolism, nutrient signalling, and neurotransmitter regulation. Changes in cell signalling and the redox homeostasis clearly define the direction of metabolism and may signal new roles for these metabolic proteins under oxidative stress. Our current understanding of these mechanisms supports a clear role for the BCAA in the supply of nitrogen for glutamate synthesis. This is of particular importance given that the BCAA, particularly leucine, can cross the blood brain barrier and provide a nitrogen source for the synthesis of the neurotransmitter, glutamate. As these aminotransferase proteins play a key role in facilitating the anaplerotic generation of glutamate, it is highly likely that their metabolism will be altered in neurodegenerative disease conditions. Therefore, understanding how these suggested shuttles are altered in disease will offer possible targets for novel therapeutic treatment to either delay onset or prevent further neuronal destruction.

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Chapter 6
Glutaminases

Javier Márquez, José M. Matés, and José A. Campos-Sandoval

Abstract Mammalian glutaminases catalyze the stoichiometric conversion of L-glutamine to L-glutamate and ammonium ions. In brain, glutaminase is considered the prevailing pathway for synthesis of the neurotransmitter pool of glutamate. Besides neurotransmission, the products of glutaminase reaction also fulfill crucial roles in energy and metabolic homeostasis in mammalian brain. In the last years, new functional roles for brain glutaminases are being uncovered by using functional genomic and proteomic approaches. Glutaminases may act as multifunctional proteins able to perform different tasks: the discovery of multiple transcript variants in neurons and glial cells, novel extramitochondrial localizations, and isoform-specific proteininteracting partners strongly support possible moonlighting functions for these proteins. In this chapter, we present a critical account of essential works on brain glutaminase 80 years after its discovery. We will highlight the impact of recent findings and thoughts in the context of the glutamate/glutamine brain homeostasis.

Keywords Glutamate • Glutamine • Glutaminase-interacting proteins • Astrocytes • Neurons

Abbreviations

AGC	Aspartate–glutamate carrier
BBB	Blood-brain barrier
EM	Electron microscopy
GA	Phosphate-activated glutaminase
GAB	*Gls2*-encoded long GA isoform
GAC	*Gls*-encoded short GA isoform

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GAD	Glutamic acid decarboxylase
GDH	Glutamate dehydrogenase
GFAP	Glial fibrillary acidic protein
GIP	Glutaminase-interacting protein
GS	Glutamine synthetase
IMM	Inner mitochondrial membrane
KGA	*Gls*-encoded long GA isoform
LGA	*Gls2*-encoded short GA isoform
NBT	Nitroblue tetrazolium
NMR	Nuclear magnetic resonance
NPCs	Neural progenitor cells
PDZ	PSD95/Dlg/ZO1 domains
TCA	Tricarboxylic acid cycle

6.1 Introduction

Glutamate (Glu) is the most important neurotransmitter in normal brain function: about 80–90 % of all brain synapses release this nonessential amino acid (Braitenberg and Schüz 1998; Attwell and Laughlin 2001). Glu does not cross the blood-brain barrier (BBB) (Hawkins 2009) and therefore must be synthesized in neurons from local precursors. The enzyme phosphate-activated glutaminase (GA) catalyzes the hydrolytic deamidation of glutamine (Gln) to Glu and ammonium ions and appears as the major Gln metabolizing enzyme in brain (Hertz 1979; Kvamme 1984). This enzyme has been considered as the principal pathway for neurotransmitter Glu production (Nicklas et al. 1987); accordingly, the bulk of Glu released at the synapses derives from precursor Gln (Bradford et al. 1978; Hamberger et al. 1979; Thanki et al. 1983; Laake et al. 1995; Hertz and Zielke 2004), while inhibition of GA depletes the stores of Glu (Conti and Minelli 1994). Nevertheless, presynaptic Glu is not always necessarily derived from Gln: synthesis from tricarboxylic acid cycle (TCA) intermediates also contributes to generation of excitatory Glu (Waagepetersen et al. 2005; Takeda et al. 2012), although the exact contribution of each source is a matter of debate and remains to be established (Kam and Nicoll 2007).

Enzymes that break the amide bond of Gln and transfer the ammonia to other specific substrates in biosynthetic pathways are known as glutamine amidotransferases (EC 2.4.2). However, these enzymes should not be confused with GA, which is a glutamine amidohydrolase (EC 3.5.1.2), a genuine hydrolytic enzyme releasing the γ-glutamyl group and ammonium into the media without transfer to any other acceptor molecule. The catalytic reaction requires no energy and is essentially irreversible: the K'_{eq} is 322 at pH 7.0 and 25 °C (Benzinger et al. 1959), which indicates that synthesis of Glu is strongly favored. Mammalian glutaminases only show reactivity toward free Gln and cannot deamidate internal Gln residues in proteins, although such activity has been reported for microbial protein glutaminases (Yamaguchi

et al. 2001). On the other hand, prokaryotic asparaginases may also deamidate free Gln and this has been used as a chemotherapeutic treatment to deplete plasma Gln levels in children suffering leukemia (Holcenberg et al. 1979). However, mammalian asparaginases (L-asparaginase-amidohydrolase, EC 3.5.1.1) do not utilize Gln as substrates (Schalk et al. 2014).

Besides the transmitter pool of Glu in excitatory synapses, there is a metabolic pool with great relevance because this amino acid is a primary metabolic fuel for brain cells (Erecinska and Silver 1990) and a precursor of many other important metabolites (Fonnum 1984). In fact, the first step in the mitochondrial breakdown of glutamine to pyruvate/lactate, a pathway known as glutaminolysis, is initiated by GA and has been largely recognized as a fundamental process for energy supply in the bioenergetics of many normal and transformed cell types (Kovacevic and McGivan 1983; Márquez et al. 2015). Furthermore, Gln can be used through glutaminolysis as an anaplerotic substrate to replenish TCA intermediates (DeBerardinis et al. 2007).

6.2 Historical Account: Pioneer Works

Krebs, in his pioneer studies on amino acids metabolism, discovered GA while studying the enzymatic hydrolysis of Gln in animal tissues (Krebs 1935). Krebs first noticed the existence of two different types of GA, classifying them as "brain type" (also present in kidney) and "hepatic type." Lately, Errera and Greenstein (1949) coined the term "phosphate-activated" GA to describe the enzymatic activity which was strongly stimulated in tissue extracts after addition of inorganic phosphate (Pi). The enzyme is widely distributed in mammalian tissues where it fulfills essential tasks related to tissue-specific function (Curthoys and Watford 1995).

The traditional terms, kidney- and liver-type isoenzymes, denoted marked differences in molecular structures, kinetic, regulatory, and immunological properties that were early detected and justified their study separately (Kovacevic and McGivan 1983). Historically, kidney-type enzymes were the first GAs isolated and characterized in mammalian tissues. Kvamme's group leads the way in this area by isolating to apparent homogeneity pig renal and pig brain GAs (Kvamme et al. 1970; Svenneby et al. 1973). Later rat kidney (Curthoys et al. 1976), cow brain (Chiu and Boeker 1979), pig brain (Nimmo and Tipton 1980), and rat brain (Haser et al. 1985; Kaneko et al. 1987) GAs were also purified. Kidney-type enzymes were isolated in highly purified states from cancer cells indeed (Quesada et al. 1988; Segura et al. 1995).

Most kidney-type enzymes undergo specific and extensive polymerization in the presence of phosphate and phosphate–borate buffers. The reversible association–dissociation equilibrium has been advantageously used to purify this GA isoform. In sharp contrast, the liver-type isoenzymes do not show aggregation and self-assembly in high molecular mass polymers. This fact, along with a remarkable susceptibility to proteolytic degradation, has hampered their purification; thus, liver-type isozymes

have been considerably more refractory to purification than their kidney-type counterparts. Actually, the isolation of pure rat liver isoform was achieved almost two decades after the first kidney-type enzyme was isolated (Heini et al. 1987; Smith and Watford 1988), although partial purifications were also previously reported (Huang and Knox 1976; Patel and McGivan 1984). Finally, it is worthy to mention that the classical terms kidney(K)-type and liver(L)-type are currently obsolete and should be avoided for the sake of coherence: the new expression data for mammalian GA do not support any more such kind of terminology (see Matés et al. 2013 for an update on GA nomenclature).

6.3 Mammalian Glutaminase Genes

The mammalian GA family members are encoded by two paralogous genes, *Gls* and *Gls2*, presumably derived from a common ancestral gene by duplication and divergent evolution (Porter et al. 2002; Pérez-Gómez et al. 2003). In humans, the *GLS* gene is located on chromosome 2 and encodes GA isozymes classically referred to as kidney-type (K-type), while *GLS2* is located on chromosome 12 and codes for liver-type (L-type) isozymes (Aledo et al. 2000). Orthologous genes have been described in rat (Chung-Bok et al. 1997) and mouse (Mock et al. 1989). The human *GLS* gene (cited in many publications with the incorrect term *GLS1*: see HUGO Gene nomenclature, http://www.genenames.org/) spans 82 kb and splits into 19 exons (Porter et al. 2002). Two different transcripts arise from this gene (Fig. 6.1): the KGA transcript, originally found in kidney, composed by 18 exons and formed by joining exons 1–14 and 16–19, and the GAC transcript which appears by alternative splicing and uses only the first 15 exons (Elgadi et al. 1999; Porter et al. 2002) (Fig. 6.1).

The human *GLS2* gene has a length of approximately 18 kb and splits into 18 exons (Pérez-Gómez et al. 2003) (Fig. 6.1). Two transcripts have been identified from the mammalian *Gls2* gene: the canonical long transcript termed GAB, formed by joining all 18 exons of the gene (Pérez-Gómez et al. 2003), and the short transcript LGA that lacks exon 1 and was originally identified in rat liver (Smith and Watford 1990) (Fig. 6.1). Human GAB transcript was isolated as a cDNA clone from ZR-75 breast cancer cells encoding a protein of 602 amino acids, which is 67 amino acids longer than rat liver LGA protein (Gómez-Fabre et al. 2000) (Fig. 6.1).

Although located in different chromosomes, the human *GLS* and *GLS2* genes share a considerable degree of sequence similarity. Apart from the additional exon present in the human *GLS* gene, the main differences in the coding sequences of both genes are located at exons 1 and 18. Exon 1 shares 62.5 % similarity, but it codes for 129 amino acids in KGA and only for 61 amino acids in GAB, accounting for most of the 67 extra amino acids of KGA protein at the N-terminal. The sequences encoded by exon 1 contain the signals involved in mitochondrial targeting and translocation processes (Shapiro et al. 1991; Gómez-Fabre et al. 2000) (Fig. 6.2). Likewise, exon 18, which codes for the C-terminal region of both proteins, shows the lowest sequence similarity (29.4 %). This region of human

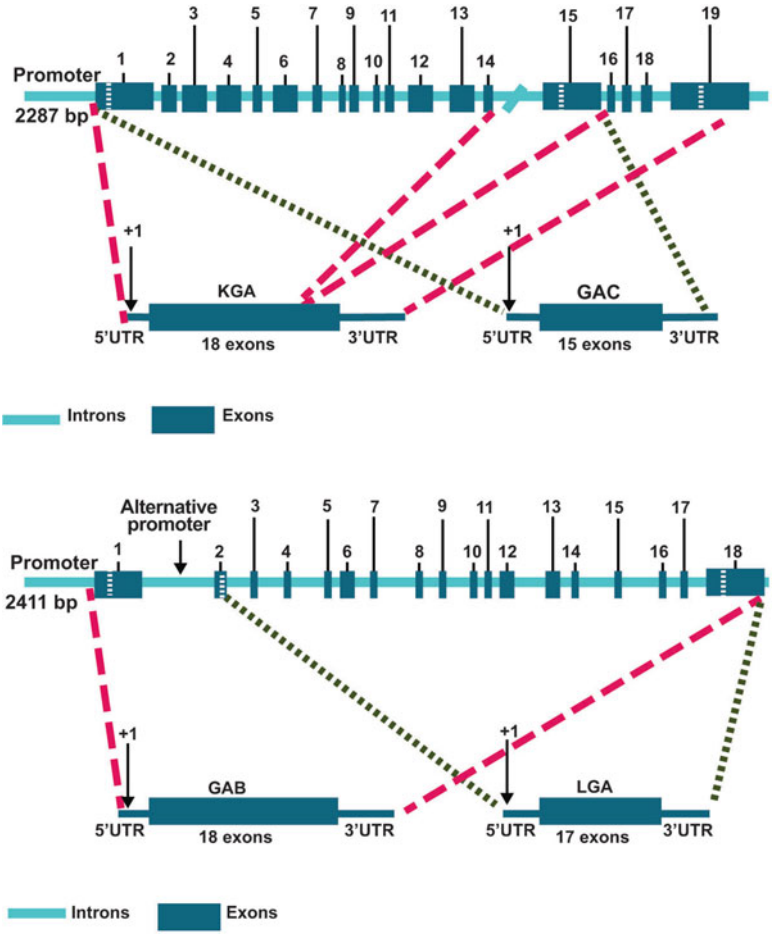

Fig. 6.1 Human glutaminase genes and mRNA transcripts. (*Top panel*) Human glutaminase *GLS* gene and alternative transcripts KGA and GAC. (*Bottom panel*) Human glutaminase *GLS2* gene and alternative transcripts GAB and LGA. Each gene is shown with introns depicted as solid light blue lines and exons as numbered dark blue boxes. The promoter regions are also indicated on the 5′-end of each gene, including the alternative promoter of the *GLS2* gene on intron 1. *Dashed red lines* indicate the exons forming KGA and GAB mRNA transcripts, while *dotted dark green lines* comprise exons involved in the generation of transcripts GAC and LGA. The transcription start site is marked by an arrow and numbered as +1

GLS2 proteins has been demonstrated to be involved in the recognition of PDZ (PSD95\Dlg\ZO1 domains) interaction modules (Olalla et al. 2001) (Fig. 6.2). Therefore, the most significant differences between human *GLS* and *GLS2* exons are located in regions involved with organelle targeting and protein–protein interactions, which may help to explain their differential function and regulation. Conversely, exons 3–17 of GLS and GLS2 mRNA transcripts have the same length and show a high sequence similarity.

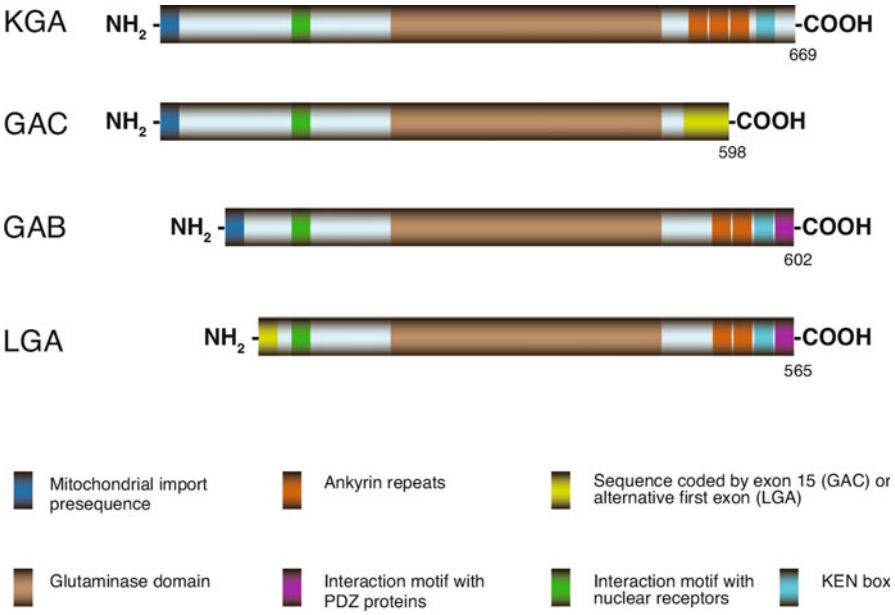

Fig. 6.2 Schematic illustration of the four human GA isoforms. The main signature sequences, motifs, and domains characterized or identified by sequence analysis are highlighted. Starting from the N-terminal end, the following domains are shown: mitochondrial import presequence, nuclear receptor interaction motif (LXXLL), glutaminase domain, ankyrin repeats, APC/C-Cdh1 (anaphase-promoting complex/cyclosome and activator protein Cdh1)-specific recognition motif (KEN) or KEN box, and PDZ protein recognition module (ESMV). The sequence coded by unique exon 15 (GAC isoform) and alternative first exon (LGA isoform) is also highlighted in *yellow* (For further information see text.)

The GLS isoform KGA is found in all tissues with GA activity with the exception of postnatal liver (Curthoys and Watford 1995; Aledo et al. 2000), although expression in liver endothelial cells has also been reported (Lohmann et al. 1999). The GAC splice variant, first isolated from a human HT-29 colon carcinoma cDNA library, is expressed predominantly in human cardiac muscle and pancreas, appreciably in placenta, kidney, and lung, but not in brain and liver (Elgadi et al. 1999). Other authors also found GAC expression in rat kidney and pig renal cells (Porter et al. 2002). On the other hand, GA transcripts derived from the *Gls2* gene were originally thought to be present in adult liver tissue and absent in extrahepatic tissues (Smith and Watford 1990; Curthoys and Watford 1995). This restricted pattern of expression was changed after new findings demonstrated the expression of GLS2 transcripts in extrahepatic tissues like brain, pancreas, and breast cancer cells (Gómez-Fabre et al. 2000). Moreover, simultaneous expression of both GLS and GLS2 isoenzymes is a more frequent event than previously thought, as has been demonstrated in human tissues (Aledo et al. 2000) and cancer cells (Turner and McGivan 2003; Pérez-Gómez et al. 2005). Therefore, the molecular portrait of GA isoform expression in mammalian tissues involves multiple transcripts even in a single cell type. The abundance of a particular GA mRNA species

may significantly change depending upon the tissue type or the developmental or metabolic state of the tissue. The physiological meaning of the existence of different species of mRNA coding for GA is not fully understood. However, it is tempting to speculate that each transcript may represent a specific target for different stimuli, the overall GA expression being the balance between these stimuli.

6.4 Glutaminase Expression in Brain

6.4.1 GA Transcripts and Proteins Expressed in Brain Cells

As mentioned before, the *Gls*-encoded KGA isoform was the first GA enzyme isolated and characterized. Furthermore, very similar molecular and kinetic properties (see next section) were found for brain and kidney KGA (Haser et al. 1985; Kvamme 1998) while, in parallel, brain KGA cDNA sequences were also available after their cloning from rat and human brain (Banner et al. 1988; Nagase et al. 1998). These early studies established the classical pattern of GA expression in mammals, where KGA was originally thought as the only glutaminase present in brain (Haser et al. 1985; Curthoys and Watford 1995). Nowadays, the pattern of GA expression in mammalian tissues has been shown to be considerably more complex. Concretely, we first reported that human brain expresses GLS2 transcripts, in addition to the KGA isoform; these transcripts were ubiquitously expressed in brain regions with the strongest signal appearing in cerebral cortex (Aledo et al. 2000; Gómez-Fabre et al. 2000). Shortly after, Northern analysis and immunocytochemistry in brain of diverse mammalian species (human, monkey, rat, cow, mouse and rabbit) confirmed simultaneous expression of GLS (KGA) and GLS2 (GAB and/or LGA) isoenzymes; interestingly, both isoforms colocalize in numerous cells throughout the brain (Olalla et al. 2002).

The existence of alternative transcripts of the *Gls2* gene was recently demonstrated in brain and liver of three mammalian species: human, rat, and mouse (Martín-Rufián et al. 2012). Two GLS2 transcript variants showing alternative first exons were amplified: the long GAB transcript, previously cloned from ZR-75 breast cancer cells (Gómez-Fabre et al. 2000), and the short LGA transcript first characterized in rat liver (Smith and Watford 1990; Chung-Bok et al. 1997). Cloning and primer extension analysis of the human brain LGA transcript, to map its transcription start site (TSS), unambiguously demonstrated that this short GLS2 isoform arises by a combination of two mechanisms of transcriptional regulation: alternative transcription initiation and alternative promoter. The LGA variant contains both the TSS and the alternative promoter in the first intron of the *Gls2* gene, which is located 7 kb away from the canonical promoter of the GAB isoform (Martín-Rufián et al. 2012) (Fig. 6.1). Therefore, three main conclusions can be drawn from these studies: (1) two alternative GLS2 transcripts, GAB and LGA, are expressed in mammalian brain (and liver); (2) GAB is a new GA isozyme and not the human orthologous of the classical rat liver LGA isozyme; and (3) the short transcript variant LGA is not liver specific and is also expressed in brain.

Quantitative measurements of GLS2 transcript variants by real-time qRT-PCR demonstrated that the ratio of the two products varied very widely between tissues and species (Martín-Rufián et al. 2012). Thus, while LGA was slightly more abundant than GAB in mouse brain, the opposite was found in rat brain where GAB mRNA level was fourfold the amount of LGA. Despite this, GLS isoforms (KGA + GAC) showed the greatest abundance in mouse and rat brain, accounting for more than 90 % of total GA transcripts and becoming the predominant GA in mammalian brain (Martín-Rufián et al. 2012). In human brain tissue and cultured neurons and astrocytes, KGA and GAC transcripts showed similar expression levels (Szeliga et al. 2008). In liver, there was also a dramatic change in the relative abundance of GLS2 transcripts, which showed to be absolutely dependent upon the species: GAB was the predominant isoform in mouse, while the short LGA transcript was the most prominent in rat. These strikingly different expression patterns observed for GLS2 transcript variants imply selective transcriptional regulatory mechanisms being operative in distinct tissues and mammalian species.

Alternative mRNA isoforms not always translate into different protein isoforms. In fact, they can be involved in the regulation of translation initiation, RNA editing, and other processes. Therefore, additional experimental evidence at the protein level is required to assess the functionality of GLS and GLS2 mRNA variants. To our knowledge, evidence supporting expression, at the protein level, of the four different GA mRNAs detected in brain tissue (KGA, GAC, GAB, and LGA) have been obtained for all of them except for GAC isozyme. Isoform-specific antibodies against whole proteins, truncated proteins, and peptide sequences were widely used to reveal KGA and GAB isoforms in mammalian brain (Kaneko et al. 1987; Aoki et al. 1991; Laake et al. 1999; Olalla et al. 2002, 2008). The human brain LGA cDNA was in vitro transcribed and translated in a rabbit reticulocyte lysate system and GA activity was consistently detected for the whole LGA translated product. Moreover, immunoblot analysis of rat brain tissue, isolated rat brain mitochondria, and SHSY-5Y human neuroblastoma cells were consistent with co-expression of both GAB and LGA proteins (Martín-Rufián et al. 2012).

6.4.2 Molecular and Kinetic Properties

The subunit composition of rat brain (and kidney) KGA has been characterized: a heterotetrameric enzyme having two different subunits of 66 kDa and 68 kDa with a 3:1 stoichiometry (Haser et al. 1985; Shapiro et al. 1987; Perera et al. 1991). These two GA polypeptides are produced in vivo from a common precursor of 74 kDa (Shapiro et al. 1991; Holcomb et al. 2000) (Table 6.1). On the other hand, the KGA protomer of pig brain has been reported to contain identical subunits of 64 kDa (Svenneby et al. 1973) or 73 kDa (Nimmo and Tipton 1980). The full length human variant GAC cDNA codes for a protein of 598 amino acids with a molecular mass of about 65 kDa (Elgadi et al. 1999) (Table 6.1). Both KGA and GAC isoforms undergo specific and extensive polymerization in the presence of phosphate and

Table 6.1 Mammalian GA genes, transcripts, and protein isoforms

Transcript/protein name	KGA/GLS	GAC/GLS	GAB/GLS2	LGA/GLS2
Gene	*Gls*	*Gls*	*Gls2*	*Gls2*
Transcript lengths	18 exons/4348 nt	15 exons/3183 nt	18 exons/2408 nt	17 exons/2026 nt
Transcriptional mechanism	Alternative splicing	Alternative splicing	Canonical	Alternative transcription initiation and alternative promoter
Transcriptional regulation	c-Myc, c-Jun	–	p53, TAp63, TAp73	–
Expression	Ubiquitous in most nonhepatic mammalian tissues, human cancer cells	Cardiac muscle, pancreas, placenta, kidney, lung, human cancer cells	Brain, pancreas, immune cells, human cancer cells	Liver, brain, human cancer cells
Human proteins	669 aa	598 aa	602 aa	565 aa
Mature subunit molecular mass	68 and 66 kDa	58 kDa	63 kDa	58 kDa
Pi dependence	High	–	Low	Low
Ammonia activation	No	No	Very low	Strong
Glutamate inhibition	Strong	–	Moderate	No
Interacting partners	Caytaxin, Bmcc1s	–	GIP, SNT	–
Drug inhibitors	BPTES, 968, ebselen, chelerythrine, apomorphine, B839, zaprinast		Alkylbenzoquinones, apomorphine	

Data are for human GA except for kinetic and expression data that were also collected from pig and rat GA. (–) Not determined

phosphate–borate buffers. Thus, the purified native enzyme may appear in three different states: (1) a protomeric and inactive state in Tris–HCl buffer formed by two subunits (dimeric form); (2) an active form by combination of two protomers in the presence of Pi (four subunits, tetrameric form); and (3) a polymeric state with molecular masses higher than 2,000,000 in the presence of phosphate–borate buffer for KGA (Godfrey et al. 1977) or with Pi concentrations as low as 20 mM for GAC (Ferreira et al. 2013). These enzymes require a polyvalent anion for activity. In vitro, inorganic phosphate (Pi) is the most prominent stimulator of GA. Furthermore, there is a correlation between tetramer formation induced by Pi and enzyme activation (Svenneby 1971, 1972; Godfrey et al. 1977; Morehouse and Curthoys 1981). The tetramer–dimer equilibrium is concentration dependent; concretely, for the GAC isoform, it has been shown to occur also in the absence of Pi just by raising

the enzyme concentration, which argues in favor of GAC having a greater tendency to oligomerize than KGA: the unique C-terminal sequence of GAC seems to be an important determinant for oligomerization (Cassago et al. 2012).

Purified brain KGA is an allosteric enzyme highly sensitive to changes in the level of Pi (Haser et al. 1985). For the purified enzyme, the activity was completely dependent on added Pi; the phosphate activation curve was sigmoidal with half-maximal activation at concentrations of 13.5 mM (Svenneby 1971) to 25 mM (Haser et al. 1985) and a Hill index of 1.4–1.5. In contrast, rat and pig brain KGA showed hyperbolic kinetics with regard to their dependence on Gln (Haser et al. 1985; Nimmo and Tipton 1981), although other authors have pointed out that this Michaelis–Menten behavior only occurs at pH 8, whereas at higher pH values the plots become concave upward indicating positive cooperativity (Kvamme 1984). Anyway, the K_M for Gln was dependent on the Pi concentration: increasing the Pi concentration from 10 mM to 150 mM decreased the K_M for Gln from 14 mM to 5 mM (Haser et al. 1985).

GLS isoforms are very sensitive to changes in the levels of Pi, but whether Pi is the true physiological stimulator of GA in brain remains to be determined. However, considering its brain concentration and the fact that it can be rapidly altered during neuronal activity, its candidature as an important physiological regulator of brain GA in vivo was postulated (Erecinska and Silver 1990). For example, in synaptosomes, the Pi activation curve of KGA was very steep and hyperbolic (Bradford and Ward 1976), instead of the sigmoidal curve seen in homogenates and purified enzyme preparations. This distinct kinetic behavior with Pi has been explained by the different intrasynaptosomal Pi concentration compared to the medium (Erecinska and Silver 1990). In addition, synaptosomes treated with KCl and veratridine, to simulate a state of increased neuronal activity in vivo, showed enhanced GA activity concomitantly with the rise in Pi, thereby authors postulated that increase in Pi was the main factor responsible for GA activation under depolarizing conditions (Erecinska et al. 1990).

The concentration of Pi in brain is 1–2 mM (Siesjö 1978; Erecinska and Silver 1989) but a sharp rise might be expected during periods of intense neuronal activation, due to the lowering of the cell energy charge after hydrolysis of high-energy compounds including nucleotide triphosphates. Interestingly, intramitochondrial levels of Pi can significantly increase in a rapid way in cellular models, like cancer cells, characterized by a strong induction of KGA/GAC activity: we found that tumor cell's mitochondria incubated with 0.5 mM Gln double their Pi concentration one minute after Gln exposure (Medina et al. 1988). Additional support for the candidature of Pi as a relevant in vivo effector of *Gls*-encoded GAs has recently come from structural data, obtained by X-ray analysis of truncated GAC proteins containing the GA domain (Cassago et al. 2012) (Fig. 6.2). They showed that Pi binds inside the catalytic pocket, resulting in allosteric stabilization of tetramers and facilitating substrate entry by outcompeting with the product, Glu, to guarantee enzyme cycling. This competence of Pi versus Glu for binding to the active site nicely explains the decreased inhibitory effect of Glu in the presence of increasing Pi concentrations (Shapiro et al. 1982). This competitive behavior between Pi and Glu was early postulated from kinetic analysis of KGA in human brain synaptosomes (Svenneby et al. 1986) and rat cortex homogenates (Bradford et al. 1984).

Main effectors of brain GA activity are, in fact, compounds that alter the Pi activation. For example, calcium activates the hydrolysis of Gln in brain synaptosomes (Kvamme et al. 1983), but it seems to affect GA indirectly by modulating the level of free intramitochondrial Pi (Erecinska et al. 1990). In fact, Ca^{2+} fails to stimulate purified brain KGA (Kvamme et al. 1983), reinforcing the notion that activation is rather an indirect effect. Another effector of possible physiological relevance is H^+. The enzyme's optimum pH is between 8 and 9; therefore, brain metabolism of Gln increases at alkaline pH (Erecinska et al. 1990), while strong reduction in GA activity was noted after lowering the pH of the incubation medium of synaptosomes (Kvamme and Olsen 1981). Of note, during increased neuronal activity the intracellular pH decreases and, hence, pH microdomains are generated in active regions, like dendrites, where these acid shifts are particularly larger (Willoughby and Schwiening 2002). Thus, it is tempting to speculate a decrease on neuronal GA function during periods of intense electrical activity. In contrast, an intracellular alkalinization is observed in mammalian astrocytes in response to repetitive neuronal activity (Chesler and Kraig 1989). Most important, in cultured astrocytes alkalinization induces Glu uptake and promotes a threefold increase in Gln content (Brookes 1997). Therefore, concrete populations of glial cells containing functional mitochondrial GA (see next section) might be activated through astrocytic alkalinization to enhance the production of endogenous Gln-derived Glu. Therefore, pH fluctuations during synaptic activity may also influence GA activity and cerebral function, being a physiologically important process during periods of intense neuronal electrical activity.

In brain, a wide variety of endogenous and exogenous effectors can modulate GA activity in the range of mM concentrations: sulphate, chloride, carboxylic acids, nucleotide triphosphates, riboflavin phosphate, acyl-CoA derivatives and the dye bromothymol blue (Erecinska and Silver 1990; Kvamme et al. 2000). The main inhibitor of brain KGA (and all GLS isoforms) was the first to be discovered: Glu, one product of the reaction (Krebs 1935). The inhibition is competitive (Chiu and Boeker 1979; Haser et al. 1985) and strongly dependent on relative Pi levels: the inhibitory effect of Glu sharply decreases at higher Pi concentration (Svenneby 1971; Shapiro et al. 1982; Campos et al. 1998), in agreement with the active site's mechanism explained before. The relative concentrations of Gln and Glu in glutamatergic terminals suggest that GA can be strongly inhibited in nerve cells as in their terminals (Kvamme et al. 2000). Furthermore, in vitro and in vivo measurements of GA activity in brain support this notion. Assuming an extracellular Gln concentration of 0.5 mM, the activity of pig brain GA in vivo with 5–8 mM Pi was calculated to be at most 5–10 % of that obtained at saturating concentrations of Gln and Pi (Kvamme 1998). Accordingly, in synaptosomal homogenates the GA rate was found to be 124 nmol/mg per min at 37 °C, whereas in intact synaptosomes it was only 7 nmol/mg per min (Ward and Bradford 1979). This dramatic decrease can be explained by Glu inhibition, since Glu concentration is at least 10 mM in intact nerve endings (Erecinska and Silver 1990) and 10 mM Glu inhibits GA by over 90 % in broken synaptosomes (Bradford et al. 1978). Moreover, a low rate of GA activity (4–5 nmol/mg per min at 28 °C) was calculated from the flux of ^{15}N from

0.5 mM [^{15}N]-Gln in nerve endings incubated at 2 mM Pi in the medium (Yudkoff et al. 1989). Finally, the in vivo GA activity in intact brain of hyperammonemic rats measured by ^{15}N nuclear magnetic resonance (NMR) is about 1 % of the reported in vitro activity shown by rat brain homogenates (Kanamori and Ross 1995). Taking together, these results suggest that GA activity in intact brain is maintained at a low level by a short-term regulation of its key effectors, the most likely ones being Pi, Glu and H^+. In conclusion, the situation is considerably more complex in vivo: GA activity will largely depend on the interplay of Gln, Glu, and Pi true concentrations in neurons and glial cells, as well as transient pH fluctuations induced by neural activity.

Conflicting data were reported for ammonium ions, the second end product of GA reaction, as effectors of brain GLS isoforms. In partially purified KGA protein isolated from pig brain (Nimmo and Tipton 1981) and cow brain (Chiu and Boeker 1979) ammonium showed no effect, as well as in cultured astrocytes and neurons (Kvamme et al. 1982; Hogstad et al. 1988). Nonetheless, inhibition of GA was reported in particulate preparations (Bradford and Ward 1976; Wallace and Dawson 1992) and homogenates (Benjamin 1981), while an activation effect was found for the purified pig brain enzyme (Svenneby 1971). In any case, the low concentration of ammonia in the CNS (<0.2 mM) makes unlikely a relevant effect of ammonia on GA activity, at least under normal physiological conditions. Other nonspecific inhibitors are compounds that react with thiol groups as well as Gln analogs like 6-diazo-5-oxonorleucine (DON) (Curthoys and Watford 1995; Campos et al. 1998).

A recently discovered GLS-specific inhibitor is BPTES [bis-2-(5-phenylacetamido-1,2,4-thiadiazol-2-yl)ethyl sulfide], a small molecule which prevents the formation of large phosphate-induced oligomers (Robinson et al. 2007). The inhibition mechanism was elucidated after structural data were recently obtained from crystallized mammalian GLS isoforms. Protein crystals were grown alone and in combination with Gln, Glu, Pi, and novel inhibitors. BPTES inhibition is achieved through an uncompetitive mechanism interfering with the Pi-induced allosteric activation: the GA domain (Fig. 6.2) of human KGA forms a highly symmetrical tetramer containing two molecules of BPTES that are positioned at the dimer/dimer interfaces; binding of BPTES triggers a major conformational change of the active site residues and also freezes the enzyme in a stable inactive tetramer (Thangavelu et al. 2012). Further, X-ray diffraction data of crystallized human and mouse GAC proteins demonstrated that BPTES inhibits this isoenzyme indeed, locking the GAC tetramer in a non-catalytic state and avoiding formation of larger active polymers (DeLaBarre et al. 2011; Ferreira et al. 2013). Other small molecules which are potent inhibitors of GLS isoenzymes are dibenzophenanthridines; in concrete, the compound 968, a bromo-benzophenanthridinone, is a potent specific inhibitor of the GAC isoform now receiving much attention as anticancer drug (Wang et al. 2010). It is also an allosteric inhibitor, but with a different mechanism to that of BPTES: it preferentially binds to an inactive monomeric state of GAC and prevents it from undergoing the required conformational changes to achieve activation (Stalnecker et al. 2015).

Much less is known with regard to the regulation of GLS2 isoforms in mammalian brain. The classical rat liver LGA isozyme has been characterized at the molecular and kinetic levels. The distinct kinetic behavior of mammalian GLS and GLS2 isozymes has been a hallmark frequently used to distinguish between GA isoforms. The main kinetic differences have been observed in the dependence of the activator Pi, low for GLS2, high for GLS; the relative affinity for the substrate Gln, higher in GLS than in GLS2 isoforms; and the inhibitory effect of Glu, a unique characteristic only reported for GLS isozymes (Kovacevic and McGivan 1983; Curthoys and Watford 1995). Also, rat liver LGA was completely dependent on the presence of ammonia, which is an obligatory activator at physiological concentrations (Verhoeven et al. 1983; Patel and McGivan 1984; Snodgrass and Lund 1984). This activation effect was largely dependent on the pH and ammonia (NH_3), rather than ammonium ions, as the activating species (McGivan and Bradford 1983). Rat liver LGA has a subunit molecular mass of 58 kDa (Heini et al. 1987; Smith and Watford 1988) (Table 6.1) and a flat pH curve with an optimum between 7.8 and 8.2.

The only GLS2 isoenzyme expressed in brain that has been characterized in a purified form is the GAB isoform. The recombinant human GAB protein was expressed in Sf9 insect cells (infected with recombinant baculovirus) and affinity purified (Campos-Sandoval et al. 2007). GAB is expressed as a precursor protein of 66 kDa and a mature, processed form, of about 63 kDa, after cleavage of the first 38-39 amino acids at the N-terminal sequence (Table 6.1). Its kinetic characterization demonstrated an allosteric behavior (Hill index of 2.7) with low affinity for Gln ($S_{0.5}$ values of 32 and 64 mM for high (150 mM) and low (5 mM) Pi, respectively), and low dependence for Pi as expected for a GLS2 isoenzyme. Surprisingly, GAB was inhibited by Glu, a characteristic only shown by GLS isoforms (Table 6.1). The inhibition has an IC_{50} value of 50 mM at low Pi concentrations (5 mM) and suboptimal Gln concentration (20 mM). These data are in the range of Ki values reported for Glu competitive inhibition of GLS isoenzymes at high Pi concentrations (Shapiro et al. 1982). The Glu inhibition of GAB disappears at high Pi concentrations (up to 100 mM Glu). This was the first report of inhibition by Glu of a purified *Gls2*-encoded GA isoform; previous studies using partially purified (Patel and McGivan 1984) or pure homogeneous (Smith and Watford 1988) LGA preparations from rat liver, as well as mitochondria isolated from human liver (Snodgrass and Lund 1984), always confirmed that liver LGA is not inhibited by Glu (up to 50 mM).

Another remarkable difference for human GAB was seen in relation with its main activators: it is scarcely activated by ammonia, unlike the liver LGA enzyme which is strictly dependent on ammonia. Partially purified LGA from rat and human liver, as well as LGA in intact and disrupted liver mitochondria, have an absolute requirement for ammonia as an obligatory activator (Häussinger and Sies 1979; Patel and McGivan 1984 and references therein). In fact, the ammonia activation has been considered the particular distinguishing feature of LGA isoforms. In liver, the physiological significance of this effect was considered in the context of feed-forward activation of hepatic GA by ammonia produced in the intestine (Häussinger and Sies 1979); thus, ammonia levels are amplified inside the mitochondria to increase flux through urea cycle via carbamoyl phosphate synthetase I (EC 6.3.4.16)

activation (Häussinger 1983). However, as mentioned before, the ammonia levels in brain are kept low under normal circumstances, whereby activation of cerebral GA isozymes by this effector does not seem to play a relevant physiological role.

The existence of GLS2 isoforms with distinctive kinetic properties different from classical K- and L-type isozymes were also noted by other authors, although using homogenates or crude cell extracts instead of purified enzymes. For example, McGivan's group found mRNAs species identical to human GAB in several colorectal tumors, but without any evidence for the presence of an enzyme with LGA-type kinetics (Turner and McGivan 2003). In conclusion, more studies are needed to further characterize GLS2 protein isoforms in mammalian brain. Tissues like brain, which express both GLS and GLS2 isoforms, are endowed with a powerful system to guarantee biosynthesis of transmitter Glu and glutaminolysis under many different conditions, including a wide range of substrate, activator, and inhibitor concentrations.

Unlike GLS isoforms, relevant structural information from X-ray diffraction data and/or NMR studies at atomic resolution has not yet been published for GLS2 isoforms; hence, the search for specific compounds that block their activity has been hampered. Nonetheless, some selective inhibitors have been recently found for GLS2 isoforms: apomorphine showed greater affinity than BPTES for GA enzymes but with similar activities toward GLS and GLS2 isoforms (Thomas et al. 2013). More recently, a drug discovery screening with natural products discovered a series of alkyl benzoquinones that preferentially inhibit GLS2 isoenzymes and display antitumor activity (Lee et al. 2014).

6.4.3 Regional, Cellular, and Subcellular Locations

One of the main histochemical markers used for identification of excitatory glutamatergic neurons has been the enzyme GA (reviewed by Kaneko 2000), as expected from its role as a main source of transmitter Glu. Nonetheless, Glu also plays important roles in brain general metabolism and it is also a precursor of inhibitory neurotransmitter γ-aminobutyric acid (GABA); for these reasons, immunoreactivity against the amino acid Glu cannot be considered specific for identification of glutamatergic neurons. Traditionally, it was thought that the only cerebral GA isoform was KGA, whereby antibodies against this isoform were the unique tools employed in most immunocytochemical studies dealing with regional, cellular, and subcellular localization of brain GA.

A survey on relevant studies dealing with the immunocytochemical localization of brain KGA (most studies done in rat) indicates expression in cerebral cortex, glutamatergic pyramidal neurons, and GABAergic interneurons (Donoghue et al. 1985; Akiyama et al. 1990; Aoki et al. 1991; Kaneko et al. 1992, 1995; Kaneko and Mizuno 1994, 1996); hippocampus, glutamatergic pyramidal and granular neurons (Altschuler et al. 1985); basal telencephalon, striatum, nucleus accumbens, cholinergic and GABAergic neurons (Aoki et al. 1991; Kaneko and Mizuno 1992;

Manns et al. 2001); cerebellum, glutamatergic granular cells (Wenthold et al. 1986; Kaneko et al. 1989; Laake et al. 1999); thalamus, glutamatergic neurons (Kaneko and Mizuno 1996); mesencephalon and rhombencephalon, catecholaminergic and serotonergic neurons, cochlear nucleus (Kaneko et al. 1990; Godfrey et al. 1994); and brainstem, spinal cord, respiratory, and motor neurons (Magnusson et al. 1986; Senba et al. 1991; Yezierski et al. 1993; Turman and Chandler 1994; Pilowsky et al. 1997).

The first reports demonstrating expression of GLS2 isoforms in mammalian brain were published 15 years ago: Northern analysis of GA transcripts in human brain indicated simultaneous expression of GLS and GLS2 mRNAs (Aledo et al. 2000; Gómez-Fabre et al. 2000). Shortly after, co-expression was also demonstrated in brain of other mammalian species such as cow, mouse, rabbit, and rat, while chicken only displayed GLS transcript (Olalla et al. 2002). This pattern of expression was in conflict with that previously reported for the rat (Smith and Watford 1990). The co-expression of both types of GA isoforms was confirmed at the protein level by biochemical and immunological approaches in rat and monkey brain (Olalla et al. 2002) (Fig. 6.3). Both isozymes, KGA and GAB/LGA (GAB antibodies do not discriminate between GLS2 isoforms and would recognize both GAB and LGA proteins), are ubiquitously expressed in brain regions with the strongest signal appearing in cerebral cortex (Olalla et al. 2002). Since Gln serves as an oxidative substrate in most cells, the wide distribution of GA throughout the mammalian brain was not unexpected. Nevertheless, GA was highly concentrated in the cerebral cortex, which is consistent with Glu as a major excitatory neurotransmitter of projection neurons in this area. An excellent correlation between the KGA and GLS2 expression patterns was observed at the regional and cellular level.

Most of the total brain GA activity is found in the crude P2 fraction, which consists of both free mitochondria and synaptosomes (Bradford and Ward 1976; Svenneby et al. 1986) and 40–60 % is retained in purified synaptosomes (Ward and Bradford 1979). GAs have been traditionally considered as mitochondrial enzymes: it was first proposed by Errera and Greenstein (1949) in liver and later shown experimentally (Guha 1961). In brain, the isoenzyme KGA has been convincingly demonstrated to be expressed in mitochondria by immunocytochemistry (Aoki et al. 1991; Laake et al. 1999; Olalla et al. 2002) (Fig. 6.3).

However, the submitochondrial localization of GA has been a very controversial matter and has not been determined conclusively. This issue has profound physiological implications in cerebral metabolism and energy homeostasis. To illustrate the discrepancies, we can say that almost all possible submitochondrial localizations have been proposed for GA, including matrix soluble (Kalra and Brosnan 1974), matrix side of the inner mitochondrial membrane (IMM) (Shapiro et al. 1985), and simultaneous presence in both halves of the IMM existing two populations of GA: one oriented in the intermembrane space (c-side) and the other in the matrix space (m-side) (Kvamme and Olsen 1979). The last authors observed an incomplete blockade of GA activity in rat brain mitochondria by Glu, ammonia and the SH-group reagent *N*-ethylmaleimide (NEM) (Kvamme and Olsen 1981; Kvamme et al. 1983) and used this evidence as the basis to propose that GA exists

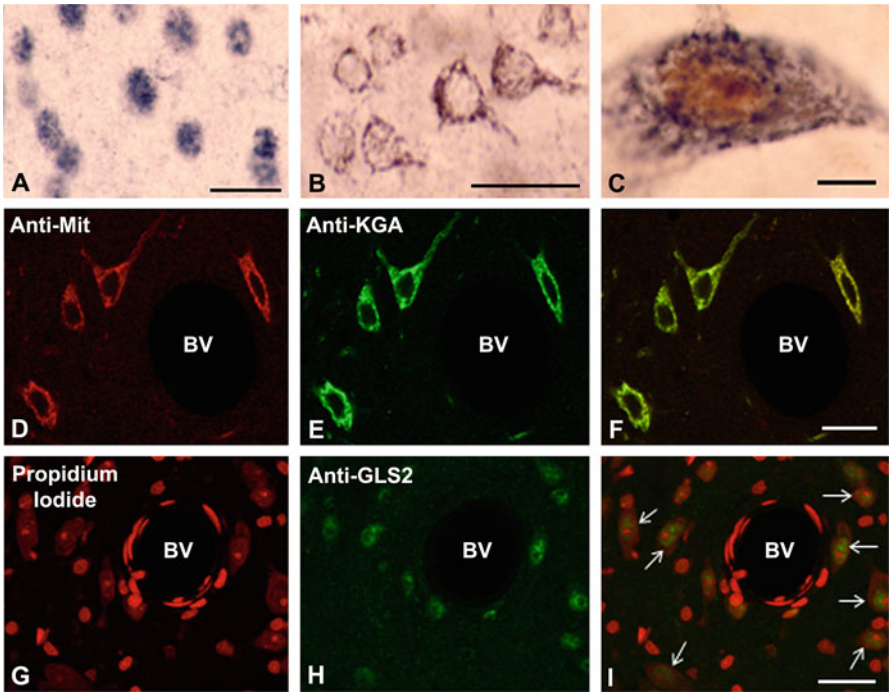

Fig. 6.3 Summary of confocal and immuno-EM results for KGA and GLS2 in neurons from mammalian brain. (**a**) Rat cortical neuronal nuclei (layer II) immunolabeled with anti-GAB isoform-specific antibody; (**b**) monkey cortical neurons displaying cytoplasmic particulate immunoreaction deposits with anti-KGA antibody; (**c**) double-labeled neuron showing granular cytoplasmic localization for KGA protein (*blue*) and nuclear localization for GLS2 protein (*brown*); (**d–f**) double-labeling immunofluorescence with anti-mitochondria (*red*, **d**), and anti-KGA (*green*, **e**) reveals a mitochondrial localization for this isoform (*yellow*, **f**); (**g–i**) double labeling with propidium iodide (*red*, **g**) and anti-GAB (*green*, **h**) shows that many cells, but not all, express nuclear GLS2 protein (**i**, *white arrows* show double-labeled cells). From (**d–i**), confocal laser scanning microscopy of rat brain sections. *BV* blood vessel. Scale bars = 20 μm (**a**), 25 μm (**b**), 10 μm (**c**), 40 μm (**d–i**). *Mit* mitochondria (Adapted from research originally published by Olalla et al. (2002) in Journal of Biological Chemistry, © the American Society for Biochemistry and Molecular Biology)

in two forms located in separate sites: the NEM-sensitive form was located at the outer surface of the IMM (c-side), whereas the insensitive form was situated at the inner surface (m-side) of the IMM. Pig brain mitochondria was also shown to contain two major forms of GA: a readily extractable soluble enzyme located in the matrix and a membrane-bound enzyme located in the IMM; only this latter enzyme was associated with mitochondria in nerve endings (Nimmo and Tipton 1979).

Nonetheless, other authors have questioned these results (Erecinska and Silver 1990), arguing that almost complete (98 %) inhibition of GA by Glu has been demonstrated in particulate preparations of brain (Krebs 1935; Bradford et al. 1978), as also happens in isolated mitochondria from kidney and tumor cells (Shapiro et al. 1982;

Campos et al. 1998). In addition, NEM crosses membranes easily and, hence, it is also hard to explain how this sulfhydryl reagent would not inhibit GA completely wherever located. For example, the inhibition pattern of Ehrlich tumor cell KGA with SH-reagents of different permeability clearly indicated that the IMM becomes a barrier to access to essential KGA residues (Aledo et al. 1997): in particulate fractions, mitochondria and mitoplasts, the membrane-permeant reagent NEM strongly inhibited KGA activity, but almost no effect was detected with membrane-impermeant reagents such as mersalyl and p-chloromercuriphenylsulphonic acid (PCMPS). However, at the same concentrations, mersalyl and PCMPS, as well as NEM, were very effective inhibitors of the soluble KGA (Aledo et al. 1997).

Later on, Kvamme and associates have suggested predominant c-side localization for the GA isoenzymes from kidney and brain, based on the inactivation by non-permeant SH-reagents and the lack of mixing between the Gln-derived Glu and the endogenous matrix Glu (Kvamme et al. 1991; Roberg et al. 1995). This location would not necessarily require a mitochondrial Gln transport while, at the same time, GA would be under the influence of the cytoplasmic Glu, Pi and Gln concentrations. Most notably, Gln-derived Glu should be imported through the IMM to be engaged in TCA reactions. In sharp contrast, all the KGA activity in kidney and tumor cell mitochondria was found to be localized on the inner surface of the IMM, and most of their functionally sensitive domain was located on the m-side of the IMM, as inferred from convergent enzymatic, immunological and chemical modification studies (Curthoys and Weiss 1974; Shapiro et al. 1985; Aledo et al. 1997).

Although submitochondrial localization of GA might differ from organism to organism, recent data support association with the inner surface of the IMM and, most notably, that the active site of GA must be facing the matrix side. Metabolic studies employing [U-^{13}C]Gln and NMR spectroscopy in cultured neurons have established that almost 50 % of the Gln metabolism involves TCA reactions (Waagepetersen et al. 2005). This result means that part of the transmitter Glu pool could be ascribed to transamination pathways but, at the same time, it is not compatible with most of the GA-derived Glu being generated outside the matrix. Similar findings were obtained using isolated rat brain mitochondria (Bak et al. 2008). In addition, an interesting observation also emerged from this last study: a 50 % decrease in the mitochondrial Glu level was detected in the presence of histidine, an inhibitor of Gln uptake in cerebral mitochondria (Albrecht et al. 2000). The authors interpreted these results as evidence of GA acting from the mitochondrial matrix; actually, Gln transport was a prerequisite for Glu formation and further metabolism in the TCA cycle (Bak et al. 2008). In fact, the location of GA's active site in the c-side of the IMM, being accessible to the cytosol, poses several questions difficult to ascertain, such as the necessity for very active mitochondrial Gln carriers, as those reported for rat kidney and tumor cells (see Matés et al. 2009 and references therein). In contrast, the generation of Glu into the matrix would facilitate their further metabolism for bioenergetics or anaplerotic purposes.

There was another noteworthy and unexpected finding concerning the subcellular location of GLS2 isoforms in brain: immunocytochemistry in monkey and rat brain revealed that GLS2-type GA immunoreactivity was mostly concentrated in neuronal nuclei (Olalla et al. 2002) (Fig. 6.3). For the first time, an extramitochondrial localization for a mammalian GA was reported, because GA enzymes were considered to be exclusively mitochondrial enzymes (Kovacevic and McGivan 1983; Curthoys and Watford 1995). Nevertheless, GLS2 was not the first neurotransmitter-synthesizing enzyme being located in the cell nucleus. In 1999, the group of Jane Rylett reported the nuclear localization of the 82 kDa form of human choline acetyltransferase [EC 2.3.1.6] (Resendes et al. 1999). We found clear isoform segregation in rat and monkey brain: KGA protein was detected in mitochondria, whereas GLS2-type GA protein was mostly localized in neuronal nuclei, albeit a minor cytoplasmic immunolabeling was also detected (Olalla et al. 2002) (Fig. 6.3). Of note, both isoforms colocalize in numerous cells throughout the brain and many neuronal cells, but not all, expressed nuclear GLS2-type GA protein (Fig. 6.3). Furthermore, the nuclear GLS2 was catalytically active, although showing kinetic characteristics atypical for classical L-type isozymes. As discussed above, the novel GAB isozyme in purified form shows mixed kinetic characteristics of GLS and GLS2-type isoforms (Campos-Sandoval et al. 2007) and seems the most plausible candidate for brain nuclear GA (de la Rosa et al. 2009). It is noteworthy that human functional GAB is targeted to both mitochondria and nucleus in Sf9 cells, and in both locations the protein was catalytically active (Campos-Sandoval et al. 2007), reinforcing the view that this isoform may be targeted to different subcellular locations, including the cell nucleus.

The expression in vivo of functional GA enzymes in astrocytes is also controversial and has been a matter of debate fueled by puzzled results. Primary cultures of astrocytes displayed efficient Gln uptake, strong GA activity (Schousboe et al. 1979; Kvamme et al. 1982), and expression of GA mRNA transcripts (Szeliga et al. 2008). However, these in vitro results were questioned arguing that GA may somehow be induced by the Gln present in the growth media and by the length of culturing (Erecinska and Silver 1990; Borg et al. 1985). On the other hand, contradictory results were obtained for the in situ expression of glial GA in brain tissue: immunohistochemical studies showed expression of KGA protein and GA activity in rat brain astrocytes (Aoki et al. 1991; Würdig and Kugler 1991), but others failed to detect immunoreactivity in non-neuronal elements such as glia and blood vessels (Kaneko et al. 1987; Akiyama et al. 1990; Laake et al. 1999). Therefore, the prevalent and currently accepted view in mammalian brain states that GA is almost exclusively confined in neuronal cells and shows a limited or no expression in glial cells, with only a few exceptions: in vitro cultured astrocytes and activated microglia cells that produce Glu in an autocrine manner through upregulation of GA expression (Takeuchi et al. 2006). Interestingly, previous studies suggested the possible existence of two GA enzyme forms in cultured astrocytes and neurons by the differences observed in kinetic properties of their main effectors (Hogstad et al. 1988).

The occurrence of GA in astrocytes in vivo has not been conclusively proved. Hence, a comprehensive study was devised to elucidate expression of GA in neuroglia and, more concretely, in astrocytes (Cardona et al. 2015) (Fig. 6.4). We demonstrated by in vivo and in vitro approaches that astrocytes express at least two GA isoforms: KGA and GLS2 (GAB/LGA), essentially considered as exclusive neuronal enzymes. Immunocytochemistry in rat and human brain tissues employing isoform-specific antibodies revealed expression of both GLS (KGA) and GLS2 (GAB/LGA) isozymes in glutamatergic and GABAergic neuronal populations as well as in astrocytes. Nevertheless, there was a different and clear subcellular distribution: KGA isoform was always present in mitochondria while GAB/LGA was preferentially detected in both nucleus and mitochondria (Fig. 6.4). Immunocytochemistry in cultured astrocytes confirmed the same pattern previously seen in brain tissue samples (Fig. 6.4). Astrocytic GA expression was also assessed

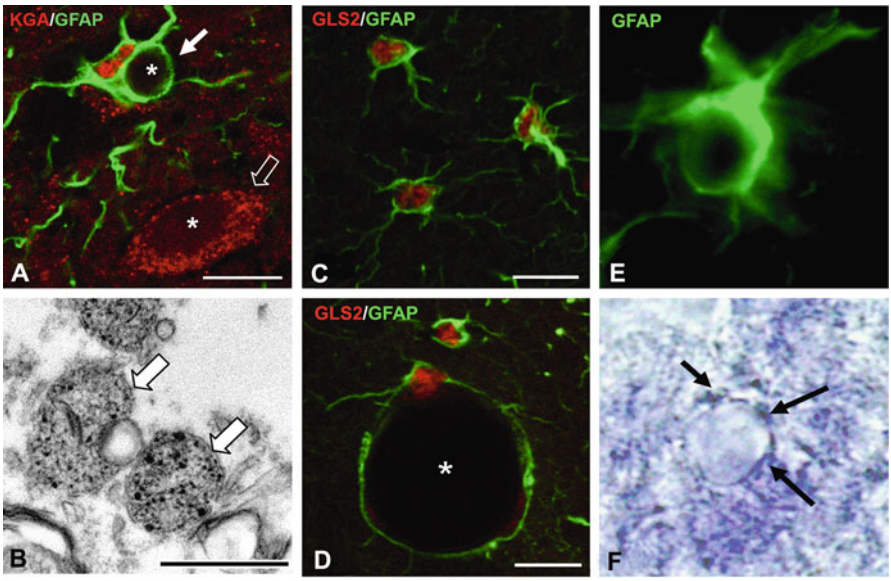

Fig. 6.4 Summary of confocal and immuno-EM microscopy results for KGA and GLS2 in astrocytes from mammalian brain. (**a**) Double immunofluorescence for KGA (*red*) and glial fibrillary acidic protein (GFAP) (*green*) in human brain temporal lobe sections. Punctate KGA immunostaining is clearly seen in the cytoplasm of neurons (*open arrows*) and astrocytes (*white arrows*). *Asterisks* (*) indicate the nuclei of neurons and astrocytes. (**b**) Immuno-EM detection of KGA in the mitochondria of rat brain astrocytes. KGA labeling was restricted to the mitochondria in astrocytes (immunoreactive mitochondria indicated with *white arrows*). (**c**, **d**) GLS2 in rat brain astrocytes: confocal microscopy and double immunofluorescence for GFAP (*green*) and GLS2 (*red*) in rat hippocampus. Merge images show the location of GAB in the nuclei (**c**, **d**) and specialized foot processes of perivascular astrocytes (**d**). *Asterisk* indicates a blood vessel. (**e**, **f**) GA activity in rat brain astrocytes. Double labeling in rat cerebellum sections for GFAP (*green*, **e**) and GA activity staining (blue formazan precipitate, **f**). In vivo GA activity appears as a granular or punctate staining (*black arrows*) in the cytoplasm of astrocytes (**f**). Scale bars = 10 μm (**a**), 0,5 μm (**b**), 20 μm (**c**–**f**) (Adapted from Cardona et al. (2015), GLIA 63:365–82)

at the mRNA level by real-time qRT-PCR: transcripts of four GA isozymes were detected, but with marked differences on their absolute copy number. The predominance of GLS isoforms over GLS2 transcripts was remarkable (ratio of 144:1). Finally, we proved that astrocytic GA proteins possess enzymatic activity by in situ activity staining: discrete populations of astrocytes were labeled in the cortex, cerebellum, and hippocampus of rat brain, demonstrating functional catalytic activity which can be ascribed to mitochondrial GA. In conclusion, subpopulations of astroglial cells possess GA activity in vivo; they are capable of generating Glu through GA pathway by utilizing Gln either internally generated or produced in neighboring cells or taken from the extracellular fluid (ECF, Fig. 6.5). Nonetheless, available experimental data predict an in vivo GA activity considerably lower in astrocytes as compared with that shown by neurons.

The presence of a non-mitochondrial brain GA in the neuronal cytoplasm has also been suggested by immunocytochemistry studies (Aoki et al. 1991). Nevertheless, the relevance of extramitochondrial GA to Gln/Glu metabolism deserves further investigations and is presently unknown, although interaction of GA with newly identified protein-interacting partners might give rise to transient sporadic appearance of GA in cytosol of neurons and astrocytes (see Fig. 6.5 and Sect. 6.6). On the other hand, it should be emphasized that the existence of a cytosolic GA in astrocytes seems unlikely taking into account the strong inhibition that synaptic Glu may exert. Most important, the simultaneous operation of GA and GS in the cytosol will give rise to a futile cycle with high energy expenditure in the form of ATP.

6.5 Role of GA in Glutamatergic Neurotransmission

The importance of GA in glutamatergic synaptic function has been largely recognized. Physiological, biochemical, immunological, and NMR spectroscopic data indicate that neurotransmitter Glu is mainly generated through GA reaction (Hertz 2004), although the relative contribution of each GA isoform to the transmitter pool is presently unknown. Quantitation of absolute mRNA levels of GA isoforms in total brain reveals a clear predominance of GLS isoforms versus GLS2 transcripts (Martín-Rufián et al. 2012). Although quantitative assessment of individual protein levels of neuronal GA isozymes is unknown, immunocytochemical results seem to support a greater abundance of KGA versus GLS2 isoforms (Olalla et al. 2002; Cardona et al. 2015). A knockout (KO) mice model for the *Gls* gene has been generated (Masson et al. 2006). Mice lacking the *Gls* gene die shortly after birth due to altered functioning of key glutamatergic neural networks, stressing the importance of GLS isozymes (mainly KGA) in glutamatergic transmission. Nevertheless, there was persistence of glutamatergic activity in null mutant mice and the quantal size was not reduced in cultured $Gls^{-/-}$ cortical neurons, suggesting that there were adequate levels of intrasynaptic Glu under conditions of basal activity; in fact, synaptic transmission was only impaired under intense stimulation (Masson et al. 2006).

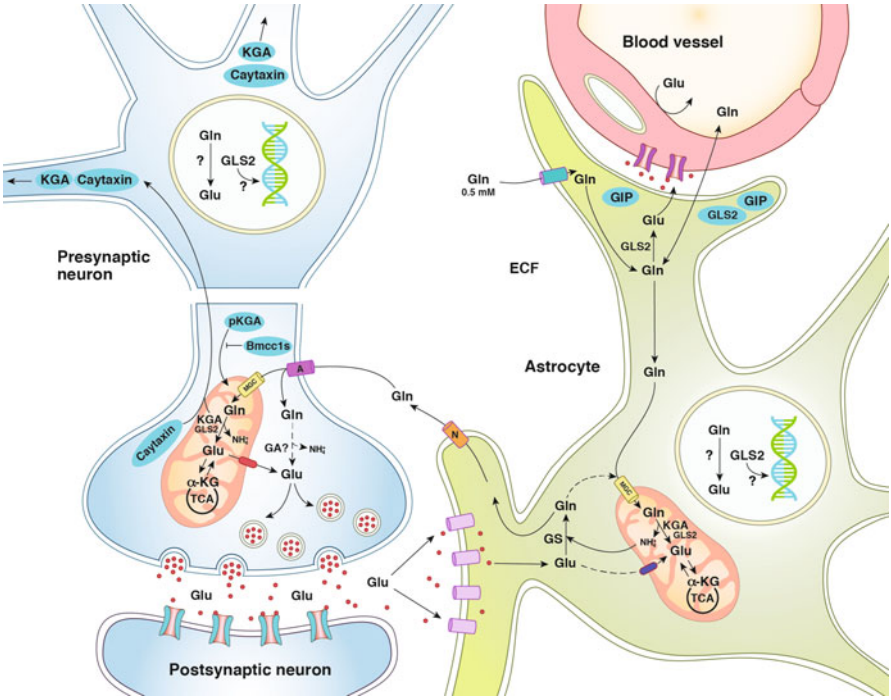

Fig. 6.5 Schematic illustration of the Glu/Gln cycle between neurones and astrocytes at the tripartite synapsis. This is a simplified model highlighting the main functions of GA isozymes, even if not enough evidence exists for a particular task. In the latter case, a question mark means "not enough evidence." For example, in neuronal nuclei the presence of GLS2 has been demonstrated; however, the nuclear function of this GA has not been fully ascertained. Two alternative nuclear functions appear: regulation of the Gln/Glu levels or transcriptional regulation (*arrow* pointing toward DNA). The relative contribution of GLS and GLS2 isoforms in the synthesis of the neurotransmitter Glu pool remains to be clarified. In neuronal body, Gln is converted to Glu by mitochondrial KGA and GLS2, whereas the existence of a cytosolic GA, which may contribute to the Glu transmitter pool, has not yet been confirmed. Synaptic Glu is primarily taken up by astrocytes, mostly converted to Gln by GS in the cytosol, and then cycled back to neurones (through N and A carriers) where GA regenerates the transmitter Glu. Exogenous Glu may be transported to glial mitochondria (*dashed arrow*) and converted to α-KG by GDH or transamination, followed by oxidation into the TCA cycle. Astroglial Gln may be exchanged with blood. Some Gln can be oxidatively degraded by astrocytic GA and TCA cycle after being transported into mitochondria through the uncharacterized MGC; the ammonium generated might be channeled to cytosolic Gln synthesis. Experimental evidence support expression of both KGA and GLS2 isoforms in astrocyte. GLS2 and GIP may interact in vivo and astrocytes are a likely anatomic substrate for their coupling in brain. The colocalization of GIP and GLS2 in perivascular end feet surrounding capillaries might be related to the regulation of the vascular tone through Glu receptors shown in endothelial cells. Caytaxin interacts with KGA and relocalizes it from mitochondria to neurites, while Bmcc1s blocks the targeting of precursor KGA (pKGA) to the mitochondria. The detailed anatomy of synapses, astrocytes, and blood vessels is not portrayed. MGC, Mitochondrial Gln Carrier; ECF, extracellular fluid; GLS2, GAB and/or LGA isoforms; KGA, *Gls*-encoded long GA isoform; α-KG, α-ketoglutarate

These results strongly suggest that alternative sources of vesicular Glu were activated in *Gls* null mutants: *Gls2*-encoded GA isoenzymes are the most suitable candidates to maintain releasable Glu stores after induction through a compensatory mechanism. On the other hand, the persistence of glutamatergic transmission in these mice would also be accounted for upregulation of other Glu-synthetic pathways, such as transamination reactions or by direct neuronal Glu reuptake.

