

Oral L-glutamine increases GABA levels in striatal tissue and extracellular fluid

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ABSTRACT We explored the possibility that circulating glutamine affects γ -aminobutyric acid (GABA) levels in rat striatal tissue and GABA concentrations in striatal extracellular fluid (ECF). Striatal microdialysates, each collected over a 20 min interval, were obtained after no treatment, oral L-glutamine (0.5 g/kg), or glutamine followed by NMDA (administered via the microdialysis probe). GABA concentrations were measured by HPLC using a stable OPA/sulfite precolumn derivatization and an electrochemical detection method. L-Glutamine administration significantly increased ECF GABA concentrations by 30%, and enhanced the response evoked by NMDA alone (70%) to 120% over baseline (all $P < 0.05$). Striatal GABA levels increased significantly 2.5 h after oral L-glutamine (e.g., from $1.76 \pm 0.04 \mu\text{mol/g}$ in vehicle-treated rats to $2.00 \pm 0.15 \mu\text{mol/g}$ in those receiving 2.0 g/kg of glutamine). Striatal glutamine levels also increased significantly, but not those of glutamate. These data suggest that GABA synthesis in, and release from, rat striatum may be regulated in part by circulating glutamine. Hence, glutamine administration may provide a useful adjunct for treating disorders (e.g., anxiety, seizures) when enhanced GABAergic transmission is desired. Moreover, the elevation in plasma and brain glutamine associated with hepatic failure may, by increasing brain GABA release, produce some of the manifestations of hepatic encephalopathy.—Wang, L., Maher, T. J., Wurtman, R. J. Oral L-glutamine increases GABA levels in striatal tissue and extracellular fluid. *FASEB J.* 21, 1227–1232 (2007)

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A NUMBER OF NEUROTRANSMITTERS exhibit the property of having their rates of synthesis and/or release depend primarily on brain levels of their amino acid precursors (1). Thus, tyrosine levels can control catecholamine synthesis in and release from neurons that are firing frequently (2, 3); serotonin's synthesis and release vary with brain tryptophan levels (4, 5). The synthesis of brain glutamate and γ -aminobutyric acid (GABA), the major central nervous system (CNS) excitatory and inhibitory neurotransmitters, respectively, are both linked to a substrate cycle involving glutamine

metabolism (6) in neurons and astroglial cells. Circulating glutamate is unable to cross the blood-brain barrier (BBB) (7) whereas glutamine can be transported into the brain by facilitated diffusion, a Na^+ -independent process (8) in competition with other neutral amino acids, or by a Na^+ -dependent process (mainly N-system transport) (9, 10), and brain glutamate serves as the precursor for GABA synthesis *in vivo* (11). The enzyme glutamate decarboxylase (GAD), which catalyzes the conversion of glutamate to GABA, has a high K_m for glutamate (2–5 mM) compared with usual glutamate levels in GABAergic neurons (1.6 mM) (12), and thus is likely unsaturated with its substrate under normal conditions (13).

The effects of providing supplemental glutamine on GABA synthesis and levels have been studied previously *in vitro*. In cultured rat pancreatic beta cells, cellular GABA content and release increased dose-dependently with increasing concentrations of glutamine (0.25–2 mM) in the medium (14); in synaptosomes prepared from substantia nigra, GABA synthesis was stimulated 3.1-fold when 500 μM glutamine was added to the incubation medium (15); and in brain slices, the rate of GABA synthesis, estimated in the presence of the GABA transaminase (GABA-T) inhibitor gabaculine (20 μM), was increased by 50% with 500 μM glutamine (16). Furthermore, inhibition of glutamine transport can deplete glutamate and GABA in brain slices (17), and nuclear magnetic resonance studies have been interpreted as showing that glutamine is the major precursor for GABA synthesis in rat *in vivo* (18). Apparently no information is available, however, about whether exogenous glutamine administration might affect brain GABA levels or release *in vivo*.

