

LIMITED CAPACITY FOR AMMONIA REMOVAL BY BRAIN IN CHRONIC LIVER FAILURE: POTENTIAL ROLE OF NITRIC OXIDE

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ABSTRACT

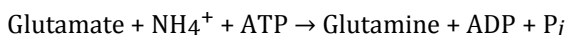
Chronic liver failure leads to hyperammonemia and consequently increased brain ammonia concentrations, resulting in hepatic encephalopathy. When the liver fails to regulate ammonia concentrations, the brain, devoid of a urea cycle, relies solely on the amidation of glutamate to glutamine through glutamine synthetase, to efficiently clear ammonia. Surprisingly, under hyperammonemic conditions, the brain is not capable of increasing its capacity to remove ammonia, which even decreases in some regions of the brain. This non-induction of glutamine synthetase in astrocytes could result from possible limiting substrates or cofactors for the enzyme, or an indirect effect of ammonia on glutamine synthetase expression. In addition, there is evidence that nitration of the enzyme resulting from exposure to nitric oxide could also be implicated. The present review summarizes these possible factors involved in limiting the increase in capacity of glutamine synthetase in brain, in chronic liver failure.

Key words: Chronic liver failure; ammonia; glutamine synthetase; nitric oxide; hepatic encephalopathy.

INTRODUCTION

There is a substantial body of evidence to suggest that ammonia has adverse effects on the brain and is therefore believed to play a major role in the pathogenesis of the neuropsychiatric disturbances observed in hepatic encephalopathy. When liver, the major ammonia-removing organ in the body, fails and is incapable of efficiently converting ammonia to urea or glutamine, hyperammonemia develops, leading to increased levels of ammonia in the brain. Unlike liver, brain is devoid of an effective urea cycle and therefore relies entirely on glutamine synthesis for the removal of blood-borne ammonia. Glutamine synthetase (GS) is strictly an astrocytic enzyme,

which is responsible for the adenosine triphosphate (ATP)-dependent amidation of glutamate to glutamine:



This specific reaction proceeds via a γ -glutamyl phosphate intermediate, and is dependent upon the presence of divalent cations such as manganese (Mn^{2+}).

GLUTAMINE SYNTHETASE IN CHRONIC LIVER FAILURE

Portal hypertension is a serious complication of chronic liver failure and is relieved either naturally from spontaneous collaterals between the portal vein and the systemic venous circulation, or surgically (as treatment) with a portacaval anastomosis or more commonly used today, transjugular intrahepatic portosystemic shunt (TIPS). In either case, portal-systemic shunting results in an increase in blood and brain ammonia, and hepatic encephalopathy develops.

In rats, 4 weeks following portacaval anastomosis, Girard *et al.* (1993) found an increase in brain ammonia and a concomitant decrease of GS activity in the brain. This finding was region selective, being observed in 3/7 brain regions (cerebral cortex, cerebellum, and hippocampus) (Fig. 1) with no change found in the other regions. Using the same model, studies by Butterworth *et al.* (1988) revealed a similar reduction in GS activity in cerebral cortex, and more recently Desjardins *et al.* (1999) reported similar findings in both cerebral cortex and cerebellum. Cooper *et al.* (1985) on the other hand did not find a decrease in GS activity and found no change in whole brain from portacaval-shunted rats. Others were unable to detect any significant increase in net glutamine synthesis in brain, following portacaval anastomosis (Cremer *et al.*, 1975; Ukida *et al.*, 1988). Many possible explanations have been forwarded (see later) to explain why the capacity of brain GS to remove ammonia is not increased to compensate for the elevated brain ammonia concentrations, in chronic liver failure. Cooper *et al.* (1985) showed that GS activity in the brain operates at near maximal capacity under normal physiological conditions and that induction does not occur. This lack of induction or, even worse, decrease of brain GS activity is a likely explanation for the precipitously high levels of brain ammonia in conditions of chronic liver failure (Butterworth *et al.*, 1988).