The homeostasis of Glu and Gln in brain should be carefully regulated due to the toxic effects elicited by an excess of Glu. The Glu/Gln cycle between neurons and astrocytes is a central pathway for neurotransmitter recycling and to deal with Glu toxicity (Hertz 1979; Berl and Clarke 1983) (Fig. 6.5). The Glu released by neurons is taken up by nearby astrocytes at the synaptic cleft through efficient Glu transport systems and then converted to Gln by glutamine synthetase (GS; EC 6.3.1.2), an enzyme exclusively located in astrocytes (Norenberg and Martínez-Hernández 1979). The Glu-derived Gln is finally exported back to neurons where GA generates neurotransmitter Glu. In vivo ^{13}C-NMR kinetic studies, metabolic modeling and flux analysis to distinguish the Glu/Gln cycle from other sources of isotopic Gln labeling (particularly Gln synthesis by glial anaplerosis), have demonstrated that the Glu/Gln cycle between astrocytes and neurones is a major pathway for neuronal Glu repletion in rat and human cerebral cortex and hippocampus (Shen et al. 1999; Lebon et al. 2002). Furthermore, these studies concluded that the rate of the Glu/Gln cycle is very high and similar in magnitude to the rate of glucose oxidation, supporting the model proposed by Magistretti and coworkers which couples neuronal activity to glucose utilization and where glial glycolytic ATP represents the major source of energy for neurotransmission (Magistretti et al. 1999; Magistretti and Pellerin 1999).

The existence of GA isoforms in astrocytes is of great importance to fully understand the bioenergetics of glial cells and, particularly, to shed light into transmitter recycling and short-term regulation of the Glu/Gln cycle. The mitochondrial location of KGA and GLS2 isoforms in astrocytes may be a control mechanism allowing broad and fine tuning of Glu production depending on their energetic needs and/or synaptic activity. In this context, it is noteworthy the significant increase in mitochondrial GLS2 detected in cultured astrocytes, using a standard culture medium containing Gln, compared with the scarce mitochondrial label observed in rat brain tissue (Cardona et al. 2015). This fact easily illustrates how astrocytes can modulate expression of GA isoforms, depending on the environmental conditions to which they are exposed, to maintain their energy and metabolic homeostasis. Mammalian KGA and GLS2 proteins possess different kinetic and regulatory properties (Table 6.1); therefore, they reach full catalytic activity at different cellular concentrations of Gln, Glu, Pi, and other effectors.

At first sight, recent data demonstrating functional expression of two GA isoforms in astrocytes are in apparent conflict with the compartmentation of the key enzymes in the Glu/Gln cycle (Norenberg and Martínez-Hernández 1979; Donoghue et al. 1985; Erecinska and Silver 1990; Kaneko et al. 1995) and seem hard to reconcile with the constant supply of synaptic Glu being received by these glial cells, which may render superfluous the existence of astrocytic GA. Nonetheless, a GA activity in astrocytes could fit well with the Glu/Gln cycle, because it would endorse

astrocytes with an endogenous mitochondrial source of Glu (and ammonium) whose synthesis from Gln does not require energy. This endogenous, non-synaptic, Gln-derived Glu could be converted through glutamate dehydrogenase (GDH) or transamination to α-ketoglutarate and then proceeds via TCA cycle to satisfy astrocytic energetic needs (Fig. 6.5). Catabolism of this endogenous Gln-derived Glu may cooperate with the strong energy and biosynthetic needs required by astrocytes, thus preserving most of the exogenous synaptic Glu to be transformed back to Gln and redirected toward neurons. In this way, astrocytes will not significantly deplete synaptic Glu stores saving most of it for Gln synthesis in the cytosol; such mechanism might be particularly relevant in periods of great synaptic activity. In addition, the ammonium generated in mitochondria by this glutaminolytic process could be channeled to Gln synthesis in cytosol, providing an additional source of nitrogen needed to recycle Glu in Gln through GS (Fig. 6.5).

The Glu/Gln cycle is a key mechanism for homeostatic control of Glu, Gln, and GABA concentrations; however, it is not always a stoichiometric pathway (McKenna 2007) because part of the exogenous Glu taken by astrocytes does not end as Gln but used to fulfill other energy and metabolic purposes instead (Sonnewald et al. 1993). Actually, it has been estimated that 10–30 % of synaptic-derived Glu is oxidized by astrocytes and must be replenished to keep the carbon balance of the Glu/Gln cycle (McKenna et al. 1996; Gamberino et al. 1997; Hutson et al. 1998). In vivo studies, using 13C-NMR spectroscopy, indicated that glial anaplerosis from glucose, through the astrocyte-specific enzyme pyruvate carboxylase (PC; EC 6.4.1.1), is the main contributor for replacing the oxidatively degraded Glu (Rothman et al. 1999; Lebon et al. 2002). In this sense, a GA activity will increase the metabolic versatility of astrocytes in such way that they do not have to exclusively rely on PC for *de novo* synthesis of Glu. The existence of a functional GA in astrocytes will diminish their anaplerotic pressure, which is important because Glu synthesis by anaplerosis would be detrimental if it is not matched by degradation through cataplerotic routes (Sonnewald 2014). Furthermore, the fast induction of GS protein in active synapse, which rapidly converts the captured synaptic Glu into Gln, means that the flux of Glu to the TCA cycle became negligible as compared with the flux through GS (Fonseca et al. 2005). Actually, these authors conclude that astrocytes adapted to a constant supply of Glu by increasing Glu uptake and GS activity in such a way that Glu was converted almost exclusively through GS. Under these conditions, the supply of endogenous Glu by GA would be a valuable resource for astrocyte bioenergetics.

An additional advantage of the existence of astrocyte GA can be related to the mitochondrial import of Glu. Generation of mitochondrial Glu in situ avoids another problem faced by synaptic cytosolic Glu: its import into astrocytic mitochondria. It has been shown that brain astrocytes do not express the main aspartate–glutamate carrier (Aralar/AGC1) (Ramos et al. 2003; Berckich et al. 2007); therefore, exogenous Glu must enter into astrocytes uniquely by the Glu/hydroxyl carrier. Studies with Aralar KO mice underscored the importance of this carrier for Glu transport in brain and skeletal muscle mitochondria (Jalil et al. 2005), while further

evidence has shown that no other Glu carrier can substitute for Aralar in those tissues (Satrústegui et al. 2007 and references therein). Notwithstanding, it should be stated that evidence for anatomic and physical linkages between the astroglial Na^+-dependent Glu transporters (GLT-1/EAAT2, GLAST/EAAT1) and mitochondria has been recently reported (Whitelaw and Robinson 2013 and references therein). On the other hand, Gln can be actively concentrated into astrocyte mitochondria using a high-affinity mitochondrial Gln carrier (Roberg et al. 1999) and then be released into the mitochondrial matrix near the GA catalytic site. The generation of Glu into the matrix facilitates its further catabolism for bioenergetics or biosynthetic purposes. Thus, Gln-derived Glu is generated into the mitochondrial matrix without the need for an additional carrier, as required for exogenous Glu.

Finally, it is noteworthy to mention the role of neuronal GA in the synthesis of GABA. The Gln-derived Glu may also contribute to the synthesis of GABA at inhibitory synapses. We have recently found expression of both GLS and GLS2 isoforms in a scarcity of Purkinje cells and in many GABAergic neurons at the molecular layer of the cerebellum, which point toward GA as an important biosynthetic source of Glu at inhibitory synapses indeed (Cardona et al. 2015). Early studies did not detect KGA immunoreactivity in GABAergic neurons of neocortex from rodents and monkey (Altschuler et al. 1984, 1985; Donoghue et al. 1985; Kaneko et al. 1992; Kaneko and Mizuno 1994). However, more recent studies presented immunocytochemical evidence of the localization of KGA in GABAergic neurons in the cat visual cortex (Van der Gucht et al. 2003) and thalamus (Fisher 2007), thereby suggesting that Gln can be a metabolic precursor for GABA synthesis. Interestingly, co-expression of KGA and glutamic acid decarboxylase (GAD; EC 4.1.1.15) was also reported in rat hippocampus and basal forebrain neurons (Kaneko and Mizuno 1994; Manns et al. 2001). GABA is produced by GAD, and two GAD isoforms, encoded by different independently regulated genes, are also expressed in brain (Bu et al. 1992). Both forms can synthesize transmitter GABA, but have different roles in the coding of information by GABA-containing neurones and different subcellular localizations (Soghomonian and Martin 1998). Thus, each of the two main neurotransmitters in the CNS—Glu and GABA—can be synthesized by two isozymes coded by distinct genes, a unique situation different from all the other neurotransmitters. Glu and GABA production must be a process exquisitely regulated to ensure a proper Glu homeostasis. Although the need for several brain GA isoforms is not completely understood, it may represent the biochemical basis to achieve this fine modulation under different physiological circumstances.

6.6 New Functions Beyond Their Role in Glutamatergic Transmission

The mammalian *Gls* and *Gls2* genes are presumed to have been derived by gene duplication of a common ancestral gene, followed by gradual changes in the sequences of both copies (Chung-Bok et al. 1997; Pérez-Gómez et al. 2003). As

paralogous proteins, GLS and GLS2 isoforms may acquire different functions during their evolution while maintaining a strong similarity in sequence and three-dimensional structure (Fig. 6.2). Furthermore, GA has been endowed with consensus protein motifs and domains that might support its role as multifunctional proteins (Fig. 6.2). We strongly support this view for GA in mammalian tissues and, with special relevance, for the nuclear localization of GLS2 isoforms in neurons and astrocytes, whose significance remains to be fully determined. The nuclear function has been related with transcriptional regulation associated to differentiation (Olalla et al. 2002; Márquez et al. 2006). First indications about this role for GLS2 isoenzymes came from cancer studies. We postulated a completely different role for GLS and GLS2 isoforms in cancer based on their relative expression patterns in human leukemia, breast cancer cells, and hepatocellular transformation: the data suggest that upregulation of GLS isoforms correlates with increased rates of proliferation, whereas prevalence of the GLS2 isoforms seems to be related with differentiated and quiescent cell states (Pérez-Gómez et al. 2005). Therefore, we proposed that the process of malignant transformation shifts the pattern of GA expression in such way that GLS becomes upregulated while GLS2 is frequently repressed. In accordance with this, human T98G glioma cells showed almost null expression of GLS2, but overexpression of human GAB induced a marked change in the cell's transcriptome correlated with a reversion of their transformed phenotype (Szeliga et al. 2009, see also chapter 9– in this book). Taking into account its presence in cell's nuclei, it has been speculated that GAB overexpression may contribute in altering the transcriptional program of glioma cells, yielding a less malignant and more differentiated phenotype, but the concrete molecular mechanisms underpinning this phenotypical change are unknown.

In this regard, important insights into GLS2 function in neuronal differentiation have been recently published (Velletri et al. 2013). The authors show that GLS2 is under the control of TAp73, a p53 family member, during neuronal differentiation of neuroblastoma cells induced by retinoic acid (Table 6.1). Indeed, GLS2 overexpression increased neurite outgrowth. Furthermore, inhibition of GA activity in cultures of mouse embryonary cortical neurons resulted in impaired neuritogenesis. In order to gain insights into the role of GLS2 in vivo, they also analyzed GLS2 expression in developing cerebellum. Interestingly, GLS2 transcript significantly increased between postnatal day 0 (P0) and P8, a development stage characterized by a massive expansion of granule cell progenitors (Velletri et al. 2013). Convergent pieces of evidence were obtained from primary human keratinocytes, where it was shown that GLS2 is a target gene regulated by the transcription factor TAp63, also belonging to the p53 family (Table 6.1). GLS2 and TAp63 expression increases during the in vitro differentiation of keratinocytes, while depletion of GLS2 inhibits skin differentiation both at molecular and cellular levels (Giacobbe et al. 2013). Altogether, these data reinforce our hypothesis relating GLS2 with cell differentiation. With regard to GLS, experimental evidence supporting a key role in the proliferation of neural cells has been reported. Thus, both GLS isoforms (KGA and GAC) were upregulated during neurogenesis of human neural progenitor cells (NPCs), and their expression pattern positively correlated with the neuronal marker MAP-2

(microtubule-associated protein 2) (Wang et al. 2014). Most important, studies of cultured human NPCs after siRNA silencing of *GLS* suggest a critical role of GLS isoforms for proliferation and survival of NPCs (Wang et al. 2014).

Another potential nuclear function for GA could be the regulation of Gln/Glu levels (Olalla et al. 2002), taking into account that Gln is a signal molecule involved in gene expression (Bungard and McGivan 2004; Häussinger and Schliess 2007). Therefore, the significance of its nuclear localization could be as simple as being an enzyme controlling in situ the Gln levels in the nucleoplasm and, thus, being indirectly involved in the expression of Gln-regulated genes (Fig. 6.5). Actually, GA activity was found in nuclear fractions isolated from rat brain, although with kinetic properties distinct from the classical *Gls2*-encoded LGA isoform (Olalla et al. 2002). Interestingly, purified human GAB isozyme showed mixed kinetic characteristics of GLS and GLS2 isoforms (Campos-Sandoval et al. 2007), in accord with the results obtained from nuclear rat brain extracts. Strikingly, no GA activity was revealed in astrocytic nuclei despite the clear immunoreactivity revealed for GLS2 isoforms; however, this result may arise because the NBT-based activity assay was not sensitive enough for low activity levels found in nucleus (Cardona et al. 2015).

In the search for discovering new physiological functions for brain GA, proteomics approaches, such as two-hybrid genetic screenings and immunoprecipitations (IP) coupled to mass spectrometry (MS) analysis, have proven to be very useful strategies for isolation of potential protein-interacting partners of GA. A yeast two-hybrid genetic assay was performed by screening a human brain cDNA library with the C-terminal region of human GAB. Two PDZ (PSD95/Dlg/ZO1) domain-containing proteins were isolated: α-1-syntrophin (SNT) and glutaminase-interacting protein (GIP), also known as tax-interacting binding protein-1 (TIP-1) (Olalla et al. 2001). One of the most common protein modules involved in scaffolding interactions are the PDZ domains, which are responsible for a wide array of protein–protein interactions in the CNS and other tissues (Kornau et al. 1995). These modules bind to short sequences at the extreme C-terminus of target proteins, thereby nucleating the formation of specific functional complexes. The last four amino acids of the C-terminal end of human GAB, ESMV, conforms a consensus sequence required for interaction with PDZ proteins (Fig. 6.2) (Olalla et al. 2001). The relevance of these residues for the human GAB–GIP binding was demonstrated by NMR and fluorescence spectroscopy studies, allowing determination of the dissociation constant (K_d) for their binding: 1.66 mM, which means a moderate affinity suitable for regulatory functions (Banerjee et al. 2008).

The physiological relevance of the GLS2–GIP interaction was first assessed by immunocytochemical studies in brain, in order to ascertain whether both proteins may be interacting partners in vivo (Olalla et al. 2008). GIP colocalizes with GLS2 in cerebral cortex astrocytes from rat brain. Double-label studies and confocal microscopy confirmed colocalization in astrocytes cell bodies and processes, including their perivascular end feet (Fig. 6.5). The presence of GIP in astrocytes was also confirmed by immuno-electron microscopy (EM), which revealed immunoreactive astrocytic end feet surrounding perivascular endothelial cells. These results strengthen the view that GA and GIP may interact in vivo and point to astrocytes as a likely anatomic substrate for their coupling in brain (Fig. 6.5).

Analysis of rat brain neurones by EM also found GIP immunoreactivity in the nuclear envelope (Olalla et al. 2008). This nuclear location agrees with previous studies on GIP which described its participation in the regulation of transcription: GIP physically interacts with beta-catenin inhibiting its transcriptional activity, being a critical component of the beta-catenin regulatory network (Kanamori et al. 2003). While human GLS2 has structural determinants needed for mitochondrial targeting (Gómez-Fabre et al. 2000), it does not possess a discernible nuclear localization signal (Fig. 6.2); therefore, the mechanism by which it reaches the nucleus is unclear. The PDZ-protein recognition motif would be implicated in GLS2 specific targeting to selective cellular locations (Olalla et al. 2008; Márquez et al. 2009). Of note, PDZ proteins have also been reported to play an important role in the targeting of protein-interacting partners to concrete subcellular localizations, including cell nucleus (Kausalya et al. 2004), and several PDZ proteins are known to localize to the nucleus (Hsueh et al. 2000). Therefore, we cannot exclude that GIP may be involved in the targeting of GLS2 to neuronal nuclei.

In addition to PDZ domains, human GAB has other sequence motifs and conserved modules that may be essential for its nuclear import (Márquez et al. 2006). For example, it has a consensus motif for interaction with nuclear receptors: the sequence LGDLL on exon 2 (residues 72–76) conform to the consensus nuclear receptor box LXXLL (Fig. 6.2). This sequence motif allows for specific interaction with nuclear receptors and is primarily found in coactivators of nuclear receptors (Heery et al. 1997). Nuclear translocation of a mitochondrial enzyme containing a mitochondrial targeting sequence, but lacking a specific nuclear targeting signal, is not without precedent: mitochondrial 3-hydroxy-3-methylglutarylCoA synthase has been detected in nuclei and its nuclear translocation seems to involve interactions with nuclear hormone receptors through its LXXLL motif (Meertens et al. 1998).

Other protein–protein interaction motifs present in the primary structure of GLS2 proteins (and KGA, but not in the GAC isoform) are two ankyrin repeats in the C-terminal region (Fig. 6.2). Ankyrin repeats are about 33 amino acids long consisting of a β-turn and two antiparallel α-helices and have been found in proteins of diverse function such as transcriptional initiators, cell cycle regulators, cytoskeletal, ion transporters and signal transducers (Sedgwick and Smerdon 1999). The ankyrin-repeat proteins mediate many important protein–protein interactions in virtually all species. Of particular relevance in this context is the existence of transcription factors (GA-binding protein) and transcriptional regulators (IkB protein family, ANCO proteins) whose ankyrin repeats are essential for proper transcriptional activity (Sedgwick and Smerdon 1999; Zhang et al. 2004). Furthermore, ankyrin-repeat motifs are critically required for nuclear localization of signaling enzymes (Hozumi et al. 2003) and transcriptional cofactors (Sedgwick and Smerdon 1999), being able to functionally substitute for a classical nuclear localization signal (Sachdev et al. 1998). Therefore, the involvement of this modular motif in the nuclear import of GLS2 will deserve further attention.

A second PDZ protein isolated as protein-interacting partner of human GAB was SNT. In brain, the PDZ domain of SNT is known to bind to protein kinases, to the neural nitric oxide synthase (nNOS), and to the water channel aquaporin-4 (AQP4) as a mechanism for selective targeting to unique subcellular sites (Adams et al. 2001). In astrocytes, it has been shown that SNT is a central organizer of the dystrophin complex, an important molecular scaffold for localization of AQP4 at the BBB (Bragg et al. 2006). SNT is the best characterized PDZ scaffold in astrocytes and is concentrated in the perivascular end feet of astrocytes anchoring the AQP4 water channel to these membranes (Neely et al. 2001). The coupling between AQP4 and SNT may be indirect and studies with KO mice for SNT have suggested that additional anchoring proteins (GIP?) may be needed for clustering AQP4 in the end feet of brain astrocytes (Amiry-Moghaddam et al. 2004). At present, the functional role of GIP in the perivascular end feet is unknown and whether GIP or SNT may act as scaffold proteins for GLS2 in astrocytes remains to be determined. Interestingly, GLS2 possesses the consensus C-terminal –SXV sequence, like the potassium channel Kir4.1, required for interaction with the dystrophin complex.

On the other hand, the localization of GIP and GLS2 in astrocytes, particularly in perivascular end feet surrounding blood vessels and capillaries, might be related to the regulation of the vascular tone (Fig. 6.5). Of note, a perivascular and pial localization of GA in rat brain was previously reported (Kaneko and Mizuno 1988). Indeed, we have found GLS2 in concrete perivascular end feet (Cardona et al. 2015), as well as colocalization of GIP and GLS2 in astrocytic processes (Olalla et al. 2008). Thus, it is tempting to speculate that GLS2 targeting to specialized foot processes of concrete perivascular astrocytes might be a plausible mechanism implicated in the regulation of the vascular function, considering the key role of astrocytes in cerebrovascular regulation (Koehler et al. 2006). The fact that Glu is a vasoactive compound (Fergus and Lee 1997) and the existence of Glu receptors in perivascular glia and vascular endothelial cells (Collard et al. 2002; Nedergaard et al. 2002) would implicate GLS2–GIP interaction in regulating the vascular tone (Fig. 6.5).

GLS isoforms can also traffic in brain and unexpected locations have been lately reported for them. The first interacting partner for a GLS protein was shown to be the brain-specific protein BNIP-H (for BNIP-2 homology) or caytaxin, a protein exclusively expressed in neural tissues and encoded by a gene associated with human cerebellar Cayman ataxia (Buschdorf et al. 2006). This protein contains a novel protein–protein interaction domain known as the BNIP-2 and Cdc42GAP homology (BCH) domain. The regional distribution of caytaxin in mouse brain broadly matched the pattern of expression previously known for KGA. In PC12 cells stimulated with nerve growth factor for 24 h to induce differentiation to neuronal phenotype, the co-transfection of KGA and caytaxin induced a specific redistribution of KGA to neurite terminals independently of, and away from, mitochondria (Fig. 6.5); therefore, caytaxin relocalized KGA from cell body to neurite terminals. Neuronal trafficking of KGA was specifically ascribed to its interaction with caytaxin, which also reduces the steady-state levels of Glu by inhibiting KGA activity (Buschdorf et al. 2006). Thus, the interaction KGA–caytaxin seems relevant for regulating the homeostasis of Glu synthesis important for proper neurotransmission and

neuronal cell growth (Fig. 6.5). For example, absence of functional caytaxin would increase Glu levels in the cell bodies of neurons leading to neurotoxicity and/or abnormal neuronal growth. In agreement with these findings, a whole-brain proteomic analysis of *Gls*-deficient mice ($Gls^{-/-}$ and $Gls^{+/-}$) has revealed a link between GLS isoforms and ATPases, ion channels, nucleic acid and lipid metabolism, calcium, Akt and retinol signaling, cytoskeletal components and protein synthesis machinery, among others. This protein network involves key players of the cellular assembly and organization, cell signaling and cell cycle (Bae et al. 2013). Thus, reduction of GLS expression by genetic knockdown of *Gls* gene in mice suggests a crucial role of these proteins in neuronal maturation and development.

A second member of the BCH domain-containing family of proteins (related to caytaxin) has been identified as direct interacting partner of KGA isoform. BMCC1 (**B**cl2, the adenovirus E1B 19 kDa interacting protein 2 and the Cdc42 GAP homology BCH **m**otif-**c**ontaining molecule at the **c**arboxy-terminal region **1**) is a large polypeptide expressed in brain which encodes several isoforms; one of them, BMCC1s, is a novel short isoform of BMCC1 predominantly expressed in the mouse brain and involved in the regulation of cytoskeleton dynamics and cell survival (Arama et al. 2012). Proteomic analysis of BMCC1s-binding proteins identified KGA as an interacting partner. When primary cultures of cortical neurons were contransfected with both proteins, the subcellular distribution of KGA was drastically modified: almost half of transfected cells lost its unique KGA mitochondrial profile, being also detected in the cytoplasm (Boulay et al. 2013). The authors concluded that BMCC1s preferentially interacts with the 74 kDa cytosolic monomeric precursor of KGA (pKGA) and, thus, it cannot reach the mitochondria for being processed to the mature catalytically active form (Fig. 6.5).

The hypothesis of brain GA isoforms being regulated by protein-interacting partners is appealing because it may provide a mechanism for both control of Glu synthesis and targeting of cerebral GA to concrete cellular compartments. In fact, GIP has been shown to inhibit GLS2-type GA activity in crude extracts of rat liver (Aledo et al. 2001), whereas two binding partners recently discovered for KGA, caytaxin and BMCC1s, were also able to inhibit the catalytic activity of KGA, although by different mechanisms: direct interaction or blocking its path to the mitochondria.

6.7 Summary

Eighty years after its discovery, the knowledge accumulated on brain GA is considerable. Significant progress has been made in this field stressing the relevance of GA, but still a number of sound questions remain to be answered. Based on novel and growing pieces of evidence summarized in this chapter, the roles of GA in cerebral function are multiple and spreading out. Nowadays, the pattern of GA expression has been shown to be considerably more complex: the old concept of only one type of GA expressed in mammalian brain (KGA) was changed at the light of overwhelming evidence demonstrating that, at least, four GA isoenzymes are expressed. The concept of an

exclusively mitochondrial location for GA was also demonstrated to be wrong as new subcellular locations (e.g., nucleus, cytosol) have been described for these proteins. Even more, the concept of GA as exclusive neuron-specific enzyme has been challenged by recent findings reporting in vivo expression of functional GA isoforms in astrocytes. The interactome of brain GA is starting to be uncovered. To reach their final destinations in brain cells, GA may interact with newly discovered scaffold proteins. Such arrangements could provide the molecular basis for selective and regulated targeting to concrete cellular locations and control of Glu synthesis by blocking GA activity. These unanticipated findings open a new avenue of research on how GA may affect the homeostasis of Glu/Gln in the tripartite synapsis. Besides their classical role in glutamatergic transmission, novel emerging roles for GA include transcriptional control, neuronal growth and differentiation, and cerebrovascular regulation. Thus, GA may be added to the growing list of moonlighting proteins, that is, proteins having a second (or third) function.

There are several key issues about GA function in brain that remain unresolved. Given our new knowledge about the presence of GA in astrocytes, it is expected that in the next years we can determine the function of GA in glial cells in physiological conditions and whether astrocytic GA contribute to the changes in brain function under pathological conditions. Further studies will undoubtedly reveal insights into the nuclear role of GA; particularly the role of GLS2 and/or Gln in the regulation of gene expression. The physiological relevance of the interaction between GA- and protein-binding partners, as well as their implications in neurological disorders, still remain to be elucidated. We expect that the function of KGA in neurogenesis and neuronal growth and development can be fully addressed in the next future as well. The expression of, at least, four GA in mammalian brain is so far unexplained. The source of neurotransmitter Glu and what is the relative contribution of each GA isoform to the transmitter pool of Glu are outstanding questions for future work, as well as elucidation of the factors which regulate GA activity in vivo, and hence Glu supply, during neuronal stimulation.

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Chapter 7
Vesicular Glutamate Uptake

Tetsufumi Ueda

Abstract Glutamate is an excitatory neurotransmitter widely used in the vertebrate central nervous systems. The synaptic transmission process is characterized by three steps: (1) presynaptic vesicular transmitter uptake, (2) presynaptic release, and (3) postsynaptic receptor activation. Presynaptic vesicular glutamate uptake plays an initial pivotal role in glutamate transmission by concentrating glutamate in the vesicular lumen prior to its release. This active glutamate transport harnesses energy derived from ATP hydrolysis, and intra- or extravesicular chloride, and is highly specific to glutamate. The uptake system consists of a vesicular glutamate transporter (VGLUT) and v-type proton-pump ATPase, which generates an electrochemical proton gradient, the driving force of the transport. The major source of ATP is likely to be supplied by glycolytic vesicle-bound enzymes, glyceraldehyde 3-phosphate dehydrogenase, and 3-phosphoglycerate kinase, rather than by mitochondrial ATP synthase. The VGLUT substrate glutamate is proposed to be synthesized by vesicle-bound aspartate amino transferase from α-ketoglutarate, not directly from glutamine. VGLUT has three isoforms, and gaged by their distributions they perform different physiological functions. The mechanism and regulation of vesicular glutamate uptake are discussed. The pharmacology of vesicular glutamate uptake is a developing field of inquiry.

Keywords Glutamate uptake • Synaptic vesicles • VGLUT • Glycolytic ATP • Glyceraldehyde-3-phophate dehydrogenase • Aspartate aminotransferase

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Abbreviations

AAT	Aspartate aminotransferase
t-ACPD	*trans*-1-amino-1,3-cyclopentanedicarboxylate
CCCP	Carbonyl cyanide *m*-chlorophenylhydrazone
CNS	Central nervous system
FCCP	Carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone
GABA	γ-Amino butyric acid
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
α-KGA	α-Ketoglutarate
IPF	Inhibitory protein factor
3-PGK	3-Phosphoglycerate kinase
VGLUT	Vesicular glutamate transporter

Brain function derives from properly integrated neural communication systems. Neuronal communication in the central nervous system (CNS) relies heavily on glutamate synaptic transmission, which is excitatory, triggering neuronal firing via activation of glutamate receptors. Intense glutamate transmission results in activation of various types of glutamate receptors, leading to synaptic modification, which is involved in memory and learning, among other functions. Preparation for glutamate transmission begins with the accumulation of this acidic amino acid into synaptic vesicles, as well as with its synthesis, in the nerve terminal prior to its release into the synaptic cleft (Fig. 7.1). Glutamate uptake into synaptic vesicles is a pivotal step in a broad definition of glutamate transmission. Important to unraveling this narrative was the discovery of the glutamate uptake system in presynaptic vesicles and of its unique properties, which contributed to the recognition of glutamate as a neurotransmitter in the central nervous system. This discovery led to the identification of vesicular glutamate transporter (VGLUT) proteins, which, in combination with the availability of their antibodies, triggered an explosion in this field of neurobiology, largely because of their implication in a variety of physiological functions. A review of VGLUTs will be followed by the discussion of the mechanism and regulation of vesicular glutamate uptake, and a proposed efficient synthesis of the VGLUT substrate and of ATP required for VGLUT function. The development of VGLUT inhibitors will be summarized. The vesicular aspartate transporter will be evaluated, as well as evidence for and against VGLUT in astrocytes. Implications of aberrant VGLUT and vesicular glutamate accumulation in various types of neurological disorders will be described. Relevant reviews (Ueda 1986; Maycox et al. 1990; Ozkan and Ueda 1998; Fremeau et al. 2004a; Thompson et al. 2005; Edwards 2007; Erickson et al. 2006; Ueda and Ikemoto 2007; Chaudhry et al. 2008; El Mestikawy et al. 2011; Omote et al. 2011; Hackett and Ueda 2015) deal with some of the advances in this field.

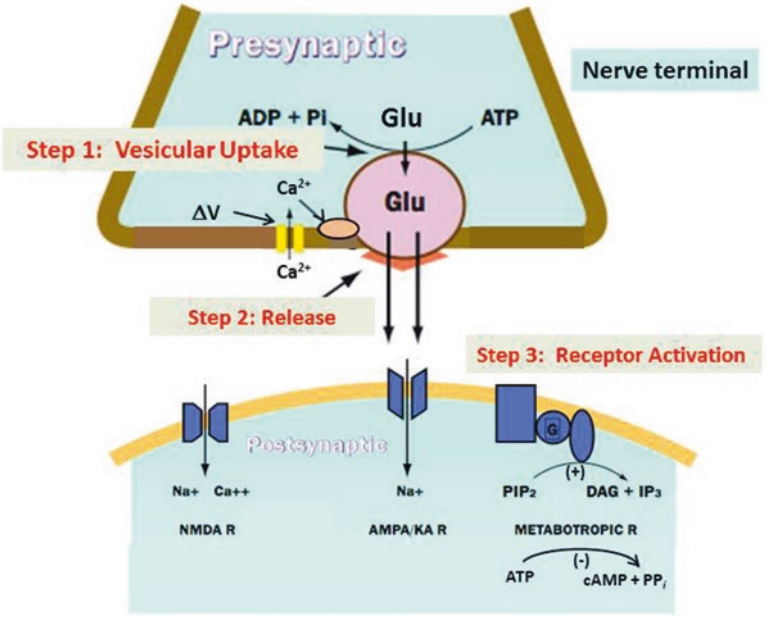

Fig. 7.1 Model of glutamate synaptic transmission. The first step in glutamate transmission is to accumulate glutamate into synaptic vesicles in an energy-dependent manner prior to its release. Upon arrival of the electrical impulse at the nerve terminal, depolarization (ΔV)-induced Ca^{2+} influx occurs. Increased rapid intracellular Ca^{2+} triggers rapid glutamate release into the synaptic cleft (step 2). Increased glutamate in the synaptic cleft activates various types of glutamate receptors, opening the Na^{+}/Ca^{2+} channels and leading to activation of various enzymes, and thereby serves as a neurotransmitter (step 3). *AMPA*, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; *DAG*, diacyl glyceride; PIP_2, phosphatidyl inositol bisphosphate; *Glu*, glutamate; *NMDA*, *N*-methyl-aspartate; IP_3, inositol triphosphate; *R*, receptor

7.1 Discovery and Significance of Vesicular Glutamate Uptake

The discovery of glutamate uptake into synaptic vesicles can be traced back to the discovery of synapsin I (formerly referred to as Protein I). Synapsin Ia and Ib were discovered, purified, and characterized as brain-specific, major synaptic endogenous substrates for cyclic AMP-dependent protein kinase by Ueda and Greengard (1997), in an investigation to understand the molecular mechanism underlying the action of monoamine neurotransmitters via cyclic AMP production at the synapse (Greengard 1976). Their primary structures were deduced by Sudhof et al. (1989, 1990). Initial immunocytochemical and subcellular distribution studies suggested that synaptic vesicles were enriched with Protein I (Bloom et al. 1979; Ueda et al. 1979). Subsequent studies by De Camilli et al. (1983) indicated that Protein I was specifically localized to synaptic vesicles, hence the name "synapsin I." Based upon this knowledge, Naito and Ueda (1982, 1983) purified brain synaptic vesicles by use

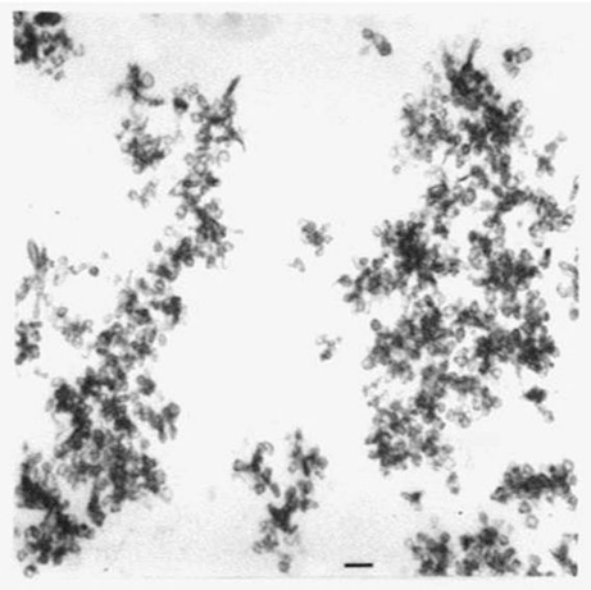

Fig. 7.2 Electronmicro graph of anti-synapsin I IgG-precipitated brain synaptic vesicles. This illustrates the purity of the preparation; it is free of mitochondria, other intracellular organelles, myelin, and plasma membranes. The bar indicates 0.1 μm. Taken from Naito and Ueda (1983)

of highly specific anti-synapsin I IgG (Naito and Ueda 1981), as shown in Fig. 7.2. The immunoprecipitated synaptic vesicle preparation exhibited pure vesicles, free of contamination from other organelles or membranes (Naito and Ueda 1982, 1983). This was the first morphologically pure synaptic vesicle preparation from the brain. Of importance, this synaptic vesicle preparation exhibited ATP-dependent, glutamate-specific uptake under the incubation conditions used (Fig. 7.3; Table 7.1) (Naito and Ueda 1982, 1983). Clear biochemical evidence was provided that synaptic vesicles are capable of accumulating glutamate in a highly specific manner. This demonstration proved key evidence for supporting the concept that glutamate functions as a neurotransmitter and served as a basis for VGLUT research.

Significantly, this evidence was obtained before glutamate had been generally accepted as a neurotransmitter. Three main barriers had prevented acceptance of glutamate's role as a neurotransmitter. The first was teleological. Glutamate occurs as a major biochemical substance, not just in neurons, but in all cells in the entire biological system. As such, it is involved in a variety of important cellular processes, such as energy and nitrogen metabolism, protein and nucleic acid synthesis, and folic acid synthesis, as well as synthesis of the inhibitory neurotransmitter GABA. In contrast, the other transmitters (acetylcholine, norepinephrine, dopamine, serotonin, and GABA) known at that time are synthesized essentially only in neurons and certain endocrine cells, and solely function as a neurotransmitter or hormone. Second, Curtis et al. (1960) showed that glutamate, when iontophoretically applied, fired all the neurons tested. All other neurotransmitters were known to act on certain, but not all, neurons. Based upon this apparent nonspecific effect, it was concluded that glutamate would not serve as a neurotransmitter. Third, evidence was lacking at the time for accumulation of glutamate in synaptic vesicles

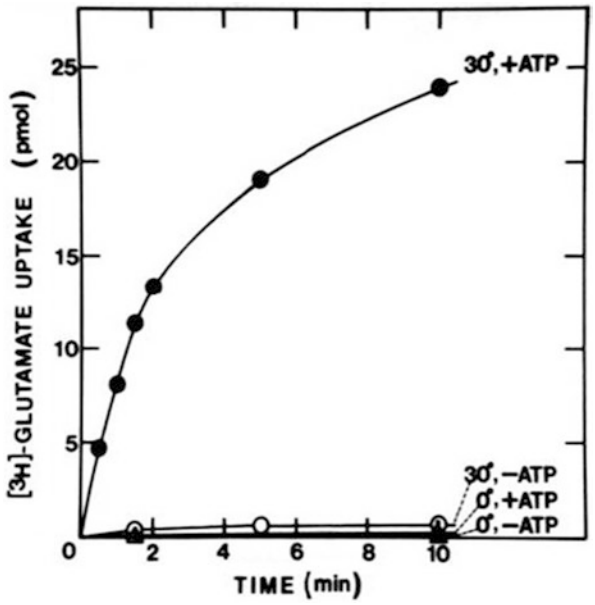

Fig. 7.3 Highly purified brain synaptic vesicles take up glutamate in an ATP-dependent and temperature-dependent manner. Anti-synapsin I IgG-precipitated synaptic vesicles (20 μg) from bovine brain were incubated at 30 or 0 °C with 50 μM [^{3}H]L-glutamate (0.4 Ci/mmol) in the absence or presence of 2 mM ATP in a solution containing 4 mM $MgSO_4$, 5 mM Tris–Cl (pH 7.4), and 320 mM sucrose. This indicates that glutamate is accumulated into synaptic vesicles against the glutamate concentration gradient, namely active transport or uphill transport. Taken from Naito and Ueda (1983)

(Mangan and Whittaker 1966; Rassin 1972; Kontro et al. 1980). Likewise, there was no evidence that synaptic vesicles have the ability to load glutamate (De Belleroche and Bradford 1973), and consequently it was proposed that glutamate is released from the cytosol, rather than from the synaptic vesicle (De Belleroche and Bradford 1977). This failed to support the notion that glutamate functions as a neurotransmitter; well-established neurotransmitters had been found concentrated in synaptic vesicles.

In retrospect, the main reason for the apparent failure of initial investigations to demonstrate enrichment of glutamate in isolated synaptic vesicles seems to have been the inability of synaptic vesicles to maintain accumulated glutamate due to the absence of an electrochemical proton gradient (Burger et al. 1989; Carlson and Ueda 1990). Likewise, the earlier failure to observe glutamate incorporation into isolated synaptic vesicles was due not only to the absence of ATP but also to the presence of a high concentration of chloride in the incubation medium used. Crucial to the clear demonstration of vesicular glutamate uptake was the presence of a low millimolar concentration of chloride as well as of ATP during the uptake, not to mention the purity of synaptic vesicle preparation; high concentrations of chloride were found to inhibit vesicular glutamate

Table 7.1 Substrate specificity for glutamate uptake into highly purified synaptic vesicles

Additions	Uptake	
	cpm	% Control
None (control)	4650 ± 100	100
L-Glutamate	1270 ± 60	27
D-Glutamate	2060 ± 360	44
L-Aspartate	4450 ± 240	96
D-Aspartate	4600 ± 130	99
L-Glutamine	5070 ± 810	109
GABA	3990 ± 40	86

Taken from Naito and Ueda (1983)
Anti-synapsin I IgG-precipitated synaptic vesicles (20 μg) from bovine brain were incubated for 1.5 min with 1 mM [^{3}H]L-glutamate (0.04 Ci/mmol) in the absence or presence of 10 mM glutamate analogues in the same medium as described in Fig. 7.3. Glutamate uptake values represent mean ± s.e.m. (n = 2 vesicle preparations). Using 50 μM [^{3}H]L-glutamate (0.4 Ci/mmol), the specific activity of glutamate uptake was determined to be 405 ± 44 pmol/min/mg (n = 7 preparations)

uptake (Naito and Ueda 1985). This is in great contrast to monoamine uptake into chromaffin granules, which requires much higher chloride concentration, such as 40 mM for maximal uptake (Johnson et al. 1979).

The low concentration chloride stimulation of vesicular glutamate uptake was an accidental discovery. In the initial investigation to characterize synapsin I-containing synaptic vesicles, the chloride concentration was kept minimal in the incubation medium for glutamate uptake, as well as in the vesicle preparation (Naito and Ueda 1983), in order to prevent synapsin I high salt-induced dissociation from vesicles (Ueda and Greengard 1997). A small stimulation by ATP of glutamate uptake in the absence of low millimolar chloride (2-fold as opposed to 40-fold in the presence of a low concentration of chloride) was reported, using crude and partially purified synaptic vesicle preparations (Disbrow et al. 1982). However, the vesicle preparations were not sufficiently pure, so that it was not clear that uptake occurred in synaptic vesicles. Moreover, glutamate specificity was not examined.

The initial clear demonstration of energy-dependent, glutamate-specific uptake into highly purified isolated synaptic vesicles (Naito and Ueda 1983), together with subsequent immunocytochemical (Storm-Mathisen et al. 1983) and biochemical studies (Burger et al. 1989) that glutamate is stored in synaptic vesicles, as well as with the demonstration of Ca^{2+}-dependent vesicular release of glutamate (Nicholls and Sihra 1986), provided strong support for the notion that glutamate plays a role as a neurotransmitter. These lines of evidence led to the subsequent identification of a VGLUT (Takamori et al. 2000; Bellocchio et al. 2000) and demonstration of its control of quantal size (Wojcik et al. 2004) and, together with pharmacological studies (Watkins and Evans 1981; Cotman et al. 1988; Monaghan

et al. 1989) and cloning of various glutamate receptors (Hollmann et al. 1989; Masu et al. 1991; Egebjerg et al. 1991; Moriyoshi et al. 1991), proved essential in establishing the neurotransmitter role of glutamate. Thus, synaptic vesicles were demonstrated to be endowed with an energy-dependent molecular machinery able to accumulate glutamate in a highly specific fashion, and this was instrumental in revising the previously held view that the common amino acid glutamate could not function as a neurotransmitter.

7.2 Characteristics of the Vesicular Glutamate Uptake System

As in the case of catecholamine and serotonin uptake into storage granules (Holz 1978; Johnson and Scarpa 1979; Carty et al. 1981; Njus et al. 1986), glutamate uptake into highly purified synaptic vesicles was shown to require ATP (Naito and Ueda 1982, 1983), which is harnessed by v-type proton-pump ATPase to generate an electrochemical proton gradient, the driving force of the uptake (Naito and Ueda 1985; Maycox et al. 1988; Shioi et al. 1989; Fykse et al. 1989; Carlson et al. 1989; Hell et al. 1990; Tabb and Ueda 1991; Tabb et al. 1992). Glutamate uptake was inhibited by the electrochemical proton gradient dissipaters carboxyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) or CCCP, as well as by the v-type proton-pump ATPase inhibitor bafilomycin, but not by mitochondrial proton-pump ATPase inhibitors. It was also inhibited by the H^+/K^+ exchanger nigericin in the presence of K^+. These observations suggested that the vesicular glutamate uptake system consists of v-type proton-pump ATPase and a VGLUT (Ueda 1986; Maycox et al. 1990; Ozkan and Ueda 1998). At the expense of ATP hydrolysis, proton-pump ATPase translocates H^+ across the vesicle membrane from the extravesicular medium to the vesicle interior, generating a membrane potential inside positive. When a membrane-permeable anion such as Cl^- is present in the extravesicular medium, the membrane potential causes anion influx, resulting in formation, for example, of HCl, and decreasing intravesicular pH, accompanied by reduction of the potential difference, namely an increase in ΔpH and a decrease in membrane potential $\Delta\Psi$. The electrochemical proton gradient $\Delta\mu_{H^+}$, defined as a combination of the electrical component $\Delta\Psi$ and the chemical component ΔpH, has been proposed to serve as the driving force for vesicular glutamate uptake. This is in contrast to cellular uptake of glutamate by the plasma membrane transporter, which utilizes the Na^+ gradient as the driving force (for review see ref. Danbolt 2001).

Vesicular glutamate uptake was found surprisingly specific to glutamate, exhibiting no significant interaction with aspartate, GABA, or glutamine (Naito and Ueda 1983, 1985). In essence, the VGLUT failed to recognize glutamate analogs which lack the free α-amino group, free α- and γ-carboxylate groups, and three-carbon skeleton between the two carboxylate groups (Naito and Ueda 1985). The difference

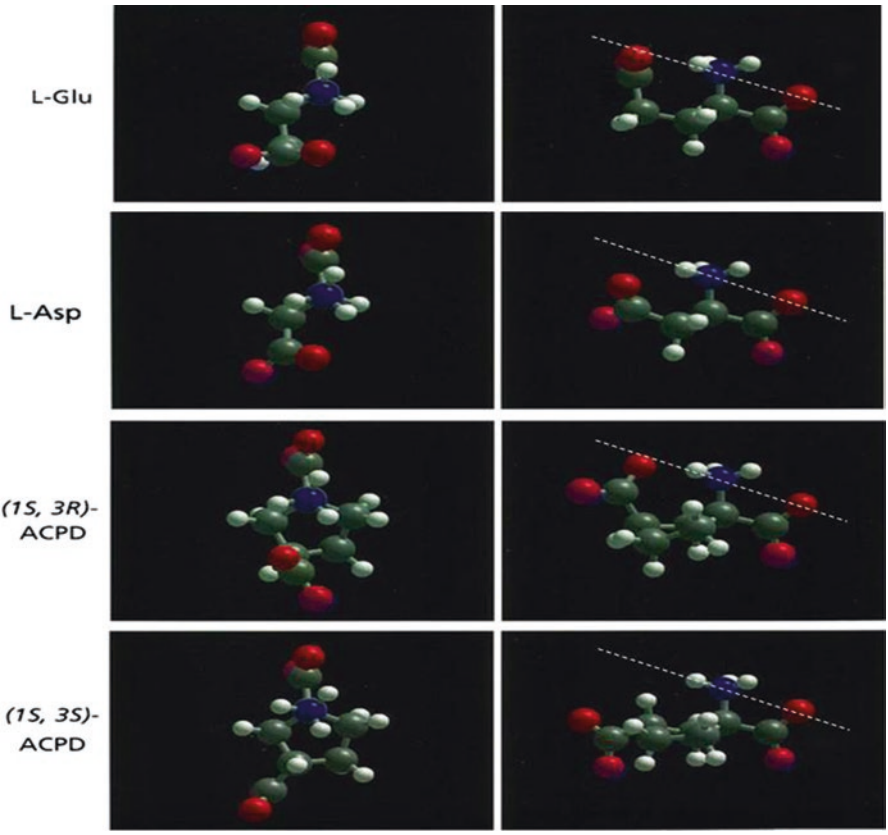

Fig. 7.4 Molecular modeling of glutamate, aspartate, and ACPD. The energy-minimized conformation of glutamate, aspartate, (*1S*, *3R*)-ACPD, and (*1S*, *3S*)-ACPD is shown. When viewed from the side, the negatively charged γ-carboxylate oxygen of glutamate is close to the line connecting its negatively charged α-carboxylate oxygen and positively charged nitrogen. The same applies to the glutamate analog (*1S*, *3R*)-ACPD, but not to the glutamate analogs, aspartate and (*1S*, *3S*)-ACPD. Color codes: *white*, hydrogen; *gray*, carbon; *blue*, nitrogen (positively charged); *red*, negatively charged oxygen of the carboxylate group. Left and right columns show top and side views, respectively. PCMODEL (Serena Software, Bloomington, IN, USA), Ball and Stick, and Mac Molecule (University of Arizona) programs were used for the molecular modeling

in three-dimensional structure between glutamate and its close analog aspartate (which has an amino group, two carboxylate groups, and the two carbon skeleton) is depicted in Fig. 7.4. VGLUT would distinguish the difference in charge distribution between glutamate and aspartate. The charge distribution of glutamate's most stable conformation (the negatively charged oxygen of the α- and γ-carboxylate groups) is similar to that of the glutamate analog (*1S*, *3R*)-ACPD bearing the three carbon skeleton as well as the free α-amino group and the free α- and γ-carboxylate groups, but substantially different from that of aspartate and (*1S*, *3S*)-ACPD. (*1S*, *3R*)-ACPD, but not (*1S*, *3S*)-ACPD, has been shown to serve as a VGLUT substrate.

This high specificity to glutamate was also observed in subsequent studies (Carlson et al. 1989; Tabb and Ueda 1991; Fisher-Bovenkerk et al. 1988; Moriyama and Yamamoto 1995). This contrasts Na^+-dependent cellular glutamate reuptake mediated by a plasma membrane transporter, which does not distinguish between glutamate and aspartate (Logan and Snyder 1972; for review see ref. Danbolt 2001). Moreover, glutamate affinity for the vesicular uptake system was found to be low ($K_m = 1.6$ mM) (Naito and Ueda 1985; Tabb and Ueda 1991), again contrasting with the high affinity for the plasma membrane glutamate transporter ($K_m = 2–66$ μM) (Ueda 1986; Danbolt 2001; Bennett et al. 1974; Kanner and Sharon 1978; Schousboe and Hertz 1981 for review). The low glutamate affinity for the vesicular transporter is compatible with high glutamate concentration in neurons (estimated to be about 10 mM), and the high affinity for the plasma membrane transporter with low concentration in the extracellular milieu (1 μM or less at rest).

Another intriguing finding was that vesicular glutamate uptake was markedly stimulated by a physiologically relevant, low millimolar intracellular concentration of chloride (Naito and Ueda 1985), as shown in Fig. 7.5a, b. However, high concentrations were found to be inhibitory. This inhibition by high chloride concentrations was attenuated by a high concentration of glutamate, suggesting that chloride competes with glutamate for the glutamate binding/transport site. The stimulatory effect of chloride was mimicked by bromide, but not by other halogens or anions (Naito and Ueda 1985; Moriyama and Yamamoto 1995). This interesting, unique property was observed in a number of studies (Fykse et al. 1989; Carlson et al. 1989; Hell et al. 1990; Tabb and Ueda 1991; Tabb et al. 1992; Fisher-Bovenkerk et al. 1988; Moriyama and Yamamoto 1995; Wolosker et al. 1996). These properties of the vesicular uptake system were demonstrated in all vertebrate brains examined (Tabb and Ueda 1991) and in a reconstituted system consisting of solubilized synaptic vesicle proteins and crude soybean phospholipid (which contains phosphatidyl choline, phosphatidyl ethanolamine, phosphatidyl serine, phosphatidyl inositol, triolein, and cholesterol); pure phosphatidyl choline, phosphatidyl ethanolamine, phosphatidyl serine, phosphatidic acid, and phosphatidyl inositol failed to support vesicular uptake (Carlson et al. 1989). These unique features render the vesicular glutamate uptake system distinct from the plasma membrane uptake system (Table 7.2) and led to a model of the vesicular glutamate uptake system (Ueda 1986), as shown in Fig. 7.6.

An electrochemical proton gradient, as opposed to a Na^+ gradient, is harnessed as a driving force to accumulate neurotransmitters into synaptic vesicles; this concept has been extended to GABA and glycine uptake (Fykse et al. 1989; Hell et al. 1990; Kish et al. 1989) as well as to acetylcholine uptake (Anderson et al. 1982; Parsons et al. 1993). Thus, it could be generalized that nonpeptide neurotransmitter uptake into synaptic vesicles occurs at the expense of an electrochemical proton gradient; the relative contribution of its two components (membrane potential and pH gradient) varies, however, depending upon the type of neurotransmitter transported (Hell et al. 1990). The electrochemical proton gradient across the inner mitochondrial membrane, originally conceived of by Mitchell (1961, 1966; Reid et al. 1966) to link electron transport and ATP synthesis, is now recognized as the

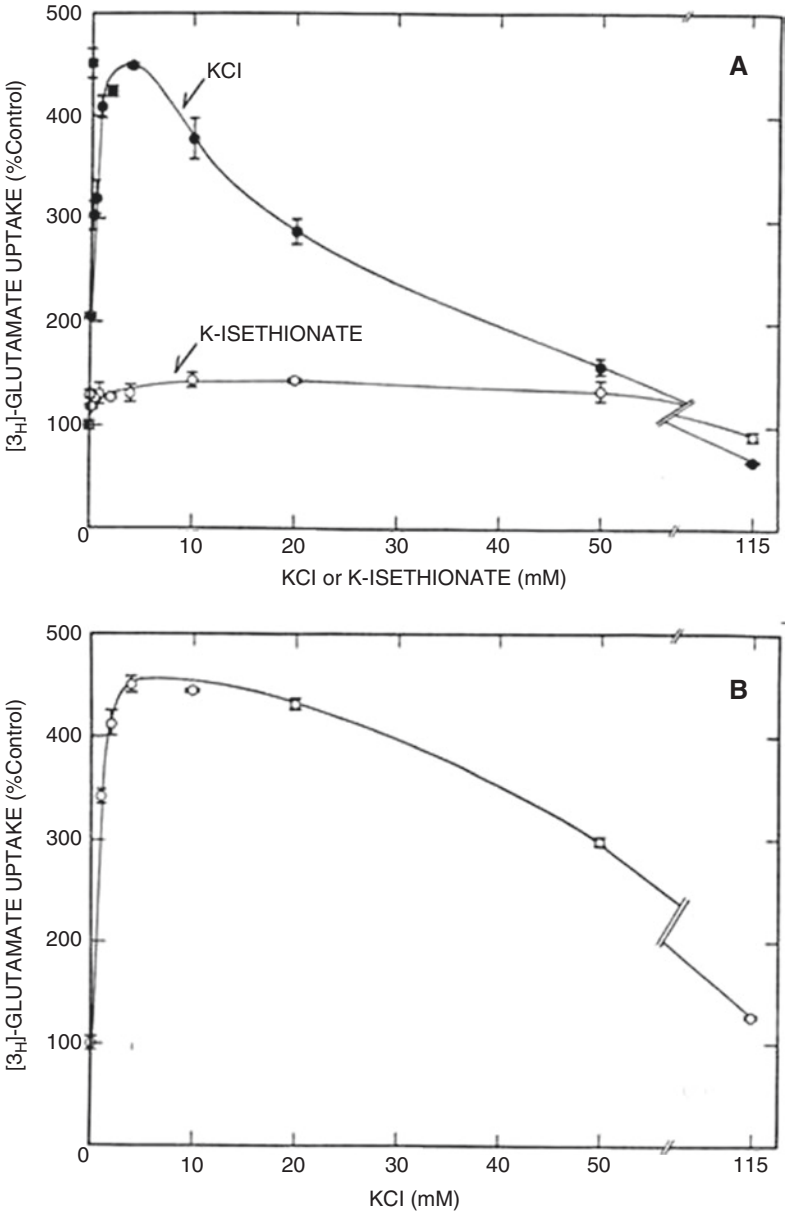

Fig. 7.5 Vesicular glutamate uptake exhibits a biphasic response to chloride. Glutamate uptake into synaptic vesicles (20 μg; purified by sucrose density gradient centrifugation) for 1.5 min at 30 °C was measured with 50 μM (**a**) or 1 mM (**b**) [^{3}H]glutamate in the presence of various concentrations of chloride or isethionate in the same solution as described in Fig. 7.3, except for replacement of 5 mM Tris–Cl and 320 mM sucrose with 5 mM Tris–maleate (pH 7.4) and 250 mM sucrose, respectively. Glutamate uptake is markedly stimulated by low concentrations of chloride, but inhibited by high concentration (**a**, **b**). The inhibition is attenuated by increasing glutamate concentration (**b**). Taken from Naito and Ueda (1985)

Table 7.2 Comparison of synaptic vesicle and plasma membrane glutamate uptake systems

	Synaptic vesicle membrane	Plasma membrane
Driving force	$\Delta\mu_H^+ = \Delta\Psi + \Delta pH$	$Na^+_o > Na^+_i$, $K^+_o > K^+_i$
Stimulator	Low mM Cl^- (1–4 mM)	High mM Cl^- (100 mM)
Affinity for glutamate	Low (K_m = 1.6 mM)	High (K_m = 2–66 μM)
Interaction with aspartate	No	Yes
Interaction with *t*-ACPD[a]	Yes	No
Glutamate transporter	VGLUT1-3	EAAT1-5

t-ACPD, *trans*-1-amino-1,3-dicarboxycyclopentane; *VGLUT*, vesicular glutamate transporter; *EAAT*, excitatory amino acid transporter (Amara and Fontana 2002)
[a]See ref. Winter and Ueda (1993)

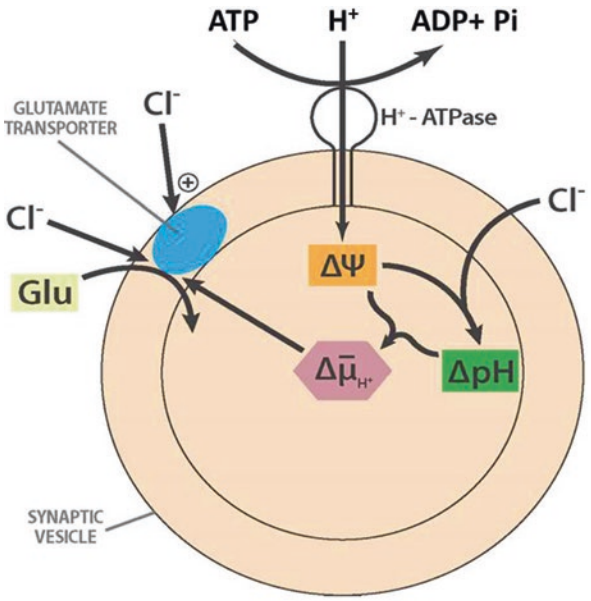

Fig. 7.6 Model of the vesicular glutamate uptake system. The uptake system consists of two functionally different components, v-type proton-pump ATPase and the vesicular glutamate transporter (VGLUT). The H^+-pump ATPase generates a membrane potential at the expense of ATP hydrolysis. In the presence of extravesicular chloride, this induces chloride influx, resulting in HCl formation in the vesicle lumen, thus generating a pH gradient across the vesicle membrane. An electrochemical proton gradient comprised of membrane potential and pH gradient serves as the driving force of vesicular glutamate uptake. Low concentration of chloride could stimulate vesicular glutamate uptake via binding to an allosteric site, as well as via pH gradient formation. High concentrations of chloride could inhibit glutamate uptake by competing with glutamate. $\Delta\mu_H^+$, electrochemical proton gradient; $\Delta\Psi$, membrane potential; *Glu*, glutamate

common currency of cellular energy. In mitochondria, it is utilized to generate ATP, whereas in intracellular organelles, such as synaptic vesicles and chromaffin granules, it is harnessed to accumulate transmitters and hormones. Thus, the synaptic vesicular neurotransmitter (NT) uptake system is thought to consist of two functionally different protein components, v-type proton-pump ATPase and a transmitter-specific transporter, in a manner analogous to antibodies (which comprise a common segment and a variable segment):

$$\begin{aligned}\text{Synaptic vesicular NT uptake system} = {} & \text{v ATPase}(\text{common}) \\ & + \text{NT v transporter}(\text{variable})\end{aligned}$$

7.3 Vesicular Glutamate Transporter

The first genetic evidence indicating VGLUT is present in glutamatergic neurons was obtained by Fisher-Bovenkerk et al. (1988). Identification of the VGLUT had remained elusive until 2000, when Takamori et al. and Bellocchio et al. (Takamori et al. 2000; Bellocchio et al. 2000) demonstrated that a brain-specific isoform of the sodium-dependent inorganic phosphate transporter (Ni et al. 1994) functioned as VGLUT. This was quickly followed by identification of two additional isoforms, designated VGLUT2 (Aihara et al. 2000; Fremeau et al. 2001; Bai et al. 2001; Takamori et al. 2001; Herzog et al. 2001; Hayashi et al. 2001; Varoqui et al. 2002) and VGLUT3 (Gras et al. 2002; Takamori et al. 2002; Fremeau et al. 2002; Schafer et al. 2002). All the isoforms were found to belong to the SLC 17 family (Reimer and Edwards 2004) and predicted to have twelve membrane-spanning regions. Little variation is seen in amino acid sequences of their greater central domain, but they largely differ in N-terminal and C-terminal regions (Fig. 7.7). As such, their biochemical functional properties are similar (Fremeau et al. 2001, 2002; Bai et al. 2001; Takamori et al. 2001, 2002; Herzog et al. 2001; Hayashi et al. 2001; Varoqui et al. 2002; Gras et al. 2002; Schafer et al. 2002). Their substrate specificity, affinity for glutamate, and chloride stimulation are essentially indistinguishable from those originally found in isolated vesicles (Naito and Ueda 1983, 1985). Detailed comparison of VGLUT1 and two biochemical characteristics has been reported by Kaneko and Fujiyama (2002). Mutational analyses (Juge et al. 2006) and computer modeling (Almqvist et al. 2007) suggest that the active site involves His^{128}, Arg^{184}, and Glu^{191} in the case of VGLUT2 and His^{120} and Arg^{176} in the case of VGLUT1. It is postulated that VGLUT1 His^{120} and Arg^{176} interact with the γ- and α-carboxyl group's negatively charged oxygen of glutamate, respectively, and VGLUT1 Glu^{183} with the positively charged α-amino group of glutamate. Proteomic studies suggest that synaptic vesicles bear 9 and 14 copies per vesicle of VGLUT1 and VGLUT2, respectively (Takamori et al. 2006). These studies also indicate that approximately 80 % of all synaptic vesicles contain VGLUTs 1 and 2, which is compatible with an early observation by Naito and Ueda (1983) suggesting that the majority of brain

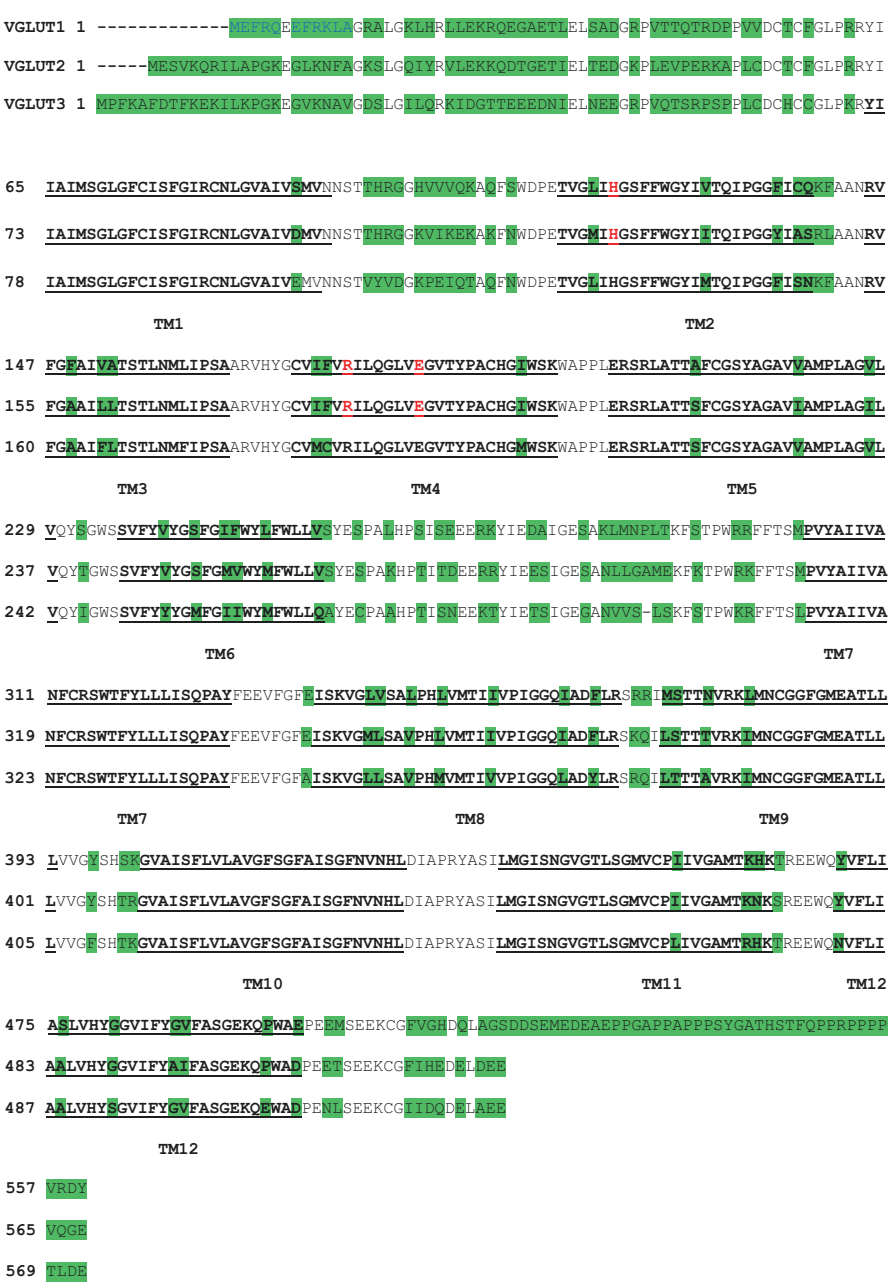

Fig. 7.7 Deduced amino acid sequence of human VGLUT1, VGLUT2, and VGLUT3. Green highlights and underlines indicate varying and transmembrane regions, respectively. Red letters indicate amino acid residues postulated to be glutamate binding at the active site (Takamori et al. 2002; Fremeau et al. 2002)

synaptic vesicles are glutamate accumulating. These lines of evidence are consistent with the notion that glutamate is the major excitatory neurotransmitter.