Plasma and brain glutamine levels can be augmented by exogenous glutamine. Giving preterm infants glutamine-supplemented parenteral nutrition (0.4 g/kg/day of glutamine) for 15 days increases their plasma glutamine levels by 50% without adverse effects (19). Giving rats L-glutamine intraperitoneally (2g/kg) increased brain and serum glutamine levels by 50% and 300%, respectively, without affecting those of glutamate

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or aspartate (20). In the current study, we determined whether brain GABA levels and GABA concentrations in ECF under conditions of spontaneous or evoked release can be increased by orally administering L-glutamine.

MATERIALS AND METHODS

Drugs and chemicals

Standards for GABA, glutamate, and L-glutamine were purchased from Sigma Chemicals (St. Louis, MO, USA). *o*-Phthalaldehyde (OPA) was purchased from Pierce Biotechnology (Rockford, IL, USA). Sodium sulfite, N-methyl-D-aspartic acid (NMDA), perchloric acid (PCA), sodium borate, GABase (a mixture of GABA-T and succinic semialdehyde dehydrogenase), GAD, and β -mercaptoethanol were purchased from Sigma Chemicals.

Animals

Sixty-six male Sprague-Dawley rats (3 months old, ~250 g) were purchased from the Charles River Laboratories (Wilmington, MA, USA). The animals were exposed to a 12 h light/dark cycle (7 AM to 7 PM). A standard rodent diet (LabDiet[®] 5P00), which lacks glutamine, and water were provided *ad libitum*. All experimental procedures were approved by the Massachusetts Institute of Technology Committee on Animal Care.

Surgery and microdialysis

Thirty-two rats (4 groups) were utilized to measure ECF GABA concentrations using *in vivo* microdialysis. Rats received vehicle (saline, 2.5 ml/kg; groups 1 and 2) or L-glutamine (0.5 g/kg; groups 3 and 4) by gavage, then half of them (groups 2 and 4) further received NMDA [500 μ M in artificial cerebrospinal fluid (aCSF)] *via* retrodialysis. L-Glutamine was mixed in saline to form a suspension (4 g to 10 ml saline); this was divided into daily doses and stored at -80°C .

Stereotaxic surgeries were carried out 1 wk prior to treatments, using CMA/11 guide cannula aimed at the right striatum [AP=+0.5, ML=-3.0, DV=3.3 mm; according to the atlas of Paxinos and Watson (21)], under ketamine and xylazine (80 and 10 mg/kg *i.p.*, respectively) anesthesia.

Microdialysis was performed on freely moving rats kept in a circular Plexiglas bowl on a rotating platform, obviating the need for a liquid swivel. Each rat was dialyzed once using a new CMA/11 probe (4 mm membrane length). ACSF containing, in mM: NaCl, 121; NaHCO₃, 25; KCl, 3.5; CaCl₂, 1.2; MgCl₂, 1.2; NaH₂PO₄, 1.0, was mixed with 95% O₂ and 5% CO₂ for 15 min. This gas mixture was used to bring the final pH close to 7.4. ACSF was then perfused through the probes continuously at a rate of 1.5 μ l/min. Samples collected during the first 2 h of microdialysis were discarded; subsequent collections over the next 4 h were for 20 min intervals. The first three samples were collected to establish baseline ECF GABA concentrations (estimated by actual GABA concentrations in the dialysates; data were not corrected for probe recoveries). Vehicle or L-glutamine were administered by gavage, and three samples were collected to study L-glutamine's effects on ECF GABA concentrations under conditions of spontaneous GABA release; then striata were perfused with aCSF or aCSF containing 500 μ M NMDA, and six additional samples were collected to study changes in ECF

GABA concentrations when GABA release was being evoked. This procedure yielded a total of 12 samples from each rat (3 baseline+3 samples under spontaneous conditions+6 evoked samples with or without NMDA=12). All experiments were carried out in a quiet cubicle. The activities of the rats were monitored for reference but not for data analysis. All samples were collected on crushed ice, then immediately frozen and kept at -80°C until HPLC analysis.

