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The Role of Glutamine Synthetase and Glutamate Dehydrogenase in Cerebral Ammonia Homeostasis

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Abstract

In the brain, glutamine synthetase (GS), which is located predominantly in astrocytes, is largely responsible for the removal of both blood-derived and metabolically generated ammonia. Thus, studies with [^{13}N]ammonia have shown that about 25% of blood-derived ammonia is removed in a single pass through the rat brain and that this ammonia is incorporated primarily into glutamine (amide) in astrocytes. Major pathways for cerebral ammonia generation include the glutaminase reaction and the glutamate dehydrogenase (GDH) reaction. The equilibrium position of the GDH-catalyzed reaction *in vitro* favors reductive amination of α -ketoglutarate at pH 7.4. Nevertheless, only a small amount of label derived from [^{13}N]ammonia in rat brain is incorporated into glutamate and the α -amine of glutamine *in vivo*. Most likely the cerebral GDH reaction is drawn normally in the direction of glutamate oxidation (ammonia production) by rapid removal of ammonia as glutamine. Linkage of glutamate/ α -ketoglutarate-utilizing aminotransferases with the GDH reaction channels excess amino acid nitrogen toward ammonia for glutamine synthesis. At high ammonia levels and/or when GS is inhibited the GDH reaction coupled with glutamate/ α -ketoglutarate-linked aminotransferases may, however, promote the flow of ammonia nitrogen toward synthesis of amino acids. Preliminary evidence suggests an important role for the purine nucleotide cycle (PNC) as an additional source of ammonia in neurons (Net reaction: L-Aspartate + GTP + H₂O \rightarrow Fumarate + GDP + P_i + NH₃) and in the beat cycle of ependyma cilia. The link of the PNC to aminotransferases and GDH/GS and its role in cerebral nitrogen metabolism under both normal and pathological (e.g. hyperammonemic encephalopathy) conditions should be a productive area for future research.

Keywords

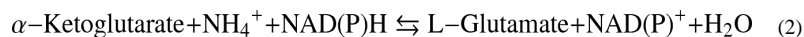
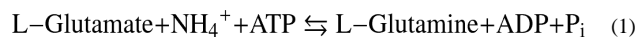
Ammonia; Aspartate aminotransferase; Glutamate dehydrogenase; Glutamine cycle; Glutamine synthetase; Purine nucleotide cycle

Introduction

I am honored to contribute an article to the special volume dedicated to Professor Leif Hertz. I have known Leif for over 25 years and we have collaborated on several occasions in the past. Leif is an expert neurochemist and an inspiration for younger neurochemists. In this article I will outline work that I began more than 30 years ago with a group of biophysicists at Memorial Sloan Kettering Cancer Center (MSKCC), New York. The results from this work are still highly relevant and interdigitate with recent concepts of nitrogen metabolism in the brain – an area to which Leif has contributed considerably.

Historical – Discovery of compartmentation of ammonia metabolism in the brain

In a classic series of experiments carried out more than half a century ago, Berl and colleagues showed that cerebral ammonia metabolism is compartmented [1]. [¹⁵N]Ammonia was continuously infused into anesthetized cats via a carotid artery. After 25 minutes of infusion, the cats were sacrificed and the ¹⁵N content of selected brain metabolites was determined. (Nitrogen in the biosphere consists of ~99.63% ¹⁴N and ~0.37% ¹⁵N.) The relative enrichment above background of ¹⁵N in these metabolites was found to be in the order: amide group of L-glutamine > amine group of L-glutamine > L-glutamate [1]. The only known route for synthesis of glutamine in mammals is via the GS reaction (Eq. 1). Moreover, at least a portion of the amine moiety of the glutamate needed for glutamine synthesis must have been derived from reductive amination of α-ketoglutarate catalyzed by GDH (Eq. 2). Thus, if the brain had acted as a single metabolic compartment, a simple product-precursor relationship dictates that the relative enrichment of ¹⁵N in the amine group of glutamine cannot exceed that of glutamate. Because ¹⁵N enrichment was greater in the amine group of glutamine than in glutamate Berl et al. proposed that ammonia entering the brain from the blood is rapidly incorporated into glutamine in a small pool of rapidly turning over glutamate (small compartment) [1]. This pool is kinetically distinct from a much larger, more slowly turning over pool of glutamate (large compartment) [1]. The authors estimated that the turnover of glutamate in the large compartment of the cat brain is of the order of hours, but less than 1 hour in the small compartment. Metabolic compartmentation of glutamate metabolism in liver could not be discerned in the experiments of Berl et al. [1]. However, as an interesting aside, it is now well established that ammonia/glutamate metabolism in the liver is indeed strongly compartmented. For example, GS in the liver is confined to a small ring of perivenous hepatocytes (zone 1 of the acinus), whereas glutaminase and urea cycle enzymes are strongly represented in perivenous hepatocytes (zone 3 of the acinus) [e.g. 2,3].



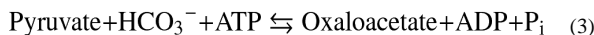
The background level of ¹⁵N in ambient nitrogen and relative insensitivity of the analytical techniques available in the late 1950s and early 1960s for isotope measurement necessitated the infusion of massive amounts of ammonia into the cats in the experiments of Berl et al. [1] in order to obtain measurable enrichments of ¹⁵N in glutamine and glutamate. This non-physiological treatment resulted in large increases in brain ammonia and glutamine, such that the animals were comatose at time of sacrifice [1]. Nevertheless, the work of Berl et al. [1] contributed substantially to our understanding of metabolic compartmentation in the brain.

Despite the large increase in brain glutamine after the infusion of [¹⁵N]ammonia there was no measurable change in the concentration of glutamate in the cat brain [1]. Part of the reason for the inability to detect a change in brain glutamate concentration in this experiment may have been due to the conversion of glutamate to glutamine in the small compartment. Changes in glutamate concentration in this compartment may have been

¹Ammonia free base (NH₃) has a pK_a of ~9.2. Thus, under normal intracellular physiological conditions (pH 7.2 – 7.4) ammonia exists predominantly (~99%) as the conjugate acid, ammonium (NH₄⁺). For convenience, unless otherwise stated, the term ammonia is used throughout the text to indicate the sum of NH₃ plus NH₄⁺.

obscured by the much larger, more slowly turning glutamate pool in the large compartment. In an accompanying publication, Berl et al. showed that the brain possesses a remarkable capacity to fix CO₂, which is apparently stimulated by elevated ammonia levels [4]. The ability of brain to anaplerotically fix substantial amounts of CO₂ (as bicarbonate) is now known to be primarily catalyzed by pyruvate carboxylase (Eq. 3), an enzyme predominantly localized to astrocytes in brain [5–10]. The findings of Berl et al. [4] have been amply verified by modern nuclear magnetic resonance (NMR) techniques. For example, Sibson et al. [11] used ¹³C-NMR to study cerebral metabolic compartmentation in rats following infusion of [2-¹³C]glucose or [5-¹³C]glucose. These authors noted that under normoammonemic conditions anaplerosis accounted for 19–26% of the carbon flux through the GS reaction, but increased to 32% during hyperammonemia [11]. In a more recent study Duarte et al [12] used ¹³C-NMR techniques to trace the cerebral metabolic fate of [1,6-¹³C]glucose in anesthetized rats. Glia were found to account for ~30% of total cerebral tricarboxylic acid (TCA) flux [12]. The anaplerotic flux through the pyruvate carboxylase reaction was estimated to be about 25% of the flux through the glial TCA cycle in these animals [12].

The brain possesses additional enzymes that are theoretically able to anaplerotically fix CO₂. These enzymes include the malic enzyme, phosphoenolpyruvate carboxykinase and propionyl carboxylase [13 and references cited therein]. However, the contribution of these enzymes to net CO₂ fixation under normoammonemic and hyperammonemic conditions in the brain remains uncertain and the malic enzyme and phosphoenolpyruvate carboxykinase may be a net source of CO₂ in the brain, rather than a net means of removal [13]. Notwithstanding these caveats, the now well established ability of the brain to fix CO₂ contributes substantially to the ability of the brain to maintain relatively constant or only slightly depressed levels of glutamate during very large increased synthesis of glutamine.



Brain uptake index (BUI) of [¹³N]ammonia in the rat

Beginning in the late 1970s my colleagues and I used the MSKCC medical cyclotron to produce [¹³N]ammonia for metabolic studies. ¹³N is a positron-emitting isotope with a t_{1/2} of 9.96 min. Annihilation of a nearby electron by the emitted positron results in the production of two coincident, or almost coincident, 511 KeV γ rays. The major disadvantage of ¹³N is its short half life. This is not, however, a problem for short-term studies, because as I shall continuously emphasize throughout this review the turnover of ammonia and certain amino acids in our experimental animal of choice (the rat) is extremely rapid. Moreover, the γ rays emitted as a consequence of ¹³N decay are easily quantitated. Finally, very high specific activities of ¹³N are theoretically possible. For comparison, the theoretical maximal specific activity of carrier-free ¹³N is 4.2 × 10⁸ times greater than that of carrier-free ¹⁴C [14]. Ammonia is a ubiquitous contaminant, however. Nevertheless, we were able to generate [¹³N]ammonia of extremely high specific activity.

As noted above, infusion of large quantities of [¹⁵N]ammonia into cats led to a marked disturbance of cerebral nitrogen homeostasis. Because of this limitation we decided to investigate ammonia metabolism and compartmentation in brain using [¹³N]ammonia instead of [¹⁵N]ammonia. We also chose rats instead of cats as the experimental animal of choice. The [¹³N]ammonia generated in the MSKCC cyclotron was of very high specific activity, such that truly tracer quantities of labeled ammonia could be administered to the rats. (For some historical perspectives on the use of ¹³N as a tracer see references [14] and [15].)