VGLUT1 and VGLUT2 have, in general, complementary distributions in the adult brain (Fremeau et al. 2001, 2004a; Herzog et al. 2001; Varoqui et al. 2002; Kaneko and Fujiyama 2002). VGLUT1 predominates in telencephalic regions, including the cerebral cortex and hippocampus, whereas VGLUT2 is primarily expressed in diencephalic and lower brain stem regions. However, in some glutamatergic nerve terminals and certain endocrine cells, VGLUT1 and VGLUT2 are colocalized (Fremeau et al. 2004a, b; Hioki et al. 2003; Morimoto et al. 2003; Hayashi et al. 2003; Bai et al. 2003; Miyazaki et al. 2003; Boulland et al. 2004; Nakamura et al. 2005; Herzog et al. 2006; Persson et al. 2006); some vesicles have both VGLUT1 and VGLUT2 (Herzog et al. 2006). Their coexpression occurs transiently at early developmental stages in multiple glutamatergic neurons (Fremeau et al. 2004b; Miyazaki et al. 2003; Boulland et al. 2004; Nakamura et al. 2005; Herzog et al. 2006); VGLUT2 is expressed first and declines, followed by VGLUT1 expression. A population of synaptic vesicles is endowed with both VGLUT1 and VGLUT2 during early development (Herzog et al. 2006); this could possibly confer greater plasticity to the control of quantal release. Electrophysiological evidence indicates that the copy number of VGLUT1 and VGLUT2 per vesicle can affect quantal size (Wojcik et al. 2004; Daniels et al. 2004; Wilson et al. 2005; Moechars et al. 2006), compatible with the notion that alteration of vesicular glutamate content can change miniature excitatory postsynaptic potential.

In contrast to the more abundant isoforms VGLUT1 and 2, VGLUT3 is expressed by a much smaller number of isolated neuronal populations (Fremeau et al. 2002, 2004a; Gras et al. 2002; Schafer et al. 2002). However, evidence indicates that VGLUT3 is expressed not only in nerve terminals but also in dendrites, a subpopulation of astrocytes, progenitor-like cells, and "nonnervous tissues" (Fremeau et al. 2002, 2004a; Gras et al. 2002; Boulland et al. 2004; Ni and Parpura 2009; Ormel et al. 2012a). Transient expression of VGLUT3 also occurs in particular cells in specific brain regions during the early postnatal period (Boulland et al. 2004; Gras et al. 2005). These observations suggest that the VGLUT isoforms are subject to differential cellular expression and/or intracellular trafficking and involved in different functions. It is interesting to note that VGLUT1 is present in photoreceptor cell terminals (Johnson et al. 2003; Sherry et al. 2003; Fyk-Kolodziej et al. 2004), whereas VGLUT3 is present in auditory inner hair cell terminals and essential for auditory coding (Seal et al. 2008; Ruel et al. 2008); however, VGLUT1 occurs in auditory nerve fiber terminals in the cochlear nucleus (Zhou et al. 2007).

VGLUT1 is mostly localized to synapses distinct from those bearing VGLUT2 and can control the reserve pool size of synaptic vesicles in excitatory nerve terminals (Fremeau et al. 2004b; Todera et al. 2007). VGLUT1 is thought to have a role in regulating the probability of transmitter release (Fremeau et al. 2001, 2004a, b; Varoqui et al. 2002; Weston et al. 2011; Herman et al. 2014) and in synaptic plasticity (Varoqui et al. 2002; Todera et al. 2007; Balschun et al. 2010) including reversal learning (Balschun et al. 2010; Granseth et al. 2015). Studies by Herman et al. (2014) suggest that the probability is affected by the

amount of glutamate accumulated in the vesicle, namely the glutamate fill state, not by the VGLUT protein amount.

In contrast, VGLUT2 appears to be present in those synapses with high release probability, which transfer information with high fidelity; these synapses are common in sensory and autonomic nervous systems (Fremeau et al. 2001; Varoqui et al. 2002). Thus, VGLUT2 is expressed in the majority of thalamus neurons (Fremeau et al. 2001, 2004a; Varoqui et al. 2002; Moechars et al. 2006); cerebellar climbing fibers (Fremeau et al. 2001; Hioki et al. 2003); blood pressure-controlling presympathetic neurons (Stornetta et al. 2002), inspiratory augmenting neurons (Stornetta et al. 2003), and chemosensory neurons innervating the main respiratory center (Weston et al. 2004), all in the brainstem; and nociception-mediating spinal cord DRG neurons (Liu et al. 2010; Rogoz et al. 2012; Ma 2014). VGLUT 3 has been found colocalized with vesicular acetylcholine, monoamine, and GABA transporters (Gras et al. 2002, 2005, 2008; Fremeau et al. 2002; Schafer et al. 2002; Boulland et al. 2004; Divito et al. 2015). This has implications for motor control, reward behavior, and neurological and psychiatric disorders (Fremeau et al. 2002; Gras et al. 2008; Divito et al. 2015).

Accumulated evidence now indicates that a subset of each VGLUT is colocalized with a subset of vesicular monoamine, GABA, and acetylcholine transporters (for review see ref. El Mestikawy et al. 2011). In addition, a subset of VGLUT2 is colocalized with a subpopulation of vesicles containing dopamine and norepinephrine and a subset of VGLUT3 with a subpopulation of serotonin-containing vesicles. Thus, VGLUT coexpression levels in nonglutamatergic vesicles could upregulate the vesicle's capacity to accumulate these transmitters, in part via glutamate influx-induced increase in pH gradient, and thereby could modulate GABAergic, cholinergic, and monoaminergic neurotransmission. Conversely, glutamate transmission could be regulated by corelease of GABA, acetylcholine, and monoamine neurotransmitters. These cophenotypes suggest interplay between glutamate transmission and other neurotransmission systems through corelease and have implications for behavioral, neurological, and mental disorders.

VGLUTs are expressed not only in the brain and certain endocrine systems (Hayashi et al. 2001, 2003; Morimoto et al. 2003; Bai et al. 2003) but also in the spinal cord (Persson et al. 2006; Todd et al. 2003; Oliveira et al. 2003; Li et al. 2003; Landry et al. 2004; Wu et al. 2004; Seal et al. 2009) and "nonnervous tissues." VGLUT2 has been shown to occur in pulmonary (sensory) nerve fiber terminals (Brouns et al. 2004) and glucagon-like peptide 1-containing granules in intestinal L cells (Uehara et al. 2006). VGLUT 1 has been observed in intrafusal muscle-spindle afferent endings (Wu et al. 2004) and motor nerve terminals of esophageal striated muscle (Kraus et al. 2004). In the bone, VGLUT1 is expressed in osteoclast cells, responsible for bone reabsorption (Morimoto et al. 2006). VGLUT3 is present in the liver (Gras et al. 2002; Fremeau et al. 2002; Boulland et al. 2004), skeletal muscle fibers, and some motor neuron terminals (Boulland et al. 2004), as well as in a small subset of dorsal root ganglion neurons innervating the spinal cord dorsal horn and mediating mechanical hypersensitivity (Seal et al. 2009). VGLUT3 is also expressed in primary sensory afferents innervating the dental pulp, controlling micro blood circulation (Zerari-Mailly et al.

2012). VGLUT occurrence in other autonomic and sensory nervous systems, including neurons innervating the colorectum (Brumovsky et al. 2011) and bladder (Brumovsky et al. 2013), as well as in the spinal cord, has recently been reviewed extensively by Brumovsky (2013). All these observations indicate VGLUT involvement not only in brain function but in other physiological functions as well.

7.4 Mechanism of Vesicular Glutamate Uptake and Modulation by Cl^- and K^+

The driving force of vesicular glutamate uptake is an electrochemical proton gradient across the synaptic vesicle membrane, analogous to catecholamine uptake into chromaffin granules (Holz 1978; Johnson and Scarpa 1979; Njus et al. 1986; Casey et al. 1977; Phillips and Allison 1978; Johnson et al. 1979; Johnson 1988). This has been demonstrated using the electrogenic proton ionophore FCCP capable of dissipating ΔpH as well as $\Delta\Psi$, the neutral H^+/K^+ exchanger nigericin (and the neutral H^+/Mg^{2+} exchanger A23187) and the electrogenic K^+ ionophore valinomycin. The relative contribution of $\Delta\Psi$ and ΔpH varies with the concentration of permeable anions such as chloride. Evidence supporting this principle has been provided with isolated synaptic vesicles (Naito and Ueda 1985; Maycox et al. 1988; Shioi et al. 1989; Fykse et al. 1989; Tabb and Ueda 1991; Tabb et al. 1992; Moriyama and Yamamoto 1995; Wolosker et al. 1996), reconstituted systems (Maycox et al. 1988; Carlson et al. 1989), cloned VGLUT1 (Takamori et al. 2000; Bellocchio et al. 2000; Schenck et al. 2009), VGLUT2 (Fremeau et al. 2001; Bai et al. 2001; Herzog et al. 2001; Varoqui et al. 2002; Juge et al. 2006), and VGLUT3 (Gras et al. 2002; Takamori et al. 2002; Fremeau et al. 2002). The critical importance of the membrane potential as the driving force has been investigated by several laboratories (Maycox et al. 1988; Shioi et al. 1989; Schenck et al. 2009; Shioi and Ueda 1990; Juge et al. 2010). The membrane potential increases affinity for glutamate (Wolosker et al. 1996; Francis S. Lee, Ph.D. Dissertation, University of Michigan, 1996), suggesting a conformational change in VGLUT. The contribution of the pH gradient has been addressed in a number of studies (Naito and Ueda 1985; Takamori et al. 2000, 2002; Tabb et al. 1992; Wolosker et al. 1996; Fremeau et al. 2001, 2002; Bai et al. 2001; Herzog et al. 2001; Varoqui et al. 2002; Gras et al. 2002; Schenck et al. 2009; Shioi and Ueda 1990).

As to the modulation by chloride, the stimulatory effect of low millimolar chloride is due to an increase in V_{max} with little change in K_m for glutamate (Naito and Ueda 1985; Wolosker et al. 1996). This chloride stimulation was initially postulated to be mediated by a conformational change in the glutamate transporter, as well as by a pH gradient produced by membrane potential-induced chloride influx (Naito and Ueda 1985). Tabb et al. (1992) have provided evidence for the generation of a small pH gradient in the presence of low millimolar chloride and suggested that an intravesicular optimal pH, produced through a small influx of chloride, is responsible for activation of VGLUT. Studies by Hartinger and Jahn (1993) provided the

first evidence supporting the notion that chloride stimulation involves its binding to an allosteric site of the glutamate transporter, followed by studies by Moriyama and Yamamoto (1995). Tabb et al. (1992), Wolosker et al. (1996), Schenck et al. (2009), and Juge et al. (2010) showed that activation of vesicular glutamate transport by an extravesicular low chloride concentration can occur in the presence of a membrane potential alone, without a pH gradient.

In elegant experiments using reconstituted liposomes with VGLUT1 and a mitochondrial type of H^+-ATPase, Schenck et al. (2009) demonstrated that intraliposomal chloride together with a membrane potential is sufficient to cause glutamate uptake. This is consistent with an earlier observation by Francis S. Lee (Ph.D. dissertation, University of Michigan, 1996) that chloride-preloaded synaptic vesicles in the presence of ATP and a high concentration of chloride exhibited a twofold increase in glutamate uptake compared to chloride-unloaded intact vesicles, when assayed in the absence of extravesicular chloride. However, in the absence of intraliposomal chloride, low millimolar extraliposomal chloride is required for maximal glutamate uptake. The requirement for intraliposomal chloride was attributed to the charge and osmotic balance maintenance enabled by chloride efflux being loosely coupled to glutamate influx, which would allow continuous glutamate uptake. Similarly, an anti-glutamate/chloride transport mechanism had previously been proposed (Wolosker et al. 1996). It remains to be determined, however, whether the intraliposomal chloride could also activate VGLUT via binding to it on the lumen side.

The stimulation by extraliposomal chloride was attributed to a pH gradient formed by VGLUT-mediated chloride influx. Nigericin (which dissipates the pH gradient) substantially reduced glutamate uptake into reconstituted liposomes with a H^+-pumping ATPase and VGLUT1 (Schenck et al. 2009), as observed with purified brain vesicles (Tabb et al. 1992). It is also noted that there is a small component of low millimolar extraliposomal chloride-stimulated glutamate uptake even in the absence of pH gradient. This could be attributed to the chloride activation via its binding to the extraliposomal side of VGLUT. VGLUT-mediated chloride influx is supported by evidence that VGLUT is capable of chloride transport in the presence of membrane potential, enabling pH gradient formation (Bellocchio et al. 2000; Schenck et al. 2009; Preobraschenski et al. 2014). However, this contrasts studies by Juge et al. (2010), in which a diffusion membrane potential was induced by K^+ in the presence of valinomycin, and chloride flux was measured in the presence of 5 mM glutamate. They showed that VGLUT was unable to mediate chloride flux and suggested that low millimolar extraliposomal chloride, together with a membrane potential alone, is sufficient for maximal vesicular glutamate uptake. Recently, in agreement with studies by Schenck et al. (2009), Preobraschenski et al. (2014) have also demonstrated the intravesicular chloride requirement for vesicular glutamate uptake in the absence of extravesicular chloride, as well as stimulation by low millimolar extravesicular chloride in the absence of intravesicular chloride. Thus, membrane potential can cause vesicular glutamate uptake in the presence of chloride in either the interior or the exterior of the vesicle.

In freshly endocytosed vesicles containing a high concentration of trapped chloride, extravesicular chloride would not be required for maximal glutamate uptake, whereas in chloride-empty vesicles a low physiological concentration of extravesicular chloride would be critical. The degree of glutamate uptake stimulation by extravesicular chloride would presumably vary with the amount of intravesicular chloride: the lower the intravesicular chloride concentration, the higher the glutamate uptake stimulation by low millimolar concentration of extravesicular chloride. This could explain variability in the extent of low millimolar chloride stimulation observed with different vesicle preparations, including those from various species (Tabb and Ueda 1991), possibly reflecting different chloride concentrations present in prepared vesicles. It is likely that, in synaptic vesicles containing low concentrations of chloride, extravesicular chloride, in addition to activating VGLUT via an allosteric binding, substantially contributes to the driving force via generation of a pH gradient induced by chloride influx. The pH gradient-dependent glutamate uptake could be due to proton efflux, possibly coupled with chloride efflux by the same mechanism of Na^+-dependent phosphate transport mediated by VGLUT (cation-dependent anion transport). The chloride efflux in this manner (if the stoichiometry is $2Cl^-/H^+$) could maintain charge and osmotic balance with glutamate uptake. An optimal intravesicular pH brought about by pH gradient formation could be involved in enhancing vesicular glutamate uptake as well.

As to the mechanism underlying the inhibition of vesicular glutamate uptake by high chloride concentration, evidence now supports the notion that chloride competes with glutamate for the transport site. This inhibition is attenuated by increasing glutamate concentration (Naito and Ueda 1985; Wolosker et al. 1996; Schenck et al. 2009). Chloride-dependent inhibition is also observed in the presence of an enhanced, constant membrane potential in the absence of pH gradient (Tabb et al. 1992; Wolosker et al. 1996). Bellocchio et al. (2000) have provided evidence that cloned VGLUT1 is capable of chloride transport and that glutamate competes for anion permeation. This was also demonstrated by Schenck et al. (2009) and Preobraschenski et al. (2014), using reconstituted proteoliposomes containing VGLUT1 and H^+-pump ATPase. Moreover, Schenck et al. (2009) showed that chloride inhibition of glutamate uptake is reduced by increasing glutamate concentration and occurs even under conditions in which the membrane potential is enhanced and the pH gradient is abolished. These lines of evidence together indicate that high chloride concentration inhibits glutamate uptake by competing with glutamate for the anion transport site.

In an effort to address the role of intravesicular H^+ in vesicular glutamate, Goh et al. (2011) found that a physiologically relevant high concentration of extravesicular K^+ stimulates vesicular glutamate uptake via K^+/H^+ exchange. The final level of accumulation, not the initial rate of uptake, is increased. It is proposed that the pH gradient (formed not only by chloride influx but also by glutamate transport) is consumed by a 5-(*N*-ethyl-*N*-isopropyl)amiloride (EIPA)-sensitive cation/H^+ exchanger (potentially NHE6), generating an additional membrane potential when vesicular H^+-pump ATPase is in operation, and that this extra membrane potential is responsible for enhanced glutamate uptake. Preobraschenski et al. (2014) confirmed the K^+/H^+ exchange activity and K^+-stimulated glutamate uptake, as well as the presence of the protein NHE6, in synaptic vesicles. They have also shown, using mitochondrial type

H^+-ATPase-loaded liposomes fused with v-H^+-ATPase-blocked synaptic vesicles, that when liposomes are devoid of chloride, the K^+ stimulation is the highest in the absence of extraliposomal chloride. However, when liposomes are filled with highly concentrated chloride, the K^+-stimulation is minimal or negligible regardless of extraliposomal chloride concentration. The same was observed using reconstituted liposomes with VGLUT1 and H^+-pump ATPase only. VGLUT itself has been proposed to be capable of mediating K^+/H^+ exchange (with high selectivity for K^+ over Na^+). This stimulation is attributed to an increase in membrane potential generated by active H^+-pump ATPase upon K^+/H^+ exchange.

Thus, under physiological conditions [in which the extracellular and intraneuronal chloride concentrations are about 150 mM and 20 mM (Billups and Attwell 2002; Price and Trussell 2006), respectively, and the intraneuronal concentration of glutamate is estimated to be 1–10 mM (Nicholls and Attwell 1990; McMahon and Nicholls 1991)], freshly endocytosed synaptic vesicles would load glutamate, initially driven by a combination of membrane potential and trapped intravesicular chloride (Fig. 7.8a). As lumen chloride decreases, a cytosolic low concentration of chloride would begin to play a role in vesicular glutamate uptake as a VGLUT activator (by directly binding to an allosteric site) as well as a pH gradient cogenerator (Fig. 7.8b, c). The pH gradient thus generated could also serve as a component of the driving force potentially through VGLUT-mediated H^+ efflux coupled with Cl^- efflux (Fig. 7.8b, c). An optimal intravesicular pH (around pH 6.8) could also be produced for maximal function of VGLUT (Fig. 7.8b, c). Cytosolic K^+ could further elevate vesicular glutamate accumulation via H^+/K^+ exchange (mediated by VGLUT and NHE6)-induced enhancement of membrane potential (which occurs with active H^+-pump ATPase) (Fig. 7.8b, c). Based upon the mechanism described above, a time course similar to that shown in Fig. 7.8d would be observed upon sequential additions of ATP, a low concentration of chloride, and a high concentration of potassium ion. In vivo, inhibition by high chloride concentration would not occur, since cytosolic chloride concentration is not high enough, and extravesicular glutamate concentration is sufficiently high to compete with low levels of cytosolic chloride. Price and Trussell have provided evidence that changing the nerve terminal cytosolic chloride concentration does not alter the amount of glutamate in synaptic vesicles in the calyx of Held, a giant glutamatergic nerve ending of the auditory brainstem (Price and Trussell 2006). Although low millimolar chloride is essential in bringing about maximal vesicular uptake in vitro, chloride may not have a modulatory role in vivo. VGLUT could be kept activated at all times with relatively constant low chloride concentration.

7.5 Energy Source for Vesicular Glutamate Uptake

In order to maintain normal synaptic transmission, in particular at the time of rapid neuronal firing when synaptic vesicles are quickly depleted of pre-accumulated glutamate, emptied vesicles demand swift reloading of glutamate. This

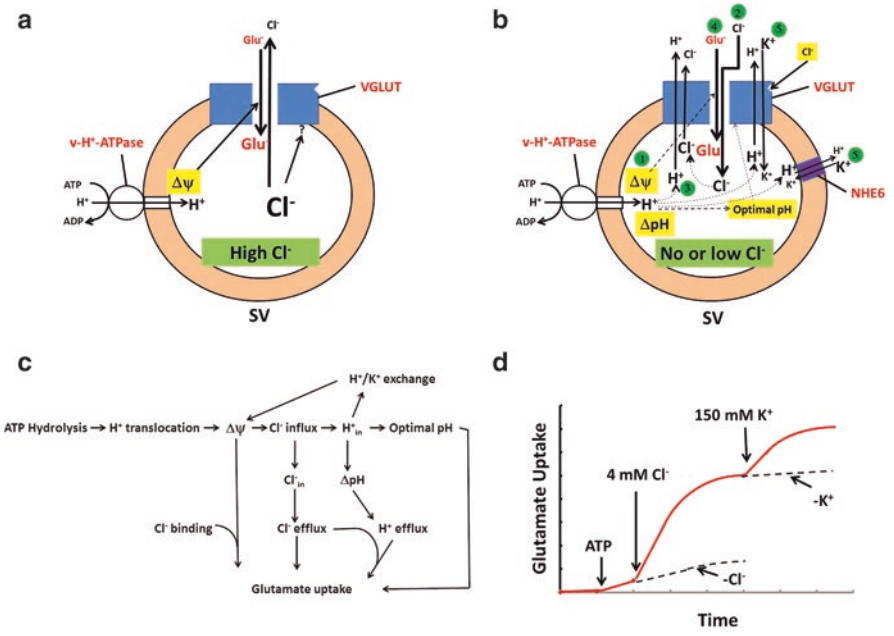

Fig. 7.8 Proposed mechanisms of vesicular glutamate uptake and its modulation by chloride. (**a**) In the presence of a high concentration of intravesicular chloride, a membrane potential generated by H^+-pump ATPase can cause glutamate influx, accumulating glutamate in the vesicle. In order to maintain ionic and osmotic balance, chloride efflux, which would be mediated by VGLUT, must occur; this would allow continuous glutamate uptake. (**b**) In the absence of intravesicular chloride or in the presence of a low concentration of chloride, a low concentration of extravesicular chloride plays a modulatory role by forming a pH gradient and providing an optimal intravesicular pH for VGLUT activity, as well as by activating VGLUT via binding to an allosteric site. VGLUT-mediated proton efflux could occur, possibly coupled with chloride efflux. This would allow ionic and osmotic balance maintenance, which would enable glutamate uptake to continue. Proton efflux could also be coupled with K^+ influx mediated by VGLUT and the cation/proton exchanger NHE6. This would enhance membrane potential when the H^+-pump ATPase is in operation, leading to an increase in glutamate uptake. $\Delta\Psi$, membrane potential; *Glu*, glutamate; *NHE*, cation/proton exchanger. (**c**) Time sequence of events from ATP hydrolysis to glutamate uptake under conditions described in (**b**). (**d**) Expected time course of vesicular glutamate uptake upon sequential additions of ATP, Cl^- and K^+. This type of time coarse would be observed as a result of sequential additions of ATP, 4 mM Cl^-, and 150 mM K^+ to isolated synaptic vesicles, reflecting the proposed mechanism shown in (**b**, **c**)

demand would be met by a readily available energy supply to fuel the loading, as well as by the availability of glutamate. How is this to be achieved?

Synaptic transmission is well known to vitally rely on glycolysis (Cox and Bachelard 1982), although mitochondria are the major source of cellular ATP production. Under hypoglycemic conditions, abnormal synaptic transmission rapidly develops, yet global cellular ATP levels are hardly changed (Cox et al. 1983; Bachelard et al. 1984). Replacement of glucose with the final glycolysis product pyruvate, which results in production of the majority of cellular ATP by mitochondria,

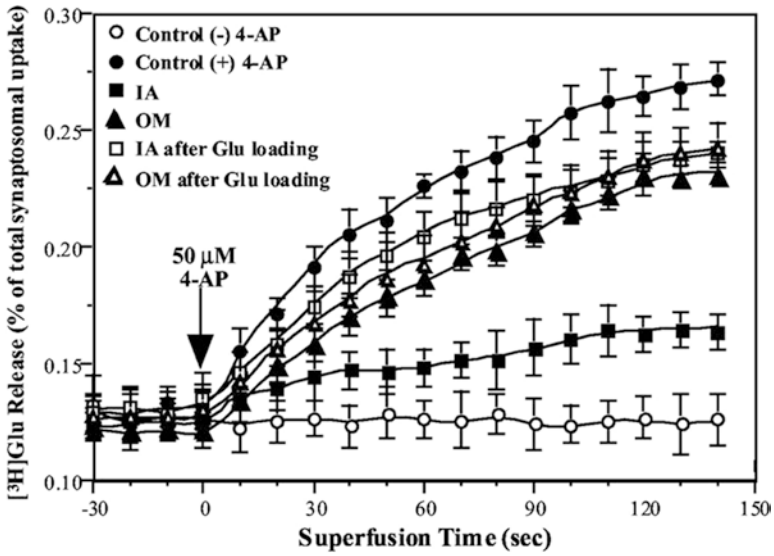

Fig. 7.9 Glycolysis-produced ATP plays a major role in providing energy required for vesicular glutamate loading, prior to its release from synaptosomes. Iodoacetate, an inhibitor of glyceraldehyde 3-phosphate dehydrogenase involved in glycolytic ATP production, reduced the amount of glutamate released in a 4-AP-induced, calcium-dependent manner from synaptosomes, which had been loaded with tritiated glutamate after exposure to iodoacetate, whereas the mitochondrial ATP synthase inhibitor oligomycin had minimal effect. The inhibitory effect of iodoacetate on glutamate is likely to reflect its effect on vesicular glutamate content, as described in the text. *4-AP*, 4-aminopyridine; *Glu*, glutamate; *IA*, iodoacetate; *OM*, oligomycin. Taken from Ikemoto et al. (2003)

does not restore normal synaptic transmission (Cox and Bachelard 1982), even though ATP tissue levels return to normal (Cox et al. 1983). Fleck et al. (1993) showed that hypoglycemic conditions which led to reduction of glutamate release did not significantly alter cellular ATP content. These observations raised the possibility that a glycolytic intermediate or a minor pool of ATP, possibly locally synthesized, might play a critical role in glutamate release and synaptic transmission. Ikemoto et al. (2003) have provided evidence suggesting that glycolytically generated ATP, rather than that produced in mitochondria, plays a major role in providing energy for vesicular glutamate accumulation. The glyceraldehyde-3-phosphate dehydrogenase (GAPDH) inhibitor iodoacetate, which is known to suppress glycolysis, reduced the amount of glutamate released from synaptosomes (pinched-off nerve endings) in a 4-aminopyridine (4-AP)-induced, calcium-dependent manner, whereas the mitochondrial ATP-synthase inhibitor oligomycin had minimal effect (Fig. 7.9). This iodoacetate-induced reduction in glutamate release could reflect its effect on vesicular glutamate content in synaptosomes. These observations are compatible with the finding by Shepherd and Harris (1998) that approximately 50 % of hippocampal CA3/CA1 nerve terminals are devoid of mitochondria; these terminals must rely exclusively on glycolytically generated ATP.

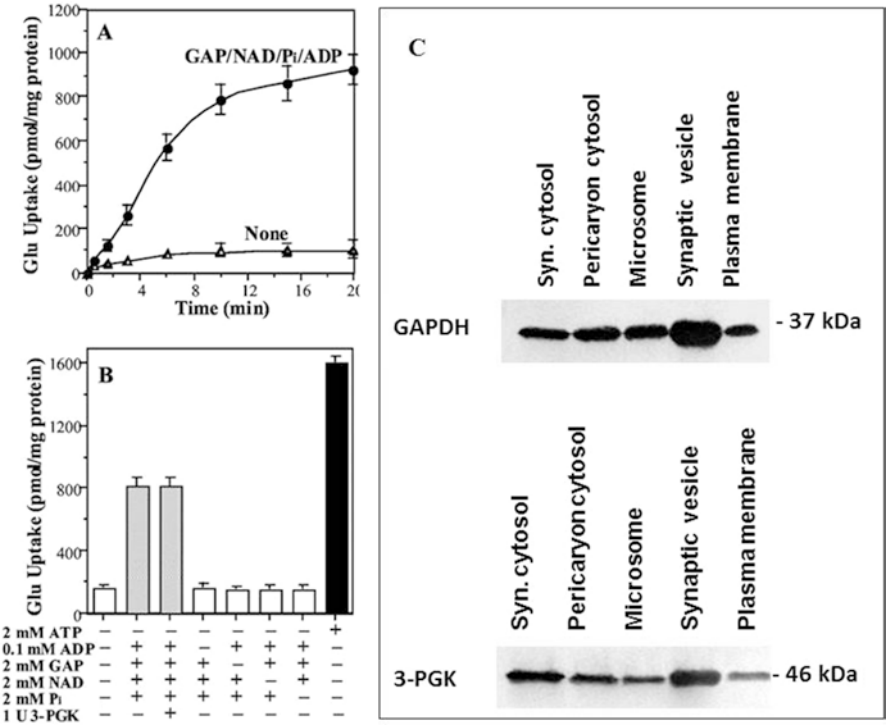

Fig. 7.10 ATP synthesized by synaptic vesicle-bound glycolytic enzymes glyceraldehyde 3-phosphate dehydrogenase and 3-phosphoglycerate kinase is harnessed by the vesicular glutamate uptake system. (**a**) The substrates for the glycolytic ATP-generating enzymes (GAP, NAD, and P_i for GAPDH and ADP for 3-PGK) support glutamate uptake into isolated synaptic vesicles. (**b**) Omission of any of these substrates abolishes vesicular glutamate uptake. (**c**) Western blots of GAPDH and 3-PGK in various subcellular fractions. GAPDH and 3-PGK are most concentrated in the synaptic vesicle fraction of all fractions tested. *GAP*, glyceraldehyde 3-phosphate; *GAPDH*, glyceraldehyde 3-phosphate dehydrogenase; *3-PGK*, 3-phosphoglycerate kinase; P_i, inorganic phosphate. Taken from Ikemoto et al. (2003)

Of importance, in searching for high-energy glycolytic intermediate-modified proteins, Ikemoto et al. (2003) found that synaptic vesicles are endowed with the glycolytic ATP-generating enzyme complex GAPDH/3-phosphoglycerate kinase (3-PGK), which is harnessed to generate energy for immediate uptake of glutamate into synaptic vesicles (Fig. 7.10a, b). Moreover, glutamate uptake into isolated synaptic vesicles was inhibited by iodoacetate. Surprisingly, both GAPDH and 3-PGK were found to be most enriched in the synaptic vesicle fraction, of all subcellular fractions examined (Fig. 7.10c). The association of GAPDH with synaptic vesicles is consistent with evidence obtained by proteomic analyses (Coughenour et al. 2004; Takamori et al. 2006; Burré et al. 2006). Further studies have suggested that a subpopulation of synaptic vesicles bears pyruvate kinase, which is also capable of supporting vesicular glutamate uptake (Ishida et al. 2009). These observations

together support the notion that locally synthesized glycolytic ATP is the major energy source harnessed for the efficient loading of glutamate into synaptic vesicles (Ueda and Ikemoto 2007). The role of locally synthesized glycolytic ATP appears not limited to vesicular glutamate uptake and most likely extended to the uptake of other transmitters at other synapses (Ishida et al. 2009). It is feasible that glycolytic ATP synthesized by membrane-bound GAPDH/3-PGK is coupled to all major cation transporters (Ueda and Ikemoto 2007). Moreover, the vital role of local synthesis of glycolytic ATP in fast axonal transport of synaptic vesicles has also been demonstrated recently (Zala et al. 2013).

7.6 Precursor of VGLUT Substrate

How do neurons produce the specific pool of glutamate for immediate use as a neurotransmitter? As the major amino acid, glutamate is involved in a number of metabolic and other cellular functions. In eukaryotes, glutamate is synthesized by three enzymes:

(a) Amino acid aminotransferase

$$\alpha \text{ ketoglutarate} + \text{amino acid} \rightleftharpoons \text{glutamate} + \alpha \text{ keto acid}$$

(b) Glutamate dehydrogenase

$$\alpha \text{ ketoglutarate} + NH_4^+ + \underset{(\text{NADPH})}{\text{NADH}} + H^+ \rightleftharpoons \text{glutamate} + \underset{(\text{NADP}^+)}{NAD^+} + H_2O$$

(c) Glutaminase

$$\text{glutamine} + H_2O \rightarrow \text{glutamate} + NH_3$$

Glutamine is widely thought to be the principal precursor for synthesis of the neurotransmitter glutamate. Bradford et al. (1978) and Hamberger et al. (1979), using radioactive glutamine and glucose, provided biochemical evidence that glutamine is the major substrate for the neurotransmitter pool of glutamate. This, together with the specific localization of glutamine synthetase in astrocytes (Martinez-Hernandez et al. 1977) and glutaminase enrichment in mitochondria within synaptosomes (Bradford and Ward 1976), as well as with the notion of the glutamate–glutamine cycle (Van den Berg and Garfinkel 1971; Benjamin and Quastel 1972), led to the concept that the glutamate–glutamine cycle plays a central role in supplying a precursor for synthesis of the neurotransmitter glutamate (Cotman et al. 1981; Shank and Aprison 1988; Schousboe et al. 1997). According to this model, astrocytes take up the majority of glutamate released from nerve terminals, followed by conversion to glutamine, which is then transported out to the extracellular space. The released

glutamine is taken up by neurons and transformed back to glutamate, largely by mitochondrial glutaminase. Glutamate thus produced and directly released to the cytosol from mitochondria had been thought to be utilized as a neurotransmitter.

This is also supported by studies (Kvamme et al. 1991; Roberg et al. 1995) indicating that phosphate-activated glutaminase is localized to the exterior of the inner mitochondrial membrane. However, other evidence indicates that the active site of this enzyme is on the matrix side of the mitochondrial inner membrane (Shapiro et al. 1985; Aledo et al. 1997; Albrecht et al. 2000; Zieminska et al. 2004; Bak et al. 2008). Moreover, Palaiologos et al. (1988) provided evidence suggesting that glutamine-derived glutamate is transformed by aspartate aminotransferase (AAT) in the mitochondrial matrix to α-ketoglutarate (Dennis et al. 1977), which is then transported out to the cytoplasm, mediated by the dicarboxylic acid exchanger (Bisaccia et al. 1985; Bolli et al. 1989). α-Ketoglutarate exported from mitochondria is then converted to glutamate, which is released in a Ca^{2+}-dependent manner. The resynthesis of glutamate was assumed to be carried out by cytosolic AAT (Palaiologos et al. 1988).

Takeda et al. (2012) have found that isolated synaptic vesicles are capable of synthesizing glutamate from α-ketoglutarate and L-aspartate (as the specific amino donor) for immediate uptake (Fig. 7.11). Vesicle-bound AAT was shown to be responsible for this synthesis. Glutamine was not an effective substrate for glutamate synthesis, consistent with the vesicle's glutaminase deficiency. These observations suggest that local synthesis of the neurotransmitter pool of glutamate could occur at the synaptic vesicle. Of interest, vesicle-bound AAT was found to belong to the mitochondrial type (GOT2), not the cytosolic isozyme (GOT1),

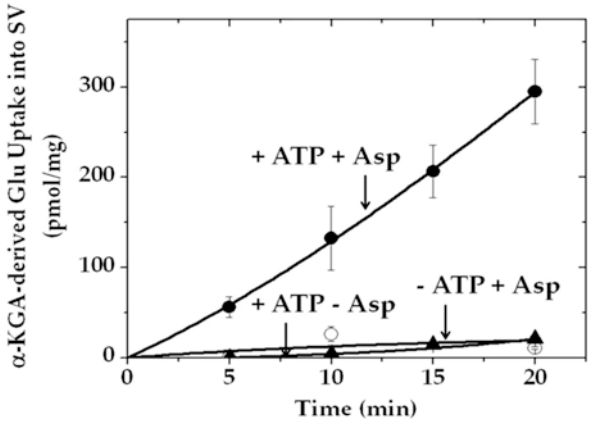

Fig. 7.11 Synaptic vesicle-bound AAT synthesizes the VGLUT substrate glutamate. When isolated synaptic vesicles were incubated with [^{14}C]α-KGA in the presence of aspartate, [^{14}C]glutamate was taken up into vesicles in an ATP-dependent manner. This was not observed in the absence of aspartate, indicating that glutamate is formed from α-KGA by AAT bound to synaptic vesicles and readily transported into vesicles. *α-KGA*, α-ketoglutarate; *Asp*, aspartate; *Glu*, glutamate. With permission from Takeda et al. (2012)

based on its kinetic and immunological properties (Takeda et al. 2012) as well as proteomic analyses (Takamori et al. 2006; Burré et al. 2006). Vesicle-bound AAT has much higher affinity for aspartate than does the cytosolic isozyme. The K_m value of vesicle-bound AAT for aspartate (0.9 mM) is substantially lower than the cellular concentration of aspartate (estimated to be 3.5–8 mM), whereas the K_m value for the cytosolic enzyme (6.7 mM) is higher than or similar to the cytosolic aspartate concentration. This suggests that vesicle-bound AAT would function at close to V_{max} in situ. These findings indicate that α-ketoglutarate can effectively serve as an immediate precursor for synthesis of the neurotransmitter glutamate. Thus, the glutamate synthesis by vesicle-bound AAT could represent an efficient mechanism for vesicular glutamate refilling and, hence, glutamate transmission.

α-Ketoglutarate to be used for synthesis of the neurotransmitter glutamate would derive mainly from nerve terminal mitochondria, where it is produced in large part from glutamine-derived glutamate (Palaiologos et al. 1988), and some possibly from glucose via the TCA cycle. In addition, it could be supplied from astrocytes (Westergaard et al. 1994), where de novo synthesis of α-ketoglutarate occurs via pyruvate carboxylation, coupled with the TCA cycle (Patel 1974; Yu et al. 1983; Shank et al. 1985, 1993; Cesar and Hamprecht 1995; Hertz et al. 2007; Serres et al. 2008; Sonnewald and Rae 2010; Morken et al. 2014; Sonnewald 2014). Synaptosomes have the capacity to take up α-ketoglutarate (Shank and Campbell 1981; Shank and Campbell 1982; Shank and Campbell 1984, Takeda and Ueda, 2016) as well as glutamine (Bradford et al. 1978), Takeda and Ueda, 2016).

Since the catalytic site of mitochondrial glutaminase is likely localized to the matrix side of the mitochondrial inner membrane (Shapiro et al. 1985; Aledo et al. 1997; Albrecht et al. 2000; Zieminska et al. 2004; Bak et al. 2008), the glutamine–glutamate conversion would occur in the matrix, where glutamate, rather than being directly released to the cytoplasm, would largely be transformed to α-ketoglutarate by mitochondrial AAT (Palaiologos et al. 1988) and glutamate dehydrogenase (Tildon et al. 1985; McKenna et al. 1993, 2000; McKenna 2007). A large part of α-ketoglutarate derived from glutamine and a small part of α-ketoglutarate from glucose would be transferred to the cytoplasm, as mentioned above. These lines of evidence taken together suggest that α-ketoglutarate can serve as the major *immediate* precursor for the neurotransmitter pool of glutamate. However, in view of immunocytochemical evidence suggesting that cytosolic AAT is associated with glutamatergic nerve terminals (Altschuler et al. 1981, 1982), the relative contribution of vesicle-bound vs. cytosolic AAT to synthesis of the neurotransmitter pool of glutamate remains to be established.

Although the glutamate–glutamine cycle is thought to play a central role in supplying the precursor of the neurotransmitter glutamate, evidence obtained over the past decade indicates that synthesis of the glutamate responsible for spontaneous synaptic transmission remains less clear (Edwards 2007). Kam and Nicoll (2007) showed that vesicular release of glutamate from neurons in culture devoid of astrocytes occurs even in the absence of glutamine in the culture medium. Masson et al. (2006) observed that genetic deletion of phosphate-activated glutaminase partially reduced, but did not abolish, evoked release, whereas it had little effect on spontaneous release of glutamate.

Moreover, alteration in the major neuronal glutamine transporter SNAT2 does not affect spontaneous glutamatergic transmission or the quantal size (Grewal et al. 2009). These lines of evidence indicate that the glutamate–glutamine cycle may not play an exclusive role in providing the immediate precursor for synthesis of the neurotransmitter pool of glutamate, in particular that of glutamate released spontaneously and responsible for generating the miniature excitatory postsynaptic current. This suggests alternative pathways for such a role. α-Ketoglutarate derived from glucose or some amino acids or odd chain fatty acids (Sonnewald 2014) via the TCA cycle could serve as such a precursor. Glutamate could also be formed from some other amino acids (Sonnewald 2014). Thus, glutamate released by an action potential is likely to derive from glutamine largely via formation of α-ketoglutarate, whereas the precursor of spontaneously released glutamate remains elusive. An effective mechanism for synthesis and vesicular loading of the neurotransmitter glutamate, as well as for ATP synthesis required for rapid vesicular glutamate uptake, is proposed in Fig. 7.12.

7.7 VGLUT Inhibitors

Excessive glutamate transmission is involved in many types of neurological pathologies. Since vesicular glutamate accumulation is an initial pivotal step in glutamate transmission, VGLUT represents a target in controlling glutamate transmission. Hence, considerable efforts have been made to find potent VGLUT inhibitors. Several classes of VGLUT inhibitors have been reported: (a) glutamate analogs such as *trans*-1-amino-1,3-cyclopentanedicarboxylate (*trans*-ACPD) and *trans*-1-amino-1,3-cyclohexanedicarboxylate (*trans*-ACHD) (Winter and Ueda 1993, 2008; Moriyama and Yamamoto 1995); (b) kynurenate analogs such as 7-chlorokynurenate and 8-hydroxy-kynurenate (Bartlett et al. 1998; Thompson et al. 2005); (c) diazo-dyes bearing disulfonic acid-substituted naphthylamine groups and a biphenyl linker, such as Trypan Blue (Fig. 7.13a) (Roseth et al. 1998; Fonnum et al. 1998) and Evans Blue (Roseth et al. 1995); (d) diazo-dyes bearing sulfonic acid-substituted stilbene and phenol groups, such as Brilliant Yellow (Fig. 7.12a), (Tamura et al. 2014); (e) new monoazo-dyes bearing a disulfonic acid-substituted naphthylamine group and a monophenyl or biphenyl group (Favre-Besse et al. 2014); (f) an inhibitory protein factor referred to as IPF (an *N*-terminal 1–25 amino acid-deleted, 135-kDa fragment of α-fordrin) (Özkan et al. 1997); (g) polyhalogenated fluorescein such as Rose Bengal and its analogs (Ogita et al. 2001; Bole and Ueda 2005; Pietrancosta et al. 2010); and (h) tetrapeptide inhibitors (e.g., D-Gln-L-Ile- D-Glu-L-Try) (Patel et al. 2007). Compounds belonging to classes (a), (b), and (h) are specific to VGLUT, but have relatively low potencies compared to the most potent inhibitors; in addition, they all lack cell membrane permeability. Trypan Blue, IPF, Rose Bengal, and Brilliant Yellow are the most potent inhibitors known (IC_{50} = 20–50 nM). Both Trypan Blue and Brilliant Yellow (Fig. 7.13a) are highly specific to VGLUT (Roseth et al. 1998; Tamura et al. 2014). However, they would not penetrate the cellular membrane, since they are highly charged. The endogenous

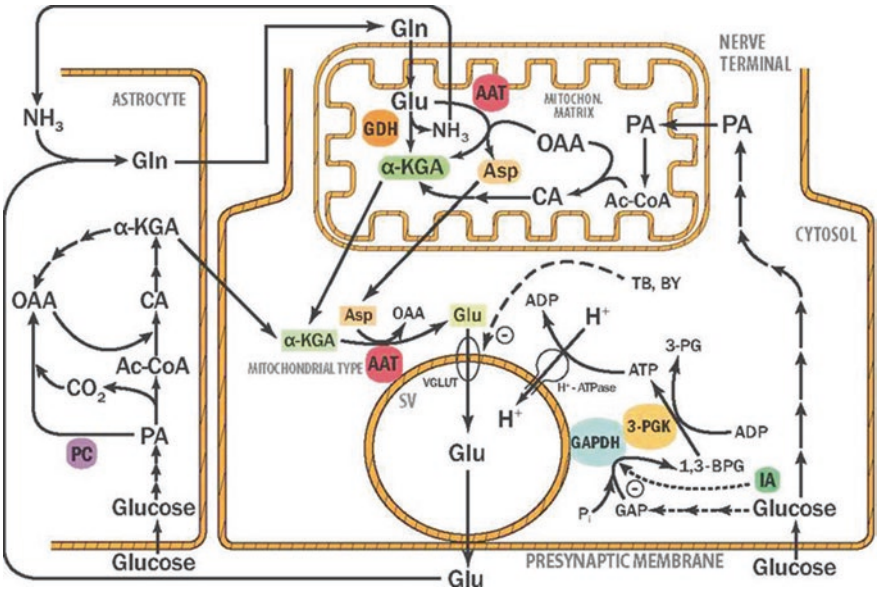

Fig. 7.12 Proposed effective mechanism for accumulating the neurotransmitter glutamate into synaptic vesicles. The VGLUT substrate glutamate is synthesized by vesicle-bound AAT from α-ketoglutarate and aspartate. Energy required for VGLUT's transport function is also generated by the vesicle-bound, glycolytic ATP-generating enzymes, GAPDH and 3-PGK. The majority of α-ketoglutarate would originate from nerve terminal mitochondria, where it is produced from glutamine-derived glutamate by AAT and GDH; this glutamine–glutamate conversion would be carried out largely by glutaminase on the matrix side of the inner membrane. An additional source of α-ketoglutarate would be nerve terminal-linked astrocytes, where it is synthesized de novo via pyruvate carboxylation and the TCA cycle. Another minor source of α-ketoglutarate could be nerve-terminal glucose, which leads to production of α-ketoglutarate via the TCA cycle. α-Ketoglutarate produced in this manner could serve as a substrate for synthesis of the transmitter glutamate responsible for spontaneous firing. This mechanism for supply of the VGLUT substrate does not depend on pre-neuronal activity, in contrast to its supply derived from glutamine, whose synthesis in astrocytes heavily depends on glutamate released upon neuronal activation. Aspartate is supplied from mitochondria in the nerve terminal. *AAT*, aspartate aminotransferase; *Ac-CoA*, acetyl coenzyme A; *α-KGA*, α-ketoglutarate; *Asp*, aspartate; *BY*, Brilliant Yellow; *GAP*, glyceraldehyde-3-phosphate; *GAPDH*, glyceraldehyde-3-phosphate dehydrogenase; *GDH*, glutamate dehydrogenase; *Glu*, glutamate; *Gln*, glutamine; *IA*, iodoacetate; *OAA*, oxaloacetic acid; *PA*, pyruvic acid; *3-PG*, 3-phosphoglycerate; *3-PGK*, 3-phosphoglycerate kinase; P_i, inorganic phosphate, *TB*, Trypan Blue

protein inhibitor IPF, though highly potent, is neither specific to VGLUT nor membrane permeable. Search for potent membrane-permeable VGLUT inhibitors resulted in the discovery of Rose Bengal. Among all known inhibitors, Rose Bengal is unique in that it is not only highly potent but also membrane permeable, leading to reduced glutamate release by inhibiting vesicular glutamate accumulation in synaptosomes (Ogita et al. 2001). However, it proved not highly specific to VGLUT; it exhibited a similar potency for vesicular uptake of GABA and monoamines (Bole and Ueda 2005; Pietrancosta et al. 2010).

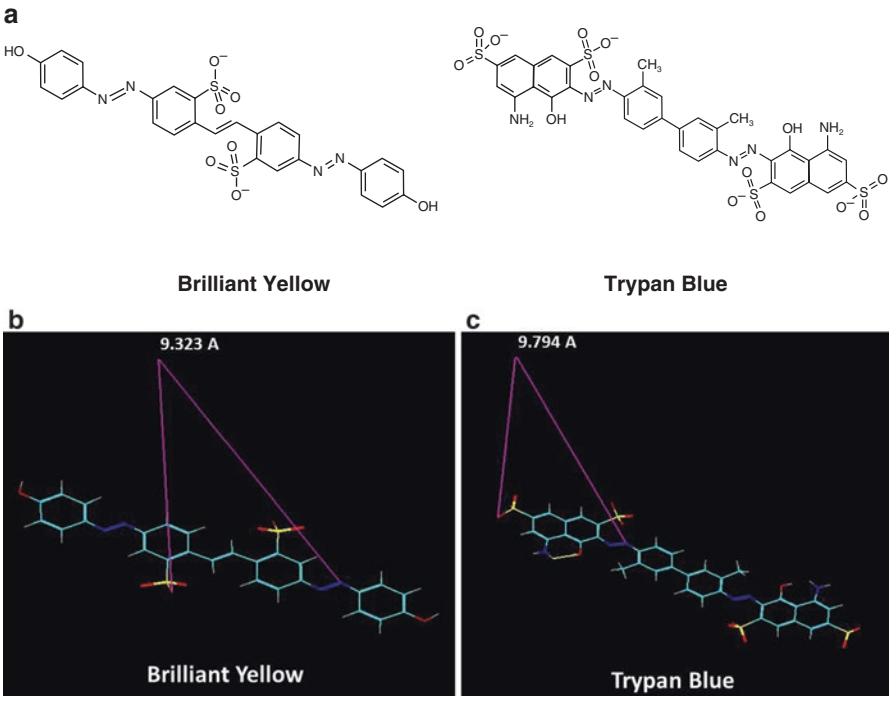

Fig. 7.13 Chemical structure and molecular modeling of Trypan Blue and Brilliant Yellow. (**a**) Chemical structure. (**b**) Molecular modeling of Brilliant Yellow. (**c**) Molecular modeling of Trypan Blue. The distance between the negatively charged oxygen and the azenyl group nitrogen linked to the phenyl group is indicated in purple. PCMODEL V 9.0 (Serena Software, Bloomington, IN, USA) was used for the molecular modeling

Recently, Rose Bengal has been reported to attenuate glutamate synaptic transmission (Neale et al. 2013, 2014). However, it required a much higher concentration than that determined in a glutamate release assay (Ogita et al. 2001). Of interest, Juge et al. (2010) have shown that ketone bodies inhibit VGLUT2 by blocking Cl^- activation, and that acetoacetate, the most potent, is capable of abolishing depolarization-induced glutamate release at millimolar concentrations. However, acetoacetate decreased not only the amplitude but also the frequency of mEPSCs. These ketone bodies were suggested responsible for the benefits of the ketogenic diet in controlling seizures (Juge et al. 2010). Other α-keto acids found in maple syrup urine disorder, such as α-keto-β-methyl-n-methyl valeric acid and α-ketoisocaproic acid, have also been shown to inhibit vesicular glutamate uptake by blocking the Cl^--activating site (Reis et al. 2000). However, these agents appear to also compete with the glutamate transport site. All the VGLUT-inhibitory keto acids reported are endogenous substances, but not potent. The most potent, highly VGLUT-specific inhibitors known today are Trypan Blue (Roseth et al. 1998) and Brilliant Yellow (Tamura et al. 2014), but these are not membrane permeable. Thus, membrane permeable, potent VGLUT-specific inhibitors, which could specifically regulate glutamate release, remain to be developed.

Molecular modeling indicates that one prominent feature shared by Brilliant Yellow and Trypan Blue is the similar distance between the azenyl group nitrogen linked to the phenyl group and the negatively charged oxygen of the sulfonate group: 9.3–9.9 A (Fig. 7.13b, c). Studies by Tamura et al. (2014) suggest that the 9.3 A distance is part of the Brilliant Yellow pharmacophore, which consists of the phenyl group-linked sulfonate group, the azenyl group, and the azenyl group-linked hydroxyphenyl group. Thus, the 9.3–9.9 A distance could represent part of the pharmacophore of VGLUT-specific, highly potent inhibitors.

Brilliant Yellow and Trypan Blue are postulated to block access of glutamate to VGLUT's active site (which are likely to involve VGLUT1 His^{120}, Arg^{176}, and Glu^{183} or VGLUT2 His^{128}, Arg^{184}, and Glu^{191}) by interacting with VGLUT1 His^{120} and Tyr^{187} or VGLUT2 His^{128} and Tyr^{195}; both Brilliant Yellow (Tamura et al. 2014) and Trypan Blue (Roseth et al. 1998) have been shown to compete with glutamate. This could occur in part via (a) the ionic bonding between histidine residue's positively charged nitrogen and the negatively charged oxygen of inhibitor's sulfonate group and (b) the hydrogen bonding between the tyrosine residue's hydroxyl group hydrogen and the inhibitor's azenyl group nitrogen linked to the phenyl group. These considerations suggest that the distance between the positively charged nitrogen of VGLUT1 His^{120} (or VGLUT2 His^{128}) and the hydroxyl group hydrogen of VGLUT1 Tyr^{187} (or VGLUT2 Tyr^{195}) is in the range of 9.3–9.8 A.

7.8 Astrocytic VGLUT

Accumulated evidence suggests that astrocytes, once thought to merely provide structural and trophic support for neurons, can modulate neuronal activity and could play an important role in information processing in the brain. Astrocytes, though electrically silent, have the capability to elevate intracellular Ca^{2+} concentrations through activation of various G-protein-coupled receptor systems (Haydon 2001). Glutamate, exogenously applied or synaptically released, can induce an oscillatory elevation of cytosolic Ca^{2+} in astrocytes, propagating calcium waves, not only within individual cells but also between adjacent astrocytes (Cornell-Bell et al. 1990; Smith 1992; Dani et al. 1992; Porter and McCarthy 1996). Increased cytosolic Ca^{2+} elicits release of glutamate from astrocytes, signaling neighboring neurons (Parpura et al. 1994; Jeftinija et al. 1996; Pasti et al. 1997; Bezzi et al. 1998; Parpura and Haydon 2000; Hassinger et al. 1995). Glutamate released from astrocytes induces neuronal Ca increase via activation of NMDA and non-NMDA receptors, and thereby can modulate synaptic transmission (Parpura et al. 1994; Hassinger et al. 1995; Araque et al. 1998; Newman and Zahs 1998; Jourdain et al. 2007).

In an effort to understand the mechanism of Ca^{2+}-dependent glutamate release from astrocytes, Montana et al. (2004) and Bezzi et al. (2004) provided evidence for the occurrence of VGLUT in astrocytic small vesicles, suggesting that this release originates from VGLUT-bearing vesicles, in agreement with the initial observation by Fremeau et al. (2002). Observations consistent with this, as well

those supporting an astrocytic exocytotic mechanism, were also made in a variety of other investigations (Crippa et al. 2006; Stenovec et al. 2007; Platel et al. 2010; Bergersen et al. 2012; Ormel et al. 2012b; Mothet et al. 2005; Marchaland et al. 2008; Zhang et al. 2004). These VGLUT-loaded vesicles are localized in astrocytes adjacent to extra-presynaptic terminals that express NMDA receptors (Jourdain et al. 2007; Montana et al. 2004; Bezzi et al. 2004).

However, VGLUT expression in astrocytes proved controversial. Some other laboratories did not detect VGLUT in astrocytes (Aihara et al. 2000; Franke et al. 2006; Graziano et al. 2008). Transcriptome analysis also failed to support astrocytic expression of VGLUT (Cahoy et al. 2008). Moreover, the ketone body acetoacetate, which was found to downregulate glutamate release from neurons (by interfering with chloride activation of VGLUT), did not inhibit glutamate release from astrocytes (Juge et al. 2010). The nonspecific binding was considered an issue (Fritschy 2008; Li et al. 2013). Tissue fixation can alter the conformation of antigen epitopes, reducing affinity for antibodies or generating nonspecific high background. More recent extensive experiments have failed to provide evidence for VGLUT expression in astrocytes (Li et al. 2013).

Evidence is overwhelming for astrocytic exocytosis involving small vesicles in culture. However, in astrocytes, lysosomes appear to be the major intracellular compartment undergoing exocytosis, which occurs at a much slower rate than does neuronal vesicular exocytosis (Li et al. 2008). Lysosomes are expected to accumulate glutamate and aspartate by sialin, a lysosome marker enzyme, capable of transporting not only sialic acid but also glutamate and aspartate (Miyaji et al. 2008). These lines of evidence suggest that the majority of exocytotically released glutamate could come from lysosomes. The functional importance of astrocytic exocytosis (as judged by physiologically relevant quantitative analyses) in information processing in the brain remains to be determined (Hamilton and Attwell 2010).

7.9 Vesicular Aspartate Uptake

Aspartate has been considered an excitatory neurotransmitter candidate (Curtis and Johnston 1974). The putative neurotransmitter role of this amino acid was supported by demonstration of its release in a calcium-dependent manner, an important criterion for the neurotransmitter candidate (Nadler et al. 1976, 1978). Fleck et al. (1993) also showed calcium-dependent aspartate release in CA1 of the hippocampus, suggesting it is achieved by exocytosis. However the molecular mechanism for vesicular storage of this amino acid has remained elusive.

Miyaji et al. (2008) demonstrated that the lysosomal membrane transporter sialin (SLC17A5) (Mancini et al. 1989) (responsible in vivo for removal of sialic acid produced in lysosomes) is able to transport aspartate into liposomes reconstituted with sialin and proton-pump ATPase. The amino acid sequence of sialin had been determined and compared with VGLUT by Verheijen et al. (1999). Sialin/vesicular aspartate transporter has an arginine residue corresponding to

VGLUT1 Arg^{176} and a glutamate residue corresponding to VGLUT1 Glu^{183}, but lacks a histidine residue corresponding to VGLUT1 His^{120}. These three amino acid residues are thought to be all required for glutamate uptake by VGLUT (Juge et al. 2006; Almqvist et al. 2007). Of interest, aspartate uptake is driven by a membrane potential, while sialic acid transport is driven by a pH gradient (Miyaji et al. 2008; Mancini et al. 1989). Similar to glutamate uptake by VGLUT, aspartate uptake exhibits a biphasic response to extravesicular chloride when ATP is used to generate a membrane potential (Miyaji et al. 2008). However, contrary to VGLUT, sialin/aspartate transporter does not distinguish between aspartate and glutamate (Miyaji et al. 2008). It appears that the sialic acid/aspartate transporter occurs in a subset of hippocampal synaptic vesicle populations, as well as in synaptic-like small vesicles in pinealocytes, but not in the majority of brain synaptic vesicles. However, Herring et al. (2015) have recently provided evidence that synaptically released aspartate is not sufficiently highly concentrated to cause preferential activation of the NMDA receptor, thus ruling out a role of aspartate in fast excitatory synaptic transmission.

Given that sialin occurs in lysosomes, aspartate and glutamate would be accumulated in lysosomes in an ATP-dependent manner. Lysosomes are the major vesicular compartment subject to calcium-dependent exocytosis in astrocytes (Li et al. 2008). However, this astrocytic exocytosis occurs at a much slower rate than that of its neuronal counterpart (Li et al. 2008). Thus, the physiological role of these acidic amino acids released from the lysosomes in synaptic transmission remains elusive.

7.10 VGLUT Pathology

VGLUT is essential for survival of mammals and perhaps other vertebrates. Homozygous knockout (KO) of VGLUT1 or VGLUT2 in mice (which would result in little or no glutamate accumulation in synaptic vesicles) is lethal. VGLUT2-lacking mice die immediately after birth as a result of respiratory failure (Moechars et al. 2006). VGLUT1 KO mice die toward the end of the postnatal third week, most likely due to diminished functioning of multiple organs including the pancreas (Wojcik et al. 2004). A variety of evidence indicates that abnormal vesicular glutamate uptake is involved in various types of neurological and mental disorders. Mentioned here are some of the studies indicating association of certain pathophysiologies with aberrant alterations in vesicular glutamate uptake or VGLUT levels.

7.10.1 Epilepsy

Several lines of evidence suggest that excess glutamate loading in synaptic vesicles is involved in some forms of epilepsy. Vesicular glutamate uptake was shown to be elevated region specifically in the cerebrum of a genetic animal model of human

complex partial seizures, in parallel with development of seizure (Lewis et al. 1997). VGLUT1 levels were found to be high in the hippocampus of temporal lobe epilepsy patients (Van der Hel et al. 2009). VGLUT1 in the hippocampus (particularly in the dentate gyrus) was also shown to be overexpressed in a seizure-prone animal model, as well as in animal models with hypoxia-induced and pilocarpin-induced seizures (Kang et al. 2005; Kim et al. 2005, 2007). VGLUT2 is increased in the cortex (but not in the thalamus) of absence-epilepsy animals (Touret et al. 2007), as well as in the hippocampus of an animal model of teratogen-induced seizure (Harrington et al. 2006). These lines of evidence all suggest that brain region-specific vesicular overloading of glutamate and excessive glutamate release underlie certain forms of seizure.

7.10.2 Schizophrenia

In schizophrenics, VGLUT1 mRNA is decreased in the hippocampus and dorsal prefrontal cortex (Eastwood and Harrison 2005), whereas VGLUT2 mRNA expression is increased in the inferior temporal gyrus (Uezato et al. 2009). Inta et al. (2012) have shown that heterozygous VGLUT1 KO mice exhibit abnormal behaviors resembling correlates of schizophrenia, such as working- and social-memory impairments and deficits in prepulse inhibition. Recently, VGLUT1 reduction in prefrontal cortex glutamatergic neurons projecting to the subcortical structure nucleus accumbens (a reward circuit component) has been proposed to underlie schizophrenia's cognitive flexibility deficit, based upon impaired reversal learning observed in heterozygous VGLUT1 KO mice (Granseth et al. 2015). These observations suggest that aberrant alterations in VGLUT1-selective vesicular uptake and release in specific brain substructures are involved in some cognitive dysfunctions apparent in schizophrenics.

7.10.3 Depression

In both major depression and bipolar depression, VGLUT1 mRNA has been reported to be decreased in the entorhinal cortex (Uezato et al. 2009), while VGLUT1 protein is increased in the lateral amygdala (Varea et al. 2012). In the middle temporal gyrus, VGLUT2 mRNA expression is also reduced in patients with major depression (Uezato et al. 2009). In an animal model of corticosterone-induced depression, VGLUT2 protein has been shown to be increased in the hippocampus but not in the amygdala (Lussier et al. 2013). Tordera et al. (2007) have observed that heterozygous VGLUT1 KO mice exhibit enhanced, depressive-like behavior. These lines of evidence suggest the involvement of abnormal vesicular glutamate uptake in specific brain areas in affective disorders as well.

7.10.4 Alzheimer's Disease

Alzheimer's disease (AD) is a complex neurodegenerative disease with severe cognitive dysfunction, its prevalence increasing among the elderly. In this dementia, VGLUT1 is decreased in the prefrontal dorsal cortex (Kashani et al. 2008). This VGLUT1 reduction is thought to play a role in progression of the cognitive decline. Recent studies suggest that hyperexcitability in the hippocampus contributes to the development of AD. Functional MRI shows that elevated hippocampal activation occurs in individuals at risk for AD.

Miller et al. (2008) proposed that aberrant hippocampal hyperactivity is predictive of the degree and rate of subsequent cognitive decline and conversion to AD. Consistent with this, Bakker et al. (2012) have provided evidence that dampening hippocampal hyperactivity in mild cognitive impairments can result in cognitive improvement. The notion that hippocampal hyperactivation is an early indicator of AD development is also supported by Putcha et al. (2011), demonstrating that hippocampal hyperactivation in mild cognitive impairments is well correlated with degeneration of "AD-signature" regions in the cortex. Compatible with these lines of evidence, Vossel et al. (2013) observed that the absence of epilepsy in patients with mild cognitive impairments and AD retards cognitive decline.