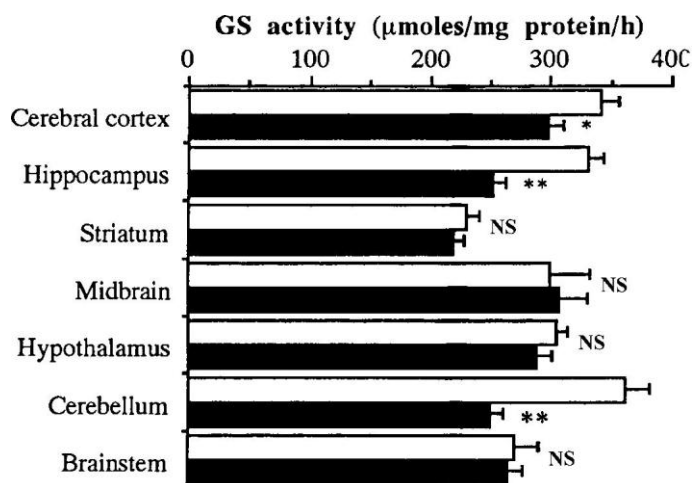


Figure 1. Effect of portacaval anastomosis on glutamine synthetase (GS) activity in different brain regions. White bars, sham-operated; black bars, portacaval anastomosis. The asterisk (*) indicates significantly different from sham-operated; NS, nonsignificant (modified from Girard *et al.*, 1993).

FACTORS INVOLVED IN LIMITING GLUTAMINE SYNTHETASE CAPACITY TO REMOVE AMMONIA

Cell (Astrocyte) Loss

Chronic liver failure does not result in significant loss of neural cells (i.e., neurons or astrocytes) in the brain (Butterworth, 2003). It is therefore highly unlikely that the reductions in the activity of GS in brain reported following portacaval anastomosis are the consequence of the loss of cells rich in this enzyme, since neuropathologic studies do not reveal evidence of astrocytic cell loss. However, alterations of the functional integrity of astrocytes have been described (Norenberg, 1987).

Decreased Protein or Gene Expression

It has been shown that the loss of activity of the GS enzyme is not associated with the loss of GS protein or gene expression in the brains of portacaval-shunted rats (Desjardins *et al.*, 1999).

Limited Concentrations of Glutamine Synthetase Enzyme Substrate

With increasing concentrations of ammonia in the brain during chronic liver failure, it is obvious that the amidation of glutamate to glutamine is not limited by the substrate ammonia in the pathogenesis of hepatic encephalopathy. However, many other factors may be altered, resulting in a decrease or non-induction of GS in astrocytes.

Glutamate is the substrate for GS, and therefore a decrease or increase in intracellular glutamate may affect GS activity. Termination of the action of glutamate in the synaptic cleft relies to a significant degree on its reuptake by high-affinity glutamate transporters

into perineuronal astrocytes where glutamate is transformed via GS into glutamine. Studies in astrocytes cultured from different brain regions of the rat demonstrate that the capacity for glutamate uptake correlates with regional GS activities (Hansson *et al.*, 1986). In chronic liver failure, using in vivo microdialysis, no increase in extracellular glutamate was observed in cerebral cortex (Rao *et al.*, 1995) or striatum (Tossmann *et al.*, 1983) of rats with portacaval anastomosis. It has been demonstrated that chronic hyperammonemia results in a region-selective loss of glutamate transporter sites. However, Desjardins and Butterworth found no change in gene expression of the astrocytic glutamate transporters EAAC-1 and EAAC-2 in cerebral cortex of rats with portacaval anastomosis (unpublished results). On the contrary, Suarez *et al.* (2000) described a significant loss of both astrocytic and neuronal glutamate transporter expression in cerebellum of portacaval-shunted rats (Suarez *et al.*, 2000). In brain tissue, concentrations of glutamate have been found to be decreased in portacaval-shunted rats (Giguere and Butterworth, 1984; Hindfelt *et al.*, 1977; Gjedde *et al.*, 1978) (Fig. 2). Together this data provides evidence to support that glutamate availability may be limited in some brain structures of portacaval-shunted rats. On the contrary, whether concentrations of glutamate in the astrocyte compartment (available to GS) are reduced remains unclear.