Sample preparation for plasma and striatal levels of glutamate, glutamine, GABA

Thirty-four rats (4 groups) were used to measure plasma and striatal glutamate, glutamine, and GABA levels after L-glutamine administration. Rats received vehicle (saline; 2.5 ml/kg) or L-glutamine (0.5, 1.0, and 2.0 g/kg). Preliminary microdialysis experiments showed that maximal GABA release evoked by NMDA occurs 2 to 3 h after L-glutamine administration; hence, in this study rats were anesthetized (with ketamine), sacrificed, and decapitated 2.5 h after dosing. Trunk blood and brain samples were then obtained.

Striata were quickly dissected on a chilled dissection board, homogenized on ice (50 mg of tissue with 1 ml 400 mM HClO₄, 50 μ M EDTA), and neutralized with 100 mM borate buffer (1:10). The homogenates were then centrifuged (14,000 rpm, 15 min, 4 $^{\circ}$ C) and filtered with Ultrafree-MC centrifugal filter units (Millipore, Billerica, MA, USA; 14,000 rpm, 1 min, 4 $^{\circ}$ C). Glutamate, glutamine, and GABA concentrations were determined by HPLC as described below.

GABA analysis

GABA concentrations in dialysates and striatal homogenates were determined using a precolumn OPA/sulfite derivatization method, according to Smith and Sharp (22). This HPLC electrochemical detection system consisted of a Shimadzu 10A HPLC pump, an Alltech 580 autosampler, an ESA Coulochem II 5100A detector, and an ESA 5014B microdialysis cell (E_1 =+150 mV, E_2 =+600 mV, E_{guard} =650 mV). Twenty microliter samples were automatically mixed with 10 μ l OPA/sulfite working derivatizing reagent; 20 min later, 25 μ l of this mixture was injected. The flow rate of the mobile phase (100 mM Na₂HPO₄; 10% acetonitrile) was 0.6 ml/min. The column (ESA MD 150, 3 \times 150 mm, 3 μ m) was kept at 40 $^{\circ}$ C. Samples were injected using an Alltech 580 autosampler, then data were collected and analyzed using an All-Chrom workstation (Alltech, Deerfield, IL, USA).

Stock solutions of the OPA/sulfite-derivatizing reagent were prepared by dissolving 22 mg OPA in 0.5 ml of absolute ethanol. To this was added 0.5 ml of 1M sodium sulfite, followed by 9 ml of 100 mM sodium borate buffer adjusted to pH 10.4 with sodium hydroxide. The working OPA/sulfite solution was prepared by diluting 50 μ l of OPA/sulfite stock solution with 5 ml of deionized water.

We validated the GABA peak in the dialysate using OPA/sulfite derivatization of samples pretreated with GABase, which destroyed the GABA. We were unable to validate the glutamate peak by converting the glutamate to GABA with GAD, then treating it with OPA/sulfite. Hence, glutamate and glutamine were measured using the OPA/ β -mercaptoethanol method as described below.

Glutamate and glutamine analysis

Glutamate and glutamine were measured as their OPA/ β -mercaptoethanol derivatives according to Donzanti and Yamamoto (23). HPLC conditions were similar to those used

for GABA determinations, except for the following: E_2 of the ESA 5014B microdialysis cell was set at + 530 mV, and the mobile phase consisted of 100 mM Na_2HPO_4 at pH 6.75 with 38% methanol. Twenty microliter samples were automatically mixed with 10 μl of the OPA/ β -mercaptoethanol working derivatizing reagent; 25 μl of this mixture was injected after waiting for 2 min.

Stock solutions of the OPA/ β -mercaptoethanol derivatizing reagent were prepared by dissolving 27 mg OPA in 1 ml of methanol. Five microliters of β -mercaptoethanol and 9 ml borate buffer (100 mM, pH 9.3) were then added. The working OPA/ β -mercaptoethanol solution was prepared by diluting 1 ml of OPA/ β -mercaptoethanol stock solution with 3 ml of borate buffer.