The hallmark pathology of this disease is abundant accumulation of extracellular β-amyloid aggregates and intracellular neurofibrillary tangles containing hyperphosphorylated tau protein. Recently, tau has been shown to mediate β-amyloid-induced cognitive deficits and hyperexcitability in the hippocampus (Roberson et al. 2007, 2011). Tau-induced pathology initially occurs in the entorhinal cortex, followed by the hippocampus, preceding spread to other regions (de Calignon et al. 2012). In a more recent study of a tau mouse model of Alzheimer's disease (TauP301L), Hunsberger et al. (2015) have shown that this mutated, misfolded tau (which coaggregates with endogenous tau) induces increased VGLUT1 expression in the hippocampus. This raises an interesting possibility that VGLUT1 over-expression could be involved in hippocampal hyperexcitability observed near the onset of AD.

7.10.5 Parkinson's Disease

In Parkinson's disease, VGLUT1 and VGLUT2 in the putamen are moderately increased, whereas VGLUT1 is substantially decreased in the prefrontal and temporal cortex; these alterations are thought to contribute to the cognitive impairments associated with the disease (Kashani et al. 2007). In an MPTP-treated monkey model of Parkinsonism, VGLUT1 in corticostriatal nerve terminals was found increased, whereas VGLUT2 in thalamostriatal nerve terminals was not changed (Raju et al. 2008). In MPTP-treated mice, Pflibsen et al. (2015) have recently reported that VGLUT1 is elevated in the dorsolateral striatum as well as in

the medial prefrontal cortex, whereas VGLUT2 is diminished in the dorsolateral striatum. Using a 6-hydroxydopamine-treated rat model, Favier et al. (2013) have provided evidence suggesting that reduction of basal ganglia VGLUT2, induced by dopamine depletion, is involved in genesis of the motor deficit associated with the disease, particularly at the advanced stage. Divito et al. (2015) have recently reported interesting findings, suggesting that VGLUT3 can affect neural mechanisms underlying motor deficit and dyskinesia in a mouse model of Parkinson's disease, raising the possibility of a novel therapeutic target.

7.10.6 Pain

In morbidity associated with inflammation, nerve injury, and trauma, mechanical pain hypersensation plays a major role. Using global VGLUT3 knockout mice, Seal et al. (2009) provided evidence suggesting that this is mediated by a small subset population of sensory neurons in the spinal cord dorsal root ganglia containing VGLUT3. Lou et al. (2013), however, did not observe major changes in acute and chronic mechanical pain in VGLUT3-depleted mice; these mice were produced through knockout of a transcription factor, which controls development of unmyelinated sensory neurons including pain-related nociceptors as well as VGLUT3 expression. Experiments with heterozygous VGLUT2 KO mice suggest that VGLUT2 plays a role in neuropathy-induced allodynia and hypersensitivity (Leo et al. 2009). Using conditional VGLUT2 KO mice, Liu et al. (2010) have provided evidence indicating that VGLUT2 in nociceptor-bearing neurons in dorsal root ganglia mediates a variety of pain sensations, including mechanical, thermal, capsaicin-evoked, inflammatory, and neuropathic. Recent evidence suggests that VGLUT2 in the thalamus, periaqueductal gray, and amygdala is involved in neuropathic pain induction (Wang et al. 2015). The involvement of VGLUT2 and VGLUT3 in nociception and pain has been extensively reviewed by Brumovsky (2013).

7.11 Summary and Concluding Remarks

Vesicular glutamate uptake is now recognized as a pivotal step in glutamate synaptic transmission, allowing glutamate to enter the neurotransmitter pathway away from its metabolic pathway. This process requires ATP and a low millimolar concentration of extravesicular chloride or high intravesicular chloride and is highly specific to glutamate. The vesicular uptake system is composed of a vesicular glutamate transporter, VGLUT, and v-type proton-pump ATPase, which generates an electrochemical proton gradient, the driving force for active transport into intracellular organelles. Energy harnessed by v-type proton-pump ATPase is likely to be largely supplied by a vesicle-bound, glycolytic ATP-synthesizing enzyme complex, GAPDH/3-phosphoglycerate kinase. The VGLUT substrate glutamate is postulated

to be synthesized by vesicle-bound AAT from α-ketoglutarate at the expense of aspartate. The majority of α-ketoglutarate would derive from mitochondria where astrocyte-derived glutamine is converted to glutamate then to α-ketoglutarate. The presence of both ATP- and glutamate-generating enzymes in the synaptic vesicle would represent an efficient mechanism for vesicular glutamate loading.

VGLUT has three isoforms. Their biochemical properties are similar, but in general they have complementary distributions not only in nervous tissue but also in nonnervous tissue, serving various physiological functions, from learning and memory formation to visual and auditory sensing as well as respiratory control and mechanical sensation. As such, aberrant VGLUT expression or vesicular glutamate uptake is involved in various types of neurological pathophysiologies.

Under physiological conditions, freshly endocytosed synaptic vesicles would take up glutamate at the expense of a high concentration of lumen chloride as well as of a membrane potential generated by H^+-pump ATPase. As the intravesicular chloride concentration decreases, a cytosolic low concentration of chloride (in the presence of a membrane potential) would begin to play a significant role in glutamate uptake by forming a pH gradient as well as activating VGLUT upon allosteric binding. An optimal intravesicular pH produced could also contribute to Cl^--enhanced glutamate uptake. Moreover, cytosolic K^+ could elevate vesicular glutamate accumulation through K^+/H^+ exchange.

In view of VGLUT involvement in a number of neurological pathophysiologies, drug development targeting VGLUT should be explored. Several types of inhibitors which act on VGLUT were discovered. The most potent and specific are Trypan Blue and Brilliant Yellow, with IC_{50} in the 20–50 nM range. However, these agents are not membrane permeable. Moreover, although they are specific to VGLUT, their selectivity among VGLUT isoforms remains to be determined.

7.11.1 Anticipated Developments

(a) Membrane-permeable VGLUT isoform-specific inhibitors and activators, which could serve as prototype therapeutic agents for treatment of certain pathological conditions involving aberrant hyper- or hypoglutamate neurotransmission.
(b) Membrane-permeable VGLUT isoform-specific agents useful for noninvasive imaging of VGLUT isoform-specific glutamatergic nerve endings in various brain regions and nonnervous tissue.
(c) Determination of the three-dimensional structure of VGLUTs and ultimately elucidation of the glutamate transport mechanism at the atomic level.

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Chapter 8
The Glutamine Transporters and Their Role in the Glutamate/GABA–Glutamine Cycle

Renata Leke and Arne Schousboe

Abstract Glutamine is a key amino acid in the CNS, playing an important role in the glutamate/GABA–glutamine cycle (GGC). In the GGC, glutamine is transferred from astrocytes to neurons, where it will replenish the inhibitory and excitatory neurotransmitter pools. Different transporters participate in this neural communication, i.e., the transporters responsible for glutamine efflux from astrocytes and influx into the neurons, such as the members of the SNAT, LAT, y$^+$LAT, and ASC families of transporters. The SNAT family consists of the transporter isoforms SNAT3 and SNAT5 that are related to efflux from the astrocytic compartment, and SNAT1 and SNAT2 that are associated with glutamine uptake into the neuronal compartment. The isoforms SNAT7 and SNAT8 do not have their role completely understood, but they likely also participate in the GGC. The isoforms LAT2 and y$^+$LAT2 facilitate the exchange of neutral amino acids and cationic amino acids (y$^+$LAT2 isoform) and have been associated with glutamine efflux from astrocytes. ASCT2 is a Na$^+$-dependent antiporter, the participation of which in the GGC also remains to be better characterized. All these isoforms are tightly regulated by transcriptional and translational mechanisms, which are induced by several determinants such as amino acid deprivation, hormones, pH, and the activity of different signaling pathways. Dysfunctional glutamine transporter activity has been associated with the pathophysiological mechanisms of certain neurologic diseases, such as Hepatic Encephalopathy and Manganism. However, there might also be other neuropathological conditions associated with an altered GGC, in which glutamine transporters are dysfunctional. Hence, it appears to be of critical importance that the physiological and pathological aspects of glutamine transporters are thoroughly investigated.

Keywords Glutamine transporters • Glutamate/GABA–glutamine cycle • SNAT • LAT • y$^+$LAT • ASC

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Abbreviations

AAR	Amino acid response
AARE	AAR elements
APC	Amino acid- polyamine- and organocations family
BCH	2-Aminobicyclo-(2-2-1)-hepatane-2-caboxylic acid
CATs	Cationic amino acid transporters
CNS	Central nervous system
DHT	Dihydrotestosterone
EAAT	Excitatory amino acid transporters
EGFR	Epidermal growth factor receptor
eIF2α	Eukaryotic initiation factor 2α
ERK	Extracellular regulated kinase
FXR	Farnesoid X receptor
GABA	Gamma-aminobutyric acid
GAT	Gamma-aminobutyric acid transporter
GGC	Glutamate/GABA–glutamine cycle
GS	Glutamine synthetase
HATs	Heteromeric amino acid transporters
HE	Hepatic encephalopathy
IPSC	Inhibitory postsynaptic potential
LATs	L-type amino acid transporters
L-DOPA	Dihydroxyphenylalanine
MAP2	Anti-micro-tubule-associated protein-2
MAPK	Mitogen-activated protein kinase
MeAIB	α-Methylamino-iso-butyric acid
Mn	Manganese
PAG	Phosphate-activated glutaminase
PI3K	Phosphatidylinositol 3-kinase
PKB	Protein kinase B
PMA	Phorbol-12-myristate-13-acetate
SGK	Glucocorticoid inducible kinase
SNAT	Solute neutral amino acid transporters
TCA	Tricarboxylic acid
TMB	Transmembrane domain
UTR	3′-untranslated region
vGAT	Vesicular gamma-aminobutyric acid transporter

8.1 Introduction

Glutamine is one of the most important amino acids in the human body, participating in several pathways and functions, such as ammonia detoxification and the production of urea in the liver, acid-base buffering in the kidneys, and as nitrogen

donor for other amino acids, purines, pyrimidines, and amino sugars (Bode 2001; McGivan and Bungard 2007). It also serves as an energy substrate since its carbon skeleton gets access to the tricarboxylic acid cycle (TCA), as described for the intestinal epithelia as well as the liver and brain (Ziegler et al. 2000; Verrey et al. 2009; Pochini et al. 2014; Schousboe et al. 2014a). Its vital role is underlined by its abundancy in the human body, where concentrations range from 2.0 to 20 mM intracellularly and 0.2 to 0.8 mM in the extracellular compartment (Bode 2001; Newsholme et al. 2003). In the central nervous system (CNS) specifically, extracellular glutamine concentrations are very low ranging from 0.13 to 0.5 mM, being the upper concentration similar to that found in the CSF in rodents and humans (Erecińska and Silver 1990; Aasly et al. 1997; Xu et al. 1998; White et al. 2004). The intracellular glutamine concentrations vary in the range 5–10 mM and can even reach 20 mM in cultured astrocytes and neurons when exposed to media containing 2 mM glutamine (Patel and Hunt 1985; Brookes 1992). This important amino acid participates in several pathways, including scavenging of ammonia, as a nitrogen donor, maintenance of redox state by glutathione, and importantly, in the glutamate/gamma-aminobutyric acid–glutamine cycle (GGC) (Bröer and Brookes 2001; Bak et al. 2006). In this cycle, the transfer of glutamine from astrocytes to neurons is a key point for replenishment of the amino acid neurotransmitter pools in the neuronal compartment, and its proper function and regulation rely upon specific transporters (Bak et al. 2006; Schousboe et al. 2013).

For that reason, the glutamine transporters are of fundamental importance in sustaining the GGC properly and they have been extensively investigated in several studies. As it will be discussed in the present chapter, glutamine carriers are known as solute neutral amino acid transporters (SNAT), system L, system y^+L, and ASC, and the isoforms of these carriers are expressed and regulated differently, depending upon the cellular compartment in which they are expressed as well as the intra and extracellular environments. Other transporters expressed in the brain have affinity for glutamine, as the isoforms B0AT2 and arpAT, but they are not involved in the GGC and therefore will not be addressed in this chapter (Pochini et al. 2014; Fotiadis et al. 2013).

Considering the importance of the GGC in the maintenance and optimal function of the excitatory and inhibitory neurotransmitter systems, it is not surprising that disturbances in the cycle have been related to different neurologic diseases. However, many of these diseases have been linked mainly to defects in the functioning of the high affinity transporters for glutamate and gamma-aminobutyric acid (GABA) as well as to derangements in the activity of glutamine synthetase (GS). However, alterations in the activity and/or expression of glutamine transporters are likely to play a significant role in the etiology of such diseases as well as the neurological symptoms associated with hepatic encephalopathy. The present chapter will provide a detailed account of the expression and regulation of the different glutamine transporters as well as a discussion of their importance for GGC in brain diseases as well as under physiological conditions.

8.2 Glutamine Transporters

Studies by Oxender and Christensen (1963) were the first to describe the existence of two different transporters called system L and A that were able to transfer glutamine between cellular compartments. The nomenclature, system L and A refers to their preference to carry the amino acids leucine and alanine, respectively (Collarini and Oxender 1987). Later, it was described that system L likely does not contribute significantly to glutamine transport across the plasma membrane in most cells, since this transporter is Na^+ independent and works as an exchanger rather than performing net uptake (Chaudhry et al. 2002a). However, system L activity has been described as part of the machinery for glutamine exchange in the CNS, which will be discussed in more detail later. System A plays an important role in glutamine transport, but also has affinity for small aliphatic amino acids. These transporters are Na^+ dependent and therefore can work against cellular concentration gradients in a concentrative fashion. Subsequently, another Na^+-dependent glutamine transport system was discovered and this was originally classified as being constituted by two isoforms Nm and Nb, found in muscle and brain tissue, respectively (Tamarappoo et al. 1997). Later, a carrier with similar functional characteristics and affinity for glutamine and to a lesser extent histidine and asparagine was found in hepatocytes. This transporter was described as being the main, if not the solely, carrier of glutamine in this tissue (Kilberg et al. 1980).

System N and A have since been thoroughly studied and different isoforms for these systems have been discovered and characterized. Both are of great importance for the transfer of glutamine into different cellular compartments, and therefore participating in the communication between neurons and astrocytes. Other transporters that have affinity for glutamine were also described in the CNS, as system L, system y^+L, and ASC, but their importance for regulating glutamine concentrations in the CNS and participation at the GGC is controversial.

System N and A isoforms, which are now denominated solute neutral amino acid transporters (SNAT), have been originally classified based on their substrate affinity, ion dependence, tolerance to Na^+ substitution by Li^+, kinetics and regulatory properties, and pH sensitivity (Bode 2001). However, in 1999 the first isoform of system N (SNAT3) was cloned, which led to the molecular characterization and better understating of its function and classification (Chaudhry et al. 1999). The cloning strategy for SNAT3 was based on its homology to the vesicular inhibitory amino acid transporter (VIAAT) family, i.e., the vesicular gamma-aminobutyric acid transporter (vGAT). SNAT3 shares the characteristics of vGAT such as cotransport of Na^+ and the exchange of lumenal protons by cytoplasmatic transmitters/amino acids. However, SNAT3 was found to be expressed in the cellular plasma membrane in contrast to vGAT, that is present in the vesicular membranes (Chaudhry et al. 1999; Gu et al. 2000). Subsequently, SNAT1 was cloned also facilitated by its sequence similarity to vGAT (Varoqui et al. 2000), followed by the other isoforms SNAT2, SNAT4, SNAT5, SNAT7 and most recently SNAT8 and based on the previously described functional characteristics, these isoforms were classified into a new family of proteins (Sugawara 2000; Yao et al. 2000; Varoqui et al. 2000; Nakanishi et al. 2001a; Hägglund et al. 2011, 2015).

Table 8.1 Glutamine transporters

Gene name	Protein name	Alias	System	Mechanism of transport	Substrate affinity
SLC38A1	SNAT1	SAT1, ATA1, SA2, NAT2	System A	Na^+-Glutamine cotransport (electrogenic)	A, S, C, M, H, Q, N
SLC38A2	SNAT2	SAT2, ATA2, SA1	System A	Na^+-Glutamine cotransport (electrogenic)	A, N, C, Q, G, H, M, P, S
SLC38A3	SNAT3	SN1, NAT	System N	Na^+-Glutamine/H^+ antiport (electroneutral)	Q, H, N
SLC38A4	SNAT4	ATA3, NAT3, SAT3	System A	Na^+-Glutamine cotransport (electrogenic)	A, N, C, G, S, T
SLC38A5	SNAT5	SN2	System N	Na^+-Glutamine/H^+ antiport (electroneutral)	G, N, A, S, Q, H
SLC38A7	SNAT7	–	System N		Q, H, A, S, N
SLC38A8	SNAT8	–	System A	Na^+-Glutamine/H^+ antiport (electroneutral)	Q, N, A, H, S
SLC7A5	LAT1	–	System L	Glutamine/amino acid antiport	Large neutral and aromatic amino acids (W,F, L, H, Q, T)
SLC7A8	LAT2	–	System L	Glutamine/amino acid antiport	Neutral amino acids
SLC7A7	y+LAT1	–	System y+L	Na^+-Glutamine/cationic amino acid antiport	Cationic and large neutral amino acids
SLC7A6	y+LAT2	–	System y+L	Na^+-Glutamine/cationic amino acid antiport	Cationic and large neutral amino acids
SLC1A5	ASCT2	–	System ASC	Glutamine/amino acid antiport	Neutral amino acids

Description of the different glutamine transporters and their gene family, mechanism of transport, and substrate affinity. The different nomenclatures used for the glutamine transporters are shown in the Alias column. *A* alanine, *C* cysteine, *F* phenylalanine, *G* glycine, *H* histidine, *L* leucine, *M* methionine, *N* Asparagine, *P* proline, *Q* glutamine, *S* serine, *T* threonine, *W* tryptophan

The glutamine transporters belong to the solute carriers 38 (SLC38) gene family and thus the superfamily of amino acid and auxin permeases (AAAP). The SLC38 family is composed of 11 members described in the human genome, 7 of which have been characterized in more detail (Table 8.1). The vesicular GABA transporter, which was the starting point for cloning the glutamine transporter, is part of the SLC32 gene family (Mackenzie and Erickson 2004). The SLC36, constituted by the proton-coupled amino acid transporters located in lysosomes and plasma membranes is also part of the AAAP superfamily (Boll et al. 2004).

The glutamine transporter isoforms have been given different nomenclature through the years, due to the simultaneous studies focusing on characterizing these proteins. However, with the advent of molecular characterization with the information obtained from the functional studies, the isoforms for glutamine transporters belonging to System N or A were accordingly identified and classified, and currently their nomenclature is based on the chronologic order in which they were discovered (Mackenzie and Erickson 2004). As seen in Table 8.1, which shows the current and older nomenclature used for these proteins, System A is constituted by SNAT1, SNAT2, SNAT4, and recently SNAT8, while System N includes SNAT3, SNAT5, and SNAT7. Their particular characteristics, focusing on those functionally related to the GGC, will be discussed in more detail in the following sections.

Regarding the protein structure of SCL38 members, their predicted topology suggests a protein with an intracellular N terminus, 11 transmembrane helices, and an extracellular C terminus (Pochini et al. 2014; Mackenzie and Erickson 2004). Studies that employed the crystallographic structure of hydantoin transporter Mhp1 as a template, transporter belonging to the APC family and sharing homology based on profile alignment and secondary structure prediction, predicted that the transmembrane domain (TMD) 1 contributes to a conserved Na^+ binding site (Bröer et al. 2009). In addition, mutations in the asparagine 76 of SNAT3, which is located in the TMD1 abolished all substrate-induced currents (Bröer et al. 2009). Mutation of N82A of SNAT2, which is localized at TMD1, also leads to complete depletion of the transporter currents and amino acid uptake (Zhang et al. 2008).

As previously mentioned, system L was identified due to its preference for leucine as substrate, but it also has affinity for other neutral amino acids such as glutamine. Contrary to system A, system L is characterized by being Na^+ independent and functioning as an antiporter of intracellular and extracellular amino acids, having affinity for branched chain and bulky neutral amino acids (Kanai et al. 1998; Fotiadis et al. 2013). The system L isoforms started to be identified molecularly in 1998 and were subsequently classified as part of the SLC7 gene family (Kanai et al. 1998; Mastroberardino et al. 1998; Rajan et al. 2000). This family of proteins is formed by two distinct subfamilies, which are named cationic amino acid transporters (CATs) and light or catalytic subunits (L-type amino acid transporters—LATs), of the heteromeric amino acid transporters (HATs) (Fotiadis et al. 2013). Both CATs and LATs are part of the large amino acid-polyamine- and organocations-(APC) family. With respect to LATs, they are known as glycoprotein associated transporters, due to the covalently association with a heavy subunit (Verrey et al. 2000). The heavy subunit can be the glycoprotein named 4F2hc, which is important for routing the transporters to the plasma membrane, whereas the light subunit is necessary for the transport activity (Palacín and Kanai 2004; Fotiadis et al. 2013). At the moment, six LATs have been identified as heterodimers with 4F2hc, i.e., LAT1 (SLC7A5), LAT2 (SLC7A8), y^+LAT1 (SLC7A7), y^+LAT2 (SCL7A6), Asc-1 (SLC7A10), and xCT (SLC7A11), all belonging to the SLC7 family. It is important to mention that the isoforms LAT1 and LAT2 are part of System L, while y^+LAT1 and y^+LAT2 constitute System y^+LAT (Table 8.1). The latter, however, represents a different transport system, mediating the exchange of cationic amino acids and neutral amino

acids in the presence of sodium (Bröer et al. 2000a; Kanai et al. 2000). From all members mentioned earlier, only those related to GGC will be discussed in more detail in the following sections.

Regarding the protein structure for the LAT proteins, as described for LAT1, a 12 TMD was predicted that is linked with the heavy subunit 4F2hc through a conserved disulfide bridge. They exhibit an intracellular terminus and are not glycosylated (Fotiadis et al. 2013). The heavy subunit is described as type II membrane N-glycoproteins, which have an intracellular N-terminal and a bulky extracellular C-terminal (Fort et al. 2007).

System ASC was originally named on the basis of its affinity for the substrates alanine, serine, and cysteine (ASC), to differentiate it from System A activity (Christensen et al. 1967). This family of transporters is characterized by the affinity for neutral amino acids and as a transport system being Na^+ dependent. The first transporter gene was isolated in 1996 from mouse testis and from human choriocarcinoma (Kekuda et al. 1996; Utsunomiya-Tate et al. 1996), and subsequently classified as part of the gene family SLC1. This gene family encompasses the five high affinity glutamate transporters (SLC1A1, SLC1A2, SLC1A3, SLC1A6, and SLC1A7) and two neutral amino acid transporters, ASCT1 (SLC1A4) and ASCT2 (SLC1A5). Among the neutral amino acid transporters, ASCT2 has affinity for glutamine, whereas ASCT1 does not transport glutamine (Shafqat et al. 1993; Arriza et al. 1993). The structure for these proteins as determined subsequent to the cloning revealed a protein with 6N-terminal domains followed by a C-terminal domain. Although the crystal structure for these transporters is unknown, it is predicted that the C terminal domain is the one that has the binding site for the amino acid to be transported (Albers et al. 2012).

8.2.1 Glutamine Transporters: Isoforms, Function, and Localization

8.2.1.1 System N, Isoforms SNAT3, SNAT5, and SNAT7

System N isoforms are characterized by a narrower substrate specificity, with affinity for glutamine, asparagine, and histidine (Chaudhry et al. 1999; Fei et al. 2000; Nakanishi et al. 2001a). These carriers function as a Na^+-glutamine symporter and H^+ antiporter, a characteristic that distinguishes them from the isoforms belonging to system A (Chaudhry et al. 1999, 2001; Bröer et al. 2002). The carriers that are members of this system also have the particular property of high tolerance to the substitution of Na^+ by Li^+ for the amino acid cotransport (Chaudhry et al. 1999; Fei et al. 2000; Nakanishi et al. 2001a, b). This feature has for a long time been used to distinguish system N from system A but later studies demonstrated that SNAT1, SNAT2, and SNAT4 also tolerate Li^+ (Albers et al. 2001; Chaudhry et al. 2002b; Gu et al. 2001a). However, more recent studies have been inconclusive regarding the tolerance of system A transporters to Li^+. As discussed by Mackenzie and coworkers, it is

likely that the affinity for Li^+ decreases upon membrane depolarization to a higher extent than that observed for Na^+ (Mackenzie and Erickson 2004; Mackenzie et al. 2003). Therefore, this criterion should be carefully taken into account when evaluating the isoforms of glutamine transporters with respect to their functionality (Chaudhry et al. 2002a; Gu et al. 2001a; Hägglund et al. 2011). System N also has the property of marked inhibition by low pH environments, with impairment of influx transport as external pH is lowered (Kilberg et al. 1980; Gu et al. 2001a).

When the first isoform of the glutamine transporter SNAT3 was cloned and characterized, it was revealed that it resembled the proteins that belong to System N, due to its properties regarding Na^+-dependent transport, affinity to glutamine, histidine, and asparagine and the fact that it is found highly expressed in hepatocytes (Chaudhry et al. 1999; Gu et al. 2000). The SNAT3 gene encodes a protein of 503 amino acids and exhibits a K_m for glutamine of 2.4 mM at pH 7.0 (Chaudhry et al. 1999; Bröer et al. 2002). Its mRNA was found expressed predominantly in liver, but also in brain, kidney, and heart (Chaudhry et al. 1999; Gu et al. 2000). In the brain, specifically, SNAT3 is found in the neocortex, cerebellum, olfactory bulb, and brain stem. At the cellular level, this isoform is abundant in astrocytes, while it is absent from neurons and oligodendrocytes (Boulland et al. 2002, 2003). With respect to transport activity, it is currently agreed that this isoform mediates the exchange of 1 glutamine molecule together with 1 Na^+ for the cotransport of 1 H^+, and therefore it acts as an electroneutral transporter (Bröer et al. 2002; Chaudhry et al. 2001). It is important to mention that ionic inward currents were observed when the SNAT3 transporter was expressed in *Xenopus oocytes*, a finding leading to confusion about the stoichiometry of this carrier. However, these currents turned out to be a consequence of uncoupled H^+ fluxes through the transporter, which were not related to the mechanisms of the amino acid transport per se (Chaudhry et al. 2001; Schneider et al. 2007). This uncoupled proton flux might be of importance for restoration of the intracellular pH thereby securing the correct amino acid transporter activity in between cellular compartments (Chaudhry et al. 2001). In addition, it was found that other cations also elicit these currents, in a fashion dependent on or independent of substrate, but its relevance is still unknown and further investigations are needed to elucidate its physiological importance (Hamdani et al. 2012; Schneider et al. 2007).

Subsequently, SNAT5 and SNAT7 were also shown to be part of system N due to their molecular and functional characteristics (Nakanishi et al. 2001a; Hägglund et al. 2011). The SNAT5 gene encodes a protein of 471 amino acids and has a sequence homology to SNAT3 of about 63 %, for the isoforms described in rats (Nakanishi et al. 2001a). Although SNAT5 shares genetic similarity with SNAT3, it differs with respect to substrate specificity, since it accepts a broader spectrum of amino acids such as glycine, asparagine, alanine, serine, glutamine, and histidine (Nakanishi et al. 2001a). With regard to glutamine, SNAT5 has a K_m of 4.1 mM, as demonstrated in the murine isoform of this transporter. It is found expressed in different organs, including brain, lung, intestines, and spleen (Nakanishi et al. 2001a), and it is described as an electroneutral transporter, i.e., analogous to its family counterpart SNAT3. In the CNS, SNAT5 is ubiquitously expressed, with higher levels in the neocortex, hippocampus, and striatum and at the cellular level, this transporter is found in astrocytes (Cubelos et al. 2005).

The isoform SNAT7 was more recently characterized as a member of system N, due to its characteristic of substrate preference toward glutamine and histidine and due to its insensitivity toward MeAIB (α-methylamino-iso-butyric acid), which is the hallmark inhibitor of the isoforms belonging to system A (Christensen et al. 1965; Hägglund et al. 2011). In addition to the amino acids mentioned earlier, SNAT7 also has affinity for other amino acids, such as alanine, serine, and asparagine (Hägglund et al. 2011). This transporter is Na^+ dependent as also described for the other members of the system N family. However, contrary to SNAT3 and SNAT5, this carrier does not tolerate Na^+ substitution by Li^+. This supports the concept that this parameter, i.e., substitution of Na^+ to Li^+, may not alone be adequate to characterize the carrier isoforms (Hägglund et al. 2011). Its gene encodes a protein of 461 amino acids and it is markedly expressed in the liver and brain. In the CNS it is ubiquitously expressed, with highest abundance in hypothalamus, basolateral amygdala, ventral tegmental area, and locus coeruleus (Hägglund et al. 2011). At the cellular level, SNAT7 is present in layers in the cortex and at the dentate gyrus and pyramidal cell layers in the hippocampus, zones that are rich in neurons. In this context, it was demonstrated that this isoform is predominantly present in the neuronal compartment, while it is absent from astrocytes (Hägglund et al. 2011).

8.2.1.2 System A, Isoforms SNAT1, SNAT2, SNAT4, and SNAT8

System A is constituted by the amino acid carriers that are Na^+-dependent electrogenic transporters. Contrary to the isoforms belonging to system N, System A transporters do not exhibit electroneutral transport, as they transport Na^+ together with the neutral amino acid without H^+ exchange (Oxender and Christensen 1963; Collarini and Oxender 1987). Therefore, System A works in a concentrative fashion against transmembrane concentration gradients, as demonstrated by its susceptibility to cell depolarization (Chaudhry et al. 2002b). With respect to the substrates accepted by System A, these include a broader range of aliphatic amino acids, such as alanine, asparagine, cysteine, glutamine, glycine, methionine, and serine (Mackenzie and Erickson 2004), i.e., differently from system N that has a restricted substrate preference.

The isoforms of system A are characterized by the competitive inhibition by a nonmetabolizable substrate analog MeAIB (Christensen et al. 1965; Christensen 1990). Such glutamine transport inhibition has been demonstrated in oocytes expressing SNAT1 (Varoqui et al. 2000), in cultured neurons (Su et al. 1997) and in rat brain in vivo (Kanamori and Ross 2006). Therefore, this analogous substrate has been used as a functional marker to distinguish the isoforms that constitute system A from those that belong to system N (Christensen et al. 1965). However, the use of MeAIB has its limitations, since it has been demonstrated that at lower concentrations it does not inhibit amino acid transport, resulting in confusion with respect to subfamily classification (Gu et al. 2001a). Therefore, the combination of functional and molecular characteristics constitutes the safer way of classifying these amino acid transporter isoforms. In analogy to system N, system A is influenced by pH, having its activity decreased at low extracellular pH, due to the competitive inhibition of H^+ to Na^+ transporter activation (Albers et al. 2001; Chaudhry et al. 2002b).

The isoform SNAT1, the gene of which encodes a protein of 486 amino acids, has glutamine as the preferred substrate (K_m of 230–300 μM), but it also transports alanine, cysteine, and asparagine (Varoqui et al. 2000; Chaudhry et al. 2002b; Albers et al. 2001). In addition, it has affinity for histidine, methionine, and serine, but since the concentration of these amino acids is very low in the cerebral spinal fluid, in comparison of that to glutamine, it is likely that under physiological conditions they are not transported by SNAT1 (Albers et al. 2001; Varoqui et al. 2000; Chaudhry et al. 2002b; Mackenzie et al. 2003). This isoform is mainly expressed in brain, but it has also been described at lower levels in heart and colon tissues (Varoqui et al. 2000; Chaudhry et al. 2002b). Other studies also verified SNAT1 in retina and placenta (Gu et al. 2001b; Wang et al. 2000). In the CNS, SNAT1 is found widespread with greater expression in the cerebellum and thalamus, followed by brainstem, cerebral cortex, hippocampus, and striatum (Varoqui et al. 2000; Melone et al. 2004). In terms of its cellular localization, it was demonstrated that this glutamine transporter isoform is mainly related to the neuronal compartment, but also described in ependymal cells lining the ventricles as well as in astrocytes (Varoqui et al. 2000; Melone et al. 2004; Mackenzie et al. 2003). Regarding its functional activity, the SNAT1 isoform has been well characterized as a unidirectional transporter that couples substrate with Na^+ in a 1:1 stoichiometry ratio (Albers et al. 2001; Mackenzie et al. 2003). As mentioned previously, the isoforms of system A exhibit sensitivity toward low pH, a characteristic shared by SNAT1, but interestingly it also exhibited greater glutamine uptake at pH 8 when compared to more acid environments (Varoqui et al. 2000). The effect of H^+ apparently results from reducing the affinity to Na^+, subsequently affecting the transporter activity, likely as a result of an allosteric effect. This is in agreement with the fact that when studying the transporter functionality it was verified that Na^+ binding is voltage dependent and occurs prior to the substrate biding and subsequent transport across the membrane (Mackenzie et al. 2003).

The isoform SNAT2, the gene of which encodes a protein with 506 amino acids, shares a very similar spectrum of the amino acids for which SNAT1 has affinity, albeit SNAT2 has a lower affinity for glutamine (K_m 1.65 mM) and higher capacity to transport alanine in comparison to SNAT1 (Reimer et al. 2000; Yao et al. 2000). In addition, SNAT2 demonstrated affinity toward proline, while this amino acid is almost not recognized by SNAT1 (Yao et al. 2000; Mackenzie et al. 2003). This carrier has a widespread expression, being found in all tissues in which its expression was tested for (Yao et al. 2000; Reimer et al. 2000). By Northern blot analysis, SNAT2 mRNA was found in heart, adrenal, skeletal muscle, stomach, lung, brain, spinal cord, kidneys, and adipose tissues, with lower expression in the spleen (Yao et al. 2000). With respect to its cellular localization in the CNS, SNAT2 protein is detected in neuron-rich cultures, while absent in astrocyte-rich cultures (Yao et al. 2000). However, a study employing in situ hybridization demonstrated mRNA expression for this transporter in certain glial cells and cells that are part of the blood–brain barrier (Reimer et al. 2000; Takanaga et al. 2002). Its functional characteristics are analogous to those described for SNAT1, i.e., electrogenic with a stoichiometry 1:1 substrate to Na^+ (Yao et al. 2000; Chaudhry et al. 2002b) and SNAT2 is inhibited by low pH by the same mechanisms as those described for its counterpart SNAT1 (Chaudhry et al. 2002b).

A third transporter SNAT4, a protein with 546 amino acids, is predominantly expressed in liver and bladder (Hatanaka et al. 2001; Sugawara et al. 2000). It is a carrier of short neutral amino acids and has low affinity for glutamine (Hatanaka et al. 2001; Gu et al. 2001a). Its mechanism of substrate transport is the same as that for the other members of this subfamily of transporters (Hatanaka et al. 2001). Since this isoform is not expressed in the CNS, its functional characteristics and role will not be discussed further.

Another isoform was recently characterized and named SNAT8. This carrier demonstrated higher preference for glutamine, alanine, arginine, histidine, and aspartate, using a Na^+-dependent cotransport mechanism of action (Hägglund et al. 2015). When analyzed in the mouse brain, SNAT8 was found expressed in the gray matter, highly expressed in thalamus, hypothalamus, amygdale, and pons, while its expression was lower in hippocampus, cerebellum, and layers of the cortex (Hägglund et al. 2015). This recently discovered isoform is found exclusively expressed in neurons, as demonstrated by in situ hybridization combined with immunohistochemistry (Hägglund et al. 2015). This expression pattern is different from that of SNAT1 and SNAT2, isoforms being present in both neurons and astrocytes. The mechanisms of transport appear similar to those described for system A isoforms, using a Na^+-dependent substrate transport and also showing intolerance to Na^+ substitution by Li^+, as demonstrated in studies using oocytes overexpressing SNAT8 (Hägglund et al. 2015). Interestingly, when flux studies employing MeAIB were performed it was observed that positive currents are generated for this transporter, contrary to the other members for this subfamily. The reason why this isoform specifically is not inhibited by MeAIB is not clear, and due to the transporter substrate profile and mechanism of transport SNAT8 was classified as part of the System A subfamily (Hägglund et al. 2015).

8.2.1.3 System L, Isoforms LAT1 and LAT2

System L is constituted by the carriers LAT1 and LAT2 that facilitate amino acid exchange in a Na^+-independent manner, with affinity toward large neutral amino acids, with a 1:1 stoichiometry for the amino acids (Kanai et al. 1998; Rossier et al. 1999). The light subunit LAT1 gene was the first one among the HATs to be cloned and characterized (Kanai et al. 1998; Mastroberardino et al. 1998). It was isolated from both rats and humans, and the only protein that has been characterized until now consists of 507 amino acids (Kanai et al. 1998; Mastroberardino et al. 1998). The isoform LAT1 is a heterodimer (4F2hc/LAT1) that exerts the exchange of large neutral amino acids in a Na^+- and pH-independent manner, demonstrating high affinity for tryptophan, phenylalanine, leucine, and histidine as substrates (Del Amo et al. 2008). This isoform has lower affinity for glutamine and threonine, and it does not recognize alanine, proline, and charged amino acids (Mastroberardino et al. 1998). In addition, the LAT1 transporter is selectively inhibited by 2-aminobicyclo-(2-2-1)-hepatane-2-caboxylic acid (BCH), which is a nonmetabolizable compound (Meier et al. 2002; Kim et al. 2008). LAT1 is an obligatory exchanger; however,

intracellular amino acid concentrations control its activity due to a high intracellular apparent affinity, in comparison to a low extracellular apparent affinity. Therefore, when in combination with other transporter systems, such as System A, it can participate in regulating the amino acid equilibrium between intra and extracellular compartments (Verrey et al. 2004).

LAT1 mRNA is found expressed in the placenta, brain, spleen, testis, colon, blood–brain barrier (Kanai et al. 1998; Wittmann et al. 2015). In the CNS, LAT1 is expressed in the micro-vessels, being responsible for the transport of dihydroxyphenylalanine (L-DOPA) across the blood–brain barrier (Kageyama et al. 2000). In addition, it has been ascribed an important role for the maintenance of large neutral amino acid and neurotransmitter amino acid homeostasis in the inner blood retinal barrier (Tomi et al. 2005). Although the LAT1 isoform has been described in cultured astrocytes and neurons (Deitmer et al. 2003), its significance for the GGC is likely minimal or absent, due to its low affinity toward glutamine as a substrate.

The second isoform LAT2 was isolated in 1999 from both human and rat tissues and consists of a protein with 535 amino acids and 12 putative transmembrane domains (Pineda et al. 1999; Segawa et al. 1999; Rossier et al. 1999). This isoform is also characterized as an exchanger of amino acids, in a Na^+-independent fashion. Contrary to its counterpart, LAT2 is sensitive to more acidic environments (Christensen 1990). In addition, LAT2 has a broad substrate preference, preferring all neutral amino acids, including the small ones alanine, glycine, and serine (Pineda et al. 1999; Bröer and Brookes 2001). Importantly, LAT2 exhibits much higher affinity for glutamine when compared to LAT1 (Rajan et al. 2000; Rossier et al. 1999; Segawa et al. 1999). This isoform, like LAT1, is inhibited by the substrate analog BCH (Kim et al. 2008). LAT2 mRNA was found expressed in kidney, placenta, brain, liver, spleen, skeletal muscle, heart, intestine, and lung (Pineda et al. 1999). As for LAT1, LAT2 is a heterodimer with 4F2hc, which is the heavy subunit responsible for anchoring the transporter in the cytosolic membrane (Rossier et al. 1999). Regarding its expression in neural cells, LAT2 is like LAT1 been described in astrocyte cultures (Nagaraja and Brookes 1996; Deitmer et al. 2003) but also in neurons and in the choroid plexus (Núñez et al. 2014).

8.2.1.4 System y^+L, Isoform y^+LAT2

The isoform y^+LAT2 also belonging to the SLC7 gene family was characterized in 1999 by Torrents and colleagues, from the human cDNA clone number KIAA0245 described in 1996 by Nagase and colleagues (1996). The human protein has 515 amino acids (Pfeiffer et al. 1999) and is predicted to exhibit 12 transmembrane domains (Torrents et al. 1998). In analogy to system L isoforms, y^+LAT2 forms a heterodimer with the heavy chain of 4F2hc via a disulfide linkage, which is related to its coexpression and activity (Kanai et al. 2000; Pfeiffer et al. 1999; Torrents et al. 1998). mRNA for this isoform was found expressed in many tissues in the mouse, such as heart, kidney, small intestine, and parotid but its highest expression was in the brain (Bröer et al. 2000a). It was also demonstrated in the rat brain

(Deitmer et al. 2003). With respect to the cellular localization of y^+LAT2, the isoform has been described in astrocytes, as demonstrated in primary cultures, albeit, the gene expression of y^+LAT2 was less abundant when compared to its counterparts LAT1 and LAT2 (Deitmer et al. 2003). In the in vitro system, y^+LAT2 was also found in cultured neurons (Bröer and Brookes 2001; Bröer et al. 2000a). The mRNA expression of y^+LAT2 has also been verified in the cortex of rats (Zielińska et al. 2011). With respect to the mechanism of transport of this isoform, it works as an exchanger of cationic amino acids and neutral amino acids, the latter being coupled to Na^+. This leads to an electroneutral transport, since the cationic amino acid compensates the charge of the ion flux that accompanies the neutral amino acid (Bröer et al. 2000a).

8.2.2 System ASC, Isoform ASCT2

The isoform ASCT2 was cloned in two independent studies, which isolated the cDNA for this transporter from both murine testis and human placenta (Kekuda et al. 1996; Utsunomiya-Tate et al. 1996). The cDNA for the human homolog encodes a polypeptide containing 541 amino acids that is described to have 10 putative transmembrane domains (Kekuda et al. 1996). ASCT2 is a transporter obligatory working as a Na^+-dependent antiporter of neutral amino acids. Differently from system N, ASCT2 does not tolerate Na^+ substitution for Li^+, and in contrast to system A and L, the substrate analogs MeAIB and BCH do not inhibit this carrier (Pingitore et al. 2013). The ASCT2 transport stoichiometry is described as 1:1 extracellular Na^+-glutamine by intracellular amino acid, but its electrical characteristics of transport are not completely clear, being described as electroneutral or electrogenic (Bröer et al. 2000b). However, recently it was described that the human ASCT2 transporter exerts the inward flux of one glutamine with one Na^+ molecule, leading to an electrogenic transport albeit the authors do not discard the possibility that more than one Na^+ ion might be involved in the transporter activity (Scalise et al. 2014). In fact, Zander and colleagues verified that the rat isoform of ASCT2 exhibited the Na^+/amino acid stoichiometry of at least 2:1, with one sodium ion binding to the amino acid–free apo form of the transporter (Zander et al. 2013).

In this sense, ASCT2 is strongly dependent on Na^+ concentrations at both intracellular and extracellular compartments, but the mechanism of action for its activation is not completely clear (Bröer et al. 2000b; Scalise et al. 2014). It has been described that Na^+ binds to the ASCT2 isoform before the amino acid association, which would favor the catalysis due to structural conformation changes in the transporter (Bröer et al. 2000b). Another study, however, pointed to Na^+ binding to the transporter after the amino acid association is complete, resulting in the complex formation and subsequent amino acids exchange (Grewer and Grabsch 2004). Recently, the effect of intracellular Na^+ was verified as part of a regulatory mechanism, likely promoting the coupling of the transporter subunits or stabilization of the protein during the transition state (Scalise et al. 2014). It is also important to mention that this transporter exhibits

substrate-induced anion conductance, which was also described for its family counterparts, the high affinity glutamate transporters (Bröer et al. 2000b). However, the physiological role of this mechanism is still not completely understood.

The preferred substrates for ASCT2 are alanine, serine, and cysteine, giving rise to its name, but also for other neutral amino acids (Utsunomiya-Tate et al. 1996; Bröer et al. 2000b). It was demonstrated that the ASCT2 transporter has asymmetric specificity for certain amino acids, being alanine, cysteine, valine, and methionine only inwardly transported, while glutamine, serine, asparagine, and threonine were transported bidirectionally (Pingitore et al. 2013). The K_m values for glutamine were of 0.097 and 1.8 mM, when measured on the external and internal sides of the proteoliposome in which the transporter was expressed for the functional studies (Pingitore et al. 2013). Regarding its expression, the mRNA for ASCT2 transporter has been described widespread throughout almost all tissues analyzed. Interestingly, earlier studies did not detect its presence in the brain (Utsunomiya-Tate et al. 1996) but subsequent studies proved that this isoform is present in the CNS (Bröer et al. 1999; Gliddon et al. 2009). In the brain, this transporter isoform was found widespread in areas such as cortex, hippocampus, striatum, and cerebellum, and at the cellular level it was described in the neuronal compartment (Gliddon et al. 2009).

8.3 Glutamine Transporters and the Glutamate/GABA–Glutamine Cycle

Transfer of glutamine from astrocytes to neurons is of fundamental importance for maintaining the proper function of glutamatergic neurotransmission (Schousboe et al. 2013; Nissen-Meyer and Chaudhry 2013). This is highlighted by the fact that during neurotransmission glutamate is taken up by astrocytes via the efficient high affinity glutamate transporters of the excitatory amino acid transporter family (EAAT), which terminate the excitatory stimulation (see Schousboe et al. 2013). Hence, neurons rely upon glutamate recycling in the astrocytic compartment for the replenishment of the glutamate neurotransmitter pool, since neurons are unable to perform de novo synthesis of glutamate due to the absence of the anaplerotic enzyme pyruvate carboxylase (Yu et al. 1983; Schousboe et al. 1997; Hertz et al. 1999). In the astrocytes glutamine can be produced either from glutamate taken up from the synaptic cleft or from glutamate synthesized de novo via anaplerosis and conversion of α-ketoglutarate by transamination (Schousboe et al. 2013). The synthesis of glutamine from these glutamate sources depends on the enzyme glutamine synthetase (GS) which is exclusively expressed in astrocytes (Norenberg and Martinez-Hernandez 1979). Subsequently, glutamine is released from the astrocytic compartment and taken up by neurons, where it will give rise to the synthesis of glutamate by the action of phosphate-activated glutaminase (PAG) and serve mainly as a precursor for the neurotransmitter pools of either glutamate or GABA. In the GABAergic synapses, the majority of the inhibitory neurotransmitter is recycled in neurons, but part of the released GABA is taken up by surrounding astrocytes, by

the activity of GAT, and therefore lost from the transmitter pool (Schousboe et al. 2013). Therefore, the neurotransmitter portion that is consumed by the other compartment is also compensated by the transfer of glutamine to replenish the neurotransmitter pool of GABA (Schousboe 2003; Patel et al. 2001; Liang et al. 2006). Hence, the glutamine transporters are of critical importance in sustaining the appropriate transfer of this neutral amino acid between the neural compartments. From the astrocytic compartment, glutamine needs to be released to the extracellular space and subsequently be taken up by carriers in the neuronal compartment. Although the exact mechanisms of transport are not completely elucidated, the transporters that have been mainly related to the action of glutamine efflux from glia are SNAT3, SNAT5, LAT2, y+LAT2, and ASCT2 (see Fig. 8.1).

The isoform SNAT3, as previously mentioned, is found confined to the astroglial compartment, specifically at the processes ensheathing glutamatergic and GABAergic synapses, and has been considered one of the major transporters involved in glutamine release from astrocytes (Chaudhry et al. 1999; Boulland et al. 2002). It has been reported that SNAT3 immunostaining was more closely associated with the GABAergic than the glutamatergic synapses, suggesting that this isoform could be linked to the transfer of glutamine from astrocytes to neurons for the synthesis of GABA (Boulland et al. 2002). This is in agreement with the fact that this transporter exhibits the ability of performing flux reversal (Chaudhry et al. 2002a). It has been described that SNAT3 activity is influenced by the intracellular Na^+ concentration and membrane potential, since cells that undergo progressive depolarization showed a switch from glutamine uptake to release. Also, the direction of flux is influenced by the glutamine concentration, efflux being favored at concentrations bellow 400 μM (Bröer et al. 2002; Chaudhry et al. 1999). The importance of SNAT3 for glutamine efflux was demonstrated in cultured astrocytes, since in the absence of extracellular amino acids this transporter was the predominant mediator, but a part was also mediated by a system independent of Na^+ (Deitmer et al. 2003). Interestingly, the presence of extracellular amino acids changed the profile of transport, glutamine efflux being greatly stimulated, indicating the participation of amino acid exchangers for this type of transport. In this sense, LAT2 seems to participate actively in this process, since histidine, leucine, and alanine stimulated glutamine efflux (Deitmer et al. 2003). In addition, the Na^+-dependent transporter isoform ASCT2 exerted an important function for glutamine release in the in vitro environment. Although this transporter mRNA was clearly demonstrated in cultured astrocytes, in previous studies ASCT2 levels were found very low in the murine brain tissue (Deitmer et al. 2003). Low mRNA levels were also observed for the transporter y^+LAT2 (Deitmer et al. 2003). However, a subsequent study described the presence of ASCT2 in astrocyte cultures and cerebral cortex homogenates at the protein level. In addition, it was demonstrated by immunolabeling that this transporter localizes at the dendrites of neurons, and it is absent from the astrocytic compartment (Gliddon et al. 2009). Therefore, ASCT2 might not participate in glutamine efflux from astrocytes, since in the adult brain this isoform is not expressed in this cellular compartment. However, it is abundant in neurons, as will be further discussed.

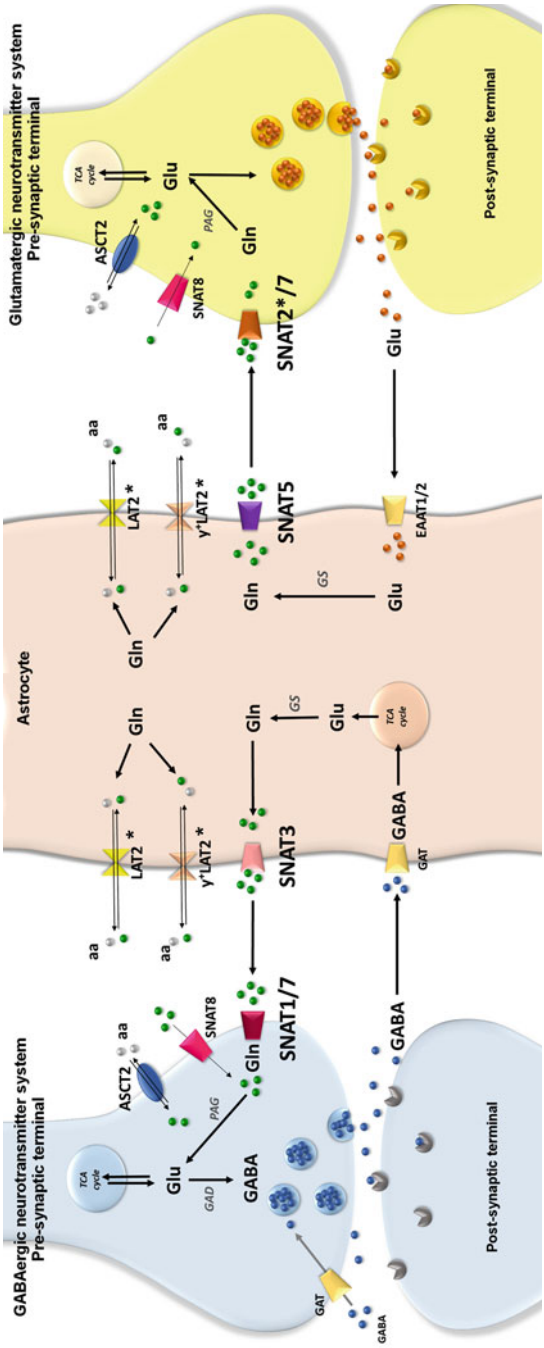
Glutamatergic neurotransmitter system
Pre-synaptic terminal
TCA cycle
Glu
ASCT2
SNAT8
PAG
Gln
SNAT2*/7
Post-synaptic terminal
Glu
aa
LAT2 *
y+LAT2 *
SNAT5
EAAT1/2
Gln
GS
Glu
Astrocyte
Gln
SNAT3
GS
Glu
TCA cycle
GABA
GAT
GABAergic neurotransmitter system
Pre-synaptic terminal
SNAT1/7
GAD
GABA
GAT
GABA
Post-synaptic terminal

Fig. 8.1 Glutamine transporters and the glutamate/GABA–glutamine cycle: Schematic representation of the glutamine transporters involved in the glutamate/GABA–glutamine cycle (GGC). In the glutamatergic synapse, the released glutamate is taken up into astrocytes by the high affinity glutamate transporters (EAAT1/2) and glutamate (Glu) is subsequently amidated by glutamine synthetase (GS), giving rise to glutamine (Gln). Thereafter, glutamine is released to the extracellular space by the action of SNAT5, and subsequently taken up by neurons by the transporter SNAT2. In the neuronal compartment glutamine is deamidated by the activity of phosphate-activated glutaminase (PAG), giving rise to glutamate. In the GABAergic neurotransmitter system, part of GABA released as neurotransmitter is taken up via gamma-aminobutyric acid transporters (GAT) into astrocytes where it will be converted to succinate, which is metabolized in the TCA cycle to α-ketoglutarate, which can be transaminated to glutamate. In the GS catalyzed reaction, glutamate is amidated to form glutamine which is released to the extracellular space by the action of SNAT3, and subsequently taken up by SNAT1 into the neuronal compartment. In the GABAergic neuron, glutamine is deamidated by PAG to form glutamate, which is decarboxylated to GABA by the action of glutamate decarboxylase (GAD) hereby replenishing the GABAergic neurotransmitter pool. Glutamate may in both the glutamatergic and the GABAergic neurotransmitter system additionally serve as an energy substrate and be metabolized in the TCA cycle. The SNAT7 isoform is also described as being responsible for transport glutamine into the neuronal compartment and has been reported to be expressed in both glutamatergic and GABAergic synapses. Its family counterpart SNAT8 might also participate in the same functions. The transporters LAT2 and y^+LAT2 have been related to glutamine efflux from the astrocytic compartment, although their cellular localization remains to be determined. The transporter ASCT2 is expressed in the neuronal compartment in vivo and might also be part of the mechanisms of glutamine uptake. Note that the transporters with an asterisk do not have their cellular localization clarified (LAT2 and y^+LAT2) or are not found at the nerve terminals (SNAT2). *aa* amino acid, *EAAT1/2* excitatory amino acid transporters 1 and 2, *GABA* gamma-aminobutyric acid, *GAD* glutamate decarboxylase, *Glu* glutamate, *Gln* glutamine, *GS* glutamine synthetase, *PAG* phosphate-activated glutaminase, *TCA* tricarboxylic acid

It is important to note that the study performed by Deitmer and coauthors (2003) specifically analyzed the efflux of glutamine from glial cells, while other studies have investigated the profile of glutamine uptake, which was very different with respect to the glutamine transporter isoforms involved in this process (Heckel et al. 2003). Uptake in cultured astrocytes was mediated by, in descending order from the highest to lowest percentage of participation, LAT2, ASCT2, y^+LAT2, and SNAT3 (Heckel et al. 2003). However, the importance of SNAT3 in cultured astrocytes was found different in previous studies, which described a significant participation of this transporter isoform (Nagaraja and Brookes 1996; Su et al. 1997). The discrepancies in these results might be a consequence of the cell culture preparation and experimental protocols employed for these studies. Furthermore, the importance of SNAT3 in relation to the glutamate/GABA–glutamine cycle is highlighted by the observation that exogenous glutamate mediates a decrease in the K_m for this isoform in astrocyte cultures (Bröer et al. 2004).

It may be noted that the observations described earlier represent results from in vitro experiments, and to which degree they are translated into an in vivo condition is unknown. Recently, the importance of SNAT3 has been demonstrated in a mutant mouse model deficient in this SNAT isoform (Chan et al. 2016). SNAT3 deficient mice had a short life span (18–20 days old) and exhibited smaller body size, lower body weight, and ataxia when compared to the wild-type mice (Chan et al. 2016). At the CNS level, the SNAT3 deficient mice showed higher brain glutamine levels, while glutamate and GABA were found reduced, demonstrating that this transporter is fundamental for sustaining the GGC for the replenishment of the neurotransmitter pools (Chan et al. 2016). In addition, an increase in SNAT1 and LAT1 expression was observed in these animals, likely as a compensatory mechanism due to the higher concentrations of glutamine (Chan et al. 2016).

With respect to the isoform SNAT5, its role for glutamine efflux is less well understood, but it has also been regarded as a transporter responsible for glutamine efflux from astrocytes. It was shown that its distribution paralleled that of the vesicular glutamate transporter vGLUT1 in the forebrain of mice (Cubelos et al. 2005). Therefore, SNAT5 has been related to the glutamatergic synapses, while SNAT3 correlated more closely with the distribution of GABAergic synapses (Cubelos et al. 2005; Rodríguez et al. 2014). In this regard, SNAT5 demonstrates partial overlap with SNAT3 according to its distribution within the brain, but also exhibits complementary distribution depending on the brain region. SNAT5 is enriched in the hippocampus and striatum, where SNAT3 has a lower expression, whereas it has low immunoreactivity in the diencephalon, brainstem, and cerebellum that are brain areas with high expression of SNAT3 (Cubelos et al. 2005; Hamdani et al. 2012). In addition, it has been described that SNAT5 is related to the modulation of glutamatergic neurotransmission by the release of glycine. This carrier isoform has under certain conditions the property of releasing glycine, which serves as a cotransmitter at NMDA receptors thereby influencing synaptic plasticity in the CNS (Hamdani et al. 2012).

Once at the extracellular space, glutamine needs to be taken up and subsequently internalized in the neuronal compartment in order to complete the GGC (see Fig. 8.1). The transporters that have been shown to be the main facilitators of this process are SNAT1, SNAT2, and SNAT7 (Chaudhry et al. 2002b; Blot et al. 2009; Hägglund et al. 2011). The isoform SNAT1 is considered one of the main transporters responsible for glutamine uptake in the neuronal compartment due to its characteristics of concentrative, Na^+-dependent transport. This isoform was found in GABAergic and glutamatergic neurons, but it is also expressed in the astrocytic compartment (Mackenzie et al. 2003; Melone et al. 2004; Varoqui et al. 2000; Solbu et al. 2010). In the adult murine brain, SNAT1 has been found in the neuronal cell body region as well as neuronal processes including axons at or near synaptic terminals (Weiss et al. 2003; Solbu et al. 2010; Brown and Mathews 2010). With respect to function, it appears that SNAT1 is mostly related to the GABAergic synapses, since it was demonstrated in brain regions that are enriched in GABAergic neurons in proximity to vGAT. In addition, the pattern of labeling for SNAT1 in GABAergic neurons resembles that for glutamate decarboxylase (GAD) responsible for the synthesis of GABA from the precursor glutamate (Solbu et al. 2010). The pattern of distribution of SNAT1 and its relation to the vesicular GABA transporter could be an indication of its relation with glutamine uptake as a prerequisite to replenish the GABA neurotransmitter pool (Solbu et al. 2010; Varoqui et al. 2000). This is in agreement with the observation that glutamine substantially contributes to the vesicular and cytoplasmatic pools of GABA in cultures of cerebral cortical neurons (Waagepetersen et al. 2001), and that inhibition of glutamine uptake by MeAIB reduces inhibitory synaptic transmission in rat hippocampal CA1 pyramidal neurons, indicating that synaptic vesicle content of GABA was reduced (Fricke et al. 2007). In line with this, it has been shown that MeAIB blocked the increase of inhibitory postsynaptic potential (mIPSC) amplitude following depolarization of hippocampal slices (Brown and Mathews 2010). These studies underline the importance of system A activity, in this case most likely the SNAT1 transporter, for the replenishment of vesicular GABA.

SNAT2, on the other hand, being ubiquitously expressed, is localized mainly in glutamatergic neurons, apparently in a manner complementary to the expression of SNAT1 (González-González et al. 2005; Jenstad et al. 2009; Solbu et al. 2010). As an example, Jenstad and coauthors (2009) described that SNAT2 immunolabeling is barely detectable in interneurons, while pyramidal neurons and hilar mossy cells, all having glutamatergic phenotypes, are clearly SNAT2 positive. In the cerebral structures of neocortex and hippocampus, high levels of SNAT1 expression are described in dispersed GABAergic interneurons, while adjacent pyramidal neurons lack immunoreactivity (Jenstad et al. 2009). Regarding the cellular localization of SNAT2 in the neuronal compartment, it has been verified that this isoform is present at the somatodendritic region of glutamatergic neurons.

Previously the participation of SNAT2 in the GGC has been questioned, since its cellular localization apart from the synaptic terminal is not in accordance with the concept of a fast and efficient glutamate neurotransmitter recycling system, and it has been suggested that SNAT2 participates in taking up glutamine for other reasons

than replenishing the neurotransmitter pool (Melone et al. 2006; Grewal et al. 2009). However, Jenstad and coauthors (2009) showed synthesis of glutamate at the expense of glutamine in the nerve endings of electrically stimulated cells from the perforant path, although they also failed to demonstrate SNAT2 expression at the nerve terminals of glutamatergic neurons (Jenstad et al. 2009). In addition, MeAIB led to depletion of glutamate, and therefore the authors suggested that SNAT2 at the dendrites might be supplying glutamatergic nerve terminals with glutamine, but the participation of other transporters in this process was not excluded. Moreover, SNAT2 has been related to glutamate retrograde signaling, which modulates neurotransmission and therefore synaptic plasticity (Jenstad et al. 2009).

The isoform SNAT7, which is part of system N, is widely expressed in both glutamatergic and GABAergic neurons in the mouse brain, while absent from astrocytes. At the cellular level, this isoform is distributed in the neuronal soma and axon, and studies of colocalization failed to demonstrate its presence at the neuronal terminals (Hägglund et al. 2011). It is possible that SNAT7 could also play a role in the GGC by acting in the uptake and recycling of glutamate/GABA (Hägglund et al. 2011), but further investigation is necessary to better understand the importance of this SNAT isoform in this context. As its family counterpart SNAT7, SNAT8 has not yet its role very well defined in the GGC. It is exclusively expressed in the neuronal compartment in the cell bodies and axons of both excitatory and inhibitory neurons (Hägglund et al. 2015). Interestingly, SNAT7 and SNAT8 were found colocalized in the same neuronal compartment and subcellular region, being apart by 40 nm or less of each other (Hägglund et al. 2015). The significance of this transporter interaction is not clear at the moment. While it has been suggested that both transporters might be important for internalization of glutamine as an energy source and participation in general biosynthesis, their function and interaction in the GGC remain to be elucidated (Hägglund et al. 2011, 2015).

Recently, another transporter has been functionally characterized in hippocampal neurons, and contrary of those described for the SNAT family of transporters, this carrier exhibits a Ca^{2+}-dependent glutamine transport activity and it is inhibited by P type voltage-gated Ca^{2+} channel blockers. Moreover, it saturates at 200 μM and has a higher affinity for glutamine (K_m 30 ± 4 μM) when compared to SNAT 1 and 2 neuronal isoforms (app 1.5–0.4 mM range). This recent discovery brings a new participant for the GGC, which contributes with the concept of a fast and concentrative transporter for the replenishment of the neurotransmitter pools of glutamate and GABA (Erickson 2016).

As previously mentioned, the isoform ASCT2, which was earlier considered important for astrocyte glutamine efflux, is absent from this cellular compartment, but largely expressed in neurons, as verified by immunohistochemistry in the adult mouse brain. This transporter isoform is found in neuronal areas that are anti-microtubule-associated protein-2 positive (MAP2), suggesting that its localization may be restricted to the dendrites, while absent in the cell bodies. In addition, ASCT2-mediated low affinity Na^+-dependent uptake of D-serine and L-glutamine was observed in synaptosomal preparations. These findings indicate that ASCT2 may be an important neuronal neutral amino acid transporter (Gliddon et al. 2009).

Therefore, as exemplified in Fig. 8.1, the transporters that have been related to glutamine uptake in the astrocytic compartment are SNAT3 and SNAT5 mostly expressed in GABAergic and glutamatergic synapses, respectively. While at the neuronal compartment, the isoforms SNAT1, SNAT2, SNAT7, and likely SNAT8 are related to glutamine uptake. SNAT1 and SNAT2 are found in the inhibitory and excitatory neurons, respectively, whereas SNAT7 and SNAT8 are expressed at both neuronal systems. It is important to mention that SNAT1 and SNAT7 are found expressed close to the synaptic cleft (Armano et al. 2002; Hägglund et al. 2011), which is in agreement with the concept of the glutamate/GABA–glutamine cycle but they are also to a considerable extent present at the soma regions of the neurons. SNAT2 is rarely found at the axonal terminals, but is abundant at the somato-dendritic regions of the neuronal compartment (Jenstad et al. 2009). SNAT8 is also localized at the soma and axonal region of the neuronal compartment, albeit its importance for the GGC is still unknown. The role of ASCT2 at the neuronal compartment and the activity of system LAT and y^+LAT remains to be better elucidated.