Brain Region	Glutamate Concentration		
	Sham Operated $\mu\text{mol/g}$	Portacaval Shunted	
		$\mu\text{mol/g}$	% Sham Operated
Cerebral cortex	11.35 \pm 1.46	5.67 \pm 1.69*	49.96*
Hippocampus	8.55 \pm 1.32	9.27 \pm 1.52	108.42
Striatum	7.96 \pm 2.90	14.54 \pm 2.12*	182.66*
Midbrain	6.89 \pm 1.15	8.60 \pm 0.68	124.82
Hypothalamus	7.94 \pm 0.71	4.68 \pm 1.20*	58.94*
Cerebellum	8.75 \pm 1.43	13.91 \pm 1.67*	158.97*
Medulla-pons	6.48 \pm 0.94	4.60 \pm 0.80*	70.99*

Figure 2. Brain glutamate concentrations following portacaval anastomosis. Results are expressed in $\mu\text{mol/g}$ wet weight tissue as mean \pm SD. The asterisk (*) indicates significantly different from sham-operated (modified from Giguere and Butterworth, 1984).

Each of the 12 active sites of GS requires two Mn^+ ions, a metallic ion whose availability is not limited in chronic liver failure. On the contrary, it has been demonstrated that increased manganese deposition occurs in the brain (more specifically in the basal ganglia) of patients with chronic liver failure (Rose *et al.*, 1999) and with spontaneous or surgically-induced portal-systemic shunting (Spahr *et al.*, 1996). Similar observations were found in rats with both portal-systemic shunting and liver dysfunction (Fig. 3). These results suggest that in chronic liver failure, manganese availability is adequate to maintain GS activity.

GS is an ATP-dependent enzyme. Although ammonia has been shown to inhibit α -ketoglutarate dehydrogenase in the tricarboxylic acid (TCA) cycle with the potential to

cause energy impairment, no evidence of energy failure has been reported in the brains of rats with chronic liver failure (Hindfelt *et al.*, 1977).

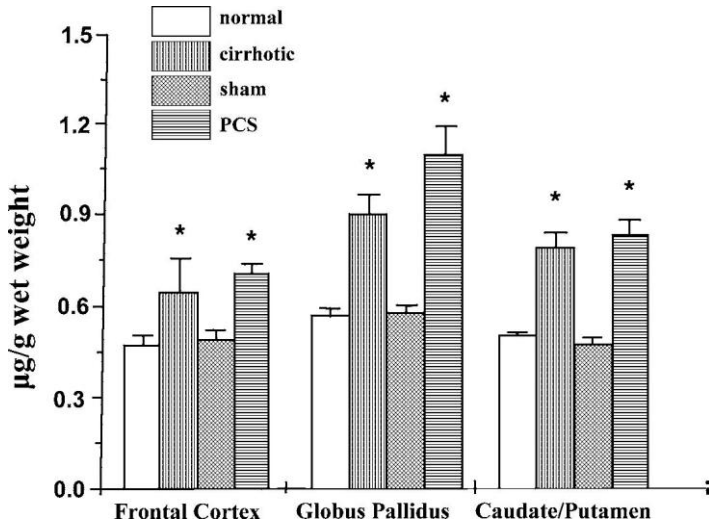


Figure 3. Manganese concentration in rat brain tissue. Data are expressed as mean \pm SEM. The asterisk (*) indicates significantly different from normal and sham-operated (modified from Rose *et al.*, 1999).

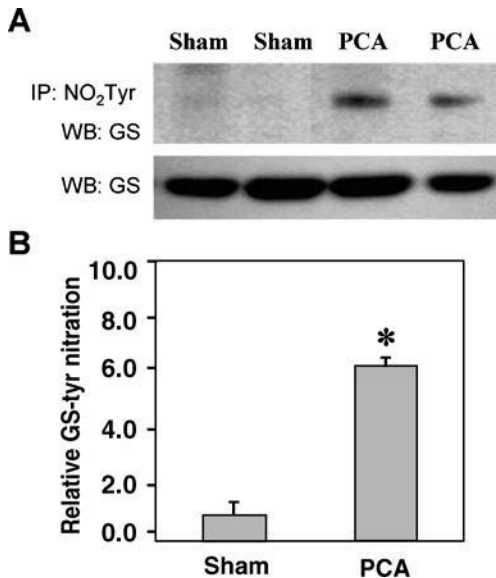
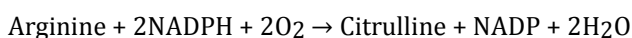


Figure 4. GS nitration in brain in chronic liver failure. PCA rats were sacrificed 4 weeks following portacaval anastomosis. (A) Brain proteins were precipitated with the anti-3-nitrotyrosine antibody and analyzed using Western blot for the presence of tyrosine-nitrated GS (NO₂Tyr-GS). (B) Densitometric quantification of tyrosine nitration of GS (modified from Schliess *et al.*, 2002).