In our first series of experiments we administered a bolus of [^{13}N]ammonia via one carotid artery and measured the BUI of [^{13}N]ammonia in awake, but restrained rats [16]. [The BUI is the single pass extraction by the brain of the test substance (expressed as a percentage) relative to a marker that is completely extracted by the brain.] The BUI for ammonia was found to be about 25% and independent of the concentration of added, unlabeled ammonia in the bolus. The BUI was found, however, to increase with increasing pH of the bolus [16]. Similar findings were reported by Lockwood et al. [17]. The relative proportion of diffusible NH_3 to NH_4^+ ($\text{p}K_a \sim 9.2$) will increase with increasing pH. Therefore, our findings [16] and those of Lockwood et al. [17] indicate that ammonia enters the rat brain largely by diffusion of the free base (NH_3). The pH dependence of [^{13}N]ammonia uptake has also been demonstrated for rhesus monkey brain [18] and dog brain [19]. Raichle and Larson used positron emission tomography (PET) to study uptake of [^{13}N]ammonia in rhesus monkey brain and concluded that uptake is largely by diffusion of the free base, but that a small portion enters as ammonium ion [18].

Metabolism of blood-derived [^{13}N]ammonia in rat brain – validation of the metabolic compartmentation of cerebral ammonia metabolism

After determining the BUI for [^{13}N]ammonia we next investigated the metabolic fate of tracer quantities of [^{13}N]ammonia in rat brain. We used a rapid isolation procedure coupled with HPLC and analysis of a portion of rat brain homogenate with glutaminase to calculate the relative amount of label in the amine *versus* amide positions of glutamine. We found that about 60% of the label was already in a metabolized form 5 seconds after carotid artery administration of a bolus of [^{13}N]ammonia [16]. Because residual [^{13}N]ammonia is likely to be present in the cerebral blood supply at time of sacrifice (5 seconds), our findings indicated that the $t_{1/2}$ for metabolism of ammonia entering the rat brain from the blood into the small compartment must have been of the order of seconds. More than 95% of the label in metabolites was found to be present in the amide position of glutamine with much smaller amounts in the amine position of glutamine and glutamate. Importantly, the relative specific activities were found to be in the order: L-[amide- ^{13}N]glutamine > L-[amine- ^{13}N]glutamine > L-[^{13}N]glutamate. Similar results were obtained when tracer quantities of [^{13}N]ammonia were infused via a carotid artery cannula for 10 min (Table 1). Thus, our results, which were obtained under truly tracer conditions, corroborated the previous findings of Berl et al. [1,4] that ammonia metabolism is compartmented in brain. The results also highlighted the previously unsuspected speed with which nitrogen can move among different metabolites in the brain.

Another example of rapid nitrogen flux is briefly mentioned here. We showed that within 20 seconds after femoral artery injection of a bolus of [^{13}N]ammonia into adult male rats almost all the label had disappeared from the circulation [20]. Moreover, of the label remaining, most was in a metabolized form [20]. The rapid exchange of nitrogen is of fundamental importance in any discussions of mechanisms of nitrogen partitioning among different amino acid pools, yet is not widely appreciated.

In other experiments [^{13}N]ammonia was continuously infused for 20 minutes into adult male rats via a carotid artery cannula [16,21]. An HPLC profile of ^{13}N metabolites in brain obtained after a 20-minute infusion is shown in Fig. 1. This figure dramatically emphasizes the importance of the GS reaction in the brain. Note the relatively small amount of radioactivity in glutamate, aspartate and GABA. The very small amount of label in urea presumably reflects the fact that a large portion of the administered [^{13}N]ammonia circumvented the brain and entered the general circulation to be converted to [^{13}N]urea in the liver, some of which was released to the general circulation.

Metabolism of cerebrospinal fluid (CSF)-derived [¹³N]ammonia in rat brain

When tracer quantities of [¹³N]ammonia were administered to the CSF of an adult male rat via an indwelling cannula metabolic compartmentation of cerebral ammonia metabolism was again observed [16]. The specific activity of brain metabolites was in the order: L-[amide-¹³N]glutamine > L-[amine-¹³N]glutamine > L-[¹³N]glutamate. Thus, ammonia entering the brain from the CSF, in addition to that entering from the blood, is metabolized in the glutamine-synthetase containing small compartment.

Anatomical basis for compartmentation of cerebral ammonia metabolism

Brain has been known for over 50 years to be a rich source of GS [22]. Moreover, sheep brain is often used as a convenient source of GS [23]. But where is the enzyme located in the brain? This question was answered by Norenberg and associates who convincingly showed that GS is largely, if not exclusively, located in astrocytes in the brain [24,25]. This work showed that, to an approximation, the small and large compartments, as defined by prior tracer and kinetic experiments, are represented by astrocytes and neurons, respectively. Astrocytic end feet surround brain capillaries and underlie the ependyma. Thus, ammonia diffusing into the brain across the blood-brain barrier (BBB) or from the CSF enters the astrocytes (small compartment) where it is efficiently incorporated into glutamine before it has a chance to diffuse further and act as a substrate of GDH in neurons (large compartment). Compartmentation of ammonia metabolism in brain would also occur if GDH occurred exclusively, or almost exclusively, in neurons rather than in astrocytes. However, as reviewed by Hertz and Schousboe GDH is well represented in both astrocytes and neurons in culture [26]. A simplified compartmentation model of cerebral ammonia metabolism is shown in Fig. 2.

Estimation of rat brain GS activity *in vivo*

Although our studies with ¹³N demonstrated unequivocally the importance of GS in the metabolism of ammonia in the brain [16], they did not provide a numerical estimate of the rate of cerebral glutamine synthesis. Several subsequent NMR studies using [¹⁵N]ammonia have, however, been used to calculate the GS flux in rat brain during hyperammonemia [e.g. 27–31]. Estimates of rat brain glutamine synthesis in these hyperammonemic animals ranged from 0.06 to 0.2 μmol/min/g [27–31]. Other estimates using ¹³C MR techniques suggested a rate in the hyperammonemic rat brain of 0.29–0.43 μmol/min/g [11,32]. Recent NMR studies from Gruetter and colleagues suggest a rate of 0.18 μmol/min/g [33] under normoammonemic conditions and 0.30 μmol/min/g under hyperammonemic conditions. Some of the differences in reported rates may be due to differences in the blood ammonia concentrations in the experimental animals. For example, in the work of Kanamori et al. the blood ammonia levels reported were from a low of 0.2 – 0.3 mM to a high of 0.8 – 1.3 mM [27–30]. At the higher blood ammonia levels the brain GS reaction apparently reached saturation. In other studies, reported blood ammonia levels were 0.95 mM [33] and maximally 0.35 – 0.40 mM [11,31,32].

Different methodologies may also account for some of the apparent discrepancies in reported cerebral glutamine synthesis rates. For example, in some studies measurements were carried out in acutely hyperammonemic rats in which the brain glutamine level was rising [31,33]. In other studies, Kanamori et al. [27–30] and Sibson et al. [11,32] used a steady state hyperammonemia model in which brain glutamine levels were maintained at a constant, but elevated level. For example, in the Sibson et al. study, blood ammonia levels were raised from ~0.05 mM (baseline) to 0.35 mM by infusion of ammonium acetate [32]. Once a steady state level of brain glutamine was established [1-¹³C]glucose was infused and NMR analysis of ¹³C-labeled metabolites was measured. Flux through the rat brain TCA

cycle under normoammonemic and hyperammonemic conditions was estimated to be 0.46 ± 0.16 and 0.57 ± 0.16 $\mu\text{mol}/\text{min}/\text{g}$, respectively (not significantly different) [32]. The rates of cerebral glutamine synthesis in anesthetized normoammonemic and hyperammonemic rats in these experiments were calculated to be 0.21 ± 0.04 and 0.43 ± 0.14 $\mu\text{mol}/\text{min}/\text{g}$, respectively ($p < 0.01$) – a two-fold increase [32]. The authors suggested that under normal conditions most of the glutamine flux is accounted for by neurotransmitter cycling between glutamate and glutamine (the glutamine cycle; see below). In contrast, during hyperammonemia, increased synthesis of glutamine leads to an increase in cerebral glutamine superimposed upon that required for neurotransmitter glutamate cycling and to increased egress of glutamine from the brain [32]. In a later report Sibson et al [11] again used NMR and $[1-^{13}\text{C}]$ glucose infusion to study cerebral glutamine metabolism in anesthetized rats and reaffirmed their previous estimate of glutamine synthesis in the brain of about 0.2 $\mu\text{mol}/\text{min}/\text{g}$ [11]. As noted above, anaplerosis was estimated to contribute 19–26% of carbon flux in the glutamine synthesis reaction. This value increased to about 32% in the hyperammonemic animals [32]. This finding again emphasizes the remarkable ability of the brain to utilize anaplerosis and to increase this process during hyperammonemia, as first revealed in the pioneering studies of Berl et al. [4].