8.4 Regulation of Glutamine Transporter Isoforms

Glutamine transporters are regulated by different mechanisms at translational as well as transcriptional levels. In the following section, this will be described for those isoforms which play important roles in the GGC, i.e., those that belong to the SNAT, ASC, and LAT family of proteins. As previously mentioned, the SNAT family of transporters is considered fundamental for the transfer of glutamine between the neuronal and astrocytic compartments. Therefore, their function is regulated by several mechanisms that modulate the expression of the transporters as well as their function. In line with this, the first mechanism for modulating the transporter activity is affecting their trafficking across the cytoplasmic membrane, which will alter the availability of the transporter at the plasma membrane and thereby increase or decrease the influx/efflux of glutamine between the intracellular and extracellular spaces. This mechanism has been well characterized for SNAT2, which is present in both plasma and internal membrane fractions from rat adipose and muscle tissues (Hyde et al. 2001, 2002), and the intracellular pool of this isoform functions as a reservoir of the transporter that can quickly respond to a higher demand. In accordance with this, it has been described that increased recruitment of SNAT2 from the cytoplasm to the plasma membrane was associated with an acute stimulus, namely, the absence of amino acids in the media (Ling et al. 2001). Moreover, in a chronic phase, amino acid starvation led to an increase in gene expression, enhancing de novo synthesis of SNAT2. This phenomenon resulted in a larger transporter activity when compared to the acute phase (Ling et al. 2001). In addition, the stability of the SNAT2 protein appears to participate in the regulation of the transporter function. This is shown by the finding that HeLa cells expressing the CMV-driven SNAT2 construct exhibited more stable SNAT2 protein during amino acid restriction. Furthermore, L6 myotubes

treated with the protein synthesis inhibitor cycloheximide exhibited decreased system A activity when these cells were incubated in a medium containing amino acids, in contrast to what was observed after incubation in an amino acid free medium, suggesting that SNAT2 is stabilized in an amino acid-dependent manner (Hyde et al. 2007). This mechanism seems to be related to a domain located at the N terminus of the transporter (Hyde et al. 2007). Besides these adaptive regulatory mechanisms, SNAT2 expression is stimulated by hormones, such as insulin, growth factors, as well as by cell stress (Guma et al. 1988; McDowell et al. 1998; Hundal et al. 1994). It has been shown that insulin increases System A transport in L6 cells by stimulating the exocytosis of SNAT2 carriers from an endosomal compartment, an event that is related to the activation of phosphatidylinositol 3-kinase (PI3K) and protein kinase B (PKB) (Hyde et al. 2002). Another parameter that influences SNAT2 availability/activity is its rate of degradation that has been described to be regulated by polyubiquitination by Nedd4-2, a specific ubiquitin ligase with HECT domain, leading to endocytosis of the transporter and subsequent proteoasomal degradation (Hatanaka et al. 2006).

The SNAT1 isoform has also been shown to be regulated at the protein level, as demonstrated by an increased transporter activity in synaptosomes and enhanced inhibitory synaptic strength as a result of an increase in metabolic demand. This may be very important for modulation of GABAergic transmission (Brown and Mathews 2010).

For those isoforms that are responsible for glutamine efflux, posttranslational regulation has been described for SNAT3 (Karinch and Lin 2002; Nissen-Meyer and Chaudhry 2013). In analogy to SNAT2, it was demonstrated in vitro and in vivo that SNAT3 is upregulated in hepatocytes by dietary restriction and serum deprivation (Gu 2005). Moreover, acute treatment of hepatocytes with insulin stimulates the recruitment of SNAT3 to the plasma membrane resulting in an increase in substrate uptake. On the other hand, chronic stimulation by this hormone led to down-regulation of the expression, a mechanism involving PI3K (Gu 2005). In addition, SNAT3 exhibits different putative phosphorylation sites that are involved in down-regulation of the activity of the transporter. In keeping with this, activation of PKC by phorbol-12-myristate-13-acetate (PMA) leads to down-regulation of SNAT3 in *Xenopus laevis* oocytes (Balkrishna et al. 2010). However, this study showed that the reduced activity of SNAT3 was not a consequence of direct phosphorylation of the transporter, but rather the result of trafficking and internalization of the transporter in a caveolin-dependent manner (Balkrishna et al. 2010). In addition, other studies explored the effect of phosphorylation and demonstrated that SNAT3 is directly phosphorylated by the protein kinases PKCα, PKCγ, and PKCδ (Sidoryk-Wegrzynowicz et al. 2011; Nissen-Meyer et al. 2011). More specifically, PKC phosphorylates SNAT3 at one single serine residue at the N-terminal of the transporter, leading to sequestration of the transporter to intracellular reservoirs, which consequently regulates the transporter activity. In addition, prolonged activation of PKC results in the degradation of the SNAT3 transporter (Nissen-Meyer et al. 2011). In analogy to SNAT2, SNAT3 is regulated by degradation in a ubiquitin ligase Nedd4-2 manner (Boehmer et al. 2003). This appears to be regulated by the

pathways involving serum and glucocorticoid inducible kinase (SGK) and PKB, which reverted the Nedd4-2 induced degradation of SNAT3 (Boehmer et al. 2003).

An alternative form of regulation of SNAT isoforms involves changes in pH. As mentioned earlier, SNAT proteins are sensitive to changes in the pH, having their activity decreased in more acidic environments, while at more alkaline pH they exhibit higher activity.

The SNAT isoforms are also tightly regulated at the transcriptional level, which consequently influences the availability of the transporters. SNAT2 transcriptional regulation has been extensively studied and several factors affect its expression, such as hypertonic conditions, amino acid deprivation, and the availability of hormones (Ling et al. 2001; Palii et al. 2004; Franchi-Gazzola et al. 2004; Kashiwagi et al. 2009). Moreover, osmotic shock which is stressful to cells has been demonstrated to result in increased mRNA levels of SNAT2 (Franchi-Gazzola et al. 2004; Kashiwagi et al. 2009). Activation of mitogen-activated protein kinase (MAPK) cascades, such as extracellular regulated kinase (ERK) is possible intracellular signaling pathways involved in this process (Matsuda et al. 1995). The intracellular signaling pathway involved in the stimulation of mRNA synthesis seems to depend upon the cell type, since other studies with L6 cells demonstrated that the up-regulation of the transcription of SNAT2 transporter did not involve PI3K, ERK, p38, and JNK pathways (Kashiwagi et al. 2009), while in CHO-K1 cells, the effect of hyperosmotic conditions was partially blocked by inhibition of p38 (Lopez-Fontanals 2003).

Amino acid deprivation has also been described to increase mRNA levels for SNAT2 and subsequently protein levels (Kashiwagi et al. 2009; Lopez-Fontanals 2003). This was verified when inhibitors of mRNA synthesis suppressed the increase in the transporter expression, demonstrating that the increase in the transporter was related to de novo synthesis of the transporter (Kashiwagi et al. 2009). It is known that amino acid deprivation involves the activation of a pathway called amino acid response (AAR), leading to the phosphorylation of the translation initiation factor eIF2α (eukaryotic initiation factor 2α), which consequently promotes increased translation of a selected number of mRNAs, including activating transcription factor 4 (ATF4) (Harding et al. 2000). ATF4, in turn, increases the transcription from a subset of target genes, including those related to AAR elements (AARE) that mediate the enhanced transcription (Kilberg et al. 2005). In relation to this, the AARE site has been identified in the SCL38A2 gene, which is located at intron 1 (Palii et al. 2004). This region together with a CAAT box is responsible for mediating the amino acid response as an enhancer, while another site, called Purine rich box acts as an inhibitor of gene transcription (Palii et al. 2004). In addition, it has been described that multiple transcription factors bind to the SNAT2 AARE and thereby contribute to the amino acid-dependent regulation, being a key role demonstrated for ATF4, likely a component that triggers the mechanism for the SNAT2 activation (Palii et al. 2006). In addition, other signaling cascades that appear to be involved in the increased transcription of SNAT2 under amino acid deprivation are ERK and JNK (Kashiwagi et al. 2009; Lopez-Fontanals 2003). Insulin has also an effect on stimulating the transcription of SNAT2, being the signaling pathway of this event related to P38 in L6 muscle cells (Kashiwagi et al. 2009).

Increases in mRNA expression have also been demonstrated for the System N isoform SNAT3 during metabolic acidosis in the cortex of kidney of acidotic rats. However, the mechanisms related to this increase in translation for SNAT3 are not completely clear but they might involve the pH-response element present in the 3′-untranslated region (UTR), and/or mRNA stabilization (Karinch and Lin 2002). Also, glucocorticoids, directly or indirectly regulate the expression of the SNAT3 transporter during chronic metabolic acidosis (Karinch et al. 2007).

It has also been described that glutamine itself can stimulate ASCT2 transporter expression in human hepatoma cells by indirectly inducing the expression of Farnesoid X receptor (FXR), which binds to an IR-1 sequence on the ASCT2 promoter region (Bungard and McGivan 2005). This leads to an increase in expression of ASCT2 mRNA and protein thereby increasing glutamine uptake, as observed in HepG2 cells (Bungard and McGivan 2005). Moreover, the signaling pathways SGK1 and SGK3 modulate ASCT2 by increasing the transporter abundance in the plasma membrane (Palmada et al. 2005). In addition, a role for the EGF signaling pathway has also been suggested. Insulin like growth factor (IGF) has an effect on stimulating the transporter activity, involving the PI3K signaling pathway (Karlsen and Serck-Hanssen 2002). Furthermore, ASCT2 is modulated by pH, its activity being increased in more acidic environments (Bröer et al. 1999).

With respect to the system L isoform LAT2, it was shown that its activity and expression were increased in skeletal muscle fibers by the hormone dihydrotestosterone (DHT). This regulation is likely mediated by epidermal growth factor receptor (EGFR) involving the activation of ERK1/2 module of the MAPK pathway (Hamdi and Mutungi 2011).

It is important to mention, however, that the regulatory mechanisms described earlier were studied in different cell types and experimental models, and for the different stimuli, the response and pathway involved in these processes vary according to the cell type studied. Therefore, the mechanisms that regulate glutamine transporters in neurons and astrocytes may be identical or similar to those that have been mentioned here but it should be emphasized that neural cells might exhibit distinct regulation involving other mechanisms.

8.5 Glutamine Transporters in Neurologic Diseases

Altered function of the GGC has been linked to the pathophysiological mechanisms of different neurological disorders, such as epilepsy, Alzheimer's disease, and stroke (Walton and Dodd 2007; Coulter and Eid 2012; Jeitner et al. 2015). In the case of epilepsy, some studies have described a decrease in the high affinity glutamate transporters expression in patients with temporal lobe epilepsy and decreased expression and activity of GS (Mathern et al. 1999; Proper et al. 2002). Patients with Alzheimer's disease also present disturbances in GS, which has been shown to be reduced at the vascular end-feet of astrocytes in the cortical brain region (Robinson 2001), as well as the function of astrocytic glutamate transporters (Li et al. 1997).

The involvement of GS in neurological disorders is further elaborated on in another chapter by Jayakumar and Norenberg (2016).

It is clear that defects in key steps of the GGC, as glutamate uptake by astrocytes and its conversion to glutamine by GS will have a significant impact on the performance of the cycle thereby affecting neurotransmitter systems. Nevertheless, the role of glutamine transporters in neurological diseases is sometimes overlooked. Although as discussed earlier, the glutamine transporter family consists of members having a great variety of properties, some isoforms share similar functional characteristics and have a widespread expression in the brain tissue. It should therefore be expected that they play important roles in the mechanisms underlying a variety of neurologic disorders.

In this regard disturbances in different glutamine transporter isoforms have been described during conditions of hyperammonemia, such as that associated with hepatic encephalopathy (HE). Increases in the ammonia concentration in the blood and CNS are a hallmark of HE, a neurologic disease that occurs due to the reduced ability of the liver to synthesize urea for the clearance of circulating blood ammonia (Albrecht and Jones 1999; Butterworth 2002). As a consequence, increased ammonia levels reach the CNS, which lacks a metabolizing system for ammonia disposal as urea. In the brain the main system responsible for ammonia detoxification is constituted by the astrocyte-specific enzyme GS catalyzing the ATP-dependent synthesis of glutamine from glutamate and ammonia. As a result, glutamine synthesis, exclusively occurring in astrocytes, is increased during this condition and since most histopathological findings of HE are found in this cell type, HE has been designated as a gliopathy (Rama Rao and Norenberg 2014; Norenberg 1998). Extracellular glutamine can be transferred from the CNS to the periphery. However, this event together with the detoxification via GS is not enough to prevent the pathological consequences, which lead to several disturbances in various neurotransmitter systems and brain energy metabolism (Butterworth 2000; Leke et al. 2011a, b; Schousboe et al. 2014b).

As mentioned earlier, HE leads to altered GS activity as well as glutamate and GABA transporter activity (Butterworth 2000; Suarez et al. 2002) and importantly an altered expression of glutamine transporters. It has been demonstrated that acute HE leads to a diminished gene expression for SNAT5, although other studies described unaltered expression at both mRNA and protein levels (Desjardins et al. 2012). Decreases in the astrocytic transporter would not be in agreement with the pathophysiological findings of acute HE, i.e., increased levels of glutamine at the extracellular space (Swain et al. 1992). On the contrary, in animals with chronic HE resulting from bile duct ligation, SNAT5 mRNA levels were found increased, which could be an adaptive response due to the increased intracellular concentrations of glutamine. However, the protein levels remained unchanged during this condition (Leke et al. 2015). The other astrocytic isoform SNAT3 has been described to be augmented in the brain tissue of rats with acute HE due to liver failure induced by thioacetamide (Zielińska et al. 2014). In addition, an increase in the brain expression and activity of y^+LAT2 has been documented in rats acutely exposed to hyperammonemia (Zielińska et al. 2011). These authors suggest that the increased y^+LAT2 function leads to enhanced efflux of arginine in exchange of extracellular

glutamine, events that are linked to an altered NO/cGMP pathway (Zielińska et al. 2011). Although the authors described that the decrease in y^+LAT2 is related to the defects in astrocytes (Skowrońska et al. 2012), it is still needed to elucidate in which cellular compartment y^+LAT2 is expressed in vivo.

Manganism is another neurologic disease that alters the function of GGC. It is characterized by accumulation of manganese (Mn) due to food intoxication or occupational exposure, leading to CNS toxicity, which culminates in a neurological brain disorder characterized by symptoms of idiopathic Parkinson's disease (Sidoryk-Wegrzynowicz and Aschner 2013). Intoxication with Mn is linked to dysfunction of different steps of GGC, such as down-regulation of GS and decreased expression of the glutamate transporters GLT-1 and GLAST (Erikson et al. 2008). A decrease in the expression of the GABA transporter GAT1 has also been shown in cerebral tissue of rats, together with increases in the extracellular GABA concentration in striatum of Mn-exposed rats (Brouillet et al. 1993). In this regard and in analogy with HE, astrocytes are the main target for Mn intoxication (Aschner et al. 1992). As a result, decreased levels for SNAT3, SNAT2, and LAT2 at mRNA and protein levels in astrocyte cultures have been demonstrated (Sidoryk-Węgrzynowicz et al. 2009). Interestingly, it was shown that SNAT3 protein during Mn exposure is completely degraded, a process that could be a result of increased activity of the ubiquitin-mediated proteolytic system (Sidoryk-Wegrzynowicz et al. 2010).

It is likely that other neurological conditions also may lead to defects in glutamine transporter expression and activity. However, the role of glutamine transporters in the pathological mechanisms of different neuropathologies, in which the dysfunction of the excitatory and/or inhibitory neurotransmitter systems and likely GGC is acknowledged, is often disregarded. Hence, for better understanding of the role of glutamine transporters in physiological and pathological conditions it appears necessary to elucidate in greater detail their role and participation in the GGC.

8.6 Concluding Remarks

It is clear from the presentation earlier that glutamine transport in the brain is extremely complex being mediated by an extraordinarily large number of different isoforms of transporters belonging to several transporter families. This complexity extends to the regulatory mechanisms and altogether this indicates that this transport capacity is of remarkable importance for brain function. Since the amino acid neurotransmitter systems account for a significant fraction of all neurotransmission and the transfer of glutamine between astrocytes and neurons is obligatory for its function, it is not surprising that the transporters responsible for this are numerous and highly regulated. Extensive research in this area will unquestionably lead to a better understanding of the mechanism of action of these transporters providing new therapeutic avenues for treatment of neurological disorders in which malfunction of the glutamine transporters is involved.

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Chapter 9
Glutamine Metabolism in Gliomas

Monika Szeliga and Jan Albrecht

Abstract By histological, morphological criteria, and malignancy, brain tumors are classified by WHO into grades I (most benign) to IV (highly malignant), and gliomas are the most frequently occurring class throughout the grades. Similar to peripheral tumors, the growth of glia-derived tumor cells largely depends on glutamine (Gln), which is vividly taken up by the cells, using mostly ASCT2 and SN1 as Gln carriers. Tumor growth-promoting effects of Gln are associated with its phosphate-activated glutaminase (GA) (specifically KGA)-mediated degradation to glutamate (Glu) and/or with its entry to the energy- and intermediate metabolite-generating pathways related to the tricarboxylic acid cycle. However, a subclass of liver-type GA are absent in glioma cells, a circumstance which allows phenotype manipulations upon their transfection to the cells. Gln-derived Glu plays a major role in promoting tumor proliferation and invasion. Glu is relatively inefficiently recycled to Gln and readily leaves the cells by exchange with the extracellular pool of the glutathione (GSH) precursor Cys mediated by xc- transporter. This results in (a) cell invasion-fostering interaction of Glu with ionotropic Glu receptors in the surrounding tissue, (b) intracellular accumulation of GSH which increases tumor resistance to radio- and chemotherapy.

Keywords Gliomas • Glutamine • Glutamate • Glutathione • Cell proliferation • Tumor invasion • Glutaminase

9.1 Introduction and Outlines

Neoplastic transformation of a tissue involves complex genetic and metabolic remodeling of the cells, wherein no single event can be considered as either decisive or dispensable in the whole process. This chapter aims at elucidating the specific roles of Gln in the biology of gliomas, the most frequently occurring class of brain tumors. Gln and its metabolites contribute outstandingly to the different aspects of

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the biology of tumors residing in, or stemming from, the central nervous system (CNS) and the peripheral tissues alike. In so far, most of the data describing the role and fate of Gln have been derived from studies on peripheral tumors. Indeed, interest in the subject commenced with the seminal observation that growth of the archetypical human epithelial cancer cell line, the HeLa cell, is better promoted by the presence of Gln than glucose in the growth medium (Reitzer et al. 1979). The Gln dependence of the growth of glia-derived tumor cells has been later confirmed in a rat C6 glioma cell line (Dolińska et al. 2001; Martin et al. 1998; Portais et al. 1996).

Studies on different cell lines or solid tumors have confirmed the role of Gln not only as a major energy fuel, but not less so as a precursor of metabolites critically involved in tumor progression. It is by now clear that all the processes, which contribute to the tumor growth-promoting effects of Gln are associated with its glutaminase-mediated degradation to Glu and/or with its entry into the energy- and intermediate metabolite-generating pathways related to the tricarboxylic acid cycle. Subsequent sections of the chapter will focus on the pertinent changes of Gln metabolism in gliomas which promote their proliferation and invasiveness. Wherever possible (in so far a few instances only), relation between distinct traits of Gln metabolism and tumor grade will be emphasized.

To position the high-grade gliomas, further referred to as glioblastomas (GB), among the diversified world of brain tumors, the chapter commences with a brief account on brain tumor classification, epidemiology, and treatment (Sect. 9.2). The next section (Sect. 9.3) characterizes Gln uptake to the tumor cells, accounting for the diverse involvement of distinct Gln transporters and presents data on the relative Gln accumulation within the tumor tissue vs. surrounding nontransformed brain tissue. Section 9.4 will be devoted to the Gln-degrading enzymes, phosphate-activated glutaminases (GAs). Diverse status and functions of GA isoforms will be emphasized. Similar to other neoplastic and metabolically active tissues, the kidney-type GA contained in glioma cells is upregulated and bears the brunt for increased Gln degradation to Glu. KGA appears as a plausible therapeutic target. By contrast, the liver-type GA which in nontransformed cells is hypothesized to be involved in the regulation of transcription (Olalla et al. 2002), is absent in glioma cells, a circumstance which allows phenotype manipulations upon their transfection to the cells. Section 9.5 will account for the role of Gln-derived Glu in promoting tumor proliferation and invasion. The tumor-promoting role of the xc- transporter, which catalyzes the exchange of intracellular Glu for the extracellular pool of the GSH precursor Cys, will be emphasized. Finally, results of magnetic resonance spectroscopic analysis of Gln fluxes via the tricarboxylic acid cycle and their role in glioma metabolism will be described in Sect. 9.6. The fates of Gln described in the chapter are summarized in Fig. 9.1.

9.2 Characteristics of Glial Tumors: Classification, Prognosis, and Treatment

Primary brain tumors comprise a heterogeneous group of neoplasms arising from the tissues of the brain or its surroundings. The world incidence rate for all primary brain tumors ranges from 8.63 to 13.56 per 100,000 per year (de Robles et al. 2015).

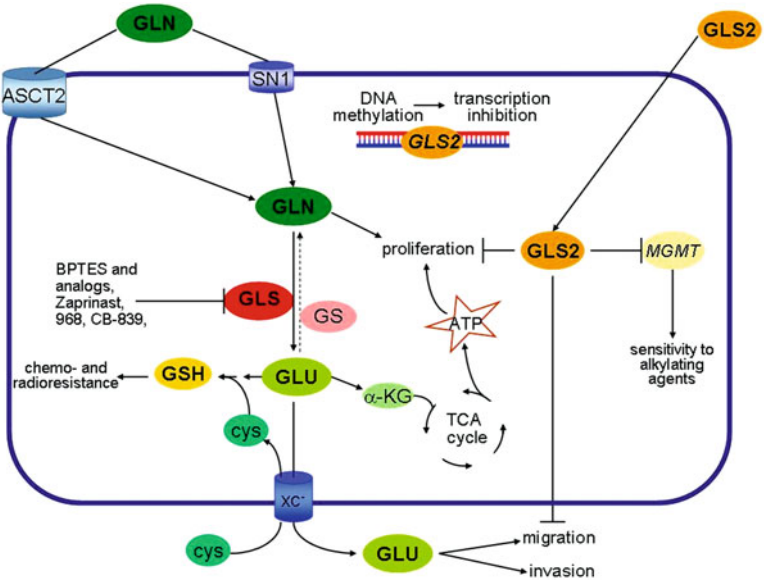

Fig. 9.1 A scheme summarizing metabolic fate and biological roles of Gln in brain tumors. Gln is taken up by ASCT2 and SN1, the two Gln carriers which abound in brain tumor cells as compared to the surrounding normal brain tissue. Gln is primarily degraded by the upregulated kidney-type glutaminase (GLS). Gln serves as a precursor for the synthesis of nucleic acids and proteins and in this way promotes cell proliferation. Excess of Gln-derived Glu is released from the cells via the xc- transporter exchanging Glu for Cys, with two key consequences: (1) increased extracellular Glu which promotes cell invasion and (2) increased intracellular GSH synthesis, responsible for increased chemo- and radioresistance. Part of Glu enters the GGC replenishing TCA cycle constituents (anaplerosis) and giving rise to other important metabolic substrates and ATP, used among other purposes in cell proliferation. Only a relatively minor proportion of Glu is recycled to Gln, because of low GS activity. Malignant brain tumors contain very low amounts of the liver-type glutaminase (GLS2), most likely due to excessive DNA methylation in the promoter region of *GLS2*. Introduction of GLS2 into the cells elicits a chain of as yet not fully described events that inhibit cell proliferation, migration, and invasion. Experimental studies point to the utility of GLS inhibitors (BPTES and analogs, zaprinast, 968, CB-839) in curbing malignant properties of the cells

The World Health Organization (WHO) classification of tumors of the central nervous system (CNS) divides the tumors by histological criteria, and based on morphological features of anaplasia assigns them a malignancy grade from I (benign) to IV (highly malignant) (Louis et al. 2007).

Gliomas arising from any type of glial cells (i.e., astrocytes, oligodendrocytes, ependymocytes) are the most common primary brain tumors accounting for more than 70 % of all neoplasms of the CNS (Ohgaki and Kleihues 2009). Pilocytic astrocytoma (WHO I) develops in children and young adults and is usually cured by resection with low risk of tumor recurrence (Fernandez et al. 2003; Tibbetts et al. 2009). Diffuse astrocytoma (WHO II) is a slow-growing tumor with a tendency to recur after surgery which is often associated with progression to high-grade gliomas. Median survival time of patients with diffuse astrocytoma ranges from 5 to 8 years

(Lind-Landström et al. 2012). Oligodendroglioma (WHO II) has better prognosis with median survival time from 10 to 17 years (Scheie et al. 2011). Mixed oligoastrocytoma (WHO II) has an intermediate prognosis compared to the other low-grade gliomas with median survival time 6 years (Scheie et al. 2011). Anaplastic astrocytoma (WHO grade III) is a rapidly growing tumor with median survival time 2–3 years (Riemenschneider and Reifenberger 2009). Glioblastoma (GB) (WHO IV) is the most frequent of all gliomas (Ohgaki and Kleihues 2009). Median survival time of patients with newly diagnosed GB is less than 10 months (Weller et al. 2013a). Grade III and IV tumors are referred to as high-grade gliomas. Standard treatment of these tumors, which consists of surgery followed by radiation plus concomitant and adjuvant chemotherapy with an alkylating agent, temozolomide (TMZ), results in only marginal prolongation of patient's survival.

Histological examination is still the basic method used in diagnosis of brain tumors. However, a growing body of evidence points to a significant role of some molecular markers as diagnostic, prognostic, or predictive factors (Hegi et al. 2006; Weller et al. 2013b). Specifically, *IDH1* and *IDH2* mutations, 1p and 19q codeletions, and *MGMT* promoter methylation are used in diagnosis of some types of brain tumors (Weller et al. 2013b). Mutations in *IDH1* and *IDH2* genes encoding isocitrate dehydrogenase 1 and 2, respectively, occur mainly in low-grade gliomas. Patients with mutated *IDH1* or *IDH2* have significantly longer overall survival than patients with wild-type *IDH* of the same histological grade (Cohen et al. 2013). Codeletion of 1p and 19q is strongly associated with oligodendroglial histology and tumors carrying this aberration are more sensitive to radio- and chemotherapy (Weller et al. 2013b). Hypermethylation of *MGMT* promoter leads to silencing of the gene coding for a DNA repair protein, O-6-methylguanine-DNA methyltransferase (MGMT), promoting resistance of tumor cells to alkylating agents. Reduced level of MGMT protein results in diminished DNA repair activity which in turn is associated with prolonged overall survival in GB patients treated with alkylating agents (Weller et al. 2013b).

The detailed description of classification, prognosis, and treatment of distinct brain tumors goes far beyond the scope of this chapter. The readers interested in this issue are encouraged to study comprehensive reports cited in this section.

9.3 Gln Uptake and Accumulation in Brain Tumor Cells

Studies on peripheral tumor cell lines (Collins et al. 1998; Wasa et al. 1996) and tumor spheroids (Pawlik et al. 2000) have equivocally pointed to increased Gln uptake as a determinant of rapid growth of transformed cells, more specifically of the increased DNA and protein synthesis in these cells (Wasa et al. 1996). The studies have emphasized the role of the neutral amino acid transporting system ASC in the upregulation of Gln transport. The ASC system variant, ASCT2 has been implicated as the predominant Gln transporting moiety in a C6 rat glioma cell line (Dolińska et al. 2003). Interestingly, diminished proliferation of C6 cells grown in

the absence of Gln paralleled the reduction in the activity of system ASC-mediated Gln uptake (Dolińska et al. 2001). While ASCT2 is likewise overexpressed in all classes of native human gliomas, the most malignant phenotypes (grades III/IV) are distinguished from the more benign tumors by abundance of mRNA coding for the glia-specific N system transporter SN1 (SNAT3) (Sidoryk et al. 2004).

Since Gln synthesis in cerebral tumors is comparatively very low (see Sect. 9.6), their Gln content is thought to mainly reflect its import from the surrounding brain tissue and/or from blood. Proton magnetic resonance spectroscopy (^{1}H MRS) carried out in high-grade glioma patients has consistently revealed elevated Gln content in the affected tissue (Li et al. 2015, and references therein). Elevation of Gln content is more pronounced and more consistently found in primary human gliomas than in brain metastases of peripheral tumors (Fan et al. 2004). Moreover, Gln content has been reported to be higher in malignant recurrences of primary tumors than in the primary tumors themselves (Lehnhardt et al. 2005). Increased Gln concentrations have also been detected in the contralateral normal-appearing white matter of GB patients suggesting that elevated Gln may serve as a marker of early neoplastic infiltration of an apparently healthy tissue (Kallenberg et al. 2009). Combined gene expression and ^{1}H NMR metabolomic analysis of nine different glioblastoma cell lines revealed their considerable heterogeneity with regard to their amino acid content and expression of pertinent transporter molecules differing in their content of different amino acids (Cuperlovic-Culf et al. 2012). The heterogeneity encompassed the Gln status: of the four distinct classes of the cell lines, only two showed elevated Gln content, which coincided with simultaneous overexpression in both, of two Gln-transporting moieties—the N system transporter SNAT1 and the L system transporter LAT1 (Cuperlovic-Culf et al. 2012). In a more general perspective, it will be of interest to see whether and in what degree, the existence of four metabolically distinct types of glioma cells in culture correspond to the four subtypes of glioblastoma identified in clinical material, recently defined by Verhaak and colleagues as proneural, neural, classical, and mesenchymal, respectively (Verhaak et al. 2010).

9.4 Diverse Roles and Status of Phosphate-Activated Glutaminase (GA) Isoforms

Apart from increased Gln uptake, a wide range of cancer cells present also elevated glutaminolysis. Phosphate-activated glutaminase (GA, EC 3.5.1.2), the initial enzyme of the glutaminolysis, metabolizes Gln to Glu and ammonia. In humans, there are two genes coding for GA isoforms: the *GLS* gene encodes kidney-type isoforms—KGA and GAC (Curthoys and Watford 1995; Elgadi et al. 1999), while the *GLS2* gene encodes liver-type isoforms—LGA and GAB (Aledo et al. 2000; de la Rosa et al. 2009). GA isoforms present distinct tissue specificity, kinetic properties, and cell localization (Aoki et al. 1991; Campos et al. 2003; Campos-Sandoval et al. 2007; Castell et al. 2004; Curthoys and Watford 1995; Elgadi et al. 1999; Olalla et al. 2002).

Impaired expression and activity of GA isoforms is a hallmark of different tumors and neoplastic cell lines (Szeliga and Obara-Michlewska 2009). *GLS* is ubiquitously overexpressed in neoplastic tissues of different origin (Huang et al. 2014; Pan et al. 2015; Pérez-Gómez et al. 2005; Turner and McGivan 2003; Yu et al. 2015) and silencing of this gene significantly reduced proliferation rate of neoplastic cells in vitro (Lobo et al. 2000; Meng et al. 2014). The expression of *GLS2* in neoplastic tissues is more diverse and ranges from its lack (Hu et al. 2010; Liu et al. 2014; Yu et al. 2015; Zhang et al. 2013) to abundance (Xiang et al. 2013). Tumor regression was obtained by both ectopic expression of *GLS2* (Hu et al. 2010; Liu et al. 2014; Zhang et al. 2013) or its silencing (Lee et al. 2014), depending on the cell type and initial status of the *GLS2*. Recent findings indicate that GA isoforms may be regulated at several levels by oncogenes or tumor suppressor genes. *GLS* expression has been shown to be indirectly upregulated by the oncogene MYC via suppression of miRNA23a/b expression in lymphoma and prostate cancer cells (Gao et al. 2009). Moreover, the other mediators of oncogenesis, Rho GTPases, have been shown to activate the NFkB transcriptional program which, in turn, elevates the activity of GAC isoform in different cancer cells (Wang et al. 2010). By contrast, the transcription of *GLS2* is regulated by tumor suppressor p53 (Hu et al. 2010; Suzuki et al. 2010). Of note, GLS2 mediates the role of p53 in antioxidant defense and energy metabolism (Hu et al. 2010; Suzuki et al. 2010).

High levels of the *GLS* isoforms and only traces of transcripts arising from the *GLS2* were found in GB tissues (Szeliga et al. 2005) and in several human GB cell lines (Cheng et al. 2011; Szeliga et al. 2009). Very recent data strongly suggest that downregulation of *GLS2* in GB may be caused by DNA methylation found in the promoter region and in the first intron of this gene (Szeliga et al. 2016). Increasing evidence suggests that GA isoforms play opposing role in GB. Overexpression of *GLS2* in GB cells reduced their proliferation and migration ability (Szeliga et al. 2009) and sensitized them to the alkylating agents, most likely through diminishing the expression of *MGMT* (Szeliga et al. 2012). On the other hand, significant decrease in GB cell proliferation was also observed after silencing of *GLS* (Martín-Rufián et al. 2014; Szeliga et al. 2014). Inhibition of *GLS* diminished GSH-dependent antioxidant capacity and triggered apoptosis mediated by mitochondrial dysfunction. Similar tendency was observed in GB cells after ectopic overexpression of GAB (Martín-Rufián et al. 2014).

Inhibition of Glu production in glioma cells offers therapeutic perspectives. A growing body of evidence shows that GLS silencing reduces proliferation of cancer cells of different origin. Thus, pharmacological inhibition of GLS seems to be a potential therapeutic approach for treating cancer. Research conducted in last decade identified some GLS-selective inhibitors increasing the possibility to block GLS with minimal off-target effects. Of these, the most deeply described is an allosteric GLS inhibitor, bis-2-(5-phenylacetamido-1,3,4-thiadiazol-2-yl)ethyl sulfide (BPTES). BPTES binds to an allosteric pocket of GLS triggering conformational changes leading to inactivation of the enzyme (DeLaBarre et al. 2011; Thangavelu et al. 2012). Antitumor activity of BPTES has been demonstrated in different models (Emadi et al. 2014; Thangavelu et al. 2012), including gliomas

(Seltzer et al. 2010). Of note, in case of glioma and leukemia, IDH mutant cells were more sensitive to BPTES as compared to the wild-type counterparts (Emadi et al. 2014; Seltzer et al. 2010). An increasing number of other GLS inhibitors, such as: analogs of BPTES, compound 968 (bromo-benzophenanthridinone), CB-839, Zaprinast, have been shown to diminish growth of some cancer cells in vitro and in vivo (Elhammali et al. 2014; Gross et al. 2014; Katt et al. 2012; Shukla et al. 2012; Wang et al. 2010). Insofar, however, none of them has been tested in gliomas in the clinical setting.

While GLS has emerged as a critical enzyme in tumorigenesis, the role of GLS2 is still elusive. It is possible that GAB isoform suppresses malignant phenotype of GB cells through its potential ability to modulate transcription (Szeliga et al. 2009). Of note, based on nuclear localization, existence of consensus motifs in the structure and identification of novel interacting proteins, GAB has been proposed to be a multifunctional protein (Márquez et al. 2006). The molecular mechanism underlying the mode of action of distinct GLS2 proteins in different cancer models in vivo and in vitro is a subject of ongoing research in different laboratories (Liu et al. 2014; Martín-Rufián et al. 2014; Szeliga et al. 2009, 2012, 2014; Xiang et al. 2013; Zhang et al. 2013).

9.5 Increased Glu Accumulation in Glioma Cells: Mechanisms and Implication for Tumor Progression and Epileptic Seizures

Excessive intra- and/or extracellular Glu accumulation has been consistently documented in rodent glioma cell lines and glioma implants (Buckingham et al. 2011; Lyons et al. 2007; Takano et al. 2001; Ye and Sontheimer 1999; Ye et al. 1999), and in human brain tumors and peritumoral tissue (Cuperlovic-Culf et al. 2012; Liubinas et al. 2014; Yuen et al. 2012). Increased Glu content and release in glial tumor cells has been ascribed to three factors: (1) decreased intracellular conversion of Glu to Gln, due to the decrease of GS activity (Rosati et al. 2009, but see Sect. 9.6 for contradictory views); (2) decreased GLU reuptake associated with downregulation, misplacement, and/or impaired RNA splicing of the astrocytic Glu transporters EAAT-2 (GLT-1) (Münch et al. 2001; Ye et al. 1999) and/or EAAT1 (Cuperlovic-Culf et al. 2012); (3) increased Glu release in exchange for cystine, due to upregulation of the Glu/Cys antiporter xc- (Ye et al. 1999). Coincident changes in the expression of EAAT-2 and xc-, originally reported for glioma cell lines, were recently documented in human brain tumor tissue (Yuen et al. 2012). Increasing body of evidence suggests that increased peritumoral accumulation of Glu promotes excitotoxicity by interacting with ionotropic and/or metabotropic Glu receptors in adjacent neurons (de Groot et al. 2008; Rzeski et al. 2001), and subsequently, tumor proliferation and invasion (reviewed by de Groot and Sontheimer 2011). One of the important clinical complications of the overexposure of brain tissue to the adjacent

glioma-derived Glu is epileptic seizures (Buckingham et al. 2011; Rosati et al. 2009; Yuen et al. 2012). Glutamatergic hyperexcitability underlying the seizures was suppressed by application of the xc- inhibitor, sulfasalazine (SAS), underscoring the role of this transporter in their pathogenesis (Yuen et al. 2012).

One other advantage of increased xc- activity for tumor growth stems from the fact that it couples increased GLU release to increased uptake of Cys, an essential precursor of GSH. GSH is a key cellular antioxidant which is present in high amounts in GBs (Li et al. 2015; Santandreu et al. 2008), ensuring defense of GB against oxidative stress, and their resistance against radio- and chemotherapy (Rocha et al. 2015, and references therein). Indeed, application of the xc- inhibitor SAS (see earlier) decreased GSH content and simultaneously slowed down tumor growth in a xenograft animal model of human glioma (Chung et al. 2005).

There are data showing that metabolic coupling of glioma cells to peritumoral astrocytes protects neurons against excitotoxic effects of glioma-derived Glu. Glioma growth rate in glioma/astrocyte cocultures (AGC) was slowed down with increasing astroglia/glioma ratio in these cultures, and growth media from cocultures with higher glioma/astrocyte ratio produced a stronger calcium signal in cultured neurons and was more toxic to these cells (Yao et al. 2014). In an experimental glioma model in rats, proliferation of glioma cells and neuronal demise were attenuated upon in situ upregulation of the astrocytic glutamate transporter GLT-1 (EAAT-2) following administration an antibiotic, thiamphenicol (Sattler et al. 2013).

9.6 Gln Fluxes via the TCA Cycle and Their Implications for Glioma Metabolism

Similar to other neoplastic cells, glioma cells support their energy demand mostly by anaerobic glycolysis subsiding to the classic pattern of Warburg effect (Warburg et al. 1927; Warburg 1956). Glioma cells also exhibit aerobic glycolysis (Marin-Valencia et al. 2012), which is facilitated by relatively high expression of hexokinase 2 isoform (Wolf et al. 2011). Nonetheless, the strong requirement of cultured glioma cells for Gln (Dolińska et al. 2001; Martin et al. 1998; Portais et al. 1996) suggested that Gln is likewise actively metabolized in these cells. However, evidence from ^{13}C NMR studies is controversial: there is no good match between the results of cell culture studies and those carried out in the in vivo setting.

Comparative analysis of ^{13}C glucose utilization in C6 glioma cells grown in the presence or absence of excess of unlabelled Gln demonstrated that while Gln is actively metabolized, it is not an energy substrate for the cells, but rather serves as a carbon donor to replenish the tricarboxylic acid cycle (Portais et al. 1996). In a study in which ^{13}C Gln metabolism was followed in the presence or absence of glucose (^{13}C labeled or unlabelled), glioblastoma cells performing anaerobic glycolysis (SF 1898 cell line), metabolized ^{13}C Gln at a rate high enough to promote efficient generation of NADPH to support fatty acid synthesis, and anaplerosis (restoration

of oxaloacetate), at the same time facilitating the use of glucose carbon for synthesis of amino acids, nucleotides, and nucleosides (DeBerardinis et al. 2007). By contrast, a very recent study demonstrated that human GB harbored in mice and human patient GBs alike, preferentially oxidized ^{13}C acetate (Mashimo et al. 2014) and less so ^{13}C glucose, but oxidation of ^{13}C Gln was virtually absent in these cells. While further studies are needed to definitely unravel the contribution of Gln to various intracellular pathways in glioma cells, at present one can safely exclude its significant role as direct energy fuel.

There is also disagreement in the literature as to whether and in what degree glioma cells are capable of glutamine synthesis. No conversion of ^{13}C glucose to Gln was detected in cultured C6 glioma cells (Portais et al. 1993) or in C6 glioma-bearing rat model (Bouzier et al. 1999). On the other hand, analysis of ^{13}C glucose metabolism in human brain GBs in situ showed that a discernable proportion of glucose-derived carbon becomes incorporated to Gln (Maher et al. 2012). Incompatibility of literature data extends to the enrichment of the content and pathophysiological role of glutamine synthetase (GS). Proteomic analysis of a representative group of human brain tumors revealed GS enrichment in astrocytomas of all grades as compared to oligodendrogliomas (Zhuang et al. 2011). In one other study, however, a lowered GS content is considered as a factor predisposing the patients to epileptic bursts associated with tumor progression (Rosati et al. 2009), most likely in relation to excessive Glu accumulation (see also Sect. 9.5). It may thus be speculated that the GS status in GBs is at least partly stage dependent. In support of this notion, in glioma cell lines, GS expression and activity increase along with cell differentiation and decreased proliferation rates (Davies and Vernadakis 1986), and a recent study disclosed a negative growth regulatory role of GS, related to upregulation of N-cadherin synthesis and subsequent strengthening of cell–cell contact (Yin et al. 2013). Irrespective of the controversies, it appears that glia-derived neoplastic cells convert relatively less glutamate to Gln than nontransformed astrocytes, rendering their intracellular glutamine cycling relatively inefficient, which causes their metabolism and growth to rely in a great degree on the delivery of extracellular Gln.

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Chapter 10
Oligodendrocytes: Development, Physiology and Glucose Metabolism

Ana I. Amaral, Joana M. Tavares, Ursula Sonnewald, and Mark R.N. Kotter

Abstract The glutamate–glutamine cycle is an outstanding example of how essential neuronal–glial interactions are for brain function. For several decades, this and other metabolic cycles in the brain have only included neurons and astrocytes but not oligodendrocytes, the myelinating cells of the central nervous system (CNS). Recent data revealed that oligodendrocytes are highly metabolically active cells in the brain and, therefore, should not be ignored. Using ^{13}C-labelled glucose in combination with nuclear magnetic resonance spectroscopy (MRS) and/or mass spectrometry (MS) it is possible to characterize metabolic functions in primary oligodendrocyte cultures. Mature rat oligodendrocytes avidly metabolize glucose in the cytosol and pyruvate derived from glucose in mitochondria. Moreover, they seem to have the ability of performing anaplerosis from pyruvate, which might enable them to synthesize metabolites de novo and transfer them to neighbouring cells. All these original findings highlight the importance of investigating oligodendrocyte metabolism separately from that of astrocytes and neurons to be able to discern the roles played by the individual partners. This is of particular importance in the white matter where the number of oligodendrocytes is considerable. The present book chapter provides some background on oligodendrocyte biology and physiology and summarizes the not very extensive information published on glucose metabolism in oligodendrocytes.

Keywords Pyruvate carboxylation • Glucose • Glutamate • Lactate • Citrate • Glutamine–glutamate cycle

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Abbreviations

ATP	Adenosine triphosphate
CNP	20,30-cyclicnucleotide 30-phosphodiesterase
CNS	Central nervous system
Fructose-6P	Fructose-6-phosphate
GA3P	Glyceraldehyde-3-phosphate
GABA	γ-Aminobutyric acid
MAG	Myelin antigen glycoprotein
MBP	Myelin basic protein
MOG	Myelin oligodendrocyte glycoprotein
MRS	Magnetic resonance spectroscopy
MS	Multiple sclerosis
OLs	Oligodendrocytes
OPCs	Oligodendrocyte precursor cells
PC	Pyruvate carboxylase
PDGFR-α	Platelet-derived growth factor receptor alpha
PEPCK	Phosphoenolpyruvate carboxy kinase
PK	Pyruvate kinase
PLP	Proteolipid protein
PPP	Pentose phosphate pathway
T3	Triiodothyronine/thyroid hormone 3
TBI	Traumatic brain injury
TCA	Tricarboxylic acid

10.1 Introduction

Oligodendrocytes make up a large proportion of the cells in the central nervous system (CNS). Although oligodendrocytes are vulnerable to hypoxic/hypoglycaemic conditions (Lyons and Kettenmann 1998; Yan and Rivkees 2006), their metabolic properties, in particular, their glucose metabolism, have not been investigated in depth (Amaral et al. 2013). In contrast, the metabolic interactions revolving around glucose between neurons and astrocytes have received considerable attention since their discovery in the 1970s (van den Berg and Garfinkel 1971). Specifically, the shuttling of glutamine–glutamate–γ-Aminobutyric acid (GABA) between astrocytes and neurons is thought to be fundamentally important for neuronal function and is the focus of other chapters in this book. Glucose is primarily metabolized to pyruvate via glycolysis in the cytosol. Stepwise conversion of a single glucose molecule into pyruvate generates two molecules of ATP. This reaction is not oxygen dependent. Glucose metabolism can also take an alternative route via a biosynthetic pathway termed phosphate pentose pathway (PPP). This complex detour bypasses several steps of glycolysis without consuming or producing ATP. In the first, oxidative phase of the PPP,

NADP+ is converted into NADPH. NADPH acts as a reducing agent that may participate in lipid and steroid synthesis or in the production of glutathione and thioredoxin, which are involved in the cell's defence mechanism against oxidative stress. In the second phase of the PPP, 5-carbon sugars are non-oxidatively synthesized. The PPP joins the glycolytic pathway at the level of glyceraldehyde-3-phosphate (GA3P) and fructose-6-phosphate (fructose-6P). Fructose-6P is subsequently converted into pyruvate, which constitutes the endpoint of both glycolysis and the PPP.

In the presence of oxygen, the pyruvate produced by glycolysis or by the PPP can be metabolized in the mitochondrial tricarboxylic acid cycle (TCA) to further produce ATP via coupling to the mitochondrial electron transport chain. Alternatively, pyruvate can be (reversibly) converted into lactate in the cytosol, which results in the production of NAD+ from NADH. Net synthesis of TCA cycle intermediates and related compounds, including glutamate and glutamine, depend on the entry of pyruvate via anaplerotic replenishment of intermediates in the TCA cycle. In the brain, this is mediated by pyruvate carboxylase (PC) (Patel 1974). PC is not expressed in neurons, but it is present in astrocytes (Yu et al. 1983; Shank et al. 1985; Cesar and Hamprecht 1995) [for review see Sonnewald and Rae (2010) and Sonnewald (2014)]. Consequently, neurons depend on astrocytes as an external source of glutamine for the production of neurotransmitters. Conversion of pyruvate by PC generates a 'new' molecule of oxaloacetate. Oxaloacetate may subsequently condense with acetyl CoA to synthesize the TCA cycle intermediate citrate which, after several steps, is converted to α-ketoglutarate from, which, glutamate can be formed by transamination or amination. In a subsequent step, glutamine synthetase, which is known to be expressed in astrocytes (Norenberg and Martinez-Hernandez 1979), is able to convert glutamate into glutamine (Fig. 10.1).

In the present chapter, we summarize the available data on how oligodendrocyte lineage cells may contribute to the metabolic interactions in the CNS. We propose that intercellular shuttling of metabolites occurs between all three major cell groups of the CNS (neurons, astrocytes and oligodendrocytes), instead of being restricted to closed-loop interactions between astrocytes and neurons (Fig. 10.1).

10.2 Oligodendrocyte Precursor Cells (OPCs): Functions and Metabolic Role in the Brain

10.2.1 Origin and Development of Oligodendrocyte Precursors

OPCs are small unipolar or bipolar cells and can be identified by the expression of the NG2 proteoglycan and platelet-derived growth factor receptor alpha (PDGFR-α), as well as the more general oligodendroglial lineage markers such as SOX10 and OLIG2 (Richardson et al. 2011) (Fig. 10.2). OPCs are scattered randomly throughout the CNS, occurring in both white matter and grey matter; they make up approximately 5–8 % of the cell population in the CNS (Levine et al. 2001).

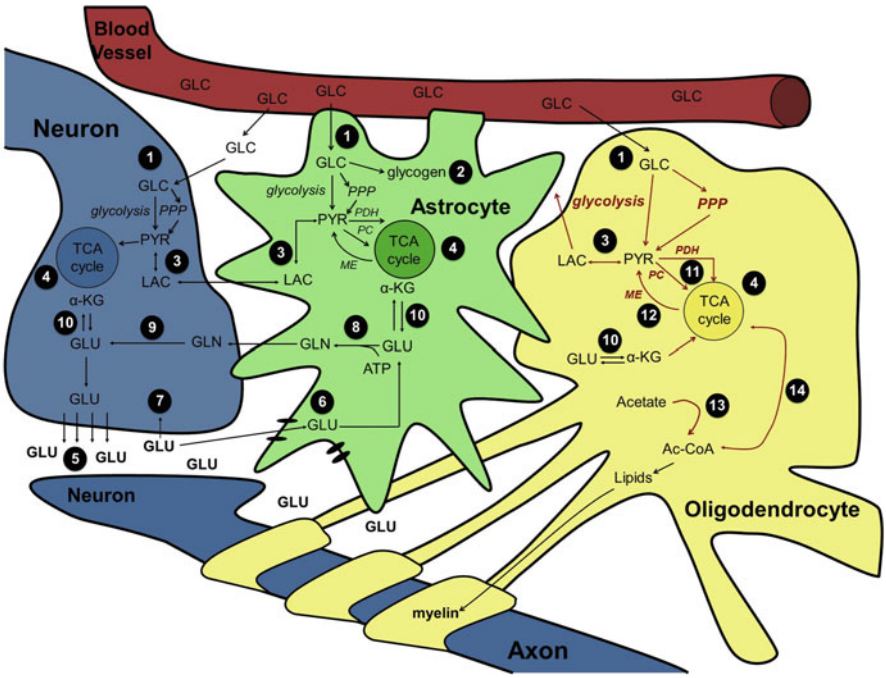

Fig. 10.1 Schematic overview of metabolic interactions between neurons–astrocytes–oligodendrocytes. Glucose (GLC) from the blood is taken up by neurons, astrocytes and oligodendrocytes and can be metabolized via glycolysis and the pentose phosphate pathway (PPP) (*1*), both giving rise to pyruvate (PYR) formation. In astrocytes, GLC can also be stored in the form of glycogen (*2*). PYR can be reduced to lactate (LAC) (*3*) which can be released and taken up by cells with lower lactate concentration or be converted into acetyl CoA (Ac CoA) and subsequently oxidized in the tricarboxylic acid (TCA) cycle (*4*). After synaptic release of glutamate (GLU) by neurons (*5*), astrocytes are responsible for the most of its uptake via specific high-affinity glutamate transporters (GLAST and GLT-1) (*6*) to prevent neuronal excitotoxicity, although some pre-synaptic re-uptake can also occur (*7*). GLU taken up by astrocytes can be converted to glutamine (GLN) (*8*) which can be transferred back to neurons where it is transformed into GLU (*9*), 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 (*10*). The pathways investigated in the present study are highlighted in red in the oligodendrocyte: after glucose is metabolized either via glycolysis only or also via the PPP, the resulting pyruvate produced can be carboxylated via pyruvate carboxylase (PC) or enter the TCA cycle after being converted to acetyl CoA via PDH (*11*). Pyruvate can be further completely oxidized if it is decarboxylated via malic enzyme (ME) (*12*), which also seems to be present in this cell type. Oligodendrocytes can also metabolize acetate into acetyl CoA (*13*) that can be then incorporated into lipids or oxidized in the TCA cycle (*14*)

Despite the widespread distribution of OPCs in the adult CNS, they originate from the ventral (Sonic Hedgehog (Shh)-dependent) and dorsal (Shh-independent) regions during embryonic development (Richardson et al. 1997; Rowitch 2004; Cai et al. 2005; Vallstedt et al. 2005). It was estimated that dorsally derived OPCs

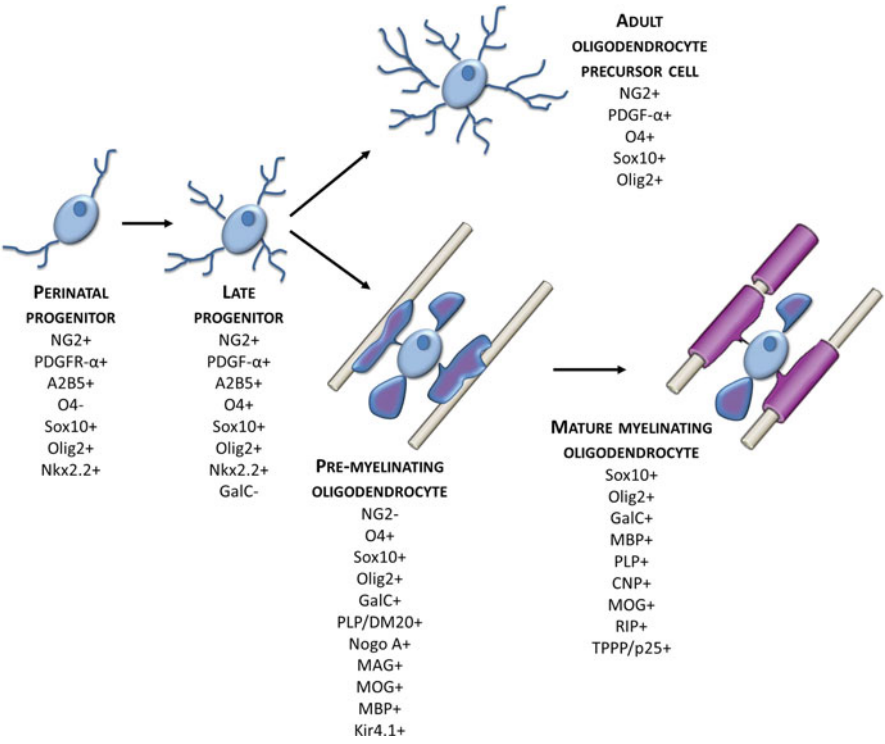

Fig. 10.2 Transcription factors and markers expressed by oligodendrocytes during differentiation. Oligodendrocyte progenitors can be identified by the lineage markers Sox10 and Olig2, in addition to the early stage-specific markers NG2, PDGFR-α and A2B5. Late stage progenitors start expressing O4, which remains being expressed throughout subsequent stages. As progenitors differentiate, they become more branched and might establish contact with axons and start myelinating. Pre-myelinating oligodendrocytes thus distinctively express lipid markers such as Galactoceramides (Galc) and myelin proteins including PLP, MBP, MAG and MOG that continue to be expressed in mature myelinating oligodendrocytes

constitute between 20 and 30 % of the total OPC number seen in the spinal cord of wild-type animals (Vallstedt et al. 2005) whereas the majority of adult oligodendrocytes in the spinal cord seems to be ventrally derived (Mitew et al. 2014). Nevertheless, it is still unclear which cellular source drives developmental myelination and which is the contribution of the different populations to different myelinated tracts in the CNS.

During the early postnatal period, OPCs migrate into the surrounding grey and white matter of the developing CNS. A proportion of OPCs engage with axons and differentiate into myelinating cells, whilst others are reserved as immature cells (adult progenitors) in the parenchyma (Fancy et al. 2011). While OPCs with distinct embryonic origins exhibit a preference to myelinate particular axonal tracts, their electrical properties seem to be comparable (Tripathi et al. 2011).

OPCs exhibit stem cell-like features as they can give rise to daughter cells, including astrocytes and oligodendrocytes, as first described by Raff and colleagues (Raff et al. 1983; Ffrench-Constant and Raff 1986; Kondo and Raff 2000). More recently, their unexpected ability to form cells that are usually associated with PNS development, namely Schwann cells, was reported (Zawadzka et al. 2010). This multipotent capacity, also confirmed by the generation of astrocytes in vivo, in Rivers et al. (2008), Tatsumi et al. (2008), and Guo et al. (2010), consolidated the view of OPCs as an internal source of cells for repair within the CNS. The potential of these cells for CNS remyelination (repair of damaged myelin) has been recently reviewed (Crawford et al. 2014).

In addition to their multipotency, OPCs are also capable of self-renewal (Rivers et al. 2008; Psachoulia et al. 2009; Kang et al. 2010). However, whether these cells have the unlimited potential of self-renewal required of a stem cell or whether their proliferation potential is limited akin to that of a precursor cell still remains to be determined (Crang et al. 1998). More recently, it has been proposed that they can also undergo asymmetrical cellular division and, as a consequence, a growing field investigates OPCs as a source for glioma formation (Sugiarto et al. 2011; Lewis and Petritsch 2013; Galvao et al. 2014).

10.2.2 OPC Migration, Differentiation and Myelination

Before myelination takes place, multiple signalling molecules and mitogens guide OPCs throughout the CNS to myelinating sites. These include the mitogenic factors PDGF and FGF-2 that stimulate cell motility; a variety of extracellular matrix molecules (e.g. fibronectin, N-cadherin, laminin) and cell adhesion molecules (e.g. polysialylated neuronal cell adhesion molecule (PSA-NCAM)) on the surface of CNS cells providing a dynamic environment of signals and cues that help guide the OPCs, as well as chemoattractant and repellent cues (see Mitew et al. 2014 for a comprehensive review).

Once OPCs have populated the neuraxis, another set of signalling pathways balancing inhibitors and effectors drive differentiation. Triiodothyronine/thyroid hormone 3 (T3) signalling in conjunction with the withdrawal of mitogenic cues regulates a crucial step for differentiation and myelin development (Barres et al. 1994). IGF-1 signalling is another positive inducer of myelination, promoting OPC differentiation and expression of myelin genes (Carson et al. 1993; Ye et al. 1995; Goddard et al. 1999) (See Mitew et al. 2014 for a full list of related references). Differentiation into post-mitotic, premyelinating oligodendrocytes generally proceeds in a caudal-to-rostral gradient in the brain but rostrocaudally in the spinal cord (Brody et al. 1987) and entails profound changes in both cellular behaviour and gene expression. The dynamic nature of oligodendrocytes being able to extend processes, sense axons and myelinate multiple axons simultaneously requires a complex and dynamic system of signalling mechanisms converging

on the oligodendrocyte to promote and coordinate each action. Although these extracellular signals have various roles in the myelination process, they can be grossly categorized into two groups: those that modulate oligodendrocyte process extension and initial axonal contact, and factors that dictate subsequent myelin thickening (Mitew et al. 2014).

To form myelin, OPCs exit the cell cycle and begin to express a subset of myelin-associated proteins (Campagnoni and Macklin 1988) such as proteolipid protein (PLP), myelin basic protein (MBP) and myelin oligodendrocyte glycoprotein (MOG), among others, typical markers of mature OL (Duchala et al. 1995) (Fig. 10.2). As part of OPC differentiation, OPCs extend processes to nearby axons; recognizing and adhering to axonal targets the processes flatten and become ensheathing. Myelin components are then synthesized and transported to the appropriate location within the sheath, axons are wrapped and the myelin membrane is compacted such that the cytoplasmic myelin leaflets are practically fused and almost devoid of cytoplasm (Snaidero et al. 2014). It is estimated that each oligodendrocyte can produce up to 40 myelin segments in multiple axons (Miron et al. 2011).

In addition to the role of signalling pathways operating during development and the early post-natal period to regulate OPC differentiation, also neurons regulate myelination via cAMP (Trajkovic et al. 2006), BDNF and neuregulin (Lundgaard et al. 2013) or synaptic activity (Wake et al. 2011; Hines et al. 2015; Mensch et al. 2015) to oligodendroglial glutamate receptors (Karadottir and Attwell 2007). However, it is still unclear in which conditions axons drive the myelination process since cultured oligodendrocytes can also myelinate synthetic nanofibres (Lee et al. 2012a). In this model, only nanofibres of >400 nm were myelinated, suggesting a size-dependent threshold for myelination. Neuronal activity seems to trigger OPC differentiation and myelination of an active neural circuit, a concept called adaptive myelination (Gibson et al. 2014), which is important, for example, to the acquisition of improved motor functions. Moreover, neuronal signalling is also being investigated in the context of myelin repair (Gautier et al. 2015), to understand how adult OPCs migrate to areas of damaged myelin and remyelinate demyelinated axons.

In vitro studies of oligodendrocyte biology were mostly based on primary rodent oligodendrocyte-enriched cultures, which are normally purified from mixed glia cultures (McCarthy and de Vellis 1980). This approach results in high yields of relatively pure (>93 %) cultures (Amaral et al. 2016). The morphological and transcriptional events that occur in vivo are recapitulated when O4+, A2B5+, O1−, CNP (20,30-cyclicnucleotide 30-phosphodiesterase)- and MBP-rodent OPCs are cultured in Sato's medium (McCarthy and de Vellis 1980). These culture conditions enable the formation of a monolayer of mature oligodendrocytes with complex-branched processes and membrane sheets, which express late stage markers, including MBP, CNP and MAG (Figs. 10.2 and 10.3). Although a significant proportion of cells (approximately 60 %) reach a mature oligodendrocyte stage, the cultures also include late stage progenitors.

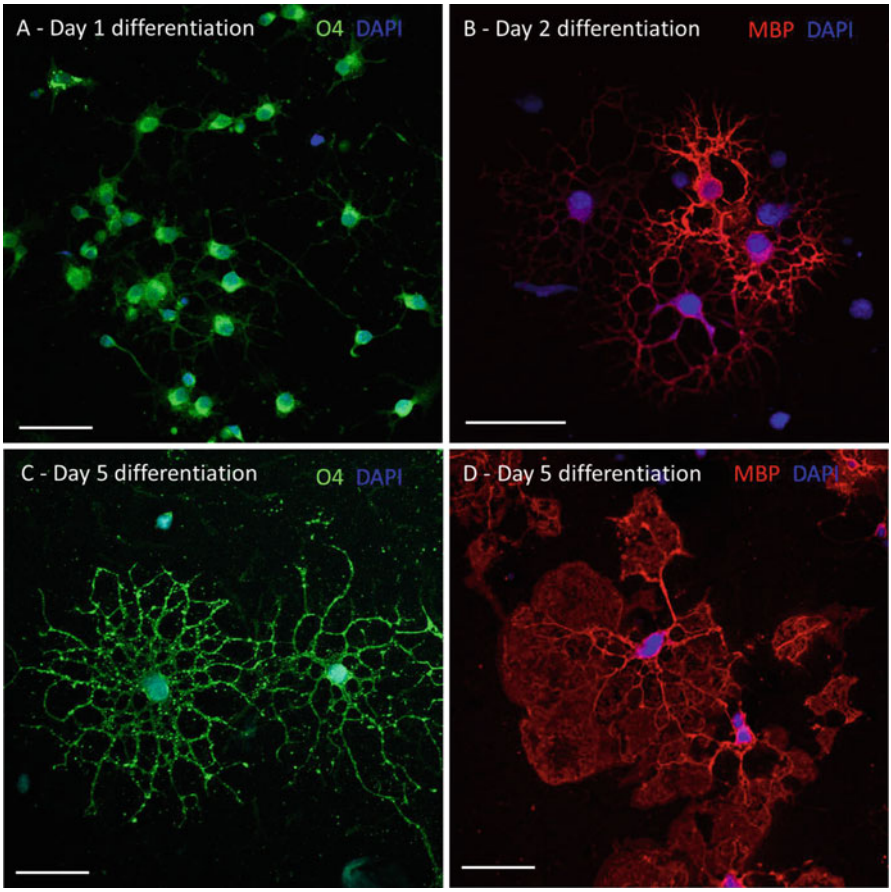

Fig. 10.3 Primary cultures of rat oligodendrocytes at different stages of differentiation. At day 1 in culture, oligodendrocyte lineage cells (O4-positive) present a simple and mostly bipolar morphology with only a few branches (**a**). At day 2 in culture, the morphology is more complex and branched, with some cells already expressing MBP (**b**). At day 5 of differentiation, cells are highly ramified and present intricate morphology and complex branched structure, as indicated by O4 staining (**c**). Above 60 % of the cells in culture at 5 days of differentiation express the mature marker myelin basic protein (MBP) (**d**)

10.2.3 Oligodendrocyte Physiology and Function

Recent data indicate that the oligodendrocyte population is heterogeneous and can differ in location, morphology, activity and function. They have a highly ramified morphology and extend processes to synapses (Bergles et al. 2000) and nodes of Ranvier (Huang et al. 2005) suggesting that they may also monitor or modulate neuronal activity. Indeed, a subpopulation expresses voltage-gated sodium and potassium channels, generates action potentials when depolarized and senses the environment by

receiving excitatory and inhibitory synaptic input from axons (Karadottir et al. 2008). This suggests that the oligodendrocyte lineage cells may process information as well as act as a reservoir of new oligodendrocytes: It is possible that subpopulations exist that are specialized to perform these different tasks (Crawford et al. 2014).