There is recent evidence to suggest that a decrease in GS activity can also result from nitration of its tyrosine residues, leading to a loss of enzymatic activity. Ammonia-

induced tyrosine nitration of many proteins has been demonstrated in both in vivo (4-week portacaval-shunted rats) (Fig. 4) and in vitro models of hyperammonemia (Schliess *et al.*, 2002). It has been demonstrated in vitro (cultured astrocytes) that an increase in GS tyrosine nitration induced by ammonia is accompanied with a ~30% decrease in GS activity (Schliess *et al.*, 2002). Tyrosine nitration is mediated by reactive nitrogen species such as peroxynitrite anion (ONOO^-) and nitrogen dioxide ($\bullet\text{NO}_2$), formed as secondary products of nitric oxide ($\bullet\text{NO}$) metabolism in the presence of oxidants including superoxide radicals (O_2^-), and hydrogen peroxide (H_2O_2). $\bullet\text{NO}$ diffuses freely across cell membranes and is believed to play a major role in the nitration of GS. Nitric oxide synthase (NOS) catalyses the production of $\bullet\text{NO}$ with the oxidation of L-arginine to L-citrulline.



An increase in $\bullet\text{NO}$ production could lead to increased production of reactive nitrogen species and hence nitration of GS, lowering its capacity to remove ammonia.

L-Arginine is the obligate substrate for NOS reaction; its intracellular availability depends exclusively on its transport. There is convincing evidence for a stimulatory effect of ammonia on cellular L-arginine uptake. For example, hyperammonemia increases the transport of L-[^3H]arginine into synaptosomes of portacaval-shunted rats, and precipitation of severe encephalopathy following ammonia administration to these rats results in a further increase in synaptosomal L-[^3H]arginine uptake (Rao *et al.*, 1997a). Furthermore, hyperammonemia without liver dysfunction also stimulates arginine uptake by increasing the V_{max} without changing the affinity of L-arginine for the transporter. It was demonstrated that increased NOS activity was not responsible for the increased L-arginine uptake (Rao *et al.*, 1997a). None of the NOS inhibitors studied (L-NAME, L-NOARG, L-NMMA) had any effect on the ammonia-induced increase in synaptosomal L-arginine uptake, suggesting that ammonia directly activates the transport of L-arginine and that inhibition of NOS has little, if any, effect on this process. Furthermore, Rao *et al.* (1995) found that increased NOS activities were the consequence of increased availability of the enzyme substrate L-arginine. Overall, ammonia-induced L-arginine uptake is an important pathway in regulating NOS activity.

There are three different genes encoding NOS: (1) *nNOS* (*NOS-1*), which until recently, was considered to be expressed only in neurons; (2) *iNOS* (*NOS-2*), the inducible form, usually found in macrophages and turned on by an appropriate stimulus; and (3) *eNOS* (*NOS-3*) found in the endothelial cells that line the lumen of blood vessels. *nNOS* and *eNOS* are constitutively expressed and synthesize $\bullet\text{NO}$ in response to increases in intracellular calcium. Rao *et al.* (1997b) reported an increased nNOS protein and mRNA expression in the brains of portacaval-shunted rats (Fig. 5).