It should also be noted that estimates of glutamine synthesis in the brain may vary depending on how the data were analyzed (e.g. a one compartment model versus a two compartment model; parameters iterated or assumed; K. L. Behar, personal communication). A detailed discussion of these parameters is beyond the scope of the present article. However, a few interesting points emerge from the NMR data. The best estimates of GS activity in the anesthetized rat brain *in vivo* under normoammonemic conditions are $\sim 0.18 - 0.2$ $\mu\text{mol}/\text{min}/\text{g}$ [11,12,32]. Under hyperammonemic conditions this value may rise to $\sim 0.3 - 0.4$ $\mu\text{mol}/\text{min}/\text{g}$ [11,33]. These calculations show that despite a modest decrease in the maximal capacity of rat brain GS in rat models of chronic [34] and acute [35,36] hyperammonemia (as determined by enzyme activity measured under assay optimal conditions) the brain enzyme is not normally saturated with ammonia and has the capacity to considerably increase the rate of production of glutamine under hyperammonemic conditions. This hypothesis is consistent with the average concentration of ammonia in normal rat brain of ~ 0.18 mM [16], a presumed lower concentration of ammonia in the small compartment, and the K_m value exhibited by brain GS for ammonia of 0.18 mM [23]. However, there is a limit to this capacity. Increased rates of glutamine synthesis cannot prevent increased accumulation of brain ammonia in various animal models of hyperammonemia [e.g. 34–37] and at end stage coma glutamine synthesis rates may reach saturation [30] or actually begin to decrease [30,36–38].

Increased cerebral glutamine is a hallmark of hyperammonemia (discussed below). Butterworth and colleagues [37] have pointed out that there is a discrepancy, however, between the accumulation of cerebral glutamine in hyperammonemic animals and rate of cerebral glutamine synthesis reported in the literature [30,35,38]. Therefore, other factors besides increased rates of glutamine synthesis may contribute to increased cerebral glutamine in hyperammonemic animals [37]. These authors have pointed out that hyperammonemia results in inhibition of the astrocyte glutamine transporter SNAT5 [37]. Thus, restricted egress of glutamine from astrocytes may account in large part for the increased brain glutamine and cell swelling/brain edema that are well established features in hyperammonemic animals. The authors also propose that such a mechanism could lead to decreased excitatory neurotransmission – a characteristic feature of the encephalopathy associated with acute liver failure [37]. This is an interesting and novel idea, and certainly an inhibition of the glutamine transporter could contribute to increased glutamine in astrocytes. However, it is probable that the glutamine synthesis reaction is still active even at end stage acute liver failure. Nevertheless, the exact roles that glutamine synthesis and inhibition of

glutamine egress play in the accumulation of glutamine in astrocytes and associated cell swelling/edema during hyperammonemia must await further study.

Comments on the use of labeled ammonia to investigate brain GS *in vivo*

Since the GS reaction requires ATP (Eq. 1) it is generally assumed that the reaction is irreversible. However, Levintow and Meister [39] showed that the reaction is, in fact, readily reversible. Thus, under optimal conditions, the back reaction (i.e. glutamine \rightarrow glutamate) is about 12% as rapid as the forward direction (i.e. glutamate \rightarrow glutamine) [39]. This reversibility could theoretically lead to an underestimate of the rate of cerebral GS when incorporation of labeled ammonia into glutamine (amide) is measured. On the other hand, another mechanism may theoretically lead to an overestimation. Thus, it has been shown, using ^{15}N as a tracer, that GS obtained from various sources catalyzes the non-productive exchange of ammonia with the amide group of glutamine (i.e. $^{15}\text{NH}_3 + \text{L-}[\textit{amide-}^{14}\text{N}]\text{glutamine} \rightleftharpoons \text{L-}[\textit{amide-}^{15}\text{N}]\text{glutamine} + ^{14}\text{NH}_3$) [40]. Wedler showed that in the presence of 0.25 mM ammonium chloride, 4 mM L-glutamate, 2 mM ATP, 5 mM ADP, 10 mM P_i , 50 mM Mg^{2+} and 20 mM glutamine the rate of exchange between ammonia and glutamine (amide) catalyzed by sheep brain GS is 70% that of the rate of exchange between glutamate and glutamine (i.e. the glutamine synthesis reaction) (Table 1 in ref 40). This finding suggests that non-productive GS-catalyzed exchange of nitrogen between ammonia and glutamine (amide) has the potential to be substantial, resulting in considerable overestimation of net glutamine synthesis rates in rat brain. However, the concentrations of glutamate and glutamine in normal rat brain are of the order 11 mM and 5 mM, respectively [e.g. 41]. Thus, since the K_m exhibited by brain GS for glutamate is 2.5 mM [23] and the affinity for glutamine is considerably less than that for glutamate (high concentrations of glutamine are required for maximal activity of glutamine as a substrate, typically 50 mM [42–44]), under normal conditions the non-productive exchange of nitrogen between ammonia and glutamine (amide) is likely to be much less than 70% relative to the net glutamine synthesis reaction. But what is the situation during hyperammonemia? During chronic hyperammonemia the concentration of brain glutamate may be unchanged [1] or decrease only slightly to moderately under a variety of hyperammonemic conditions (e.g. 41,45; see also a review by Vaquero and Butterworth [46]), but the concentration of brain glutamine can increase by 2 to 3-fold [e.g. 41,45]. Thus, during hyperammonemia the concentration of glutamine in the astrocyte compartment may be considerable. These considerations would suggest that exchange of label between ^{15}N ammonia and the amide position of astrocytic glutamine may not be inconsequential during hyperammonemia. Nevertheless, the observation that the cerebral GS rate as measured from carbon (^{13}C) flux is similar to that of the rate of label incorporation from ^{15}N ammonia into the amide position of glutamine [11,32] argues that under hyperammonemic conditions the exchange of ammonia at the amide position of glutamine is likely small relative to net glutamine synthesis *in vivo* even under hyperammonemic conditions.

At present it is not possible to estimate the correction needed to obtain the “true” rates of GS *in vivo* from studies with labeled ammonia, but it is likely that the two above mentioned opposing processes offset each other to some extent and that the overall correction needed is probably small. Nevertheless, investigators interested in modeling glutamine synthesis in brain should be aware of the reversibility of the GS reaction and possible non-productive exchange between ammonia and the amide nitrogen of glutamine.

Studies of ammonia uptake and metabolism in human brain

Our findings of rapid trapping of ^{13}N ammonia in rat brain in the amide position of glutamine, and some earlier PET studies using ^{13}N ammonia by Lockwood and colleagues

[47] have provided the basis, in part, for a recent resurgence of interest in using PET to study [^{13}N]ammonia metabolism and trapping in human brain under normal and pathological (hyperammonemic) conditions [48–52]. For example, Keiding et al. [49] showed that net metabolic flux of ammonia from blood to brain (and presumably trapping of most of the label as intracellular glutamine) in the cortex of patients with liver cirrhosis with accompanying hepatic encephalopathy (HE) was $13.4 \mu\text{mol}/\text{min}/\text{L}$ tissue. The corresponding values in patients with liver cirrhosis but without HE and in healthy controls were 7.4 and $2.6 \mu\text{mol}/\text{min}/\text{L}$ tissue, respectively [49]. The authors concluded that “increased trapping of ammonia in patients with cirrhosis with acute HE was primarily attributable to increased blood ammonia and to a minor extent to changed ammonia kinetics in the brain” [49]. These findings provide additional evidence that the GS reaction in normal brain is not saturated with ammonia and that cerebral glutamine synthesis is increased during hyperammonemia.

The increased cerebral ammonia uptake in the cirrhotic patients with HE compared to controls reflects the ready diffusion of ammonia (as NH_3) across the BBB coupled with the higher concentration of ammonia in the blood of these patients. But how readily is ammonia metabolically trapped once it enters the human brain? In the [^{13}N]ammonia PET studies there is apparently some back diffusion of [^{13}N]ammonia, but the majority of the label derived from blood-borne [^{13}N]ammonia is metabolically trapped in human brain [50,52]. This finding is consistent with the ammonia concentration in brain and the K_m value exhibited by GS toward ammonia. Reported concentrations of normal whole brain ammonia vary considerably, but, as noted above, the concentration of ammonia in rat brain is about 0.18 mM, with even lower levels probably occurring in the astrocyte compartment (reviewed in [53]). As also noted above, the K_m value exhibited by highly purified ovine GS toward ammonia is 0.18 mM [23]. Assuming that the human brain enzyme is similar to the ovine enzyme, then the GS reaction is far from saturated with ammonia in normal human brain. Therefore, assuming no major changes in ATP, glutamate and GS in the small compartment, an increased influx of ammonia into the brain will result in increased synthesis of cerebral glutamine. In fact, as discussed above, increased rates of cerebral ammonia trapping (presumably as glutamine) occur in hyperammonemic HE patients [49] and in hyperammonemic rats [11,16,33] relative to controls.

Glutamine (or glutamine plus glutamate; Glx) can now be measured in human brain *in vivo* by means of NMR spectroscopy techniques. As expected from previous studies of autopsied brain material [54] and from the discussion of the kinetic properties of GS discussed above, these techniques have shown greatly increased *in vivo* levels of brain glutamine (or Glx) in hyperammonemic/liver disease patients [55,56]. In a recent study, Mardini et al. [57] used NMR to quantify cerebral water content and selected amino acids in cirrhotics following induced hyperammonemia. Increased blood ammonia levels correlated with *in vivo* increased brain glutamine [57].

Cerebral metabolism of [^{13}N]ammonia in rats administered the GS inhibitor L-methionine-S,R-sulfoximine (MSO)

MSO has been studied extensively for many years as a potent inhibitor of GS [e.g. 58]. We made use of this property to study the cerebral metabolic fate of [^{13}N]ammonia in rats in which brain GS was inhibited. Tracer quantities of [^{13}N]ammonia were administered to MSO-treated rats via a carotid artery cannula. After a 10-min infusion the brains were rapidly frozen and label in various metabolites was determined in a portion of the frozen brain sample (Table 1). The specific activities of GDH and GS in the MSO-treated rat brain were also determined in a separate portion of the frozen brain sample. Treatment with MSO resulted in no significant change in the specific activity of cerebral GDH, whereas the specific activity of GS was reduced by about 86% (Table 1). The relative specific activity of

cerebral L-[¹³N]glutamate in the MSO-treated rats was now *greater* than that of L-[*amine*-¹³N]glutamine (Table 1). This finding shows that compartmentation of ammonia metabolism was no longer intact and that [¹³N]ammonia had diffused across the small compartment (astrocyte end feet) to the large compartment (neurons) to be acted upon therein in part by GDH. Nevertheless, the amount of label in L-[*amide*-¹³N]glutamine was still considerably greater than that in glutamate (plus glutamine amine).