Nevertheless, the main function of oligodendrocytes is to make myelin. Myelin accounts for approximately 70 % of the dry weight of the mammalian CNS (Siegel and Albers 2006) and is critical in maintaining electrical impulse conduction and maximizing its velocity. Myelin sheaths are arranged in segments separated by nodes of Ranvier, where sodium channels are clustered in high density in the axon membrane so that they can produce action potentials (Waxman and Ritchie 1993). Myelin covers and masks the internodal parts of the axon, which contain fewer sodium channels and a higher density of potassium channels, which tend to oppose the generation of action potentials (Waxman and Ritchie 1993). Myelination results in axons with high resistance and low capacitance increasing the speed of electrical conduction and improving its metabolic efficiency, thereby optimizing space and energy utilization (Waxman and Ritchie 1993). In addition, myelin sheaths play an important role in maintaining axonal integrity (Nave 2010a, b). Although deletion of single myelin genes such as PLP (Griffiths et al. 1998) or CNP (Lappe-Siefke et al. 2003) does not lead to overt structural changes of myelin sheaths, animals thus affected display enhanced chronic axonal degeneration. Therefore, myelin sheaths not only provide physiological and structural but also trophic support for axons, promoting their viability and integrity (Nave and Trapp 2008; Nave 2010a, b). These interactions may involve metabolic interactions between OL and axons via the transfer of lactate (Funfschilling et al. 2012) and, eventually, other molecules released by OLs. The disruption of such metabolic interactions might contribute to the pathology of neurodegenerative disorders such as Amyotrophic Lateral Sclerosis (Lee et al. 2012b). Therefore, a new niche of investigation has emerged on the metabolic role of OLs in the brain (Amaral et al. 2013; Hirrlinger and Nave 2014).

10.2.4 Oligodendrocyte Metabolism

Glucose is the main cerebral energy substrate. Brain glucose metabolism exhibits large regional variations at rest (consider, for example, the difference between grey and white matter) and is profoundly affected by brain activation (Siegel and Albers 2006), indicating a strong link between energy metabolism and brain function. 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 as astrocytes in the TCA cycle and had only slightly less oxidation than neurons (Edmond et al. 1987; Amaral et al. 2016). Moreover, glucose and lactate have been identified as important regulators of oligodendrocyte development and myelination (Rinholm et al. 2011). Recent findings indicate that axons and oligodendrocytes are coupled with respect to their metabolism, with oligodendrocytes providing aerobic glycolysis products to axons under conditions that cause energy deprivation (Funfschilling et al. 2012).

The recently recognized trophic and metabolic roles of myelin provide an explanation for the progressive atrophy of demyelinated axons. Conversely, they also suggest that promoting remyelination could reinstate this supportive function and prevent the neurodegenerative component of demyelinating disease (Franklin and Ffrench-Constant 2008; Crawford et al. 2014).

10.2.4.1 Glucose and Lactate Metabolism in Oligodendrocytes

A few studies have investigated glucose metabolism in oligodendrocyte lineage cells (Sanchez-Abarca et al. 2001; Rinholm et al. 2011; Amaral et al. 2016). Our recent findings indicate that glucose metabolism is very active in oligodendrocyte lineage cells, in particular, their glucose consumption rate is comparable to that of astrocytes (Amaral et al. 2016). Moreover, based on the ratios of lactate release to glucose consumption for both types of cultures, it has been reported that oligodendrocytes use a larger proportion of glucose for mitochondrial metabolism than astrocytes (Sanchez-Abarca et al. 2001; Amaral et al. 2016) although they also release substantial amounts of lactate (Amaral et al. 2016). In fact, lactate is a metabolic intermediate thought to mediate oligodendroglial trophic support to axons as proposed recently by Funfschilling et al. (2012) and Lee et al. (2012b).

Lactate is also used as substrate by oligodendrocytes. It supports myelination and oligodendrocyte development in the absence of sufficient glucose levels (Rinholm et al. 2011) and is metabolized for lipid synthesis to a larger extent than in astrocytes and neurons (Sanchez-Abarca et al. 2001). On the other hand, extracellular lactate also appears to function as a metabolic activator in oligodendrocytes (Amaral et al. 2016). Investigating glucose metabolism with isotope labelling indicated that both glucose consumption and the proportion of glucose-derived pyruvate metabolized in the mitochondria were increased in the presence of exogenous lactate (Amaral et al. 2016). A net production of lactate was observed indicating that glycolysis in oligodendrocytes is not inhibited by extracellular lactate (Amaral et al. 2016).

The Pentose Phosphate Pathway (PPP)

The PPP is a glucose shunt, which is thought to be active in neurons and astrocytes (Almeida et al. 2002; Garcia-Nogales et al. 2003; Amaral et al. 2010; Bolanos and Almeida 2010; Brekke et al. 2012). Using ^{14}C tracing techniques, Edmond et al. (1987) showed active PPP in oligodendrocyte lineage cells, whereas Sanchez-Abarca et al. (2001) reported PPP activity in immature OPC cultures as being two-fold higher than in astrocytes and four-fold higher than in neurons. We recently measured the contribution of the PPP to the synthesis of glycolytic intermediates and glutamate synthesis based on ^{13}C-tracing techniques. This demonstrated that mature oligodendrocytes use approximately 10–15 % of glucose in the PPP compared to glycolysis as observed in the labelling of the glycolytic intermediates PEP and 3PG. These values are in the range of previously published data for cortical

astrocyte cultures in a metabolic modelling study also using ^{13}C-labelled substrates (Amaral et al. 2011a, b), which contrasts with the report by Sanchez-Abarca et al. (2001). A potential explanation for the differences is that our cultures contained more mature oligodendrocytes than those used by Sanchez-Abarca et al. (2001), which may be associated with lower PPP activity, presenting a rate closer to the one observed in astrocytes (Amaral et al. 2011a). On the basis of ^{14}C tracing experiments, (Sykes et al. 1986) reported that the PPP produces more CO_2 than the TCA cycle in primary oligodendrocyte lineage cells due to its close link to de novo synthesis of fatty acids and cholesterol (Sykes et al. 1986). Since it was not feasible to measure its contribution to lipid synthesis, it is therefore possible that our approach underestimated the total use of glucose via the PPP. Furthermore, our study showed for the first time that pyruvate generated from glucose via the PPP contributes to the synthesis of acetyl CoA for oxidation and generation of metabolites in the TCA cycle in oligodendrocytes, contributing to approximately 10 % of the glutamate produced (Amaral et al. 2016). Estimation of the PPP activity on the basis of glutamate isotopomers indicated that the PPP accounted for approximately 6 % of glucose metabolism in cortical neurons and approximately 4 % in cerebellar neurons (Brekke et al. 2012). Similar to what was reported in neurons (Brekke et al. 2012), we found that oligodendrocytes incorporate ^{13}C label in glutamate produced by [1,2-^{13}C]glucose metabolism via the PPP.

Despite significant PPP activity in oligodendrocytes, it remains largely unknown whether the PPP plays a role during pathological events affecting oligodendrocyte function. Interestingly, increased activity of the enzyme transaldolase (which is part of the non-oxidative branch of the PPP and is involved in lipid and nucleotide synthesis) has been reported in oligodendrocytes in brains of Multiple Sclerosis (MS) patients compared to healthy controls (Banki et al. 1994). Banki and colleagues suggested that the PPP might be involved in the protection of myelin sheaths from oxidative stress and that transaldolase appears to be a target to break immunological tolerance and kill oligodendrocytes in MS. Altered PPP activity has also been reported in a study on traumatic brain injury (TBI) patients although the authors did not establish any correlation with oligodendrocyte function (Dusick et al. 2007). It is likely that part of PPP alterations observed in the study by Dusick et al. (2007) could be linked to extensive demyelination and remyelination that is known to occur in TBI patients (Armstrong et al. 2015).

Mitochondrial Metabolism of Pyruvate Generated from Glucose

Using different forms of ^{13}C labelled glucose our recent study also demonstrated that mature oligodendrocytes exhibit a high rate of mitochondrial metabolism (Amaral et al. 2016). These findings echo the results from previous studies by Sanchez-Abarca et al. (2001). Functional mitochondria seem to be particularly important when OPCs differentiate (Schoenfeld et al. 2010; Ziabreva et al. 2010). It is likely that OPC differentiation, which involves a complex expansion of cell morphology (Fig. 10.3), requires high levels of glucose metabolism to meet the

increased demands of ATP, citrate and amino acids required for the synthesis of cellular components. In contrast, it was proposed that myelinating oligodendrocytes are not dependent on mitochondrial activity in vivo (Funfschilling et al. 2012). The most likely explanation for the discrepancy between the high mitochondrial demands of the cultures used in Amaral et al. (2016) and the findings in vivo is the significant presence of pre-myelinating (MBP-negative) OPCs that have not yet reached fully mature stages in vitro. Our data suggest a prominent role of oligodendrocyte oxidative metabolism at the late stages of OPC differentiation, including the pre-myelinating and early myelinating stages.

Pyruvate Carboxylation

An important question with respect to mitochondrial metabolism in oligodendrocytes is whether they can replenish TCA cycle intermediates via anaplerosis. It is well established that neurons depend on astrocytes for replenishing their TCA cycle intermediates. As neurons cannot carboxylate pyruvate, external TCA cycle intermediates are required for the synthesis of amino acid neurotransmitters (McKenna et al. 2012). We therefore investigated whether and to which extent oligodendrocytes are self-sufficient with respect to the production of anaplerotic substrates. Incubation of cells with [1,2-^{13}C]glucose and subsequent mass spectrometry analysis of cell extracts indicated that pyruvate carboxylation indeed takes place in oligodendrocytes as shown by the production of [2,3-^{13}C]glutamate. This was further confirmed by label incorporation in citrate in the medium of cells incubated with [1-^{13}C]lactate, which is only possible via pyruvate carboxylation (Amaral et al. 2016). Comparable label incorporation from [1-^{13}C]lactate was found in citrate in the medium of astrocyte cultures. This suggests that lactate is metabolized in a similar way in the TCA cycle of both cell types. It must be noted that the overall contribution of pyruvate carboxylation to oligodendrocyte and astrocyte metabolism is underestimated in experiments using [1-^{13}C]lactate because the [1-^{13}C]pyruvate from [1-^{13}C]lactate competes with unlabelled pyruvate from glucose, which was also present in the incubation medium.

Pyruvate can be carboxylated to oxaloacetate by PC or to malate and NADP+ by malic enzyme. Whether PC or malic enzyme is responsible for pyruvate carboxylation in oligodendrocytes remains to be established. Murin et al. (2009) reported PC expression in cultured oligodendroglia. Whether oligodendrocytes express malic enzyme remains unknown. In neurons and astrocytes malic enzyme only works in the direction of pyruvate production (McKenna et al. 1995, 2000). Moreover, PC has been shown to be the most important anaplerotic enzyme in the brain (Patel 1974). Irrespective of which enzyme is responsible for pyruvate carboxylation, the detection of carboxylation (and thus anaplerosis) has consequences for oligodendrocyte metabolism. If oligodendrocytes (similarly to neurons) were not capable of anaplerosis they would depend on the provision of glutamine by astrocytes, which have a net production of glutamine via pyruvate carboxylation (Gamberino et al. 1997; Waagepetersen et al. 2001).

An alternative route to replenish the TCA cycle in oligodendrocytes is to use aspartate, liberated from *N*-acetyl aspartate (NAA), which in turn is supplied by neurons (Moffett et al. 2007). However, aspartate released by NAA hydrolysis in oligodendrocytes could potentially be sent back to neurons, thus avoiding the depletion of anaplerotic substrates in neurons (NAA synthesis in neurons is dependent on glutamine entry from astrocytes). Our recent study demonstrated that oligodendrocytes are capable of anaplerosis, which suggests that they are potentially independent of astrocytic pyruvate carboxylation. Whether the level of anaplerosis in oligodendrocytes is sufficient to meet their entire requirements is not known at present. It is also unclear whether aspartate is shuttled back to neurons or whether it is metabolized in oligodendrocytes. Both possibilities have previously been suggested but evidence is lacking (Baslow and Guilfoyle 2006; Moffett et al. 2007).

10.2.4.2 Acetate Metabolism

Acetyl CoA is an essential molecule in the TCA cycle. Most acetyl CoA derives from pyruvate via PDH; however, oligodendrocytes are also known to express the enzyme aspartoacylase, which catalyses the hydrolysis of NAA into aspartate and acetate (Moffett et al. 2011). NAA-derived acetate significantly contributes to myelin lipid synthesis in the CNS (Chakraborty et al. 2001) and is also thought to support oxidative metabolism during myelination (Francis et al. 2012). Furthermore, oligodendrocytes express acetyl CoA synthetase-1 (catalyses the synthesis of acetyl coenzyme A from acetate and coenzyme A), especially during postnatal brain development, supporting the contribution of acetate to lipid synthesis in these cells (Ariyannur et al. 2010). Currently, it is thought that astrocytes can convert acetate into acetyl CoA whereas neurons are not able to do so (Muir et al. 1986; Sonnewald et al. 1993). Consequently, acetate has been used extensively to assess astrocyte metabolism in the context of astrocytic-neuronal interactions in vivo (e.g. Melo et al. 2005; Morken et al. 2014; Nilsen et al. 2014), in neurospheres (Sa Santos et al. 2011) and in culture (Sonnewald et al. 1993). However, these studies did not consider potential metabolic contributions from oligodendrocytes. Our recent study demonstrated that mature oligodendrocyte cultures are able to convert acetate into acetyl CoA and oxidize it in the mitochondria as shown by the incorporation of ^{13}C label from [1,2-^{13}C]acetate into the TCA cycle intermediates malate and citrate and the amino acids glutamate and glutamine. Labelling from [1,2-^{13}C]acetate was not as pronounced as labelling from [1,2-^{13}C]glucose but nevertheless significant and comparable to that observed in astrocytes (Amaral et al. 2016). Surprisingly, we found that alanine was also labelled from [1,2-^{13}C]acetate, and that alanine M+1 enrichment was detected in oligodendrocytes incubated with [1,2-^{13}C]glucose. Both isotopologues of alanine could not have been produced without the participation of the TCA cycle and malic enzyme or pyruvate kinase (PK) and phosphoenolpyruvate carboxy kinase (PEPCK) (Cruz et al. 1998). This indicates that pyruvate recycling, a catabolic pathway (Cerdan et al. 1990; Kunnecke et al. 1993; Haberg et al. 1998; Olstad et al. 2007; Amaral et al. 2011b), is not only active in astrocytes and neurons but also in oligodendrocytes.

10.3 Conclusion

It is now clear that oligodendrocytes have an important metabolic role in the brain. Metabolic support to axons is essential and its dysfunction might be implicated in many neurological disorders, including ischemia (Bakiri et al. 2008), ALS (Lee et al. 2012a, b) and psychiatric disorders (Nave and Ehrenreich 2014), in addition to the classical myelin disorders (Fancy et al. 2010). The observation of pyruvate carboxylation activity in oligodendrocytes suggests a possible role in a glutamate–glutamine cycle (Fig. 10.1) with axons, taking into account the studies reporting glutamate release from axons targeting NMDA receptors on oligodendrocytes (Karadottir and Attwell 2007). A glutamate–glutamine cycle might also play a role in OPC differentiation and myelination in the sequence of studies showing that neuronal signalling promotes OPC differentiation in certain conditions (Wake et al. 2011; Gibson et al. 2014; Gautier et al. 2015). Further studies are required to address these particular questions.

Moreover, the stem cell features of adult OPCs suggest that metabolic remodelling is likely to occur during differentiation as in other stem cell populations. Such metabolic changes might be determinant for the correct migration, proliferation and differentiation of OPCs during development or during adulthood in contexts of remyelination. It is likely that the demand for building blocks required to expand cell processes and cytoskeleton of a bipolar cell into a complex ramified cell extending myelin sheaths is high and requires specialized energetic demand. It is also likely that damage induced by reactive oxygen species (Lassmann 2010) and glutamate in MS and ischemic injury might affect oligodendrocyte metabolic functions and contribute to an incorrect differentiation/remyelination.

Finally, the possibility of obtaining human oligodendrocytes derived from pluripotent stem cells, e.g. (Stacpoole et al. 2013; Douvaras and Fossati 2015; Gorris et al. 2015) is creating the unique opportunity of investigating metabolism in human cells, including in models of disease. The metabolic characterization of human oligodendrocytes will enable to compare rodent and human cells and investigate metabolic abnormalities linked to myelin disorders in patient-derived cells.

In conclusion, oligodendrocyte metabolism is a growing field of research and future studies will further elucidate oligodendrocyte metabolic function, both in immature and mature cells and also in vivo, to shed new light into the mechanisms of OPC differentiation and neuronal–glial interactions in physiological and disease contexts.

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Chapter 11
Dysregulation of Glutamate Cycling Mediates Methylmercury-Induced Neurotoxicity

Megan Culbreth and Michael Aschner

Abstract To examine the toxicological implications of glutamate, this chapter will focus specifically on its impact in the brain. More explicitly, it will illustrate the role glutamate plays in mediating methylmercury (MeHg)-induced neurotoxicity. In this chapter, one intends to highlight the processes that occur prior to glutamate-stimulated excitotoxicity and subsequent neurodegeneration. As such, it will emphasize three main routes by which MeHg alters glutamate homeostasis. It is essential to recognize that these effects are not mutually exclusive, and that they synergistically influence glutamate dysregulation. Furthermore, the consequences of MeHg exposure will be presented here as a direct pathway; however, it must be noted these effects occur simultaneously. First, glutamate uptake will be reviewed emphasizing the function of astrocytes. Next, the induction of oxidative stress by MeHg exposure will be discussed. This process has a two-fold effect on glutamate homeostasis by (1) inhibiting extracellular glutamate uptake and (2) altering transcription of genes vital to glutamate cycling. Finally, the impact glutamate dysregulation has on glutathione synthesis will be examined. Although this chapter centers on the link between glutamate and MeHg toxicity, it is imperative that the reader acknowledges the processes discussed here can be extended to any pro-oxidant.

Keywords Glutamate • Homeostasis • Toxicity • Cycling • Methylmercury

11.1 Introduction

Glutamate is a nonessential amino acid, deemed the primary excitatory neurotransmitter in the mammalian nervous system. Simply, glutamate is synthesized in neurons and released via exocytosis into the extracellular space, where it activates specific receptors that will propagate electrical signals. This process is essential for

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normal brain function. In order to prevent continual stimulation, glutamate must be removed from the extracellular space. Several mechanisms have evolved to mediate glutamate uptake. This chapter will focus primarily on the glutamate-aspartate transporter (GLAST or EAAT1) and the glutamate transporter-1 (GLT-1 or EAAT2). Both are predominately expressed in astrocytes (Rothstein et al. 1994; Lehre et al. 1995), the main cell responsible for glutamate uptake (Rothstein et al. 1996). Under normal conditions, glutamate levels are much higher intracellularly than extracellularly (Hamberger and Nystrom 1984). Neurotransmission results in glutamate release and subsequent activation of post-synaptic glutamate receptors. Once the signal has been disseminated, synapse-adjacent astrocytes must remove excess glutamate via GLAST and GLT-1. MeHg elicits toxicity by indirectly inhibiting GLAST and GLT-1 (Brookes and Kristt 1989; Aschner et al. 1990, 1993; Qu et al. 2003).

MeHg is a potent environmental neurotoxicant. Humans are primarily exposed to MeHg via fish consumption. Although extensive regulation has virtually eliminated high-dose MeHg exposure, there continues to be significant risk associated with low-dose exposure because the mechanism of MeHg toxicity is not fully understood. Accidental high-dose exposures in Japan (Harada 1995; Eto 2000) demonstrated the severe developmental consequences of MeHg, motivating the scientific community to further investigate its mechanism of action. Moreover, several more recent epidemiological studies of populations in which fish is a dietary staple have correlated low-dose in utero MeHg exposure to long-term developmental consequences (Davidson et al. 2008, 2010, 2011; Grandjean et al. 1997; Debes et al. 2006), further prompting the need to research MeHg toxicity. In light of these events, as well as the epidemiological studies, researchers have continued to use in vivo and in vitro models to explore the mechanism of MeHg toxicity.

It is widely accepted that MeHg exposure generates reactive oxygen species (ROS), initiating an oxidative stress response in the brain (Shanker et al. 2004). The primary ROS produced subsequent to MeHg exposure is hydrogen peroxide (H_2O_2). Increase in H_2O_2 is the direct result of MeHg inhibiting glutathione peroxidase, the enzyme responsible for detoxifying H_2O_2 to water (Franco et al. 2009). Additionally, MeHg indirectly enhances H_2O_2 production by interacting with the mitochondrial electron transfer chain, causing sporadic ROS generation (Mori et al. 2011). H_2O_2 has been shown to inhibit GLAST and GLT-1 (Sorg et al. 1997). Moreover, MeHg is lipophilic and promotes lipid peroxidation. It activates phospholipase A_2, stimulating release of arachidonic acid (AA) (Shanker et al. 2002). In turn, AA inhibits GLAST and GLT-1 (Barbour et al. 1989; Lundy and McBean 1996).

The pro-oxidant properties of MeHg are not limited to the effects on glutamate uptake. In generating reactive intermediates, MeHg activates nuclear factor erythroid 2-related factor 2 (Nrf2) (Wang et al. 2009). Nrf2 is a transcription factor that translocates to the nucleus in response to reactive oxygen species generation and upregulates phase II detoxifying enzymes and antioxidant proteins. In unstressed conditions, Nrf2 is retained in the cytosol, bound to kelch-like ECH-associated protein 1 (Keap1) (Itoh et al. 1999). Keap1 serves as an adaptor for E3-ubiquitin ligases (Kobayashi et al. 2004), which will ubiquitinate Nrf2, promoting its proteasomal degradation. Keap1 further functions as an oxidative stress

sensor (Kobayashi et al. 2006). Upon ROS generation, cysteine residues on Keap1 are modified, allowing the dissociation and subsequent nuclear translocation of Nrf2. Altering Nrf2 activity has significant implications on glutamate homeostasis. Nrf2 promotes glutathione peroxidase transcription, which as mentioned above is the enzyme required to detoxify H_2O_2.

Nrf2 further functions in glutamate homeostasis by stimulating transcription of the cystine/glutamate exchanger (X_c^-) (Sasaki et al. 2002). This system exchanges intracellular glutamate for extracellular cystine, which is necessary for glutathione (GSH) synthesis (Bannai and Kitamura 1980). GSH is essential for detoxification (Lu 2009). MeHg induces ROS generation, which in turn prevents glutamate uptake (Allen et al. 2001a). The resultant increase in extracellular glutamate inhibits X_c^-, thereby decreasing GSH synthesis and subsequent detoxification.

Although the processes discussed in this chapter are by no means exhaustive of the toxicological implications of glutamate dysregulation, they do provide a flavor for the potential effects of pro-oxidants in the brain.

11.2 Methylmercury Inhibits Glutamate Uptake

As mentioned previously, glutamate is an excitatory amino acid. Extracellular glutamate levels must be tightly regulated in order to sustain normal brain function. Astrocytes are the primary cells responsible for glutamate uptake from the extracellular space (Rothstein et al. 1996). The glutamate/aspartate transporter (GLAST) and glutamate transporter-1 (GLT-1) are the main channels through which glutamate uptake occurs in astrocytes. Both GLAST and GLT-1 are expressed ubiquitously throughout the brain and are located almost exclusively in astrocytes (Rothstein et al. 1994; Lehre et al. 1995). In vitro, glutamate stimulates concentration- and time-dependent uptake via GLAST and GLT-1 in astrocytes. Furthermore, it increases GLAST expression at the astrocyte cell surface (Duan et al. 1999).

Inorganic mercury has been found to preferentially accumulate in astrocytes subsequent to MeHg exposure (Charleston et al. 1995). As such, researchers wanted to investigate whether MeHg has any effects on glutamate homeostasis. MeHg causes a concentration- and time-dependent decrease in glutamate uptake in astrocytes (Brookes and Kristt 1989; Aschner et al. 1990; Allen et al. 2001a; Qu et al. 2003). Interestingly, intracellular glutamate concentration remained unchanged (Yin et al. 2009). MeHg also increases glutamate efflux from astrocytes (Aschner et al. 1993).

In order to understand the mechanism by which MeHg could alter glutamate uptake and efflux, GLAST and GLT-1 expression subsequent to exposure was explored. It was found that MeHg significantly increases GLT-1 mRNA expression with no effect on GLAST. Interestingly, GLT-1 protein levels were decreased, while GLAST protein levels increased (Mutkus et al. 2005). These data do not definitively reveal a link to glutamate homeostasis and MeHg toxicity, thus further investigation is warranted. It has been postulated that MeHg could alter GLT-1 and GLAST function due to its high affinity for sulfhydryl groups on cysteine residues.

11.3 Methylmercury and Glutamate Generate Reactive Oxygen Species

ROS are continually being generated intracellularly. Oxidative stress results from an imbalance between ROS production and the ability to detoxify them. It has become increasingly evident that oxidative stress is complicit in several neurodegenerative diseases including Alzheimer's disease (Liu et al. 2015), Parkinson's disease (Blesa et al. 2015), and amyotrophic lateral sclerosis (ALS) (Palomo and Manfredi 2015). ROS have potential to damage DNA, proteins, and lipids, which could be detrimental to cell function.

MeHg exposure increases ROS generation. It has been shown that intracellular H_2O_2 rises subsequent to exposure in astrocytes (Shanker et al. 2004). In addition, mitochondrial ROS formation increases. The mechanism(s) by which MeHg effects ROS production is not entirely known. MeHg has been found to decrease mitochondrial membrane potential (Yin et al. 2007). Loss of membrane potential would result in spurious electron escape from the electron transfer chain and resultant increase in ROS. MeHg has also been observed to alter the protein complexes of the electron transfer chain (Mori et al. 2011), impacting the cells' ability to produce ATP. In addition, MeHg inhibits glutathione peroxidase (GPx) activity (Franco et al. 2009). GPx reduces H_2O_2 to non-toxic H_2O.

Glutamate also enhances ROS formation. It has been demonstrated that exposure to *N*-methyl-D-aspartate (NMDA), the agonist for a neuronal glutamate receptor, increases ROS production (Dugan et al. 1995). Furthermore, glutamate was found to enrich ROS depending on NMDA receptor activation and Ca^{2+} influx (Reynolds and Hastings 1995). It is proposed that mitochondrial dysfunction is critical to glutamate-induced ROS generation.

11.4 Reactive Oxygen Species Inhibit Glutamate Uptake

As mentioned above, MeHg is a pro-oxidant, which induces a concentration- and time-dependent oxidative stress response. MeHg increases ROS generation by compromising mitochondrial membrane integrity. H_2O_2 is the most abundant ROS produced subsequent to MeHg exposure. It has been demonstrated that antioxidants including *n*-propyl gallate (Shanker et al. 2005) and superoxide dismutase (Shanker and Aschner 2003) attenuate MeHg toxicity. Furthermore, co-application of MeHg and glutamate has been shown to augment ROS formation (Amonpatumrat et al. 2008).

MeHg is also highly lipophilic. It has been found to increase cytosolic phospholipase A_2 ($cPLA_2$) mRNA and protein expression (Shanker et al. 2002). $cPLA_2$ assists in cell membrane phospholipid maintenance. Moreover, MeHg activates $cPLA_2$ resulting in AA release (Shanker et al. 2002). AA promotes ROS generation (Trotti et al. 1998; Cocco et al. 1999). The effects of MeHg on $cPLA_2$ activation and AA release are tempered by arachidonyl trifluoromethyl ketone, a specific $cPLA_2$ inhibitor (Shanker and Aschner 2003). Glutamate has also been shown to stimulate AA release (Dumuis et al. 1988; Stella et al. 1994).

As mentioned above, H_2O_2 is readily generated subsequent to MeHg exposure. H_2O_2 has been found to inhibit glutamate uptake (Sorg et al. 1997). Furthermore, catalase, an antioxidant, significantly reduced MeHg-stimulated inhibition of the uptake of ^{3}H-D-aspartate, a glutamate analog (Allen et al. 2001a). Catalase reduces H_2O_2 to non-toxic H_2O and O_2. AA has also been found to directly inhibit glutamate uptake (Barbour et al. 1989; Lundy and McBean 1996).

11.5 Reactive Oxygen Species Activate Nrf2

Nuclear factor erythroid 2-related factor 2 (Nrf2) is a transcription factor that translocates to the nucleus in response to oxidative stress. Nrf2 promotes transcription of phase II detoxifying enzymes (Itoh et al. 1997). Under normal conditions, Nrf2 is bound to kelch-like ECH-associated protein 1 (Keap1) in the cytosol. Keap1 blocks Nrf2 nuclear activation (Itoh et al. 1999). Furthermore, Keap1 serves as an adaptor for E3 ligases (Kobayashi et al. 2004), which will ubiquitinate Nrf2, marking it for proteasomal degradation. Keap1 also acts as an oxidative stress sensor (Kobayashi et al. 2006). Modification of cysteine residues on Keap1 by ROS allows for partial dissociation of Nrf2 from Keap1. Nrf2 must be further phosphorylated by protein kinase C (PKC) (Huang et al. 2002) in order to completely detach from Keap1 and move into the nucleus. For a review of Nrf2 regulation, refer to Niture et al. (2014), and see Fig. 11.1.

Nrf2 activation is crucial to the cells' ability to respond to oxidative stress. It has been demonstrated that Nrf2 knockout mice are more susceptible to oxidant-induced injury (Jiang et al. 2009; Kurzatkowski and Trombetta 2013). Furthermore, Nrf2 activation has been found to be protective in neurodegenerative models of Parkinson's

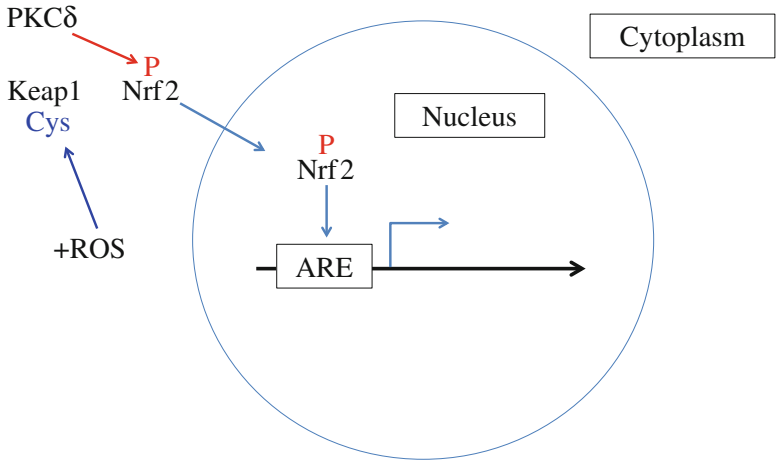

Fig. 11.1 Reactive oxygen species induce Nrf2 activation. Adapted from Niture et al. (2014). *ROS* reactive oxygen species, *Cys* cysteine, *Keap1* kelch-like ECH-associated protein 1, *Nrf2* nuclear factor erythroid 2-related factor 2, *PKCδ* protein kinase C delta, *ARE* antioxidant response element, *P* phosphorylation

(Jakel et al. 2007) and ALS (Vargas et al. 2008). It has also been shown that mutant Huntingtin gene expression significantly reduces Nrf2 activity (Jin et al. 2013). Interestingly, astrocytes were found to be the preferential site of Nrf2 activation (Kraft et al. 2004).

As mentioned previously, MeHg induces an oxidative stress response in the brain. Nrf2 has been found to partly mediate this reaction. It has been demonstrated that MeHg promotes Nrf2 nuclear translocation and subsequent upregulation of phase II detoxifying enzymes in both microglia (Ni et al. 2010) and astrocytes (Wang et al. 2009). Furthermore, Nrf2 knockout significantly increased vulnerability to MeHg with a concurrent decrease in phase II detoxifying enzyme expression (Ni et al. 2011).

11.6 Nrf2 Induces Cystine/Glutamate Antiporter Gene Expression

Nrf2 promotes the transcription of various genes required for cellular defense against oxidative stress. One such gene is xCT (Sasaki et al. 2002), the transporter component of the cystine/glutamate antiporter (X_c^-). X_c^- exchanges intracellular glutamate for extracellular cystine at a 1:1 ratio (Bannai 1986). Cystine is readily converted to cysteine, the rate-limiting precursor for glutathione (GSH) synthesis, intracellularly (Bannai and Kitamura 1980). GSH is essential for cellular detoxification (Lu 2009). Astrocytes readily transport cystine (Sagara et al. 1993) and maintain high intracellular GSH levels (Yudkoff et al. 1990).

As previously mentioned, MeHg induces Nrf2 nuclear translocation and subsequent upregulation of phase II detoxifying enzymes. It has been demonstrated that MeHg exposure stimulates xCT mRNA expression. This effect is tempered by Nrf2 knockdown in astrocytes (Wang et al. 2009; Ni et al. 2011).

Although MeHg stimulates xCT transcription, it also concurrently inhibits cystine uptake. It has been found that MeHg acts on the glutamate/aspartate transporter (X_{AG}^-) to inhibit cystine uptake in astrocytes (Allen et al. 2001b). X_{AG}^- is the primary glutamate transporter in astrocytes (Cho and Bannai 1990). It has also been demonstrated that co-application of an X_{AG}^- inhibitor and MeHg hinders cysteine uptake (Shanker et al. 2001). It must be noted that high extracellular glutamate concentration inhibits both X_c^- (Murphy et al. 1989) and X_{AG}^- (Murphy et al. 1990) transport.

11.7 Conclusion

Glutamate is the predominant excitatory neurotransmitter in the brain. As such, it must be tightly regulated in order to avoid excitotoxic injury and subsequent neurodegeneration. Several transport mechanisms have evolved to maintain glutamate homeostasis. GLAST and GLT-1 are the main channels through which glutamate uptake occurs (Zhou and Danbolt 2014). These transporters are expressed almost exclusively in astrocytes (Rothstein et al. 1994; Lehre et al. 1995). Astrocytes are

also the preferential site of MeHg accumulation in the brain (Charleston et al. 1995), rendering glutamate homeostasis a likely target for MeHg-induced toxicity.

MeHg has been observed to inhibit glutamate uptake (Brookes and Kristt 1989; Aschner et al. 1990; Allen et al. 2001a; Qu et al. 2003). This might occur by MeHg reacting with sulfhydryl groups on cysteine residues in GLAST and GLT-1, thus altering transporter function. Further research is required to confirm this hypothesis. MeHg also enhances ROS generation (Shanker et al. 2004) and AA release (Shanker et al. 2002). Both ROS (Sorg et al. 1997; Allen et al. 2001a) and AA (Barbour et al. 1989; Lundy and McBean 1996) have been demonstrated to inhibit glutamate uptake.

ROS activate Nrf2-dependent phase II detoxifying enzyme transcription. Nrf2 promotes xCT, the transporter component of the cystine/glutamate antiporter (Sasaki et al. 2002). MeHg has been shown to activate Nrf2, consequently increasing xCT transcription (Wang et al. 2009; Ni et al. 2011).

MeHg has also been found to inhibit cystine uptake via the glutamate/aspartate transporter (Allen et al. 2001b). This is mostly due to high extracellular glutamate levels as a result of MeHg-induced glutamate uptake inhibition. Cystine is a necessary precursor for GSH synthesis, the principal cellular antioxidant (Bannai and Kitamura 1980). The dynamic relationship between glutamate and MeHg is summarized in Fig. 11.2.

This chapter only highlights a portion of the potential toxicological effects of glutamate homeostasis dysfunction. However, it is important to recognize that much of what is discussed here can be applied to any pro-oxidant. Furthermore, it underscores glutamate itself as the key mediator of toxicity.

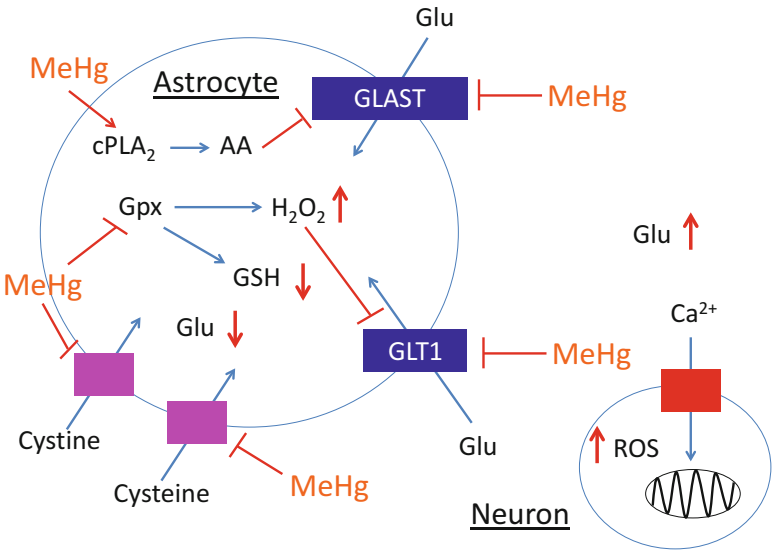

Fig. 11.2 Methylmercury alters glutamate homeostasis. Adapted from Aschner et al. (2007). *MeHg* methylmercury, *cPLA*$_2$ cytosolic phospholipase A$_2$, *AA* arachidonic acid, *Gpx* glutathione peroxidase, H_2O_2 hydrogen peroxide, *GSH* glutathione, *Glu* glutamate, *GLAST* glutamate/aspartate transporter, *GLT-1* glutamate transporter-1, *ROS* reactive oxygen species, *Ca*$^{2+}$ calcium

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Chapter 12
Astroglia, Glutamatergic Transmission and Psychiatric Diseases

Alexei Verkhratsky, Luca Steardo, Liang Peng, and Vladimir Parpura

Abstract Astrocytes are primary homeostatic cells of the central nervous system. They regulate glutamatergic transmission through the removal of glutamate from the extracellular space and by supplying neurons with glutamine. Glutamatergic transmission is generally believed to be significantly impaired in the contexts of all major neuropsychiatric diseases. In most of these neuropsychiatric diseases, astrocytes show signs of degeneration and atrophy, which is likely to be translated into reduced homeostatic capabilities. Astroglial glutamate uptake/release and glutamate homeostasis are affected in all forms of major psychiatric disorders and represent a common mechanism underlying neurotransmission disbalance, aberrant connectome and overall failure on information processing by neuronal networks, which underlie pathogenesis of neuropsychiatric diseases.

Keywords Astrocyte • Glutamatergic transmission • Neurotransmitter receptors • Glutamate synthesis • Neuropathology • Psychiatric diseases • Major depression • Schizophrenia

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12.1 The Multicellular Nature of Neural Tissue: The Neuron Doctrine Challenged

The human brain crowns hundreds of millions of years of the evolution of the nervous system, which occurred in a form of a relatively simple synaptically connected network of neurons, evenly distributed through the body of antediluvian Cnidarians and Ctenophores (Roth and Dicke 2013). This primeval nervous system bestowed a fundamental evolutionary advantage of the coordination of body parts that greatly increased the adaptability of these creatures. The evolution proceeded through the occurrence of concentrated neuronal masses or ganglia to the centralised nervous system, which coincided with the appearance of bilateral symmetry and emergence of Bilateralia. At early evolutionary stages (in, for example, nematodes), the centralised nervous system appears in a form of several ganglia localised around the oral orifice; in more advanced insects or in crustacean, the central nervous system is present in the form of a polyganglionic brain. In the more advanced Echinoderms, Hemichordate and Chordate the layered nervous system becomes the main trait, and this layered organisation characterises the brain of humans.

The connectivity between neurons is accomplished through evolutionary conserved specialised structures known as synapses. Whilst synapses come in two flavours, chemical and electrical, we shall focus our attention to chemical synapses and transmission. The presynaptic elements, mainly represented by axonal terminals, contain neurotransmitter vesicles from which neurotransmitter is released upon the action potential arrival, whereas the postsynaptic elements (often represented by dendritic spines) contain specific receptors and highly complex molecular machinery responsible for short- and long-term regulation of synaptic efficacy. Since the days of Ramón y Cajal, the synaptically connected neuronal networks are considered to represent the substrate for the brain function; this concept is known as the neuron doctrine (Glickstein 2006). The nervous system comprised from the neuronal networks and interneuronal synapses as the system for information processing and decision making epitomises the neuron doctrine that governs neuroscientific thoughts for the last hundred or so years.

The evolutionary history of the brain is, however, more complex and multifaceted. The appearance of the centralised nervous system coincided with the emergence of a second class of neural cells, the neuroglia. This event signalled the cell specialisation and division of labour between distinct elements of the nervous system. Neurons went on by perfecting the fast signalling apparatus, whereas the homeostatic functions were shifted to neuroglia. This, in the course of time, created a highly complex network of specialised neural cells which work in concert towards the brain function. The neuroglial cells by themselves specialised: some of them became brain homeostatic cells (astrocytes) and some evolved into producing the myelin sheath (oligodendrocytes), which is indispensable for making the compact interconnected brain of higher vertebrates (Parpura et al. 2012; Verkhratsky and Butt 2013). The evolving neuroglia also created the barrier between the brain

and the blood, thus imposing the immune privilege on the nervous tissue; this in turn called for resident immune cells which appeared in the form of microglia. Once the immune cells invade the brain parenchyma, they undergo the most remarkable metamorphosis which transforms the round foetal macrophages into surveillance microglia which has almost neuron-like appearance (small body with long ramified processes) and expresses the wealth of neurotransmitter receptors (Kettenmann et al. 2011). The almost revolutionary explosion of the neuroglial field in the last two decades challenged the neuron doctrine and moved attention towards a more inclusive multicellular theory of the organisation of the brain. Moreover, it became obvious that neuroglial cells contribute to all neuropathological processes and often define the progression and outcome of neurological diseases. In this short essay, we overview the role of astrocytes in neuropsychiatric disorders, specifically from the point of view of astroglial regulation of glutamatergic neurotransmission.

12.2 The Glutamatergic Synapse: The Role for Astroglial Cradle

The majority (~60–70 %) of synapses in the CNS are organised from several compartments, that include (1) the presynaptic terminal, (2) the postsynaptic neuronal part, (3) the perisynaptic astroglial process, (4) the microglial process that periodically contacts the synapse and (5) the extracellular matrix (Derouiche et al. 2002; Reichenbach et al. 2010; Dityatev and Rusakov 2011); this complex structure is often being referred to as a "multipartite" synapse (a term which represents an evolution of the tripartite and tetrapartite variants—see (Araque et al. 1999; De Leo et al. 2006; Halassa et al. 2007; Dityatev and Rusakov 2011; Nedergaard and Verkhratsky 2012)). In the complex synaptic arrangement, each component has a clearly defined role. The presynaptic terminal is packed with synaptic vesicles and proteins that underlie fast exocytosis of the neurotransmitters. The postsynaptic compartment provides a dwelling for neurotransmitter receptors and highly diverse biochemical machinery responsible for synaptic responses. The extracellular matrix exerts regulatory effects on both pre- and post-synaptic elements, whereas microglial process monitors the synaptic well-being. The role for astroglial perisynaptic process has emerged only recently, as it appeared that the astroglial compartment (dubbed the astroglial cradle (Nedergaard and Verkhratsky 2012)) controls all aspects of synaptic life, from synaptogenesis, to synaptic maturation, maintenance and extinction (Eroglu and Barres 2010; Verkhratsky and Nedergaard 2014). In particular, the perisynaptic astroglial processes contain exceptionally diverse complement of transporters that control numerous aspects of synaptic cleft homeostasis (Kirischuk et al. 2012; Parpura and Verkhratsky 2012). These transporters buffer extracellular potassium, control extracellular pH, provide reactive oxygen species scavengers and, most importantly in the context of present essay, they control the

extracellular presence of neurotransmitters. Astrocytes are fundamental for the homeostasis and metabolism of three key neurotransmitters: adenosine, glutamate and GABA (Danbolt 2001; Boison et al. 2010; Parpura and Verkhratsky 2012).

In particular, astroglial cells are indispensable elements of the glutamatergic synapse. About 80 % of glutamate, released in the course of synaptic transmission is taken up by astrocytes through the operation of plasmalemmal glutamate transporters that belong to the extended family of solute carriers (SLC). Astroglial plasmalemmal glutamate transporters are represented by two subtypes: the excitatory amino acid transporters 1 and 2 (EAAT1/SLCA3 and EAAT2/SLC1A2). These transporters utilise transmembrane gradient of Na^+ ions for glutamate translocation; transport of a single glutamate molecule (monovalent anion at physiological pH) is associated with an influx of three Na^+ ions, one H^+ ion and efflux of one K^+ ion. This stoichiometry stipulates the electrogenic effects of glutamate uptake (which results in a net inward current); the operation of plasmalemmal glutamate transporters leads to a substantial Na^+ influx that may significantly increase cytosolic Na^+ concentration hence contributing to astroglial Na^+ signalling (Kirischuk et al. 2012). Of note, the reversal potential of plasmalemmal glutamate transporters is more positive than +50 mV or even +60 mV (reflecting transmembrane distribution of relevant ions) and hence they cannot revert in physiological conditions (Kirischuk et al. 2007).

Cytosolic glutamate in astrocytes can be converted into glutamine, this reaction being catalysed (at the expanse of a single ATP) by glutamine synthetase, the astroglia-specific enzyme (Martinez-Hernandez et al. 1977), which is also fundamental for nitrogen metabolism (Rose et al. 2013). Glutamate taken up into astrocytes is not only metabolised to glutamine but, to a significant extent, is oxidatively metabolised, a process that requires the activity of glutamate dehydrogenase and the tricarboxylic acid cycle enzymes (reviewed in Kreft et al. 2012; Obel et al. 2012; McKenna 2013). Pyruvate carboxylase, an enzyme within the cycle and otherwise expressed in astrocytes but not neurons, endows these glial cells with the de novo production of glutamate from pyruvate; the latter being sourced from energy substrates, glucose and lactate (reviewed in Hertz and Zielke 2004). Nonetheless, glutamine synthetase represents a central enzyme of glutamine–glutamate shuttle; that is a coordinated system of glutamate uptake (see earlier) glutamate conversion into glutamine and glutamine transfer from astrocytes to neurons. The later transfer occurs via the glutamine transport which involves two distinct systems of amino acid transporters. The system N, comprised of Na^+-dependent neutral amino acid transporters SN1/SNAT3/SLC38A3 and SN2/SNAT5/SLC38A5, is expressed in astrocytes, where it mediates the efflux of glutamine (Nakanishi et al. 2001; Chaudhry et al. 2002). Neurons accumulate glutamine through the operation of the system A, which includes Na^+-dependent neutral amino acid transporters ATA1/SNAT1/SLC38A1 and ATA2/SNAT2/SLC38A2 (Chaudhry et al. 2002). Importantly, astroglial release of glutamine is controlled by neuronal activity, the increase of which stimulates the astrocytic secretion of glutamine (Martinez-Lozada et al. 2013). After entering the presynaptic terminals glutamine is principally hydrolysed to glutamate by phosphate-activated glutaminase, an energetically neutral conversion. Subsequently,

glutamate is accumulated within synaptic vesicles by the coordinated activity of the proton pump V-ATPase and vesicular glutamate transporters VGLUT1-3 (SLC17A7, SLC17A6 and SLC17A8, respectively), the latter linked to the chloride flux. Like neurons, astrocytes express, albeit to a lesser degree, all the three known isoforms of VGLUTs, which mediate packaging of glutamate into astroglial secretory vesicles (Montana et al. 2004); VGLUT3 emerged as a regulator of vesicular packaging of glutamate in astrocytes (Ni and Parpura 2009). The intravesicular concentration of glutamate likely reaches ~60 mM in neurons and ~20 mM in astrocytes (Montana et al. 2006). Ca^{2+}-dependent exocytosis at presynaptic terminal raises the concentration of glutamate in the synaptic cleft to ~1.1 mM (Clements et al. 1992), while in astrocytes it results in release with localised extracellular glutamate accumulation of 1–100 μM (Innocenti et al. 2000); the latter gliotransmission can lead to modulation of synaptic transmission and plasticity (Araque et al. 1999; Perez-Alvarez and Araque 2013).

12.3 The Psychiatric Diseases as Synaptic Malfunction: The Role for Glutamatergic Transmission

The human brain contains ~15 trillion of synapses, with glutamatergic and GABAergic synapses being the majority accounting for over 85 % of all synaptic contacts in the brain (Douglas and Martin 2007). Importantly, however, many synapses utilise more than one neurotransmitter (the concept of co-transmission (Burnstock 2004)), and all regions of the brain receive noradrenergic innervation (from the locus coeruleus) that controls functional activity of neurons and neuroglia alike. This intricate neurochemical concoction with its ever changing formulation is the substrate for the brain function. The psychiatric diseases reflect the general disbalance of the neurochemistry in the brain, in which the impairment of glutamatergic transmission has been receiving an increased attention, which lead to the emergence of the "glutamate" hypothesis of major depression and schizophrenia (Pittenger and Duman 2008; Racagni and Popoli 2008; Sanacora et al. 2012). At the core of this hypothesis lies an idea that glia affects glutamatergic synapses, which otherwise mediate learning, memory, cognition and emotional reactions.

Levels of glutamate have been found elevated in the cerebrospinal fluid and in the brain of patients with various forms of mood disorders, including those suffering from major depression (Altamura et al. 1993, 1995; Mitani et al. 2006; Kucukibrahimoglu et al. 2009). Levels of glutamate were also found to be increased in the post-mortem specimens of frontal and dorsolateral prefrontal cortices of depressed and bipolar patients (Hashimoto et al. 2007; Lan et al. 2009). Importantly, the treatment with antidepressants correlated with decreased levels of glutamate in the plasma (Maes et al. 1998). An impaired level of glutamate-related metabolites (the so-called *Glx index*, which included glutamate and glutamine) was found in the brains of patients with mood disorders by magnetic resonance spectroscopy. It seems that the Glx index decreases in frontal and cingulate corti-

ces and increases in occipital and parieto-occipital regions (Rosenberg et al. 2004, 2005; Sanacora et al. 2004; Yuksel and Ongur 2010; Taylor et al. 2011), see also in depth meta-analysis supporting impaired astroglial glutamate–glutamine shuttling in depression (Arnone et al. 2015).

The glutamatergic model of pathophysiology of schizophrenia was prompted by the discovery of the N-methyl-D-aspartate (NMDA) type of glutamate receptors as a molecular target for psychotomimetic compounds such as ketamine or phencyclidine (Javitt 1987; Javitt and Zukin 1991). It appeared that the inhibition of NMDA receptors can transiently reproduce some symptoms of schizophrenia, and this pharmacological model has become widely used (Olney and Farber 1995; Coyle and Tsai 2004). Considering astrocytes as the major component regulating glutamatergic transmission, their involvement in pathogenesis of psychiatric diseases seems to be highly probable.

12.4 Astroglia in Major Psychiatric Disorders

12.4.1 Psychosis Associated with Toxic Encephalopathies

Numerous toxic lesions to the brain trigger neuropsychiatric symptomatology, which can range from an acute state of confusion (the delirium) to delusions, behavioural abnormalities and impaired consciousness. Accumulating evidence links these neurotoxic disorders to impaired glutamate homeostasis associated with an astroglial failure. In encephalopathies, resulting from the exposure to heavy metals, such as lead, manganese, aluminium and/or mercury, astrocytes appear to be the primary target. These heavy metals, being accumulated into astroglial cells, lead to a substantial down-regulation of glial plasmalemmal glutamate transporters and hence to the reduction of astroglial glutamate uptake, which affects neurotransmission and causes excitotoxic neuronal damage (Suarez-Fernandez et al. 1999; Struys-Ponsar et al. 2000; Yin et al. 2007; Verkhratsky et al. 2013). Similarly, impaired glutamate homeostasis and glutamine synthesis emerge as a leading mechanism of hepatic encephalopathy, in which increased levels of ammonium in the brain affect astroglia and cause generalised homeostatic failure (Norenberg 1987; Albrecht et al. 2010; Rose et al. 2013; Haack et al. 2014; Obara-Michlewska et al. 2014). Impaired glutamate homeostasis also represents the main pathogenic factor in the development of Korsakoff syndrome (ante- and retrograde amnesia and confabulations), which is usually linked to Wernicke encephalopathy. In fact, these two disorders are so closely related that the patients are commonly diagnosed with Wernicke–Korsakoff syndrome, also called wet brain, which is a thiamine (vitamin B_1) deficiency associated with a severe excitotoxic damage secondary to alcoholism. The Wernicke encephalopathy is a primary astrogliopathy; the key pathogenetic step is a prominent down-regulation (by ~ 70 %) of the expression of astroglial plasmalemmal glutamate transporters, which leads to impaired glutamate transmission and glutamate excitotoxicity (Hazell 2009; Hazell et al. 2009).

12.4.2 Schizophrenia

In contrast to many neurological conditions, the pathophysiology of major psychiatric disorders (schizophrenia, major depressive disorder and bipolar disorder) does not include reactive astrogliosis. Rather, it seems that glial atrophy and astrodegeneration prevail in major psychiatric disorders (Verkhratsky et al. 2014). The emergence of pathologically remodelled astroglia is the common feature of the post-mortem brain of schizophrenic patients. In various brain regions a substantial decrease in astroglial numbers and glial fibrillary acidic protein (GFAP) expression along with the appearance of atrophic astroglial profiles is widely documented (Falkai and Bogerts 1986; Webster et al. 2001; Rajkowska et al. 2002; Schmitt et al. 2009). Similarly, many proteins fundamental for homeostatic function of astroglia, such as deiodinase type II, aquaporin 4, S100 protein, glutamine synthetase, plasmalemmal glutamate transporters and thrombospondin, are down-regulated in the schizophrenic brain (Xia et al. 2014). At the same time, levels of S100 protein in the cerebrospinal fluid of schizophrenic patients were often increased (Rothermundt et al. 2004, 2007, 2009); this increase may indicate the astroglial damage, with an increased leakage of S100 protein (Takahashi and Sakurai 2013). A very substantial increase in astroglial synthesis of chondroitin sulphate proteoglycans that constitute a main component of the extracellular matrix has also been identified in the amygdala and entorhinal cortex; this may affect synaptic transmission (Pantazopoulos et al. 2010).

Astroglial plasmalemmal glutamate transporters EAAT1/2 were decreased in the prefrontal cortex and hippocampus (Ohnuma et al. 2000; Bauer et al. 2008, 2010; Shan et al. 2013). At the same time, animals in which EAAT1 was genetically deleted demonstrated aberrant social behaviour and locomotor hyperactivity, resembling certain symptoms of schizophrenia (Karlsson et al. 2008, 2009). Expression of the plasmalemmal cystine-glutamate exchanger (Sxc-), which controls extrasynaptic concentration of glutamate in the CNS (Bridges et al. 2012) has been found to be up-regulated in the rodent phencyclidine model of schizophrenia (Baker et al. 2008). Aberrations in astroglia-dependent control over extracellular glutamate may instigate a profound synaptic remodelling hence affecting the spatial organisation of synaptic transmission and contributing to an overall confused information processing, which is characteristic for patients suffering from schizophrenia (Shan et al. 2014).

Pathophysiology of schizophrenia is also linked to changes in the biogenesis and catabolism of D-serine, a positive modulator of NMDA receptors. D-serine is released by both neurons and astroglia and by binding to the glycine site of NMDA receptors facilitates the receptor activation by glutamate (Wolosker 2007; Oliet and Mothet 2009). The proportional cellular sourcing (astrocytes vs. neurons) of D-serine remains a matter of debate (Balu et al. 2014). In schizophrenia, D-serine levels are decreased (Bendikov et al. 2007). Pharmacological, biochemical and genetic investigations demonstrate that, in addition to mutations and decreased expression of the NMDA receptor itself, changes may occur in the metabolism and

levels of D-serine in patients with schizophrenia (Balu and Coyle 2015). This may be linked to the reduced expression of serine racemase, an enzyme claimed to be associated with the risk of schizophrenia development (Labrie et al. 2009). A down-regulation of serine racemase has been observed in transgenic animals expressing mutant Disrupted-In-Schizophrenia-1 (DISC1) gene specifically in astrocytes (Ma et al. 2013). Alteration in the interplay of DISC1 with serine racemase leads to depletion of D-serine and consequently may instigate schizophrenia-like behaviour. Accordingly, prevailing efforts are presently directed to normalise NMDAR occupancy by enhancing D-serine levels though the administration of the co-agonist itself or by raising its synaptic availability (Balu and Coyle 2015). Along this line, preclinical and clinical investigations demonstrate that D-serine or benzoate, an inhibitor of D-serine degrading enzyme, D-amino acid oxidase (DAAO), are able to ameliorate negative and cognitive symptoms (Kantrowitz et al. 2010; Lane et al. 2013). Furthermore, peripheral levels of D-serine and the D/L-serine ratio significantly increase in patients with schizophrenia after treatment with clozapine (Yamamori et al. 2014).

Astrocytes also produce kynurenic acid, which has been implicated in pathogenesis of schizophrenia. Kynurenic acid is an endogenous competitive inhibitor of α7-containing nicotinic acetylcholine receptors and of NMDA glutamate receptors in the CNS. Kynurenic acid is synthesised from tryptophan by kynurenine aminotransferase II, which, in the CNS, is mainly expressed in astroglia (Schwarcz et al. 2012). Genetic deletion of kynurenine transferase II augmented synaptic plasticity and improved cognitive function (Potter et al. 2010). Being an NMDA receptor antagonist, kynurenic acid shares pharmacological properties with phencyclidine and ketamine, substances reported to provoke psychotic symptoms in healthy subjects, highly resembling those observed in patients (Krystal et al. 1994). The levels of kynurenic acid were increased in the post-mortem brains of psychotics (Holtze et al. 2012). The kynurenine pathway regulates the dopaminergic system, which for decades is considered to play a prominent role in the pathogenesis of schizophrenia. Increase in kynurenic acid level in the rodent brain potentiates midbrain dopaminergic neurons firing, as well as dopamine release and locomotor activity (Nilsson et al. 2006). Notably, the metabolism of kynurenic acid appears as a causal link between *Toxoplasma gondii* infection and an increased risk of schizophrenia. Seemingly, *T. gondii* infects predominantly astrocytes, and this infection results in an increased production of kynurenic acid (Schwarcz and Hunter 2007).

12.4.3 Mood Disorders

There are no major signs of glial reactivity in mood disorders; rather, and similarly to schizophrenia, astroglial atrophy and astrodegeneration dominate. In major depressive disorder as well as in bipolar disorder numbers of astrocytes are found to be reduced in the fronto-limbic brain, including orbito-frontal area, anterior cingulate, and prefrontal, entorhinal and subgenual cortices, as well as the amygdala

(Ongur et al. 1998; Rajkowska et al. 1999; Cotter et al. 2001, 2002; Bowley et al. 2002). Significant decreases in astroglial numbers were identified in prefrontal and anterior cingulate cortices in familial forms of both major depressive and bipolar disorders (Ongur et al. 1998; Cotter et al. 2001; Gittins and Harrison 2011). Expression of GFAP has been reduced in several brain regions in post-mortem tissues from depressed patients, including the prefrontal cortex (Johnston-Wilson et al. 2000; Webster et al. 2001; Si et al. 2004), amygdala (Altshuler et al. 2010), hippocampus (Muller et al. 2001) and cerebellum (Fatemi et al. 2004). Expression of GFAP was markedly depressed in the thalamus and nucleus caudatus of suicidal patients (Torres-Platas et al. 2015). Decrease in astroglial numbers and GFAP expression in the brains of patients with major depressive disorders paralleled with down-regulation of astroglia-specific proteins, in particular, those critically important for astroglial homeostatic function. Notably, expression of *SLC1A2* and *SLC1A3* genes encoding EAAT1 and EAAT2 plasmalemmal glutamate transporters as well as *GLUL* (Glutamate-Ammonia Ligase) gene encoding glutamine synthetase was found to be markedly decreased (Choudary et al. 2005), indicating impairment of astroglia-dependent glutamate homeostasis. Other astroglial genes, including those encoding S100 protein, connexins Cx30 and Cx43, and aquaporin 4 were also down-regulated in the brain tissue of patients with major depression (Bernard et al. 2011). The expression of astrocyte-specific tropomyosin-related kinase-B receptor 1, as well as connexins was reduced in orbitofrontal and prefrontal cortices of suicide victims (Ernst et al. 2009, 2011).

Similarly, decrease in astroglial numbers as well as decrease in GFAP immunoreactivity, number of GFAP-positive astroglial profiles and expression of glial-specific proteins (aquaporin 4, connexins, astroglial plasmalemmal glutamate transporters and glutamine synthetase) was observed in animal models of mood disorders (Czeh et al. 2006; Barley et al. 2009; Braun et al. 2009; Sequeira et al. 2009; Bernard et al. 2011; Rajkowska and Stockmeier 2013). In particular, chronic stress down-regulated expression of astroglia-specific Cx43 that was reflected by a decreased dye coupling between astrocytes in the prefrontal cortex. In parallel, the pharmacological blockade of gap junctions (treatment with carbenoxolone or with Cx43 mimetic peptides Gap27 and Gap26) induced anhedonia that is generally considered as one of the major symptoms of depression (Sun et al. 2012). Anhedonia was also induced by treating rats with dihydrokainic acid, an inhibitor of astroglial glutamate uptake via EAATs; this treatment also affected spatial memory (Bechtholt-Gompf et al. 2010). Finally, a treatment with antidepressants was found to affect not only neurons, but also astrocytes, in particular affecting astroglial expression of neurotransmitter receptors, ion channels and homeostatic transporters (Czeh and Di Benedetto 2013; Dong et al. 2015; Peng et al. 2015; Ren et al. 2015).

The causal role for astroglia-associated pathological changes in mood disorders was highlighted in experiments with the direct ablation of astroglial cells from the medial-prefrontal cortex of mice with L alpha-aminoadipic acid (L-AAA), an astrocyte-selective toxin, which resulted in the emergence of depressive phenotype similar to that induced by chronic stress (Banasr and Duman 2008).

Notably, the unique pattern of cell pathology in major depressive disorder characterised by prominent reductions in the density of astrocytes and expression of astroglial markers is further confirmed by in vivo brain imaging using proton magnetic resonance technique, a non-invasive procedure that allows direct detection of brain metabolites. In these studies, lower levels of myo-inositol, commonly regarded as an astroglial marker, were in the anterior cingulate cortex of depressed patients; the levels of myo-inositol increased following the treatment only in subjects responsive to therapy (Sterado, unpublished observations). These results further corroborate the role of astroglial dysfunction in the pathogenesis of depressive disorders.

12.4.4 Addictive Disorders

Astroglia contribute to addictive disorders. Morphometric studies have identified a complex astroglial response to various forms of addiction with evidence for both astrodegenerative, i.e. atrophic changes, and astroglial reactivity (Oehmichen et al. 1996; Suarez et al. 2000; Fattore et al. 2002; Armstrong et al. 2004; Büttner and Weis 2006; Weber et al. 2013). This dichotomy in astroglial reactions may reflect the length and severity of the disease. For example, astroglial numbers were reduced and GFAP-positive astroglial profiles were diminished in patients with relatively short-lived alcoholism, whereas the long-lasting alcohol dependence was associated with astroglial reactivity, manifested by increased numbers of astroglial cells, i.e. proliferation, and increased GFAP expression (Miguel-Hidalgo et al. 2002, 2006; Skuja et al. 2012). In post-mortem tissues obtained from alcoholics, reactive and hypertrophic astrocytes were observed together with areas in which numbers of GFAP-positive astrocytes were substantially decreased (Cullen and Halliday 1994; Miguel-Hidalgo 2005). Astroglial reactions were also region specific. For example, in alcoholism, the number of astrocytes was decreased in the prefrontal cortex from post-mortem tissues (Miguel-Hidalgo et al. 2006).

Similarly, complex/dichotomous astroglial responses were found in animals treated with drugs of addiction. Reactive astrogliosis and GFAP increase have been identified in animals treated with cocaine, methamphetamine and morphine (Song and Zhao 2001; Fattore et al. 2002; Bowers and Kalivas 2003; Guilarte et al. 2003; Friend and Keefe 2013). Decreased expression of GFAP and astroglial atrophy was, however, observed in animals chronically exposed to alcohol (Franke 1995; Rintala et al. 2001). Astroglial density and GFAP expression were significantly decreased in the prelimbic cortex of rats addicted to ethanol (Miguel-Hidalgo 2005). Incidentally, binge-like ethanol administration reduced astrocytic density in the adult rat medial prefrontal cortex of males but not females (Koss et al. 2012). The role of astroglia in addiction was also inferred by experiments with the astrocyte ablation using L-AAA; removing astrocytes from the prelimbic area of the prefrontal cortex increased alcohol-seeking behaviour. Same effect was produced by the inhibition of astroglial

connectivity following injection of 8-alpha-glycyrrhetinic acid, a connexon/gap-junction inhibitor (Miguel-Hidalgo 2009).

Morphological impairment of astroglia in the addictive disorders was also complemented by their functional failure, i.e. aberrant glutamate clearance and metabolism. In the context of addiction, astroglial cells down-regulated the expression of EAAT2 and Sxc^-, although overall extracellular glutamate concentration was found to be elevated probably because of disbalance between glutamate uptake (EAAT2) and glutamate release (Sxc^-) (Reissner and Kalivas 2010, 2014; Moussawi et al. 2011). Down-regulation of astroglial EAAT2 was found in various animal models of addiction, including that to cocaine, nicotine or alcohol (Knackstedt et al. 2010; Gipson et al. 2013). Increase in astroglial expression of plasmalemmal glutamate transporters following treatment with ceftriaxone, a beta-lactam antibiotic, reduced alcohol seeking in ethanol-preferring animals (Qrunfleh et al. 2013; Sari et al. 2013).

12.4.5 Huntington's Disease

Huntington's disease (HD) is an autosomal dominant inherited disease caused by the repeat of a nucleotide triplet in the exon 1 of huntingtin gene, which results in the synthesis of mutant huntingtin protein (mhtt) containing an expanded polyglutamine section in its N-terminal portion. The neurodegeneration in HD mainly occurs in striatal medium spiny neurons and in cortical pyramidal neurons (Vonsattel et al. 1985; Hedreen et al. 1991). HD shows both neurologic and psychiatric components and can represent a challenge to psychiatric services (Leroi and Michalon 1998).