Calcium is an important regulator of NOS, acting through the binding of calmodulin. Felipo and colleagues demonstrated that ammonia-induced increases of NOS activity are dependent upon N-methyl D-aspartate (NMDA) receptor activation, resulting in an opening of the ion channel and entry of calcium. This leads to activation of soluble guanylate cyclase, leading to an increased formation of cyclic guanosine monophosphate (cGMP). Part of the cGMP formed is released into the extracellular space where it can be collected and served as a marker of NMDA-receptor-mediated NOS activity. This pathway is blocked by the pre-treatment of MK-801, a glutamate (NMDA) receptor antagonist (Hermenegildo *et al.*, 1996, 2000). Overactivation of NMDA receptors leading to an increase in NMDA/NOS/cGMP pathway has been shown to occur in acute hyperammonemia (Marcaida *et al.*, 1992; Montoliu *et al.*, 2002). However, in chronic liver failure, overactivation of NMDA receptors does not occur. Peterson *et al.* demonstrated a region-selective reduction of NMDA receptor densities in brains of portacaval-shunted rats (Peterson *et al.*, 1990). It is therefore unlikely that NMDA-receptor-mediated increases of the NO-cGMP signal transduction pathway are responsible for GS nitration in chronic liver failure.

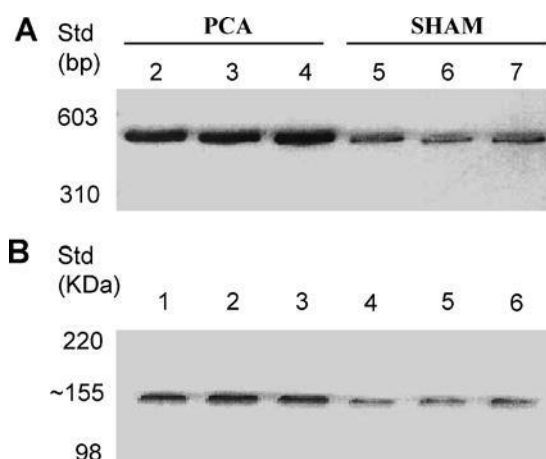


Figure 5. (A) nNOS expression (total RNA) in cerebellum of 4-week portacaval-shunted (lane 2–4) and sham-operated (lanes 5–7) rats. (B) Western blot analysis of cytosol protein from cerebellum of 4-week portacaval-shunted (lanes 1–3) and sham-operated (lanes 4–6) rats (modified from Rao *et al.*, 1997b).

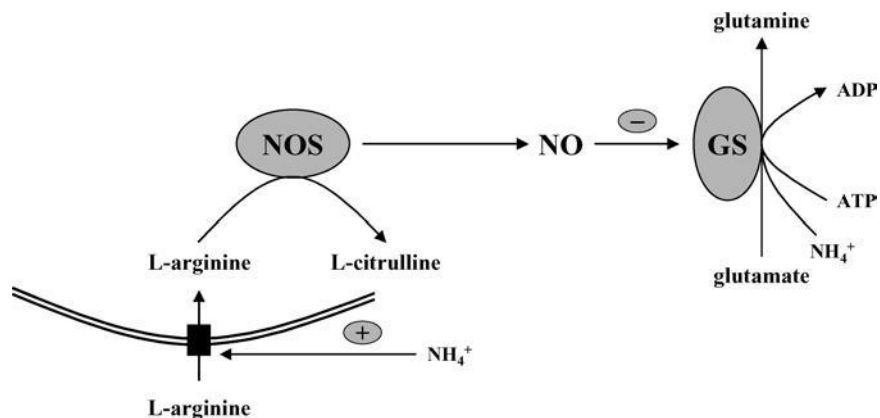


Figure 6. Hypothesis whereby ammonia stimulates L-arginine uptake into the cell, which activates NOS, resulting in an increased production of NO and consequently tyrosine nitration of GS. Even though GS is localized in astrocytes, the source of NO is yet to be defined, as NOS is found in both neurons and astrocytes (Baltrons and Garcia, 2001).

In summary, under hyperammonemic conditions, the brain is not capable of increasing its capacity to remove ammonia via its principal ammonia-removing mechanism, the astrocytic GS pathway. A review of currently available evidence supports the view that the reduction in GS capacity in brain (or lack of its induction, depending upon the brain region) in chronic liver failure does not result from a limitation in availability of substrates (NH_4^+ , glutamate) or cofactors (ATP, Mn^{2+}). The most likely explanation is GS nitration resulting from ammonia-induced increased synthesis of NO. This later mechanism appears to be independent of NMDA activation but dependent upon L-arginine (Fig. 6).

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