Table 1 shows that when the GS reaction is inhibited alternative means of removing cerebral ammonia are limited. After 10 minutes of infusion only about 20% of label was metabolically trapped in the brains of MSO-treated rats compared to that in the brains of control rats [16]. Thus, despite high inherent activity of the GDH reaction in brain (Table 1), this enzyme was not able to fully compensate for decreased GS activity and the level of cerebral ammonia increased by about 4.5 fold in the MSO-treated rats. Nevertheless, inspection of Table 1 indicates that about five times as much label is incorporated into glutamate in the MSO treated animals as compared to controls. But this label in cerebral glutamate [relative specific activity (1.4%)] is still small when compared to that in glutamine amide [100%] in untreated animals. Moreover, it should be noted that, because the GDH reaction is freely reversible incorporation of label into glutamate does not necessarily imply net synthesis.

In other experiments we showed that treatment of rats with MSO leads to a slight decrease in brain glutamate (from 12.9 mM to 10.0 mM) but to a larger decrease of brain glutamine (8.0 to 3.8 mM) [45]. Consider the specific activity of [¹³N]glutamate relative to that of [*amide*-³N]glutamine (i.e. 1.4%; from Table 1) and the larger pool size of glutamate relative to glutamine (2.6; from ref. 445). From this consideration, the comparative flux of ammonia to glutamate catalyzed by the GDH reaction in the MSO-treated rats is only about 4% (1.4 × 2.6) relative to the flux into glutamine (amide) in the brains of untreated rats. Nevertheless, as discussed below this route for ammonia assimilation may be important during maintenance of long-term nitrogen homeostasis in the hyperammonemic brain.

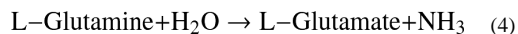
Cerebral [¹³N]ammonia metabolism in hyperammonemic rats

In a separate series of experiments we investigated the short-term (5 – 20 sec) cerebral metabolic fate of [¹³N]ammonia in hyperammonemic, portacaval shunted (PCS) adult male rats [34]. The results are shown in Table 2. Note that the incorporation of label into glutamine was slowed compared to that in controls. Nevertheless, incorporation of label in glutamine was still much greater than that in glutamate in these hyperammonemic animals. This was also true of rats injected with urease whether or not the animals had been subjected to a portacaval shunt (Table 3). (Urease treatment is a procedure for generating acute hyperammonemia in experimental animals without portacaval shunting or inhibition of GS [59].) Although the GS reaction is still the predominant route for the metabolism of blood-borne ammonia in the hyperammonemic animals, ammonia-derived nitrogen incorporation in glutamate must have increased as a result of the larger brain ammonia pool.

Our data suggest that the GS reaction is far more important in metabolizing blood-derived ammonia in rat brain than is the GDH reaction. However, the findings do not provide quantitative data on the rate of the GDH reaction. Therefore, at this point it is worth reviewing qualitative estimates of the GDH reaction in rat brain relative to the GS reaction as obtained by ¹⁵N-NMR techniques [29,31,33]. Rate values using these techniques for brain GDH coupled with plasma ammonia concentrations (shown in parenthesis) have been reported to be 0.013 – 0.020 μmol/min/g (0.9 – 1.0 mM) [29], 0.065 μmol/min/g (0.35 mM) [31] and 0.033 μmol/min/g (0.95 mM) [33] in anaesthetized rats under hyperammonemic conditions. The corresponding values for the rat brain GS reaction were reported to be

0.055, 0.20 and 0.30 $\mu\text{mol}/\text{min}/\text{g}$, respectively. The corresponding ratio of the rates of the GS reaction to the GDH reaction in rat brain among the three studies were 2.7 – 4.1, 3.1 and 9, respectively. The apparent disparities among the various ^{15}N -NMR studies may have been due in part to differences in plasma ammonia concentrations, experimental protocols and modeling. Nevertheless, the general conclusion that the GS reaction is the more important reaction than the GDH reaction in the hyperammonemic rat brain for removing ammonia is consistent with the earlier findings of Berl et al that relative enrichment of ^{15}N in brains of hyperammonemic cats after administration of [^{15}N]ammonia was in the order: glutamine (amide) > glutamine (amine) > glutamate [1]. However, it should be noted that our ^{13}N data suggest an even greater ratio of ammonia incorporation into glutamine (amide) relative to that in glutamate (Tables 1 and 2) than that obtained from the ^{15}N experiments. The higher ratio may be explained in part by the fact that the rats in our experiments were unanesthetized, blood ammonia levels were lower and infusion/sampling times were different. Nevertheless, a firm conclusion among our studies (Tables 1 and 2), previous studies of Berl et al. [1] and later ^{15}N -NMR studies [29,31,22] is that the GS reaction is a much more effective means of removing ammonia from rat brain than is the GDH reaction.

A considerable portion of the glutamate released from neurons during neurotransmission is taken up by astrocytes to be converted therein to glutamine. This flow of glutamate from neurons to astrocytes accounts for the large dilution of labeled ^{15}N at the amine position of cerebral glutamine during infusion of [^{15}N]ammonia noted in the ^{15}N -NMR studies discussed above [19,31,33]. Glutamine is released from the astrocytes to be taken up by the neurons and converted to glutamate by means of the glutaminase reaction (Eq. 4). Although probably not stoichiometric the concept of a glutamine cycle in brain is now generally accepted. See, for example, recent reviews by Albrecht et al. [60] and Hertz [61].

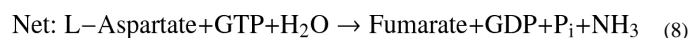
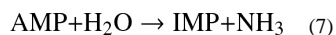
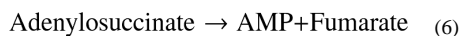


Major sources of endogenous ammonia in the brain

Cooper and Plum [53] have listed at least fourteen enzyme-catalyzed reactions that are capable of generating ammonia in the brain. Most of these reactions are probably quantitatively minor, but serve as a reminder of the central importance of ammonia in cerebral nitrogen metabolism. Here, I discuss two well established major routes and one likely major route for the production of ammonia in brain. Glutaminase is usually considered to be a major source of cerebral ammonia (Eq. 4). Both astrocytes and neurons contain glutaminase, but the enzyme specific activity is greater in neurons [e.g. 62]. As noted above, the enzyme is important in converting neuronal glutamine to glutamate as part of the glutamine cycle in brain.

The equilibrium position of the GDH reaction (Eq. 2) at physiological pH values favors reductive amination of α -ketoglutarate. As a consequence, and as also noted above, label derived from either [^{13}N]ammonia or [^{15}N]ammonia that was taken up from the blood into rat brain was incorporated in part into glutamate. However, label in glutamate was much lower than that in glutamine (amide). The very high K_m value for ammonia (~ 10 mM) exhibited by brain GDH [63] and much lower K_m value exhibited by brain GS (0.15 mM; [23]) coupled with the very low ammonia concentration in astrocytes (< 0.18 mM) [16,53] explains in part the much less effective trapping of ammonia in glutamate as compared to trapping in glutamine (amide). Despite the fact that the equilibrium position of the GDH reaction *in vitro* favors glutamate formation at neutral pH strong evidence suggests that under normal circumstances the GDH reaction in the brain is drawn in the direction of glutamate oxidation by removal of ammonia as glutamine [e.g. 61,64–66].

The purine nucleotide cycle (PNC) is likely a third major route for cerebral ammonia production. This source of ammonia is not generally considered in discussions of nitrogen metabolism in the brain, so it is important to consider the cycle here in the context of the theme of this review. This cycle was first described in detail by Lowenstein in 1972 [67]. The cycle consists of three enzyme reactions: adenylosuccinate synthetase (Eq. 5), adenylosuccinate lyase (adenylosuccinase; Eq. 6) and AMP deaminase (Eq. 7). The net reaction (Eq. 8) is the energetically favorable conversion of L-aspartate to fumarate and ammonia at the expense of GTP hydrolysis.



Lowenstein and co-workers have provided evidence that the cycle is an important source of ammonia in muscle and brain, an alternative source in kidney and of low importance in the liver [67–69]. The cycle has been suggested to act in concert with adenylate kinase (myokinase) (Eq. 9) to ensure high ratios of ATP to ADP [67,70]. This coupling protects cells against abrupt changes in the adenylate energy charge [67,70]. Such a safeguard may be important in muscle during bursts of exercise and in tissues such as the brain that maintain a high, sustained energy use.