Mhtt is present in neurons and astrocytes in brain tissue from human patients (Singhrao et al. 1998) and in mouse HD models (Shin et al. 2005; Faideau et al. 2010). Be that in human tissue or animal models, astrocytes contribute to HD through a prominent astrogliosis, which correlates with HD severity (Vonsattel et al. 1985; Gu et al. 2005; Faideau et al. 2010). Astrocytes also contribute to HD through the compromised glutamate and potassium uptake as well as an enhanced calcium-dependent glutamate release. Some or all of these contributions likely result in excitotoxicity, an important factor for neuronal death in HD (Gray 2014). Thus, EAAT2 mRNA and protein levels are decreased in human HD tissues (Cross et al. 1986; Arzberger et al. 1997), which correlate with the severity of the disease (Faideau et al. 2010). Astrocytes isolated from the cortex of the BACHD mouse model (Lee et al. 2013) showed an enhanced exocytotic glutamate release that was allied with an increased expression of pyruvate carboxylate and hence an increased *de novo* synthesis of glutamate. HD astrocytes of R6/2 and Q175 HD mouse models also have compromised potassium buffering due to a decreased expression of $K_{ir}4.1$ K^+ channels (Tong et al. 2014); this resulted in an increase of medium spiny neurons excitability in vitro. Taken together the dysregulation of glutamate and potassium homeostasis in astrocytes contributes to pathology seen in HD.

12.5 Concluding Remarks

Down-regulation of molecular pathways responsible for astroglial control over glutamate homeostasis in the brain seems to be a common feature of all major neuropsychiatric disorders. Indeed, decrease in astroglial glutamate uptake is identified in human tissue in toxic neuroencephalopathies, schizophrenia, mood and addictive disorders. Similar decrease is observed in various animal models of neuropsychiatric pathology. Failed astroglial glutamate homeostasis may therefore represent an important pathogenic step responsible for overall disbalance of neurotransmission as well as for synaptic and neuronal damage which all underlie psychiatric symptomatology; astrocytes and astroglia-specific molecules may therefore be regarded as a promising therapeutic target.

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Chapter 13
Glutamine Synthetase: Role in Neurological Disorders

Arumugam R. Jayakumar and Michael D. Norenberg

Abstract Glutamine synthetase (GS) is an ATP-dependent enzyme found in most species that synthesizes glutamine from glutamate and ammonia. In brain, GS is exclusively located in astrocytes where it serves to maintain the glutamate–glutamine cycle, as well as nitrogen metabolism. Changes in the activity of GS, as well as its gene expression, along with excitotoxicity, have been identified in a number of neurological conditions. The literature describing alterations in the activation and gene expression of GS, as well as its involvement in different neurological disorders, however, is incomplete. This review summarizes changes in GS gene expression/activity and its potential contribution to the pathogenesis of several neurological disorders, including hepatic encephalopathy, ischemia, epilepsy, Alzheimer's disease, amyotrophic lateral sclerosis, traumatic brain injury, Parkinson's disease, and astroglial neoplasms. This review also explores the possibility of targeting GS in the therapy of these conditions.

Keywords Ammonia • Astrocytes • Glutamine synthetase • Glutamine • Excitotoxicity • Neurological disorders • Neurotoxicity • Therapy

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Abbreviations

AD	Alzheimer's disease
ALS	Amyotrophic lateral sclerosis
Aβ	Beta amyloid
COX-2	Cyclooxygenase-2
EAAT	Excitatory amino acid transporter
GABA	γ-aminobutyric acid
GBM	Glioblastoma multiforme
GFAP	Glial fibrillary acidic protein
GLAST	Glutamate-aspartate transporter
GLT-1	Glutamate transporter-1
GS	Glutamine synthetase
HD	Huntington's disease
HE	Hepatic encephalopathy
iNOS	Inducible nitric oxide synthase
MSO	Methionine sulfoximine
NMDA	*N*-methyl-D-aspartate
PCA	Portacaval anastomosis
TBI	Traumatic brain injury

13.1 Introduction

Glutamine synthetase (GS) is an enzyme that is predominantly located in brain, kidney, and liver. In brain it participates in the recycling of the neurotransmitter glutamate and in the detoxification of ammonia. Glutamate, which is released by glutamatergic neurons, is taken up by astrocytes where it is converted by GS to the nontoxic amino acid glutamine. Glutamine is then released by astrocytes and taken up by neurons, where it is hydrolyzed by glutaminase to form glutamate (the "glutamate–glutamine cycle") (Hertz 2013; Van den Berg 1970; Walls et al. 2015). In this manner, GS prevents the excessive accumulation of glutamate and ammonia in synaptic regions, thereby inhibiting the development of glutamate/ammonia neurotoxicity.

Glutamate neurotoxicity has been implicated in the process of neuronal degeneration in a large number of neurological disorders. Many of these conditions are accompanied by a decline in GS expression and/or activity (see later for a detailed discussion), although contradictory observations have also been reported (see later). Considering the role of GS in glutamate and ammonia detoxification, a decline in GS expression in different neurological conditions may markedly aggravate the process of neuronal degeneration. Recent studies have indeed demonstrated that hormonal induction of GS gene expression, or the exogenous administration of purified GS to traumatized retinal glial cells, reduced the neuronal degeneration, whereas

inhibition of GS activity by methionine sulfoximine (MSO) led to neural cell death (Gorovits et al. 1997). These findings suggest that astrocytic GS is an efficacious neuroprotectant by activating a number of signaling systems that may protect neurons from the deleterious effects of both glutamate and ammonia.

13.1.1 Biochemical Properties of GS

GS catalyzes the ATP-dependent condensation of glutamate with ammonia to yield glutamine. ATP initially phosphorylates glutamate by forming an acyl-phosphate intermediate, γ-glutamyl phosphate, which then reacts with ammonia to form glutamine and inorganic phosphate. The ammonium ion binds strongly to GS only if the acyl-phosphate moiety intermediate is present. The ammonium ion, rather than ammonia, binds to GS as the binding site is polar (Liaw and Eisenberg 1994).

13.1.2 Cellular Localization of GS in the CNS

GS in brain is normally found in astrocytes (Norenberg 1979, 1983; Norenberg and Martinez-Hernandez 1979). However, in some settings (e.g., Alzheimer's disease, suicide victims, individuals with schizophrenia) reports indicate that GS may also be present in other CNS cells, including neurons, microglia, and oligodentrocytes (for review, see Anlauf and Derouiche 2013 and references therein; Bernstein et al. 2013; Nakajima et al. 2015; Robinson 2000; Yamamoto et al. 1989). The existence, as well as the significance of GS in CNS cells other than astrocytes, remains to be determined.

13.1.3 GS Activity

GS activity is regulated by adenylation (a specific tyrosine residue in each subunit of GS is modified) (Stryer et al. 2007). The rate of adenylation depends on the ratio of glutamine to α-ketoglutarate. Adenylation of GS, which is catalyzed by adenylyltransferase, involves the phosphodiester bond between the hydroxyl group of the tyrosine residue in GS and the phosphate group of an AMP nucleotide. A complex of adenylyl transferase and a regulatory protein known as PII, which may exist unmodified as PII (also known as P_A), and uridylylated PII-UMP (also known as P_D), causes an AMP molecule to either attach to, or to be removed from GS, respectively (Itzen et al. 2011). A complex of PII with adenylyl transferase that catalyzes the attachment of an AMP molecule to GS forms adenylated GS that is inactive. Alternatively, a complex of PII-UMP and adenylyl transferase may activate deadenylation which removes AMP from GS, thereby creating deadenylated GS that is active (Garrett and Grisham 2007; Itzen et al. 2011).

13.1.4 Regulation of GS Gene Expression

Recent findings indicate that the GS gene is a novel forkhead box O (FoxO) transcriptional target (van der Vos et al. 2012; van der Vos and Coffer 2012). Removal of various growth factors (e.g., basic fibroblast growth factor, transforming growth factor beta 1 and 2) in astrocytes was shown to increase GS expression and activity through the activation of the FoxO transcription factor (Chao et al. 1992; Kruchkova et al. 2001; van der Vos et al. 2012; van der Vos and Coffer 2012). Conversely, GS expression is inhibited by stimulation of the earlier growth factors, or by the selective activation of either phosphatidylinositol 3-kinase (PI3K), or by the serine/threonine-protein kinase-1 (AKT1) signaling pathway (van der Vos and Coffer 2012). Alternately, inhibition of PI3K activity induces the binding of activated FoxO to the GS promoter which increases GS transcription (van der Vos and Coffer 2012). Additionally, increased GS levels inhibit the mammalian target of rapamycin signaling by blocking its lysosomal translocation. Glucocorticoid receptor-mediated cell signaling also appears to be involved in the regulation of GS gene expression (Gorovits et al. 1996; Vardimon et al. 1988; Zhang and Young 1991).

13.2 Role of GS in Neurological Conditions

13.2.1 Hepatic Encephalopathy

Hepatic encephalopathy (HE) is a major neurological complication in patients with severe liver disease, which presents in acute and chronic forms. Acute HE usually occurs following massive liver necrosis due to viral hepatitis, acetaminophen toxicity, or exposure to other hepatotoxins. It presents with severe brain edema (Ede and Williams 1986; Hoofnagle et al. 1995), increase in intracranial pressure, and brain herniation resulting in a high mortality rate (Capocaccia and Angelico 1991). Chronic HE is generally a consequence of cirrhosis of the liver and presents with a wide range of neurological symptoms, including altered mood, increased irritability, alterations in the sleep/wake cycle, changes in muscle tone, and cognitive impairments (Jones and Weissenborn 1997; Lockwood et al. 1991). While the molecular basis underlying HE remains incompletely understood, astrocytes are generally recognized to play a critical role in its pathogenesis (Norenberg 1977; Norenberg et al. 2007). It is generally accepted that elevated blood and brain ammonia levels, due to the inability of the injured liver to adequately detoxify ammonia by the synthesis of urea, is a crucial factor in the pathogenesis of HE. Once in brain, ammonia is first metabolized by GS to form glutamine in astrocytes. A number of events are then triggered that ultimately culminates into HE (for review, see Norenberg et al. 2007).

Evidence for the involvement of GS in ammonia toxicity was first demonstrated by Warren and Schenker (1964), who observed that methionine sulfoximine (MSO), an inhibitor of GS, protected mice from the development of ammonia-induced

coma. MSO was also shown to reverse the biochemical manifestations of ammonia toxicity (e.g., depressed glucose consumption and increased blood–brain barrier transport of aromatic amino acids in hyperammonemic rats) (Hawkins et al. 1993). Pathophysiological manifestations of HE, including increased cerebral blood flow (Master et al. 1999), increased brain water content and intracranial pressure (Blei et al. 1994; Tanigami et al. 2005), astrocytic control of extracellular K^+ activity (Sugimoto et al. 1997), and impaired vascular reactivity to CO_2 (Hirata et al. 1996, 1999; Takahashi et al. 1992) were also abated by MSO, presumably by preventing the accumulation of glutamine.

Additionally, studies have shown that GS levels are increased in ammonia-treated cultured astrocytes (Suarez et al. 2002; Waniewski 1992), and in animal models of acute HE (Suarez et al. 2002; Takahashi et al. 1991). Increased GS mRNA was also reported in the thioacetamide-induced rat model of acute HE (Thomas et al. 1988). Moreover, inhibition of GS was found to be an effective means of reducing the ammonia-induced astrocyte swelling, as well as the brain edema in rats with acute HE (Bender and Norenberg 1996; Tanigami et al. 2005; Willard-Mack et al. 1996).

These findings suggest that increased GS expression and/or activity are involved in the development of brain edema in acute HE, and that inhibition of GS with MSO may serve as an important treatment modality for hyperammonemia. However, the use of MSO is problematic as MSO is well known to induce seizures likely by inducing hyperammonemia (see also Section c, epilepsy). The potential seizure-inducing effect of MSO is well established, as this agent, especially the L–S isomer, binds to the active sites of GS leading to an irreversible inhibition of the enzyme (Rowe and Meister 1970). The effect of MSO on seizure induction may also be mediated by means other than GS inhibition, as MSO is also known to inhibit glutamate cysteine ligase (Richman et al. 1973; Schatz and Sellinger 1975), and to increase ornithine decarboxylase activity (Di Giacomo et al. 1997), both of which have been shown to be associated with epileptogenesis (Baudry et al. 1986; Cárdenas-Rodríguez et al. 2014; Herberg et al. 1992; Jarrett et al. 2008). While MSO is beneficial in preventing glutamine accumulation and the subsequent increase in brain edema/intracranial pressure/coma in HE, MSO alone cannot be used to treat HE. It is possible that a combinatorial approach (MSO plus an anticonvulsant) may be more efficacious in diminishing the symptoms of HE.

While the earlier findings strongly suggest a critical role of increased GS in the pathogenesis of acute HE, a few studies, however, indicate that GS protein levels or activity are decreased in ammonia-treated astrocyte cultures (Leite et al. 2006) as well as in chronic and acute HE (Desjardins et al. 1999; Girard et al. 1993; Hilgier 1981; Kanamori et al. 1993; Lavoie et al. 1987), and that such decreased activity was shown to be promoted, in part, through increased protein tyrosine nitration of GS by ammonia, the peripheral benzodiazepine receptor and cytokines (Häussinger et al. 2005).

The reason for the differences in GS protein expression and activity (increased or decreased) in HE is unclear. It is possible that the duration of hyperammonemia may have varied in these studies, resulting in differences in GS protein expression and activity. In this regard, Desjardins et al. (1999) found decreased GS activity in cerebral cortex at 4 weeks after portacaval anastomosis (PCA, a model of hyper-

ammonemia), whereas increased GS-immunoreactivity was demonstrated after long-term PCA (6 months) (Suárez et al. 1996, 1997). It should be emphasized that brain ammonia concentrations were different in these two studies (0.5–0.1 mM, respectively) that may, in part, explain the differential expression and/or activity of GS in HE. Additionally, while glutamate and ammonia bind to active sites of several GS subunits for the production of glutamine, these subunits also contain binding sites for other amino acids (glycine, alanine, histidine, tryptophan), as well as for nucleotides (including adenosine monophosphate and cytidine triphosphate). Binding of these molecules inhibits GS activity. It is thus possible that a rise in these molecules in various neurological conditions (see later) may contribute to the inhibition of GS.

GS present in organs other than brain (e.g., skeletal muscle) has also been considered as potential ammonia-lowering strategies for HE. For instance, GS in skeletal muscle has the capacity to remove ammonia (Chatauret et al. 2006; Desjardins et al. 1999; Dam et al. 2011; Jalan et al. 2007; Rose et al. 1998, 1999; Zieve et al. 1986). Additionally, a gene therapy approach (delivery of the GS gene by a viral vector) in muscle tissue in acute hyperammonemic rats was shown to prevent the development of hyperammonemic symptoms (Torres-Vega et al. 2015).

The earlier findings strongly suggest that GS protein expression or activity is altered in HE. It is possible that increased GS may detoxify ammonia and glutamate resulting in high levels of glutamine which may contribute to the astrocyte swelling/brain edema. However, inhibiting GS with MSO to prevent edema may increase the concentrations of ammonia in brain, resulting in seizure activity (Fig. 13.1). Accordingly, when treating the brain edema associated with acute HE with MSO, the possibility of concurrently using an anticonvulsant should be considered.

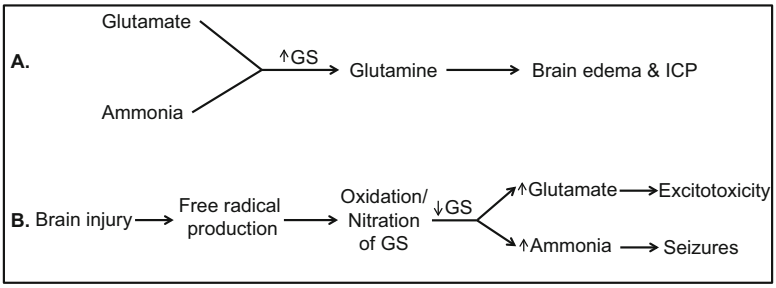

Fig. 13.1 Altered glutamine synthetase (GS) expression/activity in neurological disorders. (**a**) Increased GS detoxifies ammonia and glutamate resulting in high levels of glutamine, which may contribute to the astrocyte swelling/brain edema in various neurological conditions, including hepatic encephalopathy, ischemic stroke, and traumatic brain injury. (**b**) In conditions with a decrease in GS, or inhibiting GS with MSO to prevent the ammonia-mediated edema, may increase the concentrations of ammonia and glutamate in brain, resulting in seizure activity

13.2.2 Ischemia

Brain ischemia leads to decreased brain energy metabolism, a reduction in metabolic rates, and energy crisis. During ischemia, a massive release of glutamate from synaptic vesicles is known to occur (Benveniste et al. 1984; Drejer et al. 1985), and such increase was shown to be a major cause of neuronal degeneration (by excitotoxicity) (Dugan and Choi 1999). The accumulation of synaptically released glutamate was prevented, in part, by the astrocytic glutamate uptake system, wherein GS plays a critical role.

Immunohistochemical analysis of the gerbil hippocampus following transient ischemia showed that on day 2 after ischemia, and more prominently on day 3, astrocytes intensely stained for GS, especially in the CA1 region (Tanaka et al. 1992), and such increase was associated with increased GS protein levels (Hertz and Zielke 2004; Fujioka et al. 2003; Lee et al. 2003). Spectrophotometric analysis of frozen sections of cerebellum from children who died with acute or chronic hypoxic–ischemic insults also showed that GS activities were elevated. GS was also upregulated in retinal Müller cells after middle cerebral artery occlusion (Allen et al. 2014). Additionally, following ischemia, treatment of rats with total oligomeric flavonoids (e.g., quercetin, polyphenols), or telmisartan (an angiotensin II receptor antagonist), resulted in a decrease in GS activity, reduction in neurological deficits, and a reversal of anxiogenic behavior (Sunil et al. 2011; Justin et al. 2014). The earlier findings suggest that GS protein expression and activity are increased in brains of experimental animals and in patients with brain ischemia, and that such increase in GS activity likely contributes to the development of brain injury, presumably by increasing glutamine levels. It should be noted that increased glutamine is known to initiate a number of signaling pathways (e.g., free radical generation, activation of mitogen-activated protein kinases, as well as various transcription factors), all of which have been shown to result in astrocyte dysfunction (Norenberg et al. 2007; Rama Rao et al. 2012), that may also have contributed the pathogenesis of ischemic brain injury.

However, there is also evidence indicating that a reduction in GS activity may contribute to neuronal injury postischemia. Exposure of spinal cords to high amount of GS (15 Units) was shown to prevent GABA-A receptor-mediated axonal depression after ischemia, possibly by an ischemia-induced accumulation of ammonia (Matsumoto et al. 2008). Excess ammonia is known to potentiate the $GABA_A$ receptor response due to GABA released from the hypoxia-injured spinal cord (Takahashi et al. 1993). Oxygen-glucose deprivation (OGD) was also shown to decrease GS expression, as well as to increase extracellular glutamate level, both of which can lead to excitotoxicity (Lee et al. 2010; Wang et al. 2013). Additionally, GS expression and activity were decreased after middle cerebral artery occlusion in rats, and memantine, a highly efficacious NMDA receptor channel blocker, as well as ceftriaxone, an activator of the GLT-1 subtype of glutamate transporter expression, were both shown to prevent such reduction in GS expression/activity. The memantine and ceftriaxone effect in reversing GS expression and activity after middle cerebral

artery occlusion in rats was also associated with a reversal of altered neurological and behavioral deficits following ischemia (Babu and Ramanathan 2009; Verma et al. 2010). These findings suggest that a reduction in GS expression and activity contributes to ischemic brain injury, likely by increasing both brain ammonia concentration and excitotoxicity.

A significant decrease in GS activity was also observed after retinal ischemic injury in rats (Fernandez et al. 2009). Similarly, Lee et al.. (2010) also found that GS was rapidly lost from astrocytes in regions susceptible to ischemic damage following hypoxia in neonatal pigs, while an examination of brain cortical and hippocampal sections showed that the loss of GS preceded any overt neuronal injury. Moreover, the decreased survival rate following ischemic brain injury was shown to be potentiated by pretreatment of ischemic rats with MSO (Di Giacomo et al. 1997), suggesting that inhibition of GS expression/activity contributes to the decreased survival of rats following ischemic brain injury. Altogether, these findings strongly suggest that decreased GS protein expression and activity, resulting in increased extracellular glutamate and ammonia levels, contribute to the development of ischemic brain injury.

While both increased and decreased GS protein expression and activity have been reported in ischemic brain injury, the reason for these differences, as well as their contribution to ischemic brain injury, remains unclear. The duration of ischemia (i.e., transient vs. prolonged) may be responsible for changes in GS expression/activity. For example, short- or long-term ischemic injury to brain is well known to cause free radical production, and such increase may have contributed to the altered GS expression/activity. In support of the view that the duration of ischemia may be responsible for the differences in GS activity (increase and decrease after short- or long-term ischemia, respectively), GS activity and free radical production were shown to be increased following transient ischemia (Petito et al. 1992; Phillis 1994). Since oxidative stress is known to increase GS activity (Bondy et al. 1998), it is possible that free radicals generated by transient ischemia may have enhanced GS expression.

However, ischemia-induced oxidative stress at later stages may have initiated alterations in key proteins, in particular GS, that may have contributed to the reduced GS expression and activity (Butterfield et al. 1997; Castegna et al. 2011; Oliver et al. 1990). Studies have indeed shown that GS is sensitive to severe oxidative damage (Floyd 1990; Nakamura and Stadtman 1984; Oliver et al. 1990), and that GS protein oxidation is known to be increased in brain by 75 % after long-term ischemic injury (Akinmoladun et al. 2015; Dorfman et al. 2013; Floyd 1990; Nakamura and Stadtman 1984; Oliver et al. 1990; Safwen et al. 2015) but not after transient ischemia (15 min) (Folbergrová et al. 1993; Pahlmark et al. 1993). In aggregate, these findings suggest that the duration of ischemic injury may partly determine the status of GS expression/activity following ischemic brain injury.

It should also be highlighted that a significant increase in brain ammonia levels, as well as glutamate concentrations, was observed after brain ischemic insult (Hołownia et al. 1994; Thorn and Heimann 1958), which may have played a role in the alterations in GS expression observed following CNS ischemia. Together, these findings suggest that regulation of GS expression/activity following ischemia may represent a useful therapeutic approach for the treatment of ischemic CNS injury.

13.2.3 Epilepsy

Epilepsy is a common neurological disorder globally affecting approximately 65 million people (Ngugi et al. 2010; Thurman et al. 2011). While the cause of most cases of epilepsy is unknown, brain trauma, stroke, tumors, and drug and alcohol abuse have been shown to significantly contribute to its development.

Dysfunctional astrocytes have been strongly implicated in seizure pathogenesis (Crunelli et al. 2015). The seizure producing effect of MSO, likely by the inhibition of GS, was first demonstrated by Reiner et al. 1950, and subsequently by Proler and Kellaway 1962, 1965; Warren and Schenker 1964; and Johnson et al. 1965. Further, studies have shown reduced GS activity in the hippocampus in association with temporal-lobe epilepsy (Eid et al. 2008), that MSO exacerbated epileptic seizures (Chung and Johnson 1984), and that continuous intra-hippocampal infusion of MSO in rats resulting in a loss of GS activity, led to increased extracellular glutamate concentrations which were shown to contribute to recurrent seizure activity (for review, see Eid et al. 2013; Wang et al. 2009).

Additional support implicating the involvement of GS in epileptogenesis is derived from findings showing reduced brain levels/activity of GS in rats with genetic absence epilepsy (Dutuit et al. 2000), in amygdala-kindled seizures (Tiffany-Castiglioni et al. 1990), as well as in hippocampal regions associated with neuron loss in patients with temporal lobe epilepsy (van der Hel et al. 2005). A reduction in GS activity was also shown to increase the susceptibility to experimental febrile seizures (van Gassen et al. 2009). Altogether, these findings indicate that reduced GS expression or activity is indeed involved in epileptogenesis (See Eid et al. 2013 for review).

However, there are reports that increased GS activity may also be associated with submaximal seizure activity (Tiffany-Castiglioni et al. 1990), and that a transient upregulation of GS in the dentate gyrus was associated with amygdala-kindling seizures in rats (Sun et al. 2013). While the reason for discrepancies in GS protein expression/activity associated with epileptogenesis is unclear, it is possible that the duration of seizures may have varied in these studies, which might have generated the production of free radicals and increased brain lactate concentration, both of which may have resulted in a neurotransmitter imbalance (e.g., NMDA; see ALS section) (Kosenko et al. 2003) that may have altered GS expression. Differences in the experimental models used in these studies may also be responsible for the variations in GS expression/activity relative to epileptogenesis.

As noted in the "Hepatic encephalopathy section," the seizure-inducing effect of MSO is well established as this amino acid binds to the active sites of GS and thereby irreversibly inhibits the enzyme, resulting in convulsions (Rowe and Meister 1970). As noted earlier in "HE section" (page 9) the effect of MSO on seizure induction may also be mediated by the inhibition of glutamate cysteine ligase (Griffith and Meister 1978, 1979) or by increase in ornithine decarboxylase activity (Di Giacomo et al. 1997, and references therein), both of which have been shown to be associated with epileptogenesis (Di Giacomo et al. 1997, and references therein; Griffith and Meister 1978, 1979). These studies collectively suggest that GS inhibition, along with reduced glutamate cysteine ligase and

increased ornithine decarboxylase activities, is involved in epileptogenesis. Accordingly, the restoration of GS activity, possibly by treatment with memantine or ceftriaxone, agents known to reverse GS expression and activity in other conditions (e.g., ischemia) (Babu and Ramanathan 2009; Verma et al. 2010), may represent a novel approach for the prevention of epileptic seizure activity.

13.2.4 Traumatic Brain Injury

Traumatic brain injury (TBI) is a common neurological disorder that represents a leading cause of morbidity and mortality (Coronado et al. 2011; Harrison-Felix et al. 2006), which presents in acute and chronic forms. Brain edema and associated increased intracranial pressure in the early phase of TBI are major consequences of acute trauma, whereas more subtle neuronal injury, leading to neurobehavioral and cognitive impairments occurring years after repetitive episodes of head trauma, are major consequences of chronic TBI.

The molecular mechanisms responsible for TBI are incompletely understood. Following TBI, neuronal survival is strongly influenced by the functions of astrocytes, including glutamate uptake and release, and the release of substrates necessary for neuronal energy metabolism (e.g., lactate, glutamate, and GABA) (Bak et al. 2006; Pascual et al. 1998). Considering the role of GS in the conversion of glutamate to glutamine, increased or decreased GS expression in astrocytes may affect the concentration of glutamate (both intracellular and extracellular) potentially resulting in adverse effects on neurons caused by excitotoxicity.

A marked reduction in brain GS immunoreactivity was identified following fluid percussion injury in rats (Zhao et al. 2003). On the other hand, Gorovits and colleagues demonstrated that elevation of GS expression in astrocytes by cortisol treatment protected against neuronal degeneration in injured retinal tissue, whereas inhibition of GS activity (by MSO) led to increased neuronal cell death (Gorovits et al. 1997). These investigators also showed that the addition of purified GS to injured retinal tissue resulted in a decline in neuronal death. Of interest, a recent report by Zou et al. (2011) noted that suppression of astrocytic GS with siRNA increased astrocyte migration into the site of injury in a scratch model of TBI, and such migration may have contributed to the neuronal protection following TBI. The earlier findings suggest an important role of altered GS activity in TBI, and that increasing GS activity may represent a useful therapeutic approach for TBI.

13.2.5 Alzheimer's Disease

Alzheimer's disease (AD) is a neurodegenerative disorder characterized by cognitive dysfunction, memory loss, diminished orientation and judgment, inadequate language expression, and impaired calculation ability (Battaglia et al. 2007;

Cummings 2004; Selkoe 2001). Neurofibrillary tangles, intra- and extracellular accumulation of β-amyloid (Aβ) (neuritic plaques), as well as an increase in intraneuronal hyperphosphorylated Tau protein, are histopathological hallmarks of AD (Veerhuis 2011). Neuritic plaques are frequently associated with GFAP-positive activated astrocytes (Nagele et al. 2004). While the means by which Aβ causes neurotoxicity in AD is unclear, altered glutamatergic signaling, due to changes in the expression of glutamate transporters (e.g., GLAST, GLT-1), phosphate-activated glutaminase, changes in ionotropic and metabotropic glutamate receptors, as well as in GS, all appear to be critically involved (Burbaeva et al. 2005; Miguel-Hidalgo et al. 2002; Revett et al. 2013).

A recent finding suggests that astrocytes play an important role in the clearance of the Aβ peptides, potentially preventing plaque formation (Nielsen et al. 2009; Shibata et al. 2000; Wyss-Coray et al. 2003). Exposure of cultured astrocytes to Aβ peptides was also shown to decrease glutamate uptake, ultimately leading to oxidative stress and the subsequent activation of various intracellular signaling cascades (Agostinho et al. 2010; Matos et al. 2008). Considering the critical role of GS in glutamate metabolism, altered GS expression in astrocytes may affect the concentrations of glutamate and ammonia, both of which may adversely affect neurons, resulting in neuronal death.

Decreased GS activity and protein level were identified in postmortem brains from patients with AD (Butterfield et al. 1997; Le Prince et al. 1995; Olabarria et al. 2011; Robinson 2000). Additionally, Aβ peptides were shown to directly inhibit GS activity in vitro (Aksenov et al. 1996). Decreased GS activity in AD may also be due to the oxidation of GS by free radicals (Butterfield et al. 1997; Castegna et al. 2011; Oliver et al. 1990; Smith et al. 1991). Conversely, Aβ peptides were shown to enhance GS activity (Pike et al. 1996). While the reason for the differences in GS protein expression and activity (increased and decreased) in AD is unclear, it is likely that the concentration of Aβ peptides, the peptide sequences used (i.e., 1–42 vs. 35–42), or the duration of Aβ exposure to neuronal cells may have contributed to the differences in GS protein expression and activity reported in AD. The earlier findings suggest that alterations in GS expression/activity may provide valuable insights into the pathogenetic mechanisms of AD that may have therapeutic implications for this condition.

13.2.6 Amyotrophic Lateral Sclerosis

Amyotrophic lateral sclerosis (ALS) is a progressive disorder of motor neurons, in which both upper and lower motor neurons are affected leading to muscle atrophy. While the molecular mechanism responsible for ALS is unknown, astrocytes appear to play a role, as an SOD1 gene mutation has been identified in astrocytes in ALS which was shown to result in a disturbance in astrocyte–motor neuron interactions, resulting in a loss of motor neurons (Bame et al. 2012; Ghoddoussi et al. 2010).

Studies of brain tissue from patients with ALS, as well as from transgenic mouse models of ALS, have provided strong evidence that astroglial abnormalities precede the onset of clinical disease (for review, see Radford et al. 2015). These abnormalities include a loss of the astrocytic glutamate transporter GLT-1 protein expression and activity (up to 95 %) (Bristol and Rothstein 1996).

Increased expression of immune and inflammatory markers was identified in brains of patients with ALS (Chiu et al. 2008, 2009). Studies have shown that pro-inflammatory mediators (e.g., cytokines, iNOS, COX-2) released by microglia and astrocytes may result in NMDA-mediated neurotoxicity, possibly by phosphorylation of the NMDA receptor, resulting in the cell death of motor neurons (Hensley et al. 2006; Hewett et al. 1994). Dysfunctional astrocytes may also contribute to excitotoxic damage by decreasing glutamate transport or by actively releasing the excitotoxic amino acids, suggesting that astrocytic glutamate metabolism may be affected in ALS which may contribute to its pathogenesis.

Consistent with the earlier metabolic derangements in ALS, there is increasing evidence that elevated GS levels are involved in the mechanism of ALS, as MSO lowers brain glutamine levels, as well as improves the survival of transgenic mice that overexpresses the mutant human SOD1(G93A) gene (a mice model of ALS) (Bame et al. 2012; Ghoddoussi et al. 2010). Further, levels of GS were significantly increased in the CSF of patients with ALS (Tumani et al. 1999), suggesting that an astroglial dysfunction may have contributed to the development of ALS. While these studies examined the potential role of GS in ALS, a more comprehensive understanding of the interactions between motor neuron death and the precise role of astrocytic GS in this process is required.

13.2.7 Huntington's Disease

Huntington's disease (HD) is a fatal autosomal dominant neurodegenerative disease that results from an expanded DNA trinucleotide CAG repeats in the gene encoding for the huntingtin protein (for review, see Walker 2007). Neuropathological changes include atrophy of the caudate/putamen, along with a concomitant neuronal loss and astrogliosis.

The mutant huntingtin protein is known to aggregate in neurons of transgenic mouse models of HD (Davies et al. 1997). Recent evidence suggests that mutant huntingtin protein is also present in the nuclei of astrocytes, a phenomenon that becomes more prominent with age, and which is associated with a downregulation of glutamate transporters (especially GLT-1) in these cells (Shin et al. 2005).

A potential cause-and-effect relationship implicating astrocytes in the neurotoxicity associated with HD was reported following the observation that in a neuron–astrocyte coculture system, in which wild-type astrocytes were found to protect cortical neurons against the mutant huntingtin-mediated neurotoxicity, whereas astrocytes expressing the mutant huntingtin displayed an exacerbation of neuronal injury (Shin et al. 2005). These observations suggest that dysfunctional astrocytes

containing the mutant huntingtin contribute to the neuronal abnormalities in HD, possibly due to a disturbance of glutamate–glutamine cycle, wherein GS plays a major role.

In support of the earlier mechanistic view, GS activity was found to be reduced in the frontal and temporal cortices, putamen and cerebellum of patients with HD (Carter 1981, 1982, 1983). Decreased GS immunoreactivity was also identified in transgenic animal models of HD (Behrens et al. 2002). These findings suggest that a reduction in astrocytic GS activity may contribute to the pathogenesis of HD. However, other studies have failed to identify significant changes in glutamate metabolic pathways, or in GS levels in brains from patients with HD (Behrens et al. 2002; Perry 1981). Additional information is needed regarding the precise role of astrocytic GS activity in the pathogenesis of HD.

13.2.8 CNS Neoplasms

GS has been strongly implicated in the growth of astrocytic neoplasms as immunohistochemical analysis of GS in autopsied brain tumor tissue showed that GS was overexpressed in astrocytomas, but not in other CNS neoplasms (Pilkington and Lantos 1982). Additionally, overexpression of GS correlated well with tumor progression (Loeppen et al. 2002; McCormick et al. 1990).

While the contribution of GS to tumor initiation or progression is unclear, the overexpression of GS has been shown to enhance mutations in β-catenin. As enhanced β-catenin activity has been strongly implicated in tumor progression (Thompson et al. 2011), it is possible that GS-mediated promotion of β-catenin mutations may contribute to the progression of astrocytomas.

In contrast to the increased GS activity in lower grade astrocytomas (Grades I–III), several reports indicate that in glioblastoma multiforme (GBM, astrocytoma Grade IV) GS levels are decreased (Rosati et al. 2009, 2013). This finding correlated with the development of seizures (Rosati et al. 2013). Even though epilepsy in patients with glioma is multifactorial, it is conceivable that a downregulation of GS may result in epileptogenesis in patients with GBM by the slowing of the glutamate–glutamine cycle. The regulation of GS activity might constitute a novel approach for the treatment of the seizure disorder associated with GBM.

13.2.9 Other Neurological Disorders

Inhibition of GS activity by MSO resulting in a dysfunction of the glutamate–glutamine cycle was shown to result in depressive-like behaviors in mice (Lee et al. 2013). Brain GS level, as well as its activity was also reduced in rats after ethanol exposure (Babu and Ramanathan 2009), as well as in the cerebral cortex from individuals with major depressive disorders (Choudary et al. 2005;

Miguel-Hidalgo et al. 2010; Rajkowska and Stockmeier 2013; Toro et al. 2006), and suicidal behavior (Kalkman 2011; Klempan et al. 2009; Sequeira et al. 2009). While the reason for the decreased GS protein expression and activity in these conditions is unclear, inflammation may have played a role as inflammation is a common factor involved in these conditions and inflammatory cytokines are well known to decrease GS expression/activity by increasing its oxidation/nitration (Häussinger et al. 2005).

While decreased GS expression/activity has been identified in many neurological conditions, increased expression/activity of GS was also found in some neurological disorders. Increased GS expression was identified in brains of patients with schizophrenia (Kim et al. 2007; Bruneau et al. 2005; Burbaeva et al. 2005), in rat brain following sleep deprivation (Levental et al. 1972; Sallanon et al. 1992), as well as in rat brain after exposure to valproic acid (VPA), an animal model of autism (Bristot Silvestrin et al. 2013). While there is substantial evidence that GS expression or activity is increased or decreased in these conditions, the precise significance of changes in GS in the mechanisms of these disorders remains to be determined.

13.3 Summary

GS plays a major role in the glutamate–glutamine cycle in the CNS, by preventing the excessive accumulation of glutamate and ammonia in synaptic regions, and thereby inhibiting the development of glutamate/ammonia neurotoxicity. Increased GS levels have been identified in number of neurological conditions, including hepatic encephalopathy, ischemic stroke, traumatic brain injury, epilepsy, neoplasms, Alzheimer's disease, amyotrophic lateral sclerosis, schizophrenia, sleep disorders, and autism spectrum disorders, while inhibiting GS activity with MSO was shown to improve some of these conditions. In contrast, decreased GS expression/activity was identified in hepatic encephalopathy, ischemic stroke, traumatic brain injury, epilepsy, brain neoplasms, and in Alzheimer's disease. Such reversal of GS activity by genetic manipulation was further shown to reduce the clinical progression of these conditions.

While the reason for the differences in GS expression/activity and their involvement in the development of various neurological disorders remain unclear, it is possible that a number of factors, including the duration or severity of insult or the concentration of agents used in these studies to induce a disease process (e.g., Aβ peptides, ammonia), or the animal model used, may have varied in these studies, possibly accounting for the differences in GS protein expression and activity identified. A better understanding of the precise role of GS in these neurological conditions and its involvement in disease progression should facilitate the identification of novel agents to treat these disorders.

With regard to the current treatment strategy to prevent GS-mediated neurological conditions, inhibition of GS by MSO was shown to diminish clinical features (e.g.,

brain edema, cognitive dysfunction) in various conditions (e.g., hepatic encephalopathy, traumatic brain injury, epilepsy, ischemia, and amyotrophic lateral sclerosis). Nevertheless, the use of MSO, however, is problematic as MSO is well known to induce seizures. It is possible, however, that a combinatorial therapy (MSO along with an anticonvulsant) may represent an efficacious approach for the treatment of these disorders. Additionally, when GS levels are found to be decreased in some conditions (e.g., traumatic brain injury, ischemia, Alzheimer's disease, epilepsy), induction of GS gene expression, or the exogenous administration of purified GS may provide a useful therapeutic approach for the management of these disorders (Fig. 13.1).

We suggest that alterations in GS expression/activity are critically involved in the pathogenesis of many neurological and neuropsychiatric conditions. A better understanding of the precise role of GS in these disorders may provide valuable insights into disease pathogenesis that hopefully will lead to improved therapeutic outcomes for these debilitating conditions.

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Chapter 14
The Glutamate–Glutamine Cycle in Epilepsy

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Abstract Epilepsy is a complex, multifactorial disease characterized by spontaneous recurrent seizures and an increased incidence of comorbid conditions such as anxiety, depression, cognitive dysfunction, and sudden unexpected death. About 70 million people worldwide are estimated to suffer from epilepsy, and up to one-third of all people with epilepsy are expected to be refractory to current medications. Development of more effective and specific antiepileptic interventions is therefore requisite. Perturbations in the brain's glutamate–glutamine cycle, such as increased extracellular levels of glutamate, loss of astroglial glutamine synthetase, and changes in glutaminase and glutamate dehydrogenase, are frequently encountered in patients with epilepsy. Hence, manipulations of discrete glutamate–glutamine cycle components may represent novel approaches to treat the disease. The goal of his review is to discuss some of the glutamate–glutamine cycle components that are altered in epilepsy, particularly neurotransmitters and metabolites, enzymes, amino acid transporters, and glutamate receptors. We will also review approaches that potentially could be used in humans to target the glutamate–glutamine cycle. Examples of such approaches are treatment with glutamate receptor blockers, glutamate scavenging, dietary intervention, and hypothermia.

Keywords Epilepsy • Glutaminase • Glutamine synthetase • Glutamate receptors • Glutamine–glutamate cycle

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Abbreviations

AIDA	1-aminoindan-1,5-dicarboxylic acid
ALT	Alanine aminotransferase (alanine transaminase)
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
AST	Aspartate aminotransferase (aspartate transaminase)
BGT	Betaine/gamma-aminobutyric acid transporter
CA1-3	Cornu ammonis subfields 1-3 of the hippocampus
CGP 37849	(E)-(±)-2-amino-4-methyl-5-phosphono-3-pentenoic acid
CGP 39551	(E)-(±)-2-amino-4-methyl-5-phosphono-3-pentenoic acid ethyl ester
CGS 19755	cis-4-[phosphomethyl]-piperidine-2-carboxylic acid
CNQS	6-cyano-7-nitroquinoxaline-2,3-dione
CNS	Central nervous system
D-AP5 (D-APV APV)	D-2-amino-5-phosphonovaleric acid
DMCM	Methyl-6,7-dimethoxy-4-ethyl-beta--carboline-3-carboxylate
DNQX	6,7-dinitroquinoxaline-2,3-dione
EAAT	Excitatory amino acid transporters
GABA	Gamma-aminobutyric acid
GAD	Glutamic acid decarboxylase
GAT	Gamma-aminobutyric acid transporter
GLS	Glutaminase
GLUL	Glutamate ammonia ligase
GluR1-4	AMPA receptor subunits 1-4
GluR5-7	Kainate receptor subunits 5-7
GYKI 52466	4-(8-methyl-9H-1,3-dioxolo[4,5-h][2,3]benzodiazepin-5-yl)-benzenamine
GYKI 53773 (talampanel LY 300164)	(8R)-7-Acetyl-5-(4-aminophenyl)-8,9-dihydro-8-methyl-7H-1,3-dioxolo[4,5-h][2,3]benzodiazepine
KA1-2	Kainate receptor subunits 1-2
LY 274614	((4-)3 SR 4a RS, 6 SR, 8a SR-6-(phosphonomethyl)-l,2,3,4,4a,5,6,7,8,8a-decahydroisoquinoline-3-carboxylic acid

LY 341495	2-[(1S,2S)-2-carboxycyclopropyl]-3-(9H-xanthen-9-yl)-D-alanine
LY 382884	(3S,4aR,6S,8aR)-6-[(4-carboxyphenyl)methyl]-decahydroisoquinoline-3-carboxylic acid
mGluR	Metabotropic glutamate receptor
MK-801 (dizocilpine)	[5R,10S]-[+]-5-methyl-10,11- dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine
MPEP	2-methyl-6-(phenylethynyl)pyridine
MSG	Monosodium glutamate
MSOP	(RS)-α-methylserine-O-phosphate
MTLE	Mesial temporal lobe epilepsy
NBQX	2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo[f]quinoxaline-2,3-dione
NMDA	n-methyl-D-aspartate
NPC 17742	2R,4R,5S-(2-amino-4,5-(1 2-cyclohexyl)-7-phosphonoheptanoic acid
NR1-3	*N*-methyl-D-aspartate receptor subunits
NVP-AAM077	[[[(1S)-1-(4-bromophenyl)ethyl]amino](1,2,3,4-tetrahydro-2,3-dioxo-5-quinoxalinyl)methyl] phosphonic acid
PPDA	(2S*,3R*)-1-(Phenanthren-2-carbonyl) piperazine-2,3-dicarboxylic acid
Ro 63-1908 (Co 101244 PD 174494)	1-[2-(4-Hydroxyphenoxy)ethyl]-4-[(4-methylphenyl)methyl]-4-piperidinol
SDZ EAB-515	(S)-α-amino-2′-chloro-5-(phosphonomethyl)[1,1′-biphenyl]-3-propanoic acid
SIB-1893	(E)-2-methyl-6-(2-phenylethenyl) pyridine
SNAT	System-A-transporter
TCA	Tricarboxylic acid
UPB-302	2-{[3-[(2S)-2-amino-2-carboxyethyl]-2,6-dioxo-3,6-dihydropyrimidin-1(2H)-yl]methyl}benzoic acid
YM872	[2,3-dioxo-7-(1H-imidazol-1-yl)-6-nitro-1,2,3,4-tetrahydro-1-quinoxalinyl]-acetic acid

14.1 Introduction

14.1.1 Glutamate: From Umami Taste to Major Neurotransmitter

In his search for the chemical basis of the umami flavor, the Japanese chemist Kikunae Ikeda purified, in the late 1800s, a crystalline substance from brown seaweed (*Laminaria japonica*). The substance turned out to be monosodium glutamate (MSG)—the carboxylate sodium salt of the alpha-amino acid glutamic acid, which had been isolated by Ritthausen in 1866 (Ritthausen 1866). Ikeda was granted a patent for his discovery in 1908 (Patent 14805, Japan Patent Office, Tokyo, Japan) and industrial production of MSG began shortly after and eventually resulted in widespread use of the amino acids as an umami flavor enhancer in food production and cooking. The annual production of MSG was estimated to be two million metric tons per year in 2009 (Sano 2009).

Subsequent studies identified glutamate as one of the most abundant amino acids in biology, being present in high concentrations in nearly all prokaryotes and eukaryotes, including humans. Particularly high levels of the amino acid were found in skeletal muscle, kidney, liver, and brain, with concentrations in the latter ranging from 5 to 15 mmol/kg wet weight (Schousboe 1981). Approximately 50 years after Ikeda's discovery, Krebs and others demonstrated that glutamate was closely related to the tricarboxylic acid (TCA) cycle as glutamate can be interconverted to the TCA intermediate alpha-ketoglutarate via the reversible glutamate dehydrogenase reaction (Krebs 1935) (Fig. 14.2). Stern next discovered that brain tissue had an exceedingly high glutamate uptake activity, suggesting that the amino acid was actively shuttled between tissue compartments (Stern et al. 1949). Then, in 1954 Hayashi made the seminal discovery that glutamate excites neural tissue, a finding that initiated a new field of research by launching numerous studies aimed at understanding the role of glutamate in neurotransmission and brain pathology (Hayashi 1954).

The current state of knowledge is that glutamate is the primary small molecule neurotransmitter responsible for fast excitatory signaling in the CNS (Fonnum 1984). Glutamate is released preferentially from axon terminals into the synaptic cleft where the transmitter binds to several types of glutamate receptors. However, release from other cell types, particularly astrocytes may occur as well, although the mechanism of release is intensely debated (Li et al. 2013; Nedergaard and Verkhratsky 2012). To ensure high fidelity encoding of the synaptic glutamate response, glutamate is rapidly cleared from the synaptic cleft by diffusion into the surrounding extracellular space and by uptake via excitatory amino acid transporters (EAATs), which are present on the surface of several cell types, particularly astrocytes and neurons (Danbolt 2001; 2016a). Effective clearance of extracellular glutamate is also important because sustained high levels of the neurotransmitter can lead to seizures and neuronal death, a phenomenon known as excitotoxicity (Choi and Hartley 1993; Nadler et al. 1978; Olney et al. 1986).

14.1.2 The Glutamate–Glutamine Cycle is Critical for Clearance and Synthesis of Neurotransmitter Glutamate

The notion that glutamate needs to be effectively cleared from the extracellular space and replenished in axon terminals is underscored by a series of compartmentalized processes collectively known as the glutamate–glutamine cycle (Fig. 14.1). This cycle consists of the following principal components. (1) Exocytosis of glutamate from axon terminals into the synaptic cleft. (2) Binding of glutamate to postsynaptic glutamate receptors. (3) Diffusion of glutamate away from the synapse with possible binding to extrasynaptic receptors and uptake into astrocytes or neurons via EAATs. (4) Conversion of glutamate and ammonia to glutamine in the astroglial cytoplasm via the enzyme glutamine synthetase. (5) Shuttling of glutamine to neurons via glutamine transporters. (6) Conversion of glutamine to glutamate and ammonia via the enzyme glutaminase. And finally, (7) concentration of glutamate in synaptic vesicles via vesicular glutamate transporters. The biological advantages of the cycle are twofold. First, neurotransmitter glutamate is economically recycled from glutamine. Second, the

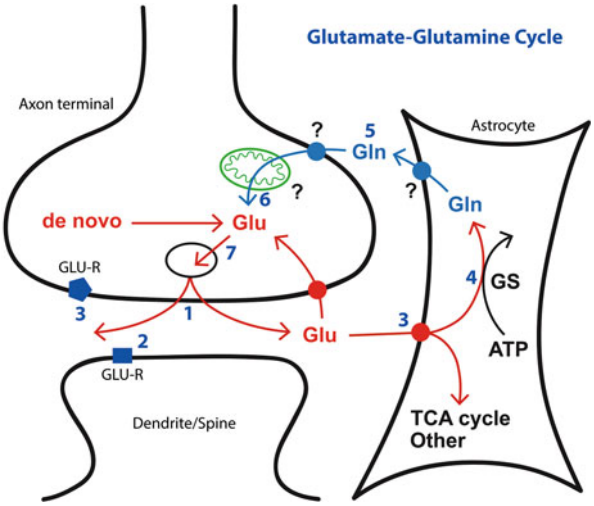

Fig 14.1 Simplified diagram of the glutamate–glutamine cycle, illustrating the flux of glutamate (Glu) and glutamine (Gln) between neurons and astrocytes. (1) Glutamate is released from vesicles in the axon terminals into the synaptic cleft (2) where the amino acid binds to glutamate receptors (GLU-R). (3) Glutamate diffuses rapidly away from the synapse followed by possible binding to extrasynaptic GLU-R and uptake into astrocytes and neurons via excitatory amino acid transporters. (4) Glutamate in astrocytes is converted to glutamine via the enzyme glutamine synthetase (GS). (5) Glutamine is next shuttled from astrocytes to neurons via glutamine transporters, which are not well defined. (6) Once in the neuron, glutamine is converted to glutamate by glutaminase, which is particularly enriched in mitochondria. (7) Finally, glutamate produced de novo or from glutamine is concentrated in synaptic vesicles via vesicular glutamate transporters, thus completing the cycle

relatively "inert" glutamine rather than the excitatory glutamate is shuttled back to the neuron, thus maintaining low extracellular levels of glutamate. However, it is important to note that the glutamate–glutamine cycle is not stoichiometric as a substantial portion of the absorbed glutamate is degraded via the TCA cycle, or modified by transaminases, rather than being converted to glutamine (McKenna 2007).

14.1.3 The Glutamate–Glutamine Cycle is Part of an Extensive Cellular, Molecular, and Metabolic Homeostatic Network

As detailed in several chapters throughout this volume, the glutamate–glutamine cycle does not operate isolated from other biological processes—it is affected by numerous metabolic and signaling pathways within and outside the CNS, as illustrated in Fig. 14.2. Brain metabolic pathways that are known to affect, depend on, or intercede with the glutamate–glutamine pathway include: the TCA cycle and glutamate dehydrogenase reaction (Hudson and Daniel 1993; Krebs 1935), the gamma-aminobutyric acid (GABA) synthetic pathway (Erlander et al. 1991), the metabolism of ammonia (Benjamin and Quastel 1975; Folbergrova et al. 1969; Krebs 1935), the synthesis of glutathione (Dringen 2000), the branched-chain amino acid pathway (Hutson et al. 1998; Leith et al. 2001), the alanine aminotransferase (a.k.a. alanine transaminase, ALT) and aspartate aminotransferase (a.k.a. aspartate transaminase, AST) pathways (Karmen et al. 1955), the pyruvate carboxylase pathway (Shank et al. 1985), the malic enzyme pathway (Hassel 2001), the glutaminase II (i.e., glutamine transaminase–ώ-amidase) pathway (Cooper and Kuhara 2014), and the isocitrate dehydrogenase pathway (Ceccarelli et al. 2002; Guo et al. 2011). Brain signaling events in the brain that are known to affect the glutamate–glutamine cycle, include the EphA5-EphrinA5 pathway (Szepietowska et al. 2012) and the mTORC1/S6K1 pathway, among others (Csibi et al. 2014). Several peripheral metabolites can also affect the glutamate–glutamine cycle, particularly branched-chain amino acids (Kanamori et al. 1998) and acetate (Patel et al. 2010) (Fig. 14.2).

The glutamate–glutamine cycle is highly compartmentalized and tightly regulated to ensure effective extracellular clearance and replenishment of neurotransmitter glutamate, particularly during periods of intense neuronal activity. However, the homeostasis of the cycle can be challenged during conditions of disease, such as brain inflammation (Gras et al. 2006), traumatic brain injury (Bartnik-Olson et al. 2013), hypoxia/ischemia (Haberg et al. 2006), and epilepsy (Eid et al. 2004; Petroff et al. 2002), with abnormal synaptic transmission, seizures, and neuronal loss as some of the deleterious consequences. It is important to understand how these conditions affect the glutamate–glutamine cycle, because such information may lead to novel therapeutic interventions for a wide range of hitherto intractable CNS disorders. The remainder of this chapter will be devoted to the role of the glutamate–glutamine cycle, particularly in the pathophysiology of epilepsy (Fig. 14.2).

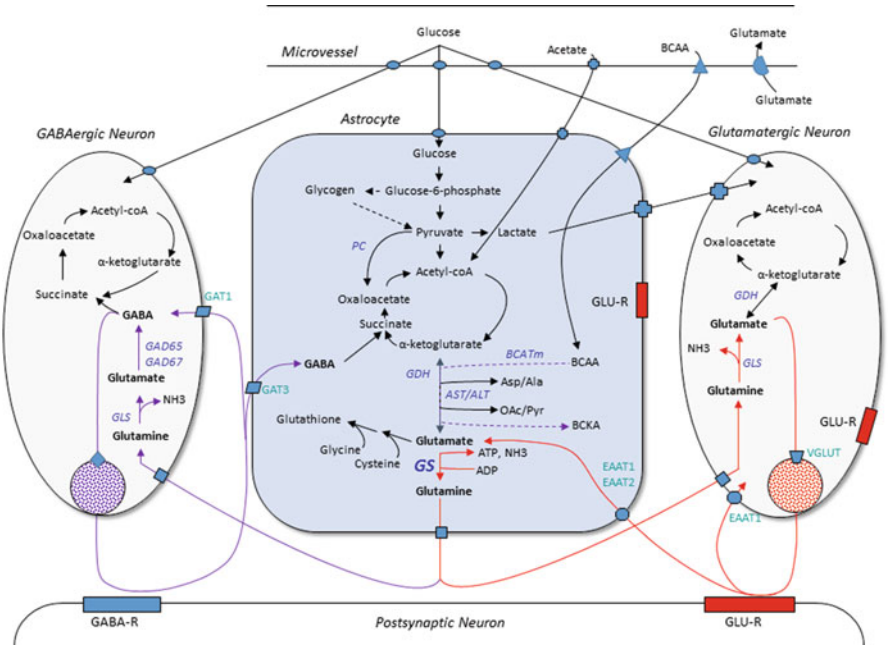

Fig 14.2 The glutamate–glutamine cycle is part of a complex metabolic network. Some of the network components are illustrated here and further discussed in the text. Transmembrane transporter molecules are depicted as blue shapes (triangle, cross, ellipse, etc.). Enzymes are indicated in *blue italics*. *ALT* alanine aminotransferase, *AST* aspartate aminotransferase, *ATP* adenosine triphosphate, *ADP* adenosine diphosphate, *BCAA* branched-chain amino acid, *BCATm* branched-chain aminotransferase mitochondrial isoform, *BCKA* branched chain keto acid, *coA* coenzyme A, *EAAT1*-2 excitatory amino acid transporter subtypes 1 and 2, *GABA* gamma-aminobutyric acid, *GABA*-R GABA-receptor, *GAD65*/67 glutamic acid decarboxylase isoforms 65 and 67, *GAT1-3* GABA transporters subtypes 1-3, *GDH* glutamate dehydrogenase, *GLS* glutaminase, *GLU-R* glutamate receptor, *GS* glutamine synthetase, *PC* pyruvate carboxylase, *VGLUT* vesicular glutamate receptor

14.2 The Glutamate–Glutamine Cycle in Epilepsy

14.2.1 A Brief Introduction to Epilepsy

Epilepsy is a chronic disorder of the CNS with a prevalence of approximately 1 % of the general population (Hauser et al. 1993). The hallmarks of epilepsy are spontaneous recurrent seizures and an increased incidence of comorbid conditions, particularly anxiety, depression, cognitive impairment, and sudden unexpected death (Tellez-Zenteno et al. 2007). Furthermore, up to 40 % of all people with epilepsy cannot control their disease with medications (Shorvon 1996), which often have significant side effects due to poor pharmacological specificity. A better understanding of the causative mechanisms of epilepsy is important because such understanding will likely facilitate the discovery of more efficacious and specific therapies.

This chapter will focus on the glutamate–glutamine cycle in mesial temporal lobe epilepsy (MTLE), which is one of the most common forms of medically refractory epilepsies. MTLE is of particular interest because perturbations in the glutamate–glutamine cycle are closely associated with the pathophysiology of the disease (Alvestad et al. 2011; During and Spencer 1993; Eid et al. 2004, 2008b; Petroff et al. 2002).

14.2.2 Glutamate

Numerous studies in laboratory animals have shown that increased extracellular brain glutamate or excessive stimulation of postsynaptic glutamate receptors can trigger seizures. Subcutaneous administration of MSG (4 g/kg) to a 1-week-old rhesus monkey (*Macaca mulatta*) lead to severe convulsions and neuronal damage, presumably due to entry of glutamate into the brain via a lesser developed blood–brain barrier in the infant primate (Olney et al. 1972). Systemic administration of relatively high doses of MSG to adult cats (~8 g/kg) (Goodman et al. 1946), adult rats (3.4 g/kg) (Bhagavan et al. 1971), or mice (5–7 g/kg) (Olney et al. 1972) led to severe convulsions and sometimes death. However, it is important to note that the dose of MSG required to induce seizures via the systemic route is quite high due to the effective barrier against glutamate transport from the blood to the brain and the remarkably efficient clearance of extracellular brain glutamate via the EAATs (Danbolt 2001). However, some glutamate receptor agonists such as kainic acid and domoic acid readily cross the blood–brain barrier and can result in seizures and neuronal damage at considerably lower systemic concentrations (Nadler and Cuthbertson 1980; Nadler et al. 1978; Perl et al. 1990).

Increased brain glutamate has also been associated with human epilepsy. Using intracerebral microdialysis in patients with MTLE, During and Spencer discovered in the early 1990s that glutamate increased rapidly in the extracellular compartment of the brain during a spontaneous seizure (During and Spencer 1993). The increase was most pronounced in the hippocampal formation that was the focus of the seizures, and glutamate levels remained high for several minutes after the cessation of the seizure, suggesting impaired extracellular clearance of the transmitter (During and Spencer 1993). In a subsequent study, Cavus et al. demonstrated that extracellular glutamate was chronically elevated in the seizure onset zone in patients with several types of epilepsies, including MTLE and neocortical epilepsies, indicating that the extracellular glutamate excess is a chronic phenomenon that involves several types of focal (i.e., localization-related) epilepsies (Cavus et al. 2005).

Based on our knowledge of the actions of glutamate and glutamate receptor agonists on laboratory animals, it is tempting to speculate that the extracellular glutamate excess is causally related to the triggering of seizures and loss of neurons in human epilepsy. However, the role of the extracellular glutamate excess is not as straightforward as one would think. First, due to the size of the dialysis probe, brain microdialysis is thought to preferentially sample the extrasynaptic extracellular space, rather than the much smaller synaptic extracellular space (van der Zeyden et al. 2008). This is an

important distinction because synaptic glutamate may induce a different cellular response (i.e., excitation via ionotropic glutamate receptors) than extrasynaptic glutamate (i.e., inhibition via presynaptic metabotropic glutamate receptors or glial responses via glial glutamate receptors (Scofield et al. 2015)). Second, epileptic seizures are intermittent phenomena while the extracellular glutamate excess is chronic. Third, extracellular glutamate is increased in many CNS conditions, such as stroke (Davalos et al. 2000) and traumatic brain injury (Hinzman et al. 2016); however, not all patients with these conditions experience seizures or develop epilepsy. However, it is reasonable to conclude that increased extracellular brain glutamate definitely causes seizures and neuron loss under certain conditions, and that increased extracellular glutamate likely has significant effects on brain physiology.

14.2.3 Gamma-Aminobutyric Acid

GABA is synthesized from glutamate via the enzyme glutamic acid decarboxylase of which there are two main isoforms in the mammalian brain, GAD65 and GAD67 (Erlander et al. 1991). GABA is the major inhibitory neurotransmitter in the CNS (Capogna and Pearce 2011; DeFelipe 1993); however, the amino acid is also involved in cellular signaling in several organs, including the endocrine pancreas and gut (Erdo and Wolff 1990). In the CNS, GABA binds to ligand-gated $GABA_A$ receptors (Capogna and Pearce 2011) and metabotropic $GABA_B$ receptors (Chen et al. 2005), thereby mediating a variety of effects, such as neuronal inhibition, neuronal excitation, cell differentiation, neurite growth, and synapse formation (Erdo and Wolff 1990). Therapeutic or experimental manipulation of the GABA system is commonly used to modulate seizures. $GABA_A$ receptor agonists such as barbiturates and benzodiazepines are powerful anticonvulsants, and drugs with other actions on the GABA system such as gabapentin and vigabatrin reduce the frequency of many types of seizures (Petroff 2002). The $GABA_A$ antagonist bicuculline (Meldrum and Nilsson 1976) and $GABA_A$ channel blocker picrotoxin (Davidson and Barbeau 1975) both induce seizures in laboratory animals.

While GABA-modulating drugs can have dramatic effects on acute seizures, studies in humans and laboratory animals suggest that perturbations in the GABA system may play a role in the development of epilepsy and manifestation of epileptic (i.e., spontaneous recurrent) seizures. For example, extracellular GABA levels measured by microdialysis increase less in the epileptogenic hippocampal formation than in the nonepileptogenic hippocampal formation during a spontaneous seizure in patients with MTLE (During and Spencer 1993). Moreover, analysis of the GABA response reveals that the glutamate-induced calcium-independent release of GABA in the epileptogenic hippocampal formation is significantly decreased compared to the nonepileptogenic hippocampal formation in these patients (During et al. 1995). Finally, changes in the density, cytoarchitecture, molecular phenotype, and electrophysiological properties of GABAergic neurons have been reported in patients with epilepsy as well as in several animal models of

the disease (de Lanerolle et al. 1989; Kobayashi and Buckmaster 2003; Margerison and Corsellis 1966; Mathern et al. 1995). However, it is important to note that the perturbations of the GABA system in epilepsy are complicated by the facts that the transmitter sometimes is excitatory (Ben-Ari et al. 2012) and that free GABA in the brain is in chemical equilibrium with homocarnosine, a dipeptide of GABA and histidine (Pisano et al. 1961), and with pyrrolidinone, the internal lactam of GABA (Seiler 1980). Homocarnosine and pyrrolidinone are particularly abundant in the *human* brain versus the rodent brain (Petroff et al. 1999).

14.2.4 Glutamine

With respect to the glutamate–glutamine cycle, glutamine has been viewed traditionally as a neurologically inert metabolite that merely shuttles and recycles glutamate from astrocytes to neurons (Benjamin and Quastel 1975; van den Berg and Garfinkel 1971). However, the first indication that glutamine was implicated in the pathophysiology of epilepsy, possibly via modulation of the glutamate–glutamine cycle, came from hippocampal slice recordings. Blocking the astroglial synthesis of glutamine by inhibiting glutamine synthetase resulted in slowing of epileptiform activity (Bacci et al. 2002). Laake et al. had previously shown that glutamate became depleted from axon terminals after inhibition of glutamine synthetase in organotypical cultures of the hippocampus, suggesting that decreased release of synaptic glutamate might explain the antiepileptiform properties of glutamine *in vitro* (Laake et al. 1999).

However, the idea that decreased glutamine inhibits epileptiform activity has been challenged by more recent studies of hippocampal glutamine synthetase inhibition *in vivo*. Such inhibition leads to decreased extracellular levels of hippocampal glutamine, recurrent seizures, and preferential loss of neurons in the hippocampal formation (Eid et al. 2008a; Wang et al. 2009). Moreover, humans with mutations in the glutamine synthetase gene exhibit decreased plasma and cerebrospinal fluid glutamine and severe epilepsy (Haberle et al. 2005, 2006, 2011). Finally, Kanamori and Ross showed that spontaneous seizures in the kainic acid rat model of MTLE were associated with a significant *decrease* in the extracellular level of hippocampal glutamine while the level of extracellular glutamate increased (Kanamori and Ross 2011).