Evidence for the importance of the cycle in brain is as follows: Schultz and Lowenstein [68] showed that the activity of adenylate deaminase (Eq. 7) in cell-free rat brain extracts is sufficient to account for maximal rates of ammonia production previously reported in the literature. They further showed that the activities of the other two enzymes of the cycle, namely adenylosuccinate synthetase (Eq. 5) and adenylosuccinate lyase (Eq. 6) are almost sufficient in combination with adenylate deaminase to account for this maximal ammonia production rate. Schultz and Lowenstein stated that according to the literature a) deamidation of glutamine (the glutaminase reaction; Eq. 4) can only account for ~5% of ammonia formation noted in brain slices and b) administration of MSO to mice results in an increase in ammonia before any fall in glutamine can be observed [references quoted in 68]. In a later extension of this work, the authors showed that within 10 sec after administration of electroshock treatment to rats the cerebral [ATP]/[ADP] ratio fell to a third of the pre-shock value accompanied by a three-fold increase in ammonia [69]. The total adenine nucleotide plus adenosine content of brain decreased and an equivalent amount of hypoxanthine-containing compounds appeared. Adenosine, inosine, and hypoxanthine accumulated, and there was a transitory accumulation of adenylosuccinate and IMP. The contents of ATP and creatine phosphate, and the [ATP]/[ADP] ratio, were rapidly restored to control values [69]. Schultz and Lowenstein [69] assumed that the GDH reaction is in equilibrium in rat brain. From this assumption and a calculation of the mitochondrial NADH/NAD⁺ ratio the authors concluded that the GDH reaction is not sufficient to account for the maximal rate of ammonia formation after electroshock treatment and may even have been a net source of ammonia removal under these conditions [69].



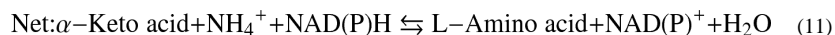
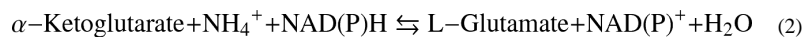
In summary, according to Schultz and Lowenstein [68,69] the glutaminase and GDH reactions cannot account for maximal rates of ammonia production in brain, whereas the maximal rate of the PNC can approach maximal rates of ammonia production. However, Schultz and Lowenstein may have overstated their case. The glutaminase and GDH reactions must account for a considerable portion of endogenous cerebral ammonia production. Thus, the demonstration by NMR techniques of a functioning glutamine cycle in brain predicated an approximate stoichiometry between glutamine synthesis and glutaminase reactions. Moreover, as I have emphasized throughout this review, continuous removal of ammonia by the GS reaction will ensure a prominent role for the GDH reaction in converting glutamate to ammonia. Nevertheless, Schultz and Lowenstein make a strong case for the PNC as an important ancillary source of cerebral ammonia. If the authors are correct then the cycle is convolved with glutaminase and the GDH reaction in the production of endogenous ammonia in the brain. Thus, it is curious that the findings of these authors have been largely ignored since the original publications more than 35 years ago.

One of the very few studies published since the mid 1970s related to the PNC in brain is that of Hamprecht and colleagues [71]. AMP deaminase mRNA was detected prominently in ventricular ependymal cells, choroid plexus and some neuronal populations. Immunohistochemical staining of rat brain sections revealed a similar distribution of the protein. Staining was strikingly intense in ependymal cells and their associated cilia. No staining could be detected in astrocytes. AMP deaminase was suggested to be important for maintaining ATP for the ependyma cilia beat [71]. It will be important to follow up this study to localize and characterize the other two enzymes of the cycle in brain (i.e. adenylosuccinate synthase and adenylosuccinate lyase).

Possible metabolic connections of the PNC with aminotransferases and GDH are discussed below.

The role of GDH in assimilating and dissimilating ammonia – transreamination and transdeamination

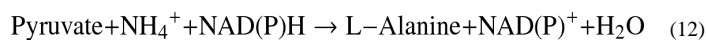
I recently reviewed this subject [11], but a further discussion is provided here. The terms transreamination and transdeamination were introduced in 1957 by Braunstein [72]. These terms are not often used, but I use them here because they exemplify informative concepts. (For an appreciation of Braunstein, a historical account of the discovery of transamination, and the biological roles of transamination see Cooper and Meister [73].) Braunstein pointed out that coupling of the GDH reaction (Eq. 2) to an α -ketoglutarate/L-glutamate-linked transaminase (aminotransferase) (Eq. 10) can be used to incorporate ammonia nitrogen into an amino acid (transreamination) or to remove it (transdeamination). The net reaction is shown in Eq. 11. Transreamination is the forward reaction of Eq. 11 and transdeamination is the reverse direction.



Recent work has shown that GDH is tightly associated with an aminotransferase in brain. Thus, Hutson and co-workers have shown that the mitochondrial enzyme GDH 1 (but not GDH 2) is part of a supranuclear complex (metabolon) that also includes the mitochondrial

form of the branched chain amino acid (BCAA) aminotransferase [74]. (Most mammals possess only the GDH 1 isoenzyme. However, some human cell types, including astrocytes, possess an additional isoenzyme – GDH 2 [75]. Most of the discussion in the present review pertains to GDH 1.) According to Hutson et al. the close juxtapositioning of the mitochondrial BCAA aminotransferase to GDH facilitates the channeling of nitrogen from the BCAAs toward ammonia [74] – i.e. transdeamination, the reverse direction of Eq. 11.

In a recent review I emphasized that even under hyperammonemic conditions the GDH reaction cannot compete quantitatively over the short term with the GS reaction as a means of ammonia removal in rat brain [14], and I continue to emphasize that fact here. Recently, however, it was suggested that synthesis of alanine, presumably via a transamination mechanism involving GDH, may be important in addition to the GS reaction for the removal of cerebral ammonia during hyperammonemia [10,76] For example, Dadsetan et al. [10] reported that inhibition of GS with MSO in co-cultures of astrocytes and neurons resulted in increased channeling of ammonia toward alanine via coupling of alanine aminotransferase to the GDH reaction. In this case the direction of Eq. 11 is toward transamination (Eq. 12).



In other work from the same group, Leke et al. [76] investigated metabolic processes in co-cultures of neurons and astrocytes (model of the GABAergic system) exposed to 5 mM ammonium chloride. The authors reported ammonia-stimulated increase of the TCA cycle and increased synthesis and release of alanine [76]. But, how important is alanine production for removal of ammonia in the normal and hyperammonemic brain *in vivo*? As indicated above, the relative short-term flux of label derived from [¹³N]ammonia into brain glutamate, although considerably greater in MSO-treated rats than in untreated rats, is still relatively low. Moreover, in the HPLC system shown in Fig. 1 alanine elutes at about 7 minutes (immediately after glutamine). In the experiment shown in Fig. 1 labeled alanine cannot be detected in the rat brain after continuous intracarotid infusion of [¹³N]ammonia for 20 minutes. Finally, the specific activity of alanine aminotransferase in rat brain (even when measured under optimal conditions) is relatively low. For example, Benuck et al. reported a specific activity for transamination of L-alanine with α-ketoglutarate under optimal conditions of ~76 μmol/h/100 mg protein in adult rat brain [77]. In contrast, the authors found maximal transamination rates with aspartate and glutamate of ~ 2,700 and 15,000 μmol/h/100 mg protein. Since the alanine aminotransferase reaction is freely reversible the optimal activity of alanine aminotransferase with pyruvate as substrate is not likely to be much different from that with alanine i.e. the inherent alanine aminotransferase reaction in rat brain is likely to be at least two orders of magnitude slower than that of the aspartate aminotransferase (AAT) reaction.

Measurable uptake of alanine across the rat BBB cannot be detected [78], yet L-alanine is present in rodent brain at a concentration of ~1 mM [79]. Thus, L-alanine in the rat brain is most likely synthesized *de novo* via transamination reactions. However, our inability to detect label in cerebral alanine after intracarotid infusion of [¹³N]ammonia suggests that the nitrogen for astrocytic L-alanine is not obtained from blood-derived ammonia via a transamination pathway. But what is the fate of the ammonia generated from metabolic reactions in the large compartment (neurons) that does not readily mix with [¹³N]ammonia derived from the blood? Because ammonia diffuses very rapidly it is likely that the bulk of the ammonia generated in the neurons is metabolized very rapidly to glutamine in the astrocytes. However, because the neurons contain considerable GDH activity and the reaction is reversible some ammonia is likely to be incorporated into glutamate in the neuronal compartment, especially under hyperammonemic conditions [80]. Thus, as

suggested by Dadsetan et al. [10] alanine could accumulate over time as a defense against persistent hyperammonemia. This is because nitrogen shuttles rapidly between glutamine and glutamate through the glutamine cycle whereas, although at a much slower rate, alanine can build up over time. In this context, it is interesting to note that *in vivo* cerebral microdialysis in acute liver failure (ALF) patients with persistent hyperammonemia revealed, as expected, marked increases in glutamine [81,82], but alanine was also markedly elevated [82].

Coupling of AAT to the GDH reaction

The AAT-catalyzed reaction is shown in Eq. 13. Most tissues contain very high levels of mitochondrial aspartate aminotransferase (mAAT) and cytosolic aspartate aminotransferase (cAAT) isozymes [83]. These isozymes, along with mitochondrial malate dehydrogenase, cytosolic malate dehydrogenase, a mitochondrial aspartate/glutamate antiporter and a malate/ α -ketoglutarate antiporter, are important components of the malate-aspartate shuttle (MAS). Because mitochondria are impervious to NADH generated in the cytosol during glycolysis, a shuttle such as the MAS is needed to transport reducing equivalents into the mitochondrion in lieu of NADH. Work from our laboratory, including a joint collaboration with Hertz and colleagues [84–86] has suggested an important role for the MAS in the brain (for reviews see for example [87,88]).

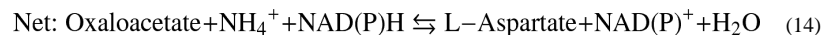
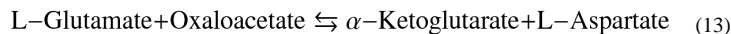
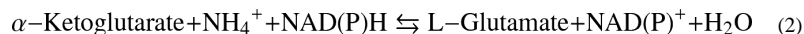
In addition, mAAT and cAAT are crucial for the maintenance of nitrogen balance. As a result of the high activity of both isozymes of AAT the components of the AAT reaction are in thermodynamic equilibrium in liver [89,90] and brain [91]. The very high activity of these enzymes is evident from our studies of the metabolism of [^{13}N]ammonia in rat liver. After portal vein injection of a bolus of [^{13}N]ammonia in anesthetized rats label rapidly appeared in hepatic glutamate [92]. Impressively, once label appeared in glutamate, aspartate was almost immediately labeled. However, a few minutes were required for label to totally equilibrate between glutamate and aspartate pools. This time to reach equilibrium results from the fact that label that was initially rapidly equilibrated between glutamate and aspartate in the liver mitochondria required several minutes to re-equilibrate between mitochondrial and cytosolic pools of glutamate and aspartate [92]. Our data suggest that nitrogen is extremely rapidly shared between glutamate and aspartate in both the cytosolic and mitochondrial pools in the rat liver (seconds or less).

Even though the amount of label present in cerebral glutamate after carotid artery administration of [^{13}N]ammonia to rats was very small relative to that in glutamine (amide) we consistently noted that once label appeared in glutamate, label could also be detected in aspartate (Figure 1). However, because the relative amount of label in glutamate was relatively small compared to that in glutamine (Figure 1; Tables 1–3) it was difficult to estimate the rate of exchange of label between glutamate and aspartate in rat brain. Nevertheless, since the specific activities of mAAT and cAAT in rat brain are similar, or even higher, to those in rat liver [83] it is likely that equilibration of nitrogen between glutamate and aspartate, as in the rat liver, must be extremely rapid in rat brain – also seconds or less².

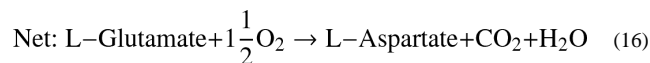
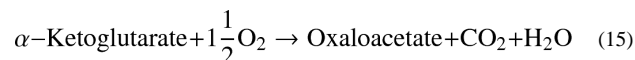
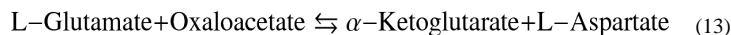
Coupling of the GDH reaction (Eq. 2) to the AAT (Eq. 13) represents a special case of transreamination/transdeamination in which ammonia can be incorporated into aspartate or aspartate can act as a source of ammonia (eq. 14). Since aspartate is also a component of the

²This finding appears to have been the basis for the model developed by the Shulman laboratory for the NMR measurement of TCA flux using [^{13}C]glucose [cf. 11, 31,32]. Due to the very rapid exchange of nitrogen in the AAT reaction (considerably faster than carbon flux through the TCA cycle) label in glutamate is a good proxy for that in the much smaller pool of α -ketoglutarate

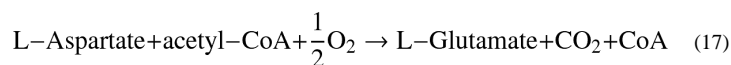
net reaction of the PNC (Eq. 8), it is important to emphasize at this point that both aspartate and glutamate are key components contributing to the maintenance of ammonia and nitrogen homeostasis in the brain.



The α -keto acid substrates of AAT, namely α -ketoglutarate and oxaloacetate, are components of the TCA cycle, and mAAT is of high activity in brain mitochondria (83). It is therefore possible to combine the section of the TCA cycle that directs carbon from α -ketoglutarate to oxaloacetate (formally depicted in Eq. 15) with the AAT-catalyzed reaction (Eq. 13) such that glutamate is readily oxidized to aspartate (Eq. 16). This conversion of glutamate to aspartate was first recognized more than 60 years ago (93) and under certain circumstances may be almost stoichiometric.



By coupling the AAT reaction to other enzymes of the TCA cycle it is also possible to utilize aspartate as a source of glutamate (Eq 17). Other pathways from aspartate to glutamate that employ in part TCA cycle enzymes (and GDH) are possible (e.g. Eq. 18). (For details of the enzyme-catalyzed steps involved in the overall transformations of aspartate to glutamate shown in Eqs. 13 – to 18 see refs 94 and 95.) The important point here is that carbon metabolism through the TCA cycle is intimately connected to amino acid nitrogen metabolism via the mAAT reaction.

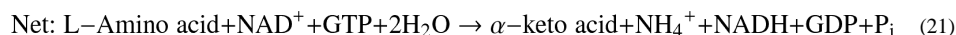
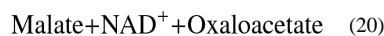
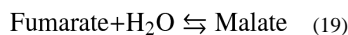
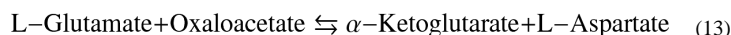
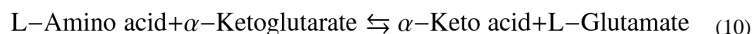
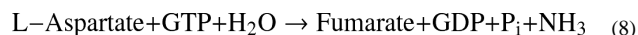


The respective quantitative roles of AAT and GDH in cerebral glutamate metabolism are occasionally debated (see, for example, the discussion in ref. 96.) In my opinion the arguments are moot. The AAT isozymes are so active that their metabolic rate coefficients are virtually zero and the enzymes exert little or no directional metabolic control. Thus, these isozymes act as metabolic conduits passively directing the flow of nitrogen toward or away from glutamate/ α -ketoglutarate or aspartate/oxaloacetate as dictated by ancillary enzymes such as GDH and the needs of the cells.

Pardo et al. recently suggested that brain glutamine synthesis requires neuronal-derived aspartate [97]. However, in a recent commentary Hertz [98] has pointed out that because aspartate is an excitatory neurotransmitter it is unsuitable as an agent for group transfer from neurons to astrocytes. Hertz has suggested an alternative model in which aspartate is crucial for glutamate/glutamine formation in astrocytes that circumvents the need for aspartate transport from neurons to astrocytes [98]. The points I discuss above emphasize connections between glutamate and aspartate carbon and nitrogen that can occur in the same cellular compartment.

Coupling of aminotransferase reactions and TCA cycle to the PNC

Lowenstein and Goodman (99) have pointed out that coupling of the PNC (Eq. 8) to an α -ketoglutarate/L-glutamate-linked aminotransferase (Eq. 10), AAT (Eq. 13), fumarase (Eq. 19) and malate dehydrogenase (Eq. 20) results in a more favorable deamination of an amino acid (Eq. 22) as a result of the hydrolysis of GTP than the transdeamination reaction shown in the back reaction of Eq. 11.



Note that if Eq. 10 is omitted the net reaction is the oxidative deamination of glutamate coupled to the hydrolysis of GTP (Eq. 22). In this pathway the oxidative deamination of glutamate is energetically more favorable than is the GDH reaction (Eq. 2, reverse reaction) as a result of the hydrolysis of GTP (99). These equations again serve to emphasize the interconnection of TCA cycle/energy metabolism with nitrogen metabolism.



Integration of major pathways of cerebral nitrogen metabolism

An integration of the various metabolic pathways discussed in this review is shown in Fig. 3. The figure emphasizes the central important of ammonia in the maintenance of nitrogen homeostasis. For simplification, cellular and subcellular compartments, transporters and non-nitrogen co-substrates of key enzymes are omitted. Ammonia (shown as NH_3) arising through metabolic reactions (three of which are depicted here) or entering the brain by diffusion from the blood or CSF is rapidly incorporated into the amide position of glutamine by the action of astrocytic GS (1). At the same time, glutamine is rapidly transported to the neurons and converted therein to glutamate in a reaction catalyzed by glutaminase as part of the glutamine cycle (2). Some glutamine may also be lost to the circulation and CSF as a

means of maintaining nitrogen balance resulting from net cerebral uptake of ammonia and amino acids (not shown here, but discussed in ref. 53). Because the reaction is reversible, the GDH reaction (3) may be either a source or sink for ammonia. However, under normal conditions cerebral GDH catalyzes net ammonia production as a result of rapid removal of ammonia catalyzed by GS [glutamate → ammonia → glutamine (amide)]. Since the AAT reaction (4) is in rapid thermodynamic equilibrium aspartate nitrogen may be directed toward ammonia production via the GDH reaction coupled to GS [aspartate → glutamate → ammonia → glutamine (amide)]. Other amino acids, such as alanine and the BCAAs may also be a source of ammonia via the transdeamination pathway coupled to GS (5) [amino acid (amine) → glutamate → ammonia → glutamine (amide)]. Finally, conversion of aspartate nitrogen to ammonia by the PNC (6) is energetically favorable. Thus, association of α -ketoglutarate/glutamate-linked aminotransferases to AAT and the PNC represents an alternative to the transdeamination pathway for conversion of amino acid amine nitrogen to ammonia and hence to glutamine (amide). The sequence of nitrogen transfers is as follows: amino acid (e.g. alanine, BCAAs) → glutamate → aspartate → ammonia → glutamine.

As noted above, work by Schultz and Lowenstein (68–69) has definitely established the occurrence of the PNC in brain. Although these workers provided strong evidence that this pathway is an important source of ammonia in brain the work has been largely ignored over the 35 years since first published. However, there is now strong evidence that the pathway may be important in neurons and ependymal cells, perhaps as a safety mechanism for maintaining ATP and adenylate energy charge (69,70).