Thus, the role of glutamine in epilepsy is complex and the mechanism by which glutamine modulates epilepsy may involve processes other than simply affecting the supply of vesicular glutamate. In fact, glutamine is critical for a variety of biological processes that have linked to neuronal excitability, neurodegeneration, and epilepsy, such as polyglutamine expansion (Banfi et al. 1994; La Spada et al. 1994), ammonia detoxification (Martinez-Hernandez et al. 1977), astrocyte swelling (Albrecht and Norenberg 2006), cell signaling (Marc Rhoads and Wu 2009), and epigenetic regulation (Simpson et al. 2012). All of these processes should be kept in mind when considering possible mechanisms of the glutamine effects in epilepsy.

14.2.5 Enzymes

Several enzymes determine the flux through glutamate–glutamine cycle, and many are altered in epilepsy (Fig. 14.2). In the following, we will focus on three that are altered in human epilepsy, i.e., glutamine synthetase, glutaminase, and glutamate dehydrogenase.

14.2.5.1 Glutamine Synthetase

Glutamate ammonia ligase (GLUL), which encodes for glutamine synthetase (EC 6.3.1.2), is one of the oldest genes known to exist, likely dating more than 1000 million years back in time (Kumada et al. 1993). There are several variants of the gene, which encode for three classes of enzymes. Class I are specific for prokaryotes and consist of 12 identical subunits arranged as two face-to-face hexameric rings, with each subunit having an active catalytic site (Almassy et al. 1986; Liaw and Eisenberg 1994). Class II are specific for eukaryotes and some bacteria and consist of ten identical subunits arranged as two face-to-face pentameric rings (Krajewski et al. 2008). Class III has only been found in a small number of bacteria, e.g., *Bacteroides fragilis and Butyrivibrio fibrisolvens*, and consist of 12 subunits arranged in a similar fashion as the Class I enzyme despite less than 10 % sequence homology (van Rooyen et al. 2006). Glutamine synthetase is present in all extant life forms and catalyzes the ATP-dependent ligation of glutamate and ammonia to glutamine (Fig.14.1). Even though glutamine synthetase is essential for life and therefore widely distributed in nature, the enzyme is restrictively expressed in mammals, being limited to specific cell populations in the liver (Bode et al. 2000; Haussinger 1990; Krebs 1935), kidney (Janicki and Goldstein 1969; Krebs 1935), pancreas (Zhou et al. 2014), adipose tissue (Kuo and Darnell 1989; Palmieri et al. 2014), skin (Danielyan et al. 2009), and CNS (Krebs 1935; Martinez-Hernandez et al. 1977). The astrocyte is the main, if not the only type of cell, expressing glutamine synthetase in the normal adult CNS (Martinez-Hernandez et al. 1977).

The importance of glutamine synthetase for life is reflected by the findings that whole body deletion of the glutamine synthetase gene in mice is embryonically lethal (He et al. 2007) whereas deletion preferentially targeting astrocytes leads to death within a few days after birth (He et al. 2010). Only three cases of whole body GLUL mutations resulting in severe glutamine synthetase deficiency have been described in humans, thus far (Haberle et al. 2005, 2006, 2011). All patients exhibited brain malformations and epilepsy, and two patients developed multiorgan failure, which resulted in death within the first month of life (Haberle et al. 2005, 2006, 2011). Heterozygous deletions in GLUL are compatible with life; however, the susceptibility to experimental febrile seizures is increased (van Gassen et al. 2009). Reduced expression or decreased activity of glutamine synthetase has also been reported to occur in a variety of CNS conditions such as major depression, Alzheimer's disease (Gunnersen and

Haley 1992; Robinson 2001; Tumani et al. 1999), schizophrenia (Bruneau et al. 2005), hepatic encephalopathy (Brusilow et al. 2010; Haussinger et al. 1994, 2000), MTLE (Eid et al. 2004; van der Hel et al. 2005), and neocortical epilepsies (Steffens et al. 2005).

While loss or inhibition of glutamine synthetase leads to a variety of signs and symptoms, depending on the degree of pathology and site of action, seizures and epilepsy often occur, as evident in patients with inherited GLUL mutations (Haberle et al. 2005, 2006, 2011). Moreover, ingestion of domoic acid by California sea lions leads to degradation of GS in astrocytes, followed several weeks later by epileptic seizures and brain pathologies that resemble those of human MTLE (Madl et al. 2014). In the juvenile pilocarpine model of MTLE, glutamine synthetase is lost from astrocytes in the dentate hilus several weeks prior to the onset of epilepsy, whereas the expression of glutamate transporters is unaffected (van der Hel et al. 2014). In the unilateral intracortical kainic acid injection model of epilepsy, astroglial glutamine synthetase is reduced in CA1 of the ipsilateral vs. contralateral hippocampus, 3–7 days after injection, several weeks before the onset of epilepsy (Jordan 2014). If glutamine synthetase is inhibited in the rat hippocampus using the irreversible enzyme blocker methionine sulfoximine, the animals develop spontaneous recurrent seizures and brain damage, similar to human MTLE (Eid et al. 2008a; Wang et al. 2009). Finally, glutamine synthetase inhibition in the amygdala results in epilepsy and depressive-like behaviors in rats, suggesting a possible causal relationship between the glutamate–glutamine cycle in the amygdala and comorbid depression in MTLE (Gruenbaum et al. 2015).

While most studies show *decreases* in glutamine synthetase in epilepsy, glutamine synthetase protein increases temporarily in the kainic acid and kindling models of MTLE (Hammer et al. 2008; Sun et al. 2013), and partial blockade of this increase, using very low doses of methionine sulfoximine, or small interfering RNAs against glutamine synthetase, *slows* the progression of epilepsy (Sun et al. 2013). This seemingly contradictory role of glutamine synthetase in epilepsy may be related to issues such as the dose, duration, and spatial distribution of glutamine synthetase alterations, and the fact that glutamine synthetase changes can modulate both glutamatergic and GABAergic neurotransmission, with potential opposing effects (Liang et al. 2006).

14.2.5.2 Glutaminase

Glutaminase (EC 3.5.1.2) catalyzes the hydrolysis of glutamine to glutamate and ammonia (Fig. 14.2). Four isoforms of glutaminase are known to exist in humans. GLS1 encodes two kidney-type glutaminases and GLS2 encodes two liver-type glutaminases (Campos-Sandoval et al. 2015). GLS1 is the predominant glutaminase transcript expressed in the mammalian brain and initial immunogold electron microscopy studies of perfusion fixed rat brains suggested that the enzyme was preferentially localized to neuronal mitochondria (Laake et al. 1999). However, recent studies by Cardona et al. have shown that astrocytes express GLS1 transcripts and active enzyme in the mitochondria and GLS2 transcripts and active enzyme in the mitochondria and

nucleus (Cardona et al. 2015). Moreover, glutaminase is overexpressed in many tumor cells, including malignant gliomas, and strategies aimed at manipulating glutaminase are being explored as a possible treatment for cancer (Cheng et al. 2011).

Using immunohistochemistry and immunogold electron microscopy, we found that the expression of kidney-type glutaminase was increased in surviving neurons in the epileptogenic hippocampal formation in patients with MTLE (Eid et al. 2007). The glutaminase was enriched in mitochondria, and the concentration of the enzyme was correlated with the concentration of mitochondrial glutamate (Eid et al. 2007). We concluded that the increased glutaminase might result in an enhanced capacity for glutamate synthesis in neuronal subsets in the epileptogenic hippocampal formation, thereby possibly contributing to the extracellular glutamate excess in human MTLE (Eid et al. 2007).

14.2.5.3 Glutamate Dehydrogenase

Glutamate dehydrogenase (EC 1.4.1.2) is present in most prokaryotes and in the mitochondria of eukaryotes, and catalyzes the reversible interconversion of glutamate to alpha-ketoglutarate (Dennis et al. 1977). Using enzyme activity measurements of surgically resected brain tissue from patients with medically intractable MTLE we discovered that the glutamate dehydrogenase activity was significantly decreased in the temporal cortex in MTLE patients who had concomitant mesial temporal sclerosis (Gloor 1991; Sommer 1880), i.e., gliosis and patterned loss of neurons in the hippocampal formation, entorhinal cortex, and amygdala (Malthankar-Phatak et al. 2006). There was a nonsignificant trend toward lower glutamate dehydrogenase activity in the hippocampus of these patients. The enzyme activity in the cortex and hippocampus of patients with MTLE and concomitant mesial temporal sclerosis was negatively correlated with the duration since the first intractable seizure (Malthankar-Phatak et al. 2006). The activity of citrate synthase was not changed in the tissue, suggesting that the loss of glutamate dehydrogenase was not due to mitochondrial dysfunction or cell death. We proposed that the loss in glutamate dehydrogenase would lead to decreased oxidation of glutamate with accumulation of mitochondrial glutamate in neurons and astrocytes. The concomitant loss of glutamine synthetase in hippocampal astrocytes in MTLE is expected to further enhance the accumulation of glutamate in these cells, as suggested by quantitative immunogold electron microscopy studies of astrocytes and neurons in the glutamine synthetase-deficient hippocampus in epileptic rats (Eid et al. 2004).

14.2.6 Excitatory Amino Acid Transporters

The mammalian genome contains five genes encoding glutamate (i.e., excitatory amino acid) transporters or EAATs (for reviews, see (Danbolt 2001; Grewer et al. 2014; Vandenberg and Ryan 2013; Zhou and Danbolt 2013)): EAAT1 (GLAST;

slc1a3), EAAT2 (GLT-1; slc1a2), EAAT3 (EAAC1; slc1a1), EAAT4 (slc1a6), and EAAT5 (slc1a7). All the EAATs catalyze coupled transport of $1H^+$, $3Na^+$, and $1K^+$ with 1 substrate molecule. L-glutamate and DL-aspartate are transported with similar affinities while D-glutamate is not. Dihydrokainate selectively blocks EAAT2 (Arriza et al. 1994). EAAT2 was the first glutamate transporter to be isolated in functional form (Danbolt et al. 1990) and localized immunocytochemically (Danbolt et al. 1992; Levy et al. 1993; Danbolt 2016b). It is the most important of the glutamate transporters and accounts for 1 % of total forebrain protein (Lehre and Danbolt 1998) and about 95 % of the total glutamate uptake activity in the normal young adult rat brain (Haugeto et al. 1996). In agreement, deletion of the EAAT2 gene in mice caused an almost complete loss (about 95 %) of glutamate uptake activity and spontaneous epilepsy starting at about 3 weeks of age (Otis and Kavanaugh 2000; Tanaka et al. 1997; Zhou et al. 2014). EAAT2 deficient mice become spontaneously epileptic about 3 weeks after birth and about half of them die suddenly within the subsequent 1–2 weeks (Tanaka et al. 1997; Zhou et al. 2014). The cellular localization of EAAT2 has been much debated as the EAAT2 protein was initially only detected in astrocytes despite presence of EAAT2 mRNA in several neuronal populations (for a review, see (Danbolt et al. 2016a)). It is now clear that there is EAAT2 in nerve terminals in CA1 of the hippocampus (stratum radiatum), at least in the rat where nerve terminal EAAT2 accounts for about 1/10 of the total EAAT2 in young adult rat hippocampus CA1 and all of the nerve terminal glutamate uptake activity (Furness et al. 2008). Several variants of EAAT2 exist due to variable splicing (Grewer and Rauen 2005). The most abundant form is EAAT2a (GLT1a) which is the predominant form both in astrocytes and in axon terminals (Holmseth et al. 2009). The significance of EAAT2 in terminals is unknown; however, selective deletion of EAAT2 in astrocytes is sufficient to create a lethal phenotype (Danbolt et al. 2016a; Petr et al. 2015).

EAAT1 is also highly expressed in the CNS (Lehre and Danbolt 1998), but in contrast to the other glutamate transporters, EAAT1 is selective for astrocytes (Lehre et al. 1995, 1997; Rauen et al. 1996; Regan et al. 2007). Mice lacking EAAT1 (Watase et al. 1998) develop normally but have disturbed functions in the cerebellum, eye, and inner ear (Hakuba et al. 2000; Watase et al. 1998) (for more references see (Zhou and Danbolt 2014)) and display poor nesting behavior, abnormal sociability, reduced alcohol intake, and reward (Karlsson et al. 2009, 2012; Stoffel et al. 2004; Watase et al. 1998). Lack of EAAT1 does not lead to spontaneous seizures like those seen in connection with EAAT2 deficiency, but when seizures are initiated, then lack of EAAT1 increases seizure duration and severity (Watanabe et al. 1999). In humans, mutations in EAAT1 are associated with episodic ataxia (Broer and Palacin 2011; de Vries et al. 2009; Jen et al. 2005).

EAAT3 is selectively expressed in neurons throughout the CNS, but it is only targeted to the cell bodies and dendrites (Holmseth et al. 2012). Within the CNS, EAAT3 is found in the highest concentrations in the hippocampus followed by the neocortex. However, the total tissue content in young adult rat brains is about 100 times lower than that of EAAT2 (Holmseth et al. 2012) explaining why mice lacking EAAT3 (Peghini et al. 1997) do not show any overt neurological phenotype. EAAT3

is highly expressed in the proximal tubules of the kidney, and humans lacking EAAT3 develop dicarboxylic aminoaciduria just like the knockout mice (Bailey et al. 2011). Interestingly, human EAAT3 polymorphisms are associated with obsessive–compulsive disorders (Brandl et al. 2012; Walitza et al. 2010).

Less is known about EAAT4 and EAAT5. These transporters are quite different from those of the other EAATs and may function more like inhibitory glutamate receptors (Dehnes et al. 1998; Schneider et al. 2014; Veruki et al. 2006) due to their very high chloride conductance (Gameiro et al. 2011; Mim et al. 2005). EAAT5 is mostly expressed in the retina (Arriza et al. 1997; Dalet et al. 2012; Eliasof et al. 1998), while EAAT4 is predominantly expressed in the dendrites and cell bodies belonging to the cerebellar Purkinje cells (Dehnes et al. 1998) as well as in a subset of forebrain neurons (de Vivo et al. 2010; Massie et al. 2008). EAAT4 is thereby believed to be neuron selective within the central nervous system, but it does not appear to be of critical importance as EAAT4 knockout mice are viable and appear normal (Huang et al. 2004) albeit with some alteration of receptor activation (Nikkuni et al. 2007).

14.2.7 Glutamine Transporters

The mammalian genome contains at least 14 transporter molecules that can transport glutamine across cell membranes (Bhutia and Ganapathy 2015); however, the mechanism whereby glutamine can enter nerve terminals is not well understood. To date, glutamine transporters have not been identified in nerve terminals in the CNS at sufficiently high expression levels to keep up with glutamate release (Conti and Melone 2006; Mackenzie and Erickson 2004). The system-A glutamine transporters SNAT2 (SAT2; slc38a2) and SNAT1 (SAT1; GlnT; slc38a1) have been reported to be expressed in dendrites and cell bodies of neurons but have not been found in terminals (Conti and Melone 2006; Jenstad et al. 2009; Solbu et al. 2010) and may therefore not be important for glutamatergic activity (Grewal et al. 2009). SNAT3 (slc38a3) is mostly present in the liver whereas the levels in the CNS are very low (Chan et al. 2016; Varoqui and Erickson 2002). SNAT5 is present in the CNS, but is, like SNAT3, predominantly an efflux transporter (Nakanishi et al. 2001; Scalise et al. 2016). Recently, SNAT7 (slc38a7) and SNAT8 (slc38a7) have been reported to be expressed in neurons and may be present in axon terminals (Hagglund et al. 2011, 2015); however, knockout mice have not yet been produced. This means neither the specificity of the antibodies used to localize the transporters nor the functional importance of these transporters has been verified. Slc7a5 may be present at the blood–brain barrier but not in nerve terminals (Dolgodilina et al. 2015). In conclusion, our knowledge about glutamine transporters in the CNS has large gaps that justify further investigation. The reader is referred to Chapter 8 by Leke and Schousboe for a more detailed account of glutamine transporter in the CNS.

14.2.8 GABA Transporters

GABA transporter 1 (GAT1; slc6a1) was the first neurotransmitter transporter to be purified (Radian et al. 1986) and cloned (Conti et al. 2004; Guastella et al. 1990; Zhou and Danbolt 2013). By homology screening it was soon realized that GAT1 was the first member of a whole new family (Eulenburg and Gomeza 2010; Gether et al. 2006; Kanner 2006) which comprises 20 different transporters including another three GABA transporters (GAT2, slc6a13; GAT3, slc6a11; and BGT1, slc6a12) (Liu et al. 1993; Yamauchi et al. 1992). Although there is some GAT1 in astrocytes, particularly in thalamus, the highest levels of GAT1 are found in GABAergic nerve terminals such as the basket cell terminals around the base of the Purkinje cells. Most GABAergic terminals are labeled with the exception of striatonigral and Purkinje cell axon terminals (for reviews, see (Conti et al. 2004; Zhou and Danbolt 2013)). In contrast, GAT3 is considered to be selectively expressed in astrocytes throughout the CNS (for reviews, see (Conti et al. 2004; Zhou and Danbolt 2013)). The highest GAT1 levels are in the cerebral cortex while the highest GAT3 levels are in the brainstem (Conti et al. 2004; Evans et al. 1996).

GAT1 is the major GABA transporter, and deletion of the GAT1 gene leads to reduced aggression (Liu et al. 2007b), hypoalgesia (Xu et al. 2008), reduced anxiety and depression-like behaviors (Liu et al. 2007a), and altered behavioral responses to ethanol (Cai et al. 2006). GAT1 gene deletion also leads to tremor, ataxia, nervousness, and increased GABA-induced tonic conductance in cerebellum (Chiu et al. 2005). The latter signs and symptoms resemble the adverse effects of tiagabine (Vossler et al. 2013), which is a highly selective GAT1 blocker (Quandt et al. 2013). Defects in GAT1 may be associated with panic and anxiety disorders in humans (Thoeringer et al. 2009).

The two last GABA transporters, GAT2 and BGT1 (Zhou et al. 2012a, b), are predominantly expressed in hepatocytes in the liver, but also in the basolateral membranes of the proximal tubules and collecting ducts in the kidney. The transporters are also found in the leptomeninges, and in very low, and probably insignificant concentrations in the brain tissue proper. Deletions of the GAT2 and BGT1 genes in mice do not lead to epilepsy (Lehre et al. 2011; Zhou et al. 2012a).

14.2.9 Glutamate Receptors

14.2.9.1 Introduction

The glutamate receptors are divided into ionotropic and metabotropic types. The ionotropic receptors are cation channels composed of numerous subunits and are named after the structural analogs of glutamate that selectively activate

the receptor. The ionotropic glutamate receptors include α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic (AMPA), N-methyl-D-aspartate (NMDA), and 2-carboxy-3-carboxymethyl-4-isopropenylpyrrolidine (kainate) receptors. In contrast to the ionotropic glutamate receptors, the metabotropic glutamate receptors function through G-protein coupling and are subdivided into three groups based both on the second messenger system that they are coupled to, as well as on pharmacological profile. Group I metabotropic glutamate receptors (mGluR1 and mGluR5) are coupled to Gq and thus activate phospholipase C, while Group II glutamate receptors (mGluR2, mGluR3) are coupled to Gi/o and thus inhibit adenylyl cyclase. Group III metabotropic glutamate receptors (mGluR4, mGluR6, mGluR7, mGluR8) are also Gi/o coupled but are categorized based on their pharmacological profile.

14.2.9.2 AMPA Receptors

The AMPA receptor is composed of four subunits (GluR1-4) arranged in a configuration that allows for a channel in the center through which cations can flow. The receptor is permeable to both Na^+ and K^+ ions. If the AMPA receptor lacks a GluR2 subunit, as is the case with AMPA receptors localized on astrocytes and many inhibitory neurons in the amygdala and hippocampus, then the AMPA receptor is also permeable to Ca^{2+} (Geiger et al. 1995; Isa et al. 1996; Mahanty and Sah 1998). Such AMPA receptors are highly expressed in the hippocampal astrocytes of epilepsy patients with Ammon's horn sclerosis (Seifert et al. 2004).

14.2.9.3 NMDA Receptors

Much like the AMPA receptor, the NMDA receptor is also a cation channel permeable to Na^+ and K^+. It can also be Ca^{2+} permeable depending on the subunit composition. The NMDA receptor is composed of seven subunits, NR1, NR2A-D, and NR3A-B. Functional NMDA receptors appear to be made up of at least one NR1 subunit and one NR2 subunit, or one NR1 subunit with both NR2 and NR3 subunits. When expressed with the NR1 and NR2 subunits, the NR3 subunit suppresses Ca^{2+} permeability (Matsuda et al. 2002; Nishi et al. 2001), although expression of the NR3 subunit is mainly limited to the developmental stage, as well as to somatic motor neurons of the brain stem and spinal cord during adulthood (Nishi et al. 2001).

The NMDA receptor requires binding of two agonists: one at the glycine-binding site on the NR1 subunit (Hirai et al. 1996; Kew et al. 2000; Kuryatov et al. 1994; Wafford et al. 1995), and one at the glutamate-binding site on the NR2 subunit (Anson et al. 1998; Laube et al. 1997). Electrophysiological studies demonstrate that NMDA receptor activation requires occupation of two independent

glycine sites and two independent glutamate sites (Benveniste and Mayer 1991; Clements and Westbrook 1991), suggesting that a functional NMDA receptor consists of two NR1 subunits and two NR2 subunits. In receptors containing an NR3 subunit, it has been speculated that an NR3 subunit substitutes for one of the NR2 subunits (Kew and Kemp 2005).

14.2.9.4 Kainate Receptors

The kainate receptor is a tetrameric structure that is composed of two subunit families, GluR5-7 and KA1-2. The GluR subunits, but not the KA subunits, can form a homomeric structure. When the KA subunits are included in the heteromeric kainate receptor, they become a high-affinity binding site (Kew and Kemp 2005). The kainate receptor is permeable to Na^+ and K^+, but also can be permeable to Ca^{2+} when expressing the unedited isoforms of the GluR5 or 6 subunits (Kohler et al. 1993). In the hippocampus, GluR6 appears to be an important subunit in both pre- and postsynaptic kainate receptors (Contractor et al. 2000). The GluR5 subunit is abundant in the temporal lobe and, in the amygdala, it is implicated in playing a role in epileptogenesis (Rogawski et al. 2001, 2003).

14.2.9.5 Metabotropic Receptors

The metabotropic glutamate receptors are categorized into three groups based on second messenger system coupling and pharmacological characteristics. The Group I mGluRs, which include mGluR1 and MGluR5 are Gq-coupled receptors; activation of these receptors leads to the stimulation of phospholipase C, which induces the production of diacylglycerol and inositol trisphosphate. Diacylglycerol activates protein kinase C, which leads to a downstream cascade of cellular events, and production of inositol trisphosphate leads to the release of Ca^{2+} from intracellular stores. Group I mGluRs are located primarily postsynaptically on neurons (Manahan-Vaughan 1997), as well as on astrocytes (Porter and McCarthy 1995, 1996); induced intracellular Ca^{2+} release is believed to lead to the release of glutamate from astrocytes, and induce excitatory postsynaptic potentials in neurons. Group II mGluRs include mGluR2 and mGluR3. They are coupled to Gi/o, which when activated, leads to the inhibition of adenylyl cyclase. Group II mGluRs are typically located presynaptically, and activation of these receptors leads to inhibition of neurotransmitter release (Manahan-Vaughan 1997). Group III mGluR receptors include mGluR4, 6, 7, and 8. Much like the Group II receptors, the Group III mGluR receptors are located on neurons, are also coupled to Gi/o, and are also inhibitory in nature (Gereau and Conn 1995). Their categorization is also based on their pharmacological characteristics. As a whole, the metabotropic glutamate receptors have demonstrated a role in epilepsy. For

instance, the group I mGluR5 and group II mGluR 2/3 receptors are upregulated in MTLE (Das et al. 2012). Less is known of the role of Group I mGluR1 receptor and group III mGluR receptors in epilepsy.

14.3 Therapeutic Targeting of the Glutamate–Glutamine Cycle

14.3.1 Introduction

As discussed in the preceding sections and in other chapters of this volume, the glutamate–glutamine cycle is perturbed in a number of CNS disorders, justifying the need for therapeutic targeting of the different cycle component. However, such targeting will be challenging for several reasons. First, the glutamate–glutamine cycle is critical for normal brain physiology and for the physiology of several other organs such as the liver, kidney, and pancreas. Second, many of the enzymes, transporters, and metabolites of the glutamate–glutamine cycle are not unique to the cycle but are critically involved in other pathways of the body. Third, the blood–brain barrier will limit many therapeutic options. In the remainder of this chapter, we will discuss several approaches for possible therapeutic targeting. Emphasis will be placed on approaches that is already in clinical use or that can readily be translated to clinical use.

14.3.2 Glutamate Receptor Antagonists

The reader is referred to Table 14.1 for an overview of the most commonly used glutamate receptor blockers with effects on seizures and epilepsy. The full names of the receptor blockers are provided in *Abbreviations*.

14.3.2.1 AMPA Receptor Blockers

Competitive AMPA antagonists have poor selectivity for the AMPA receptor, for they also bind with a similar affinity to the kainate receptor. Somewhat selective competitive antagonists include CNQX, DNQX, NBQX, and PNQX. These antagonists have only modest selectivity for the AMPA-binding site over the glycine-binding site of the NMDA receptor (Kew and Kemp 2005). YM872 is the most selective competitive AMPA antagonist, which fully suppresses amygdala-kindled seizures (Hara et al. 2006). GYKI 52466 is a selective noncompetitive AMPA receptor antagonist that blocks intrahippocampal dendrotoxin K-induced seizures and hippocampal neuronal cell loss. GYKI 53773 is a selective AMPA antagonist that is protective against status epilepticus in a kainic acid-induced neonatal status epilepticus model (Dhir and Chavda 2016).

Table 14.1 Glutamate receptor blockers with effects on seizures

Mechanism of action	Antagonist	Seizure model exhibiting anticonvulsant effect of antagonist	Citation
AMPA receptor antagonist: competitive binding to the glutamate site	YM872	Amygdala kindling	Hara et al. (2006)
AMPA receptor antagonist: noncompetitive binding to the glutamate site	GYKI 52466	Dendrotoxin K	Bagetta et al. (1996)
NMDA receptor antagonist: competitive binding to the glutamate site	D-AP5	Sound	Chapman et al. (1991), Gaoni et al. (1994)
		Strychnine	McAllister (1992)
	CGS 19755	Sound	Chapman et al. (1991)
		Maximal electroshock	Ferkany et al. (1993)
		Hippocampal kindling	Katsumori et al. (1998)
		Lidocaine	McFarlane et al. (1994)
	CGP 37849 & CGP 39551	Sound	Chapman et al. (1991)
		Maximal electroshock	Fagg et al. (1990)
		Light	Chapman et al. (1991)
	NPC 17742	Maximal electroshock	Ferkany et al. (1993)
		Pentylenetetrazole	Ferkany et al. (1993)
	LY 274614 & GYKI 52466	Dendrotoxin K	Bagetta et al. (1996)
	SDZ EAB-515	Maximal electroshock	Urwyler et al. (1996)

NMDA channel blocker	MK-801	Maximal electroshock	Barton et al. (2003), Kleinrok et al. (1995)
		Imipenem	De Sarro et al. (1995)
		Sound	Gill et al. (2002)
		Pentylenetetrazole	Jiang et al. (2004), Lukomskaya et al. (2004)
		Strychnine	McAllister (1992)
		D,L-homocysteine thiolactone	Rasic-Markovic et al. (2011)
		Tetramethylenedisulfotetramine	Shakarjian et al. (2015)
		Dimethoxy-4-ethyl-beta-carboline-3-carboxylate	Tsuda et al. (1997)
		Hippocampal stimulation	Yen et al. (2004)
	Memantine	Pentylenetetrazole	Lukomskaya et al. (2004), Mares and Mikulecka (2009)
		Kainic acid	Serdyuk et al. (2014)
NMDA receptor antagonist: binding to NR2B subunit	Conantokin-R	Sound	White et al. (2000)
		Maximal electroshock	White et al. (2000)
		Pentylenetetrazole	White et al. (2000)
	Traxoprodil	Pentylenetetrazole	Naspolini et al. (2012)
	Ro 63-1908	Sound	Gill et al. (2002)
	Ifenprodil	Pentylenetetrazole	Mares and Mikulecka (2009)
		Strychnine	McAllister (1992)
		Maximal electroshock	Kleinrok et al. (1995), Zarnowski et al. (1994)
		Hippocampal stimulation	Yen et al. (2004)
Kainate receptor antagonist: competitive binding with high affinity for the GluR5 subunit	LY 382884	Maximal electroshock	Barton et al. (2003)
	UBP302	Soman	Apland et al. (2014)

(continued)

Table 14.1 (continued)

Mechanism of action	Antagonist	Seizure model exhibiting anticonvulsant effect of antagonist	Citation
mGluR1 receptor antagonist	AIDA	Acoustic stimulation	Bashkatova et al. (2015), Chapman et al. (1999)
		Pentylenetetrazole	Mares et al. (2010), Thomsen and Dalby (1998)
		Pentetrazole	Watanabe et al. (2010, 2011)
mGluR5 receptor antagonist	MPEP	Sound	Chapman et al. (2000), Bashkatova et al. (2015)
		Maximal electroshock	Barton et al. (2003)
		Pentylenetetrazole	Mares et al. (2010)

14.3.2.2 NMDA Receptor Blockers

NMDA receptor blockers that are competitive antagonists at the glutamate recognition site, only show modest receptor subtype selectivity, but generally show anticonvulsant effects. For example, D-AP5 blocks sound-induced seizures in mice (Chapman et al. 1991; Gaoni et al. 1994) and spinal seizures induced by strychnine (McAllister 1992). CGS 19755, another competitive NMDA antagonist demonstrates anticonvulsant effect against sound-induced seizures in mice (Chapman et al. 1991) and maximum electroshock-induced convulsions in rats (Ferkany et al. 1993). In addition, CGS 19755 reduces the seizures in the partially kindled hippocampal model for epileptic seizures (Katsumori et al. 1998) and increases the dose necessary for lidocaine to induce seizures in rats (McFarlane et al. 1994). CGP 37849 and CGP 39551 block convulsions in the sound-induced (Chapman et al. 1991) and maximal electroshock-induced models of seizures in mice (Fagg et al. 1990). They also reduce photically induced myoclonus in baboons (Chapman et al. 1991). NPC 17742 antagonizes maximal electroshock and pentylenetetrazole-induced seizures (Ferkany et al. 1993). LY 274614 and GYKI 52466 block seizures and protect against hippocampal neuronal loss caused by dendrotoxin K (Bagetta et al. 1996). SDZ EAB-515 blocks maximal electroshock-induced seizures (Urwyler et al. 1996).

NMDA ion channel blockers are among the most commonly used glutamate receptor blockers and include MK-801 (dizocilpine), memantine, and ketamine. MK-801, which is widely used in research, has strong anticonvulsive effects in response to maximal electroshock treatment (Barton et al. 2003; Kleinrok et al. 1995), imipenem (De Sarro et al. 1995), sound (Gill et al. 2002), pentylenetetrazole (Jiang et al. 2004; Lukomskaya et al. 2004), strychnine (McAllister 1992), D,L-homocysteine thiolactone (Rasic-Markovic et al. 2011), tetramethylenedisulfotetramine (Shakarjian et al. 2015), dimethoxy-4-ethyl-beta-carboline-3-carboxylate (Tsuda et al. 1997), and hippocampal stimulation (Yen et al. 2004). Memantine reduces seizures in the pentylenetetrazole (Lukomskaya et al. 2004; Mares and Mikulecka 2009) and kainic acid models (Serdyuk et al. 2014). Finally, ketamine is used in humans to stop refractory status epilepticus (for reviews, see (Fang and Wang 2015; Zeiler 2015)).

With respect to pharmacological agents that show subunit selectivity, NVP-AAM077 shows high selectivity for NR1/NR2A, rather than NR1/NR2B (Chaperon et al. 2003). With respect to the NR2C and NR2D subunits, PPDA is the most potent and selective NR2C/NR2D-preferring antagonist yet reported (Feng et al. 2004). To our knowledge, little data has been collected to determine the effects of these NR1/NR2A, NR2C, and NR2D subunit selective antagonists on seizures. Numerous pharmacological agents that antagonize the NR2B subunit have been tested with respect to their anticonvulsant activity. For example, the NR2B antagonist, conantokin-R (Con-R), prevents sound-induced tonic extension seizures in mice and seizures induced by maximal stimulation, and blocks seizures induced by pentylenetetrazole (White et al. 2000). The highly selective NR2B antagonist, traxoprodil has demonstrated an ability to decrease pentylenetetrazole-induced seizures (Naspolini et al. 2012). The NR2B selective antagonist, Ro 63-1908, is an active inhibitor of sound-induced seizures in mice (Gill et al. 2002). While the earlier NR2B antagonists have

demonstrated consistent anticonvulsant effects in the studies tested, mixed results have been obtained with NR2B antagonist, ifenprodil. While ifenprodil has demonstrated anticonvulsant effects (Hrncic et al. 2009; Mares and Mikulecka 2009) (Kleinrok et al. 1995; McAllister 1992; Zarnowski et al. 1994) (Yen et al. 2004), some studies have failed to show such effects. For example, ifenprodil failed to antagonize electrical seizures (Burket et al. 2010) and failed to prevent imipenem-induced convulsions in some studies (De Sarro et al. 1995). In addition, ifenprodil did not block DMCM-induced seizures in mice (Tsuda et al. 1997). In certain instances, ifenprodil had a proconvulsant effect. For example, ifenprodil decreased the latency to the first seizure onset and increased the median number of seizure episodes induced by homocysteine thiolactone (Rasic-Markovic et al. 2011). In addition, at low doses, ifenprodil enhanced amygdala kindling acquisition, and at high doses, blocked amygdala kindling acquisition (Yourick et al. 1999). The results from these studies suggest that the role of the NR2B receptor subunit in anticonvulsant behavior may be mediated in some aspects by the level of activity at the site of ligand binding.

14.3.2.3 Kainate Receptor Blockers

Competitive kainate receptor antagonists show poor selectivity between AMPA receptors and kainate receptors. LY 382884 is a selective kainate receptor antagonist that exhibits high affinity binding at the GluR5 subunit but not at the GluR6 or AMPA-specific subunits. The antagonist protects against maximal electroshock-induced seizures (Barton et al. 2003). UBP-302, another selective GluR5 kainate receptor antagonist reduces the duration of soman-induced status epilepticus and protects against neuronal loss in the hippocampus and amygdala in this model (Apland et al. 2014).

14.3.2.4 Metabotropic Receptor Blockers

AIDA is a group I mGluR antagonist that is selective for mGluR1 versus mGluR5 (Kew and Kemp 2005). AIDA suppresses sound-induced clonic seizures in mice and rats (Bashkatova et al. 2015; Chapman et al. 1999), as well as seizures induced by pentylenetetrazole (Mares et al. 2010; Thomsen and Dalby 1998) SIB-1893 and MPEP are group I mGluR antagonists with selectivity for mGluR5 over mGluR1. MPEP inhibits sound-induced seizures in mice (Chapman et al. 2000), and seizures induced by maximal electroshock treatment (Barton et al. 2003) and by pentylenetetrazole (Mares et al. 2010).

With respect to the group II mGluRs, LY341495 exhibits nanomolar affinity at both mGluR2 and mGluR3 (Kingston et al. 1998). While selective for group II receptors, LY341495 also exhibits some activity at the other group III mGluRs as well as on the group I mGluRs (Kingston et al. 1998). Little is known on the effect of group II mGluR antagonists on seizures. MSOP is a selective group III mGluR antagonist, which is a proconvulsant in mice (Chapman et al. 2001).

14.3.2.5 Use of Glutamate Receptor Blockers in Humans

In recent years, there has been great interest in studying the effects of glutamate receptor antagonists on seizures and brain pathology damage after an acute brain insult (Ikonomidou and Turski 2002). NMDA receptor antagonists have demonstrated neuroprotective effects in animal models of stroke and traumatic brain injury (Lee et al. 1999; McCulloch 1992); however, clinical trials were stopped early because they either had no therapeutic effect in humans or worsened outcomes (Ikonomidou and Turski 2002; Muir 2006). The deleterious effects of NMDA receptor antagonism were thought to be due to interference with normal glutamate signaling, neuronal integrity, and neuronal repair after injury (Ikonomidou and Turski 2002; Zlotnik et al. 2009). In treating epilepsy, however, anticonvulsant effects have been effectively demonstrated with several types of glutamate receptor antagonists. Antagonists of group I mGluRs have demonstrated anticonvulsant and anxiolytic effects in both developing and adult rodents (Mares et al. 2010). Ketamine has been shown to effectively and safely control drug-resistant status epilepticus in both children and adults (Fang and Wang 2015). Furthermore, the anticonvulsant effects of decanoic acid, which increases in the plasma during the ketogenic diet, are though to result from noncompetitive AMPA receptor antagonism (Chang et al. 2016).

14.3.3 Glutamate Scavenging

14.3.3.1 Metabolic Scavenging

Many studies have argued that reducing the extracellular brain glutamate excess in epilepsy may effectively reduce seizure activity and prevent seizure-induced neuronal loss (Eid et al. 2008a, c; Lee et al. 2007). The brain has several mechanisms by which glutamate is removed from the extracellular compartment. In addition to the EAATs located on astrocytes and neurons (see Sect. 14.2.6 and Figs. 14.1 and 14.2), sodium-dependent glutamate transporters present on the antiluminal side of brain capillaries provide another mechanism of reducing excess glutamate from the extracellular space (Fig. 14.2) (O'Kane et al. 1999). In areas of the brain where extracellular glutamate is pathologically increased, glutamate is rapidly transported into the blood, down its concentration gradient via facilitated diffusion (Marliss et al. 1971). Several studies have shown that this brain-to-blood glutamate efflux is a physiological process, which may be an important mechanism by which excess glutamate is removed from pathological areas of the brain (Gottlieb et al. 2003; Hosoya et al. 1999).

Studies have shown that increasing the concentration gradient of glutamate between the brain and the blood can effectively increase the rate of brain-to-blood glutamate efflux (Gottlieb et al. 2003). Thus, decreasing blood glutamate levels is a mechanism by which extracellular brain glutamate may be reduced. The increased gradient has

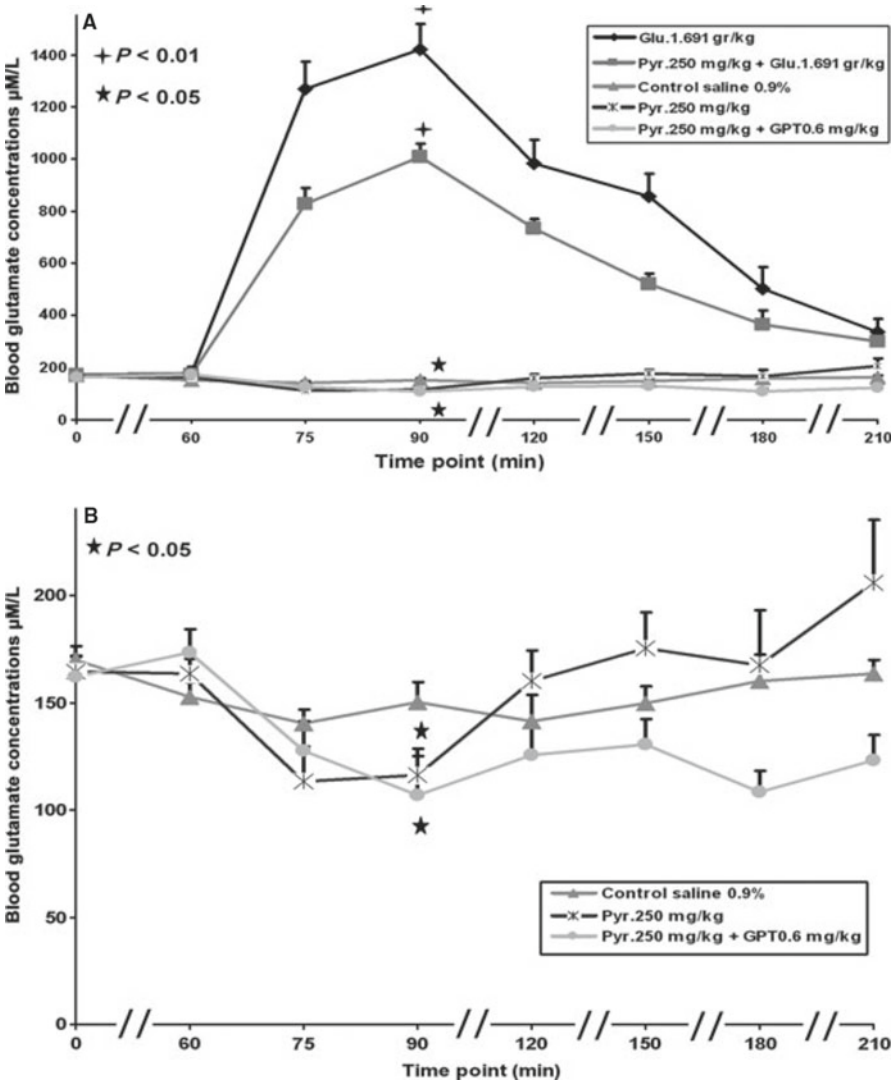

Fig 14.3 Administration of blood glutamate scavengers decrease blood glutamate concentrations over time in a rat model of stroke. Rats administered pyruvate or pyruvate + GPT demonstrate significantly lower blood glutamate concentrations compared with rats administered saline (control) or glutamate. B) Expanded scale of the lower part of A. (*$p < 0.05$, + $p < 0.01$). Adapted from Boyko et al. Boyko et al. (2013). See text for details

been experimentally demonstrated by peripheral administration of oxaloacetate and pyruvate, which are substrates for aspartate aminotransferase (AST) and alanine aminotransferase (ALT), respectively, converting glutamate to α-ketoglutarate (Fig. 14.3).

Animal studies of various CNS insult paradigms have demonstrated the neuroprotective effects of blood glutamate scavenging, with the strongest evidence in

models of traumatic brain injury and stroke (Boyko et al. 2014; Leibowitz et al. 2012). Peripheral administration of blood glutamate scavengers after traumatic brain injury reduced blood glutamate concentrations, improved neurological outcomes, and reduced hippocampal neuronal loss (Zlotnik et al. 2007, 2008, 2009, 2012) (Fig. 14.4). After an ischemic stroke, peripheral administration of blood glutamate scavengers decreased the concentration of brain and blood glutamate, reduced the infarct size and cerebral edema, improved neurological outcomes, and reduced the mortality rate (Boyko et al. 2011; Campos et al. 2011; Nagy et al. 2009). In a rodent model of subarachnoid hemorrhage, administration of blood glutamate scavengers reduced blood and brain glutamate, decreased blood–brain barrier disruption, reduced cerebral edema, and improved the neurological outcome (Boyko et al. 2012). Blood glutamate scavengers may also be neuroprotective in animal models of glioma (Ruban et al. 2012) and in migraine headaches (Campos et al. 2013).

It is possible that blood glutamate scavenging may be an effective therapeutic approach to treat epilepsy. In one study, rats were treated with a single systemic injection of oxaloacetate and pyruvate 30 min after induction of status epilepticus with pilocarpine (Carvalho et al. 2011). Despite showing no reduction in seizures, the study demonstrated prevention of seizure-induced neuronal loss in CA1 of the hippocampus. Furthermore, administration of glutamate scavengers in animal models of organophosphate intoxication resulted in rapid reduction of brain glutamate levels and neuroprotection, but failed to demonstrate a reduction in seizure activity (Ruban et al. 2013, 2015).

Because the rate of brain-to-blood glutamate efflux is dependent on a concentration gradient between the brain and blood, glutamate scavengers are thought to reduce elevated glutamate concentrations preferentially in areas of pathological glutamate excess, with minimal effects on physiological glutamate concentrations (Gottlieb et al. 2003). Furthermore, the rate of efflux from the brain to blood slows as the concentration in the brain approaches that in the blood. Hence, blood glutamate scavengers do not decrease brain concentrations below physiological concentrations, and unlike NMDA receptor antagonists, do not appear to interfere with normal synaptic transmission (Zlotnik et al. 2009).

Despite promising studies in animal models, metabolic glutamate scavenging has limitations. First, the neuroprotective effects of glutamate scavengers are evident only when administered within a short therapeutic window (Boyko et al. 2011; Zlotnik et al. 2007, 2008). The short therapeutic window may limit the utility of scavengers in the treatment of epilepsy, where seizures and brain glutamate surges occur unpredictably (During and Spencer 1993). Second, while the administration of blood glutamate scavengers within 1 h of experimentally-induced traumatic brain injury reduces hippocampal neuronal loss (Zlotnik et al. 2012), it is less clear what effect blood glutamate scavengers would have on hippocampal pathology if administered at later time points. Third, despite reducing brain glutamate levels and protecting against neuronal loss in epilepsy models, blood glutamate scavengers have not demonstrated antiepileptic effects (Carvalho et al. 2011). Therefore, blood glutamate scavengers may be more effective in reducing the secondary brain damage associated with pathologically increased brain glutamate and less effective in treating seizures.

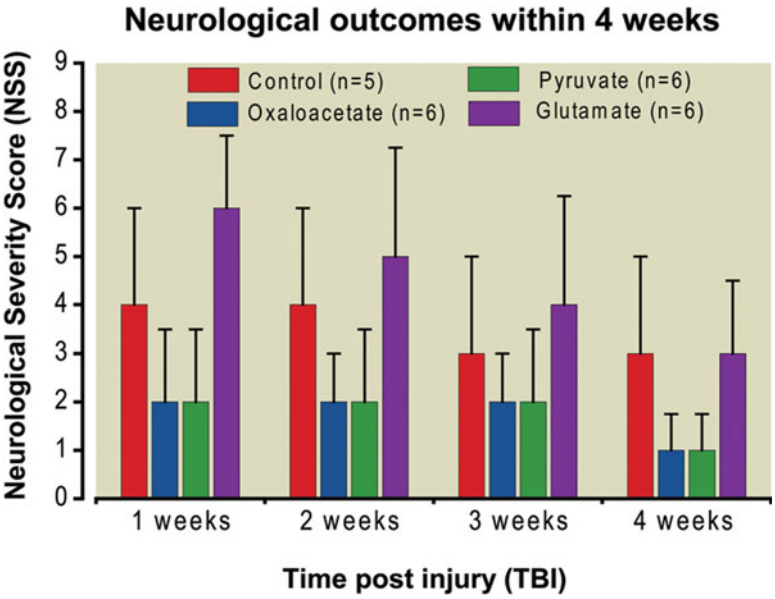

Fig 14.4 Administration of blood glutamate scavengers results in improved neurological outcomes and neuroprotection. (**a**) Administration of the blood glutamate scavengers oxaloacetate and pyruvate results in improved neurological severity score 1–4 weeks after traumatic brain injury (TBI). Adapted from Zlotnik et al. (Zlotnik et al. 2012)

Fourth, whether systemic administration of pyruvate and oxaloacetate at doses needed for brain glutamate scavenging can be safely performed in humans has not yet been established (Boyko et al. 2014).

14.3.3.2 Extracorporeal Methods

The use of extracorporeal methods to scavenge blood glutamate and thereby reduce glutamate-mediated excitotoxicity has gained increased attention in recent years (Zhumadilov et al. 2015). These methods can eliminate pathologically elevated glutamate from the blood, and like glutamate scavengers, can increase the brain-to-blood glutamate efflux. Although the effects of extracorporeal glutamate scavenging has not yet been studied in a setting of epilepsy, the blood glutamate-reducing effects of hemodialysis, peritoneal dialysis, and hemofiltration have been extensively studied in humans and animals (Boyko et al. 2014; Hu et al. 2014; Rogachev et al. 2012, 2013). These methods may be useful in the treatment of refractory epilepsies, particularly refractory status epilepticus.

In conditions where glutamate is pathologically elevated in the brain and blood, studies have shown that dialyzing the blood can eliminate the excess glutamate. A recent study demonstrated that hemodialysis (Rogachev et al. 2012) and peritoneal dialysis (Godino Mdel et al. 2013; Rogachev et al. 2013) can effectively reduce blood

glutamate concentrations in patients with chronic kidney disease. Similarly, in a rat model of stroke, the use of peritoneal dialysis was associated with a reduction in blood glutamate levels and a decrease in the cortical infarct area (Godino Mdel et al. 2013).

Despite the effectiveness of lowering blood glutamate concentrations, the hemodialysis procedure requires that patients are anticoagulated. Moreover, hemodialysis is frequently complicated by hypotension. These issues limit the use of hemodialysis in unstable patients and patients with acute brain injury. In contrast, peritoneal dialysis does not require anticoagulation, nor does it cause hemodynamic instability. The insertion of a peritoneal dialysis catheter is safe, minimally invasive, and can be performed at the bedside in approximately 20 min.

Hemofiltration is an attractive alternative extracorporeal method of blood glutamate scavenging. Hemofiltration is unique in that it uses properties of convection to create a positive hydrostatic pressure across a filtering membrane to drive solutes from the blood to the filtrate compartment (Friedrich et al. 2012). Studies have demonstrated that hemofiltration is superior to hemodialysis in clearing medium and larger sized molecules, and may therefore be more effective than hemodialysis in clearing large inflammatory cytokines (Ronco et al. 2003). Hemofiltration requires minimal anticoagulation and results in less hemodynamic instability compared with hemodialysis. Furthermore, hemodialysis typically lasts only 4 h, whereas hemofiltration may be employed continuously for several days. Although there are currently no published reports that examine the effects of hemofiltration on blood glutamate levels and neurological outcomes, two clinical trials are currently underway (Zhumadilov et al. 2015).

In conclusion, extracorporeal methods of blood glutamate scavenging have several favorable properties compared with pharmacological blood glutamate scavengers (Zhumadilov et al. 2015). First, whereas pharmacological agents exploit reversible enzymatic processes, extracorporeal methods definitively remove glutamate from the blood. Second, the timing of pharmacological methods is limited by their pharmacokinetic properties, whereas extracorporeal methods can be employed over several hours or days after an acute brain injury. Third, pharmacological agents may have unknown side effects and actions outside their target area, whereas extracorporeal methods are widely used, reliable, and their peripheral effects are well established and predictable.

14.3.4 Ketogenic Diet

The ketogenic diet has long been known to have antiepileptic properties, and a recent Cochrane review concluded that the diet resulted in seizure control comparable to antiepileptic drugs in children with epilepsy (Levy et al. 2012). Interestingly, although the effects of the ketogenic diet have been known since the 1930s, the underlying mechanisms remain unclear, but may activation of adenosine triphosphate-sensitive potassium channels, inhibition of the mTOR pathway, and inhibition of glutamatergic neurotransmission (Danial et al. 2013).

Studies have demonstrated that acetoacetate and beta-hydroxybutyrate inhibit vesicular glutamate transporters, which function in loading glutamate in vesicles

at the glutamatergic neuronal synapse (Juge et al. 2010). The glutamatergic synaptic vesicles require chloride ions outside the vesicle and are dependent on the membrane potential. Both ketones block the transport of glutamate into the synaptic vesicles by altering the dependence of the transporters on chloride, thereby shifting the chloride dependence to higher concentrations. Of the two ketones, acetoacetate is approximately ten times more potent than beta-hydroxybutyrate. Moreover, acetoacetate inhibits glutamate release from cultured hippocampal neurons but not astrocytes and reduces the frequency of excitatory postsynaptic currents in CA1 pyramidal neurons. Acetoacetate reduced seizures in a rodent model of epilepsy and decreased glutamate release during the seizures. Studies have shown that the ketogenic diet does not enhance glutamate reuptake into presynaptic neurons (Bough et al. 2007). Moreover, while acetoacetate had no effect on modulating postsynaptic currents in GABAergic neurons, cerebrospinal fluid levels of GABA were elevated in children 4 months after starting the ketogenic diet (Dahlin et al. 2005).

14.3.5 Branched-Chain Amino Acids

It has been postulated that blood-derived branched-chain amino acids (i.e., leucine, isoleucine, and valine) account for 20 % of de novo synthesis of brain glutamate (Yudkoff 1997). The synthesis occurs via transfer of the branched-chain amino acids' amino group to α-ketoglutarate in the mitochondria of astrocytes, in a reaction catalyzed by branched-chain aminotransferase (Fig. 14.2). Branched-chain amino acids are abundant in the blood, particularly after a meal, and readily enter the brain via the large neutral amino acid transporter, thus providing an important mechanistic link between peripheral metabolism and brain glutamate homeostasis. However, the role of branched-chain amino acids on glutamate metabolism and seizure activity is poorly understood.

There is increasing evidence to suggest that the branched-chain amino acids are critical for several physiological functions in the brain, such as the synthesis of glutamate (and thus glutamine and GABA), intracellular signaling, immune modulation, and mitochondrial health (Blomstrand et al. 2006; De Simone et al. 2013; Lynch and Adams 2014; Rothman et al. 2012). However, there are conflicting data on the role of branched-chain amino acids in the pathophysiology of brain disorders such as epilepsy. While some studies have suggested that branched-chain amino acids may contribute to increased extracellular glutamate and neuronal apoptosis (Alvestad et al. 2011), other studies have demonstrated that branched-chain amino acids and their analogs increase seizure threshold in humans and animal models (Dhaher et al. 2014; Dufour et al. 1999). Importantly, the neuroprotective and anticonvulsant effects of branched-chain amino acids are thought to require glutamate metabolism to glutamine in astrocytes by glutamine synthetase, which would reduce intracellular and extracellular glutamate, increase glutamine clearance to the blood, and increase GABA production in inhibitory neurons (Yudkoff et al. 2004).

The reader is referred to the chapter by Hutson and XXX in this volume for a more comprehensive discussion of the role of branched-chain amino acids in the glutamate–glutamine cycle.

14.3.6 Hypothermia

Therapeutic hypothermia has been used since 1963 to treat a wide range of intracranial pathologies (Bennett et al. 2014) and preclinical studies have demonstrated that hypothermia is effective in improving neurological outcomes in animal models of traumatic brain injury, stroke, and cerebral ischemia (Erecinska et al. 2003; Lampe and Becker 2011). Therapeutic hypothermia reduces the seizure activity in animal models of epilepsy (Motamedi et al. 2006) and is also effective in treating several conditions in humans including refractory status epilepticus (Bennett et al. 2014; Zeiler et al. 2015), hypoxic–ischemic encephalopathy (Azzopardi et al. 2014; Boylan et al. 2015; Orbach et al. 2014), and acute encephalitis and encephalopathy (Imataka et al. 2014).

A recent systematic review identified 13 studies (10 manuscripts and 3 meeting abstracts) in which therapeutic hypothermia was used to treat 40 patients with refractory status epilepticus (Zeiler et al. 2015). Patients were externally cooled to a core temperature of 33 °C, and hypothermia was maintained for a median of 48 h. The studies demonstrated a 15 % reduction in seizures with 62.5 % of patients experiencing complete seizure cessation. About 20 % did not respond to treatment. Therapeutic hypothermia in pediatric epilepsies has resulted in decreased seizure burden during and after refractory status epilepticus and has been suggested to prevent refractory status epilepticus relapse (Guilliams et al. 2013).

Hypoxic–ischemic brain injury is a common cause of seizures in the neonatal period. A recent systematic review examined the evidence from 11 randomized controlled trials, and concluded that therapeutic hypothermia reduces mortality and neurodevelopmental disability in survivors with hypoxic–ischemic encephalopathy (Jacobs et al. 2013). Several recent studies have showed that therapeutic hypothermia may be effective in reducing seizure activity in neonates with hypoxic–ischemic encephalopathy, resulting in a lower seizure burden and shorter seizure duration (Azzopardi et al. 2014). One study observed that cooled neonates with moderate encephalopathy had fewer than half the incidence of seizures compared with neonates that were not cooled, whereas cooling had no effect in neonates with severe encephalopathy (Orbach et al. 2014). Despite a reduction in seizure activity, some studies suggest that therapeutic hypothermia may not reduce the overall need for antiepileptic drugs or duration of treatment (Low et al. 2012).

Several mechanisms have been proposed to explain the antiseizure and neuroprotective effects of therapeutic hypothermia (Motamedi et al. 2013). Hypothermia is known to alter the potassium ion exchange via the sodium–potassium pump, thereby inhibiting the propagation of action potentials (Volgushev et al. 2000). Hypothermia also reduces the cerebral metabolic rate, the intracranial pressure, the release of inflammatory mediators in the brain, and the extent of reperfusion injury (Kentner et al. 2002; Lampe

and Becker 2011). Recent studies have suggested that the effects of hyperthermia on seizures and neuronal viability may be glutamate mediated. Hypothermia is thought to reduce glutamate-mediated excitotoxicity by altering the glutamate-binding affinity and synaptic release of glutamate, thereby reducing neurotransmission and epileptic activity (Boucher et al. 2010; Yang et al. 2005). In humans and animal models of stroke and traumatic brain injury, therapeutic hypothermia reduced the glutamate concentration in the extracellular fluid of the brain (Berger et al. 2002, 2004; Winfree et al. 1996).

Boyko demonstrated a strong correlation between body temperature and blood glutamate levels in naïve rats (Boyko et al. 2013). Induction of mild and moderate hypothermia resulted in decreased blood glutamate concentrations (Fig. 14.5) and increased AST concentrations. These findings suggest that the hypothermia-mediated improvement in neurological outcomes might be due to a blood glutamate scavenging mechanism, by creating a more favorable concentration gradient between the brain and blood and increasing the brain to blood glutamate efflux.

Deep hypothermia (20 °C) lasting 30 min in rats terminates refractory status epilepticus within 12 min, reduces seizure activity upon rewarming, and protects against status epilepticus-induced neuronal loss in most animals (Niquet et al. 2015). However, deep hypothermia can also have deleterious effects, as evidenced by increased blood glutamate concentrations, increased extracellular glutamate in cultured astrocytes, overstimulation of glutamate receptors, and worsened neurological outcomes (Bissonnette et al. 1999; Boyko et al. 2013). The worsened neurological outcomes have been attributed to reductions in cerebral blood flow and metabolic rate (Ehrlich et al. 2002). Some authors have postulated that deep hypothermia may cause hepatic and skeletal muscle damage, resulting in increased cellular permeability and increases in blood glutamate concentrations (Blair et al. 1961). This postulate is supported by the observation that rats and rabbits subjected to hypothermia have significantly increased creatinine kinase, lactate dehydrogenase, lipase, and amylase (Altland et al. 1974; Arakawa et al. 1997).

While therapeutic hypothermia is becoming a standard treatment for hypoxia-induced encephalopathy in infants, its use in refractory status epilepticus is increasing. Despite strong preclinical evidence and some clinical evidence, more prospective clinical trials are warranted to examine the effects of therapeutic hypothermia on seizure activity. A major factor that has limited the use of therapeutic hypothermia to treat seizures and other intracranial pathologies has been its risk of complications, including venous thrombosis, infection, cardiac arrhythmias, electrolyte disturbances, and coagulopathy (Zeiler et al. 2015). In neonates with hypoxia-induced encephalopathy, common complications include sinus bradycardia and thrombocytopenia (Jacobs et al. 2013).

14.4 Concluding Remarks

The glutamate–glutamine cycle is perturbed in epilepsy, and manipulations of the different cycle components can stop seizures and prevent seizure-induced neuronal loss. Therapeutic targeting of the cycle in humans is presently limited to a few approaches, such as administration of ketamine, GABA-receptor antagonists (e.g.,

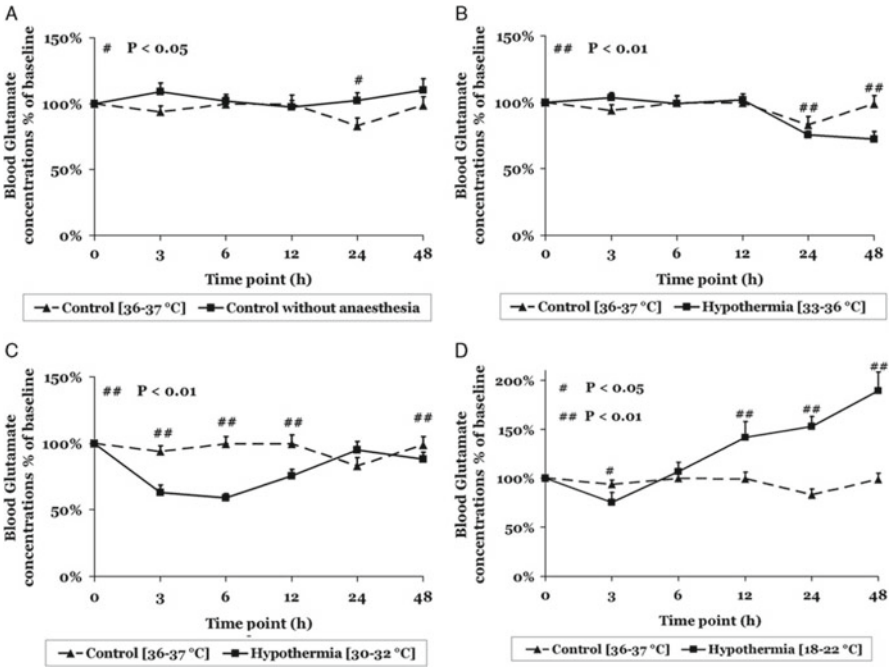

Fig 14.5 Blood glutamate concentration decreases after hypothermia. (**a**) Glutamate is reduced in the control group at 24 h ($p < 0.05$). (**b**) Mild hypothermia (33–36 °C) results in decreased glutamate levels at 24 and 48 h after initiation of cooling ($p < 0.01$). (**c**) Moderate hypothermia (30–32 °C) results in decreased glutamate levels by 3 h after initiation of cooling ($p < 0.01$). The glutamate effect is sustained for 12 h. (**d**) Deep hypothermia results in a sustained increase of blood glutamate from 12 to 48 h ($p < 0.01$). Adapted from Boyko et al. (2013)

barbiturates and benzodiazepines), hypothermia, blood glutamate scavengers, and dialysis/hemoperfusion. However, all of these approaches have significant shortcomings such as: (a) markedly affecting normal brain physiology (e.g., ketamine, GABA-receptor antagonists), (b) not being suitable for chronic use or for use outside a health care facility (e.g., blood glutamate scavengers, hemodialysis, hemoperfusion, hypothermia), and (c) exhibiting poor specificity toward the glutamate–glutamine cycle, thereby increasing the risk of adverse effects (e.g., dialysis/hemoperfusion, hypothermia). The lack of effective and specific therapies is largely due to several critical gaps in our understanding of the glutamate–glutamine cycle in health and disease. Areas of markedly deficient knowledge include glutamine transport mechanisms, regulation of key metabolic enzymes, the role of the cycle in cell signaling and differentiation, and the role of peripheral metabolism in the cycle homeostasis. Further studies are required to resolve these issues and to reveal additional therapeutic targets for glutamate–glutamine cycle-related CNS disorders, such as epilepsy, traumatic brain injury, stroke, and hepatic encephalopathy.

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About the Editors

Arne Schousboe obtained his M.Sc. in biochemistry from the University of Copenhagen in 1968 at a time where an M.Sc. was considered equivalent to a Ph.D. He subsequently (1978) earned his Doctor of Science (D.Sc.) degree also from the University of Copenhagen. After a postdoc period with Dr. Eugene Roberts at the Department of Neuroscience, City of Hope National Medical Center in Los Angeles 1972–1973, he came back to the University of Copenhagen to resume a tenured position as Associate Professor at the Medical Faculty. In 1990 he moved to the Royal Danish School of Pharmacy, Copenhagen, as a Full Professor of Biochemistry and has remained in this position also when this Institution changed to become an independent University and subsequently a Faculty of Pharmaceutical Sciences, University of Copenhagen, which lately was incorporated in the Faculty of Health and Medical Sciences still at the University of Copenhagen. He has served as the Department Chair from 2005 to 2010 and is now part-time Professor of Neuropharmacology in the Department of Drug Design and Pharmacology at the University of Copenhagen. He has been working on astrocyte function focusing on amino acid neurotransmission during the past more than 40 years and is currently

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engaged in studies of glutamate and GABA homeostasis and metabolism. He has published over 550 papers on these and related topics. He has served on the Editorial Board on numerous neuroscience journals over the years and is currently the Editor-in-Chief of Neurochemical Research and Editor of the book series Advances in Neurobiology.

Ursula Sonnewald received her Ph.D. in Chemistry at the University of Saskatchewan, Saskatoon (Canada), and subsequent to studies in Hamburg (Germany) and Canada; postdoctoral periods were spent in Salt Lake City, University of Utah, USA, in Copenhagen at the Carlsberg Research Center, Denmark, and at The Brookhaven National Laboratory in Upton, New York, USA. For 5 years she worked in the CNS Department of Novo Nordisk developing antiepileptic drugs. During this period she synthesized the GABA uptake inhibitor Tiagabine, which is on the market as an antiepileptic drug and as a tool for basic research on GABA function and metabolism. In 1990 she moved to Norway and was amongst the first in the area of metabolic studies of brain cell metabolism using magnetic resonance spectroscopy (MRS) in collaboration with the experts (Drs. Arne Schousboe and Niels Westergaard) on nervous cell cultures. Since 1996 she has been a full Professor in the Department of Neuroscience at the Norwegian University of Science and Technology. She applied MRS to animal models of epilepsy, stroke, schizophrenia, Alzheimer's, and other neurological disorders. Exciting new results have been obtained pertinent to the area of neurodegeneration. She has developed tools to analyze mitochondrial function and has applied those to study dysfunction in the aforementioned diseases.

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