Under normal conditions, cycling of nitrogen among ammonia, glutamate and glutamine (amide) reaches a quasi-steady state such that whole brain values of ammonia, glutamate and glutamine are about 0.18 mM, 10–12 mM and 3–5 mM, respectively. The concentrations of cerebral aspartate and alanine are about 2 mM and ~1 mM, respectively (see for example reference 100). However, when the steady state is perturbed during acute or chronic hyperammonemia, the flow of nitrogen among the major metabolites shown in figure 3 may be considerably altered. For example, brain glutamine and ammonia may rise substantially, whereas glutamate levels are less affected. From tracer studies the GS reaction is still the favored route for short-term removal of ammonia under hyperammonemic conditions. However, the glutaminase reaction and the glutamine cycle are still quite active, so that ammonia nitrogen can eventually flow to glutamate and to alanine even though kinetically the alanine aminotransferase reaction is probably much slower than the GS reaction *in vivo*. Eventually a new steady state is reached during hyperammonemia in which some waste nitrogen is redirected toward alanine via the transamination reaction involving alanine aminotransferase and GDH (10,76).

Conclusions

Our previous work, beginning more than 30 years ago with [^{13}N]ammonia showing that the major route for ammonia metabolism in normal and hyperammonemic rat brain is via the GS reaction, has stood the test of time. NMR techniques have now shown that this route for cerebral ammonia metabolism also predominates in normal and hyperammonemic human brain. The knowledge that [^{13}N]ammonia is metabolically trapped in human brain (presumably as L-[amide- ^{13}N]glutamine) serves as a basis for new techniques of PET modeling of [^{13}N]ammonia uptake and metabolism in normal and hyperammonemic human brain. Our original data showed that the GDH reaction is not quantitatively a major route for short-term removal of cerebral ammonia are consistent with the hypothesis that the cerebral GDH reaction normally favors oxidative deamination of glutamate. However, under conditions of prolonged hyperammonemia, it is possible that the GDH reaction contributes to a long-term increase in brain alanine as an alternative mechanism to the GS reaction as a

means of removing excess nitrogen. Finally, this review emphasizes the importance of aminotransferases linked to the GDH reaction for the homeostasis of brain ammonia and the most likely important role of the PNC as a source of ammonia. The role of the PNC in brain nitrogen metabolism is a fruitful area for future research.

Acknowledgments

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Abbreviations

| | |
|--------------|--|
| AAT | Aspartate aminotransferase |
| AMP | Adenosine monophosphate |
| BBB | Blood-brain barrier |
| BCA | Branched-chain amino acid |
| BUI | Brain uptake index |
| cAAT | Cytosolic aspartate aminotransferase |
| CSF | Cerebrospinal fluid |
| GDH | Glutamate dehydrogenase |
| GS | Glutamine synthetase |
| HE | Hepatic encephalopathy |
| HPLC | High performance liquid chromatography |
| IMP | Inosine monophosphate |
| mAAAT | Mitochondrial aspartate aminotransferase |
| MAS | Malate-aspartate shuttle |
| MSKCC | Memorial Sloan Kettering Cancer center |
| NMR | Nuclear magnetic resonance |
| MSO | L-Methionine- <i>S,R</i> -sulfoximine |
| PCS | Portacaval shunt |
| PET | Positron emission tomography |
| TCA | Tricarboxylic acid |

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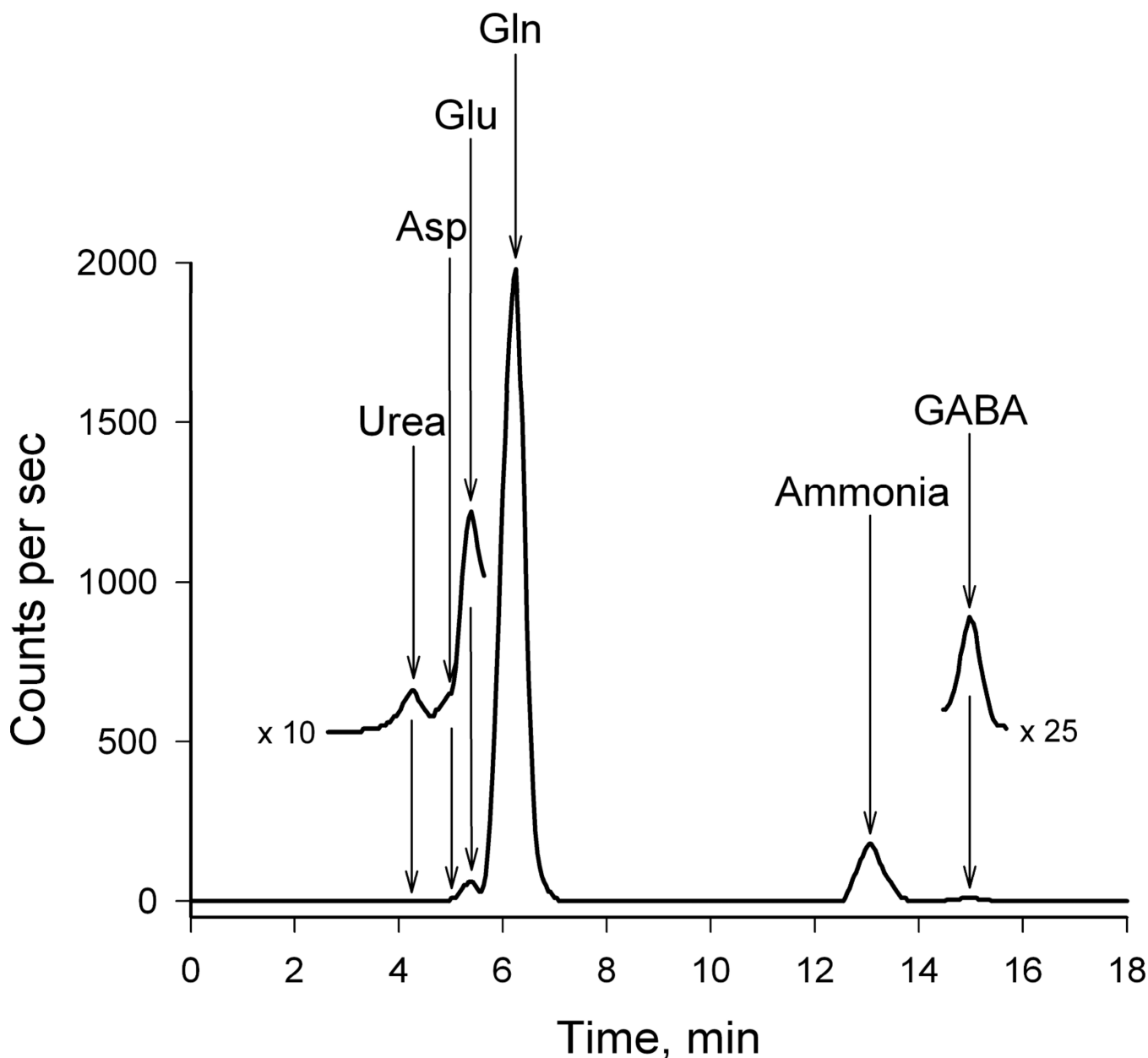


Fig. 1. HPLC profile of labeled metabolites in rat brain after a 20-minute infusion of [^{13}N]ammonia. The [^{13}N]ammonia in isotonic saline was administered intracarotidally at a rate of 0.15 ml/min into an adult male rat. At the end of the infusion the rat was sacrificed and the label in a deproteinized fraction (0.02 ml) of the brain was analyzed by HPLC by means of a cation exchange column as described in [21]. All counts in the HPLC profile were decay corrected back to an arbitrary time (start of HPLC). Modified from [21].

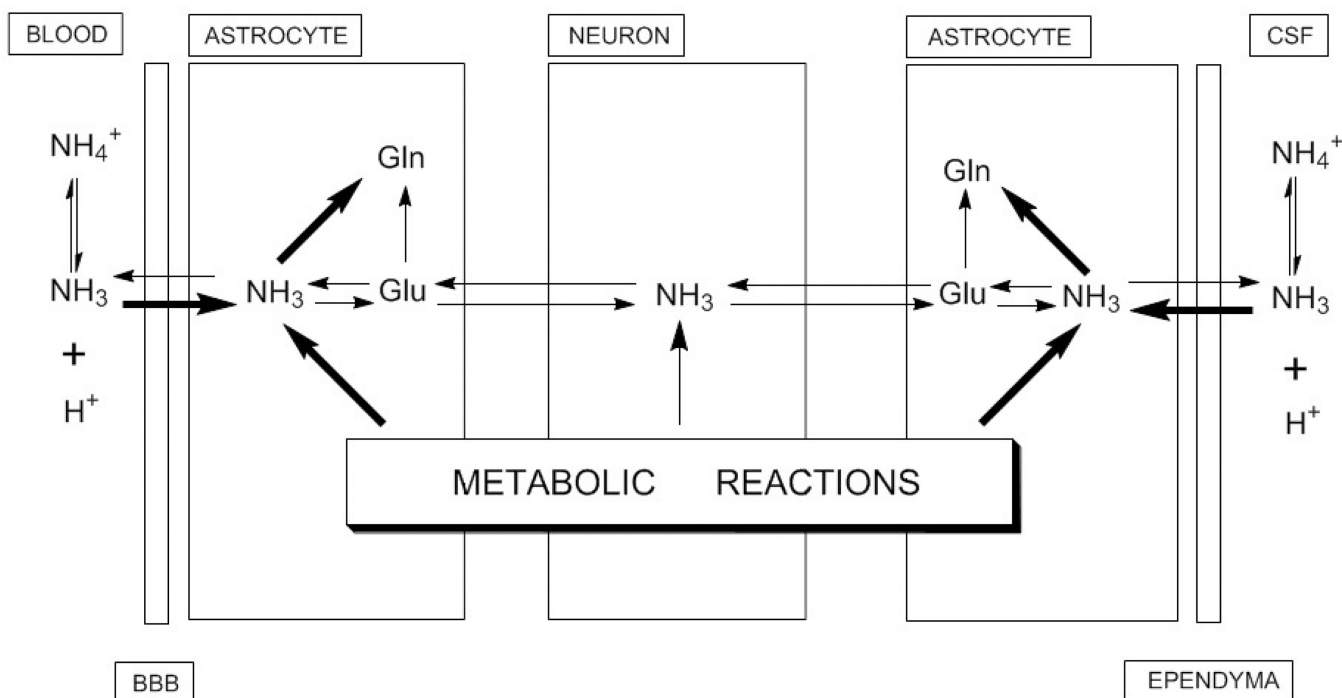


Fig. 2.

Compartmentation of ammonia metabolism in the brain. Ammonia entering the brain by diffusion from either the blood or CSF is rapidly metabolized in the small compartment (astrocytes) predominately to glutamine. Ammonia generated endogenously in both the neuronal and astrocytic compartments is also rapidly incorporated into glutamine within the astrocytic compartment. For convenience the protonated form of ammonia (ammonium, NH_4^+) is not shown in the brain compartments. The thickness of the arrows indicates the relative contribution of the GS reaction [$\text{NH}_3 \rightarrow$ glutamine Gln] and GDH reaction [$\text{NH}_3 \rightarrow$ glutamate (Glu)] to the metabolism of endogenously produced ammonia and blood/CSF-derived ammonia. Under normal conditions, the GDH reaction plays a minor role in metabolizing brain ammonia. In the neurons the GDH reaction is a mechanism for net oxidative deamination of glutamate under normal conditions. The GDH reaction is therefore not shown as a means of removing ammonia in the neuronal compartment.

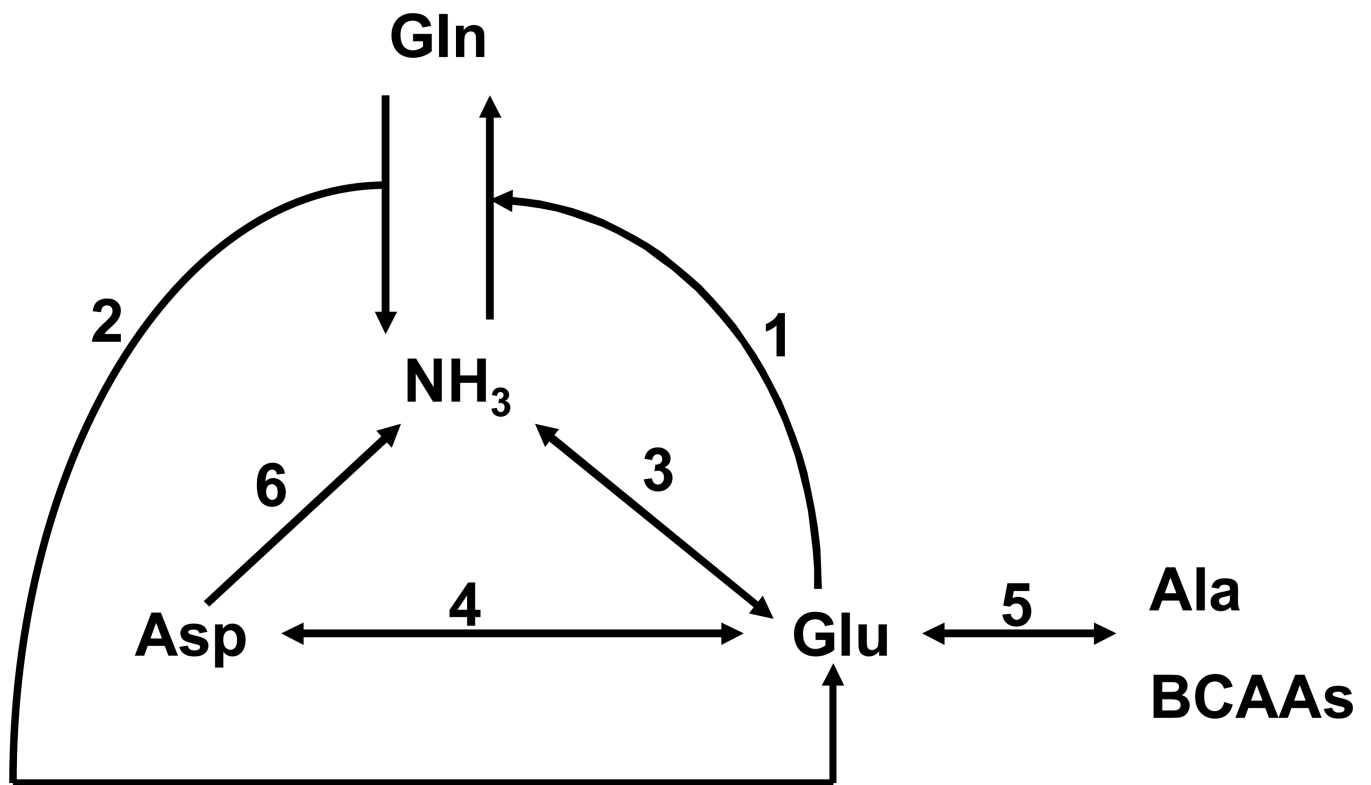


Fig. 3. Major routes for nitrogen metabolism in the brain and the central role of ammonia. Key to enzyme pathways: **1**, GS; **2**, glutaminase; **3**, GDH; **4**, AAT; **5**, alanine and branched-chain amino acid (BCAA) aminotransferases; **6**, net reaction of the PNC.

Table 1

Relative specific activities of ^{13}N -metabolites in rat brain after a 10 min-carotid artery infusion of [^{13}N]ammonia

| Metabolite | Controls (n = 4) | MSO-treated (n = 4) | P |
|-------------------|---------------------|------------------------|-------|
| Glutamine (amide) | [100 ± 29] | 8.0 ± 2.3 | <0.03 |
| Glutamine (amine) | 1.2 ± 0.4 | 0.6 ± 0.2 | NS |
| Glutamate | 0.26 ± 0.07 | 1.4 ± 0.2 | <0.03 |
| Aspartate | 0.56 ± 0.14 | 1.4 ± 0.6 | NS |

Tracer quantities of [^{13}N]ammonia were administered to immobilized rats for 10 min by continuous infusion via a carotid artery. At the end of the infusion, brains were rapidly frozen, deproteinized and analyzed for ^{13}N -labeled metabolites. Endogenous ammonia levels in the brains of control and MSO-treated rats were 0.181 ± 0.014 (n = 12) and 0.818 ± 0.098 (n = 12) mM, respectively. The specific activities of cerebral GDH in the control and MSO-treated rats were 196 ± 6 and 185 ± 4 $\mu\text{mol/h/g}$ wet weight, respectively; the specific activities of cerebral GS in control and MSO-treated rats were 54.2 ± 2.5 and 7.5 ± 2.6 $\mu\text{mol/h/g}$ wet weight, respectively (n = 12–24). From [16] as modified in [14]. The data reported in this table and in tables 2 and 3 are the means \pm SEM.

Table 2
 ^{13}N -Metabolite profile in rat brain after carotid artery injection of a bolus of [^{13}N]ammonia into portacaval-shunted (PCS) rats

| Treatment | Time after Injection (sec) | n | Percent of Label in Various Compounds | | |
|-----------|----------------------------|---|---------------------------------------|------------|-----------|
| | | | Ammonia | Glutamine | Glutamate |
| None | 5 | 5 | 46.8 ± 0.9 | 52.2 ± 0.8 | 1.0 ± 0.2 |
| None | 8 | 3 | 32.3 ± 1.6 | 66.3 ± 1.8 | 1.4 ± 0.1 |
| 14-wk PCS | 5 | 3 | 61.5 ± 0.9 | 38.0 ± 0.3 | 0.6 ± 0.3 |
| 14-wk PCS | 20 | 3 | 22.9 ± 2.5 | 72.7 ± 2.8 | 1.1 ± 0.2 |

Ammonia concentrations (μM) in control rat blood, PCS rat blood, control brain and PCS brain were 59 ± 8 (n = 19), 158 ± 31 (n = 24), 176 ± 25 (n = 14), 274 ± 26 (n = 14), respectively. From [16] as modified in [14].

Table 3

^{13}N -Metabolite profile in brain after carotid artery injection of a bolus of [^{13}N]ammonia into urease-treated rats

| Treatment | Time after Injection (sec) | n | Percent of Label in Various Compounds | | |
|-------------------|----------------------------|---|---------------------------------------|------------|-----------|
| | | | Ammonia | Glutamine | Glutamate |
| None | 5 | 5 | 44.6 ± 1.5 | 54.2 ± 1.8 | 1.3 ± 0.4 |
| 8-wk PCS | 5 | 5 | 60.0 ± 2.4 | 38.5 ± 2.2 | 1.3 ± 0.2 |
| Urease | 5 | 3 | 66.3 ± 6.2 | 32.8 ± 6.0 | 1.0 ± 0.1 |
| 8-wk PCS + urease | 5 | 2 | 60.7, 69.0 | 38.2, 29.6 | 1.1, 1.4 |

Ammonia concentrations (μM) in control rat blood, blood from urease-treated rats, control brain and brains from urease-treated rats were 59 ± 8 (n = 19), 322 ± 99 (n = 7), 176 ± 25 (n = 14), 424 ± 49 (n = 14), respectively. Ammonia levels in blood and brain from urease treated PCS rats were not measured. From [16] as modified in [14].