Data analysis

Statistical analyses were carried out using SPSS 12.0. Data are represented as means \pm SEs. Student's *t* test (2-tailed), 1-way, and 2-way ANOVA with repeated measurements were used to assess the statistical significance of effects. Tukey *post hoc* analyses were used when appropriate. The significance level was set at $P < 0.05$.

RESULTS

Effects of oral L-glutamine on striatal ECF GABA concentrations measured by microdialysis

Striatal baseline GABA concentrations in microdialysates did not differ ($F_{3, 29}=0.05$; $P>0.05$) among the four groups of rats prior to oral administration of vehicle or L-glutamine. Average values were 21 ± 1 nM (43.3 ± 2.1 pg/20 μl).

The effects of L-glutamine (0.5 g/kg) on striatal ECF GABA concentrations, estimated by its concentrations in dialysates, are shown in **Fig. 1A** (spontaneous release condition), and **B** (spontaneous release, followed by evoked release with NMDA). Each data point represents the sample collected during the preceding 20 min. During the first hour of collection, GABA baseline concentrations in each rat were established, pooled, and set as 100%. At the start of the second hour, glutamine or vehicle was administered orally. Two-way ANOVA with repeated measurements (glutamine or vehicle as between-subject factors and time as within-subject factors) revealed that glutamine increased ECF GABA concentrations compared with those after vehicle ($F_{1,9}=6.02$, $P<0.05$, Fig. 1A). The maximal increase (by 30% over baseline, $P<0.05$) occurred around the start of the 3rd hour (*i.e.*, 1 h after rats received glutamine; Fig. 1A). NMDA alone (500 μM in aCSF, perfused continuously during the 3rd and 4th hours) significantly increased ECF GABA concentrations, causing a maximal 70% rise ($P<0.01$; Fig. 1B). Among animals that had also received glutamine, the NMDA increased ECF GABA concentrations, causing a maximal 120% rise ($P<0.01$; Fig. 1B). L-Glutamine treatment increased ECF GABA concentrations under NMDA-stimulated conditions ($t=2.68$, $P<0.05$, Fig. 1B).

Changes in ECF GABA concentrations (δ value) between baseline samples during the first hour of collection and those thereafter are presented in Fig. 1C (spontaneous conditions; data collected during the first hour were omitted) and Fig. 1D (evoked conditions; data collected during the first 2 h were omitted). Horizontal axes indicate time (hours) of the microdialysis experiments. Data represent the average δ values per hour obtained by pooling the three 20 min interval samples; thus each data point represents the sample collected during the preceding hour. At the start of the second hour, vehicle or glutamine were administered (Fig. 1C); NMDA was perfused during the 3rd and 4th hours to rats that had or had not received glutamine (Fig. 1D). Two-way ANOVA using time (hours) and groups (glutamine or vehicle) as the factors revealed a significant effect of glutamine on ECF GABA concentrations under both spontaneous ($F_{1,41}=5.8$, $P<0.05$) and evoked conditions ($F_{1,29}=4.3$, $P<0.05$).

Effects of oral L-glutamine on striatal glutamate, glutamine, and GABA tissue levels

The effects of oral L-glutamine on striatal glutamate, glutamine, and GABA tissue levels are shown in **Table 1**. In vehicle-treated animals, striatal glutamate, glutamine, and GABA tissue levels were 11.25 ± 0.19 $\mu\text{mol/g}$, 5.60 ± 0.11 $\mu\text{mol/g}$, and 1.76 ± 0.04 $\mu\text{mol/g}$ (wet weight of tissue), respectively. Oral L-glutamine administration (0.5 g/kg) significantly increased striatal glutamine by 5% ($F_{2,29}=6.0$, $P<0.01$) and GABA levels by 11% ($F_{3,27}=3.5$, $P<0.05$) without affecting those of glutamate ($F_{2,29}=1.4$, $P>0.05$) (1-way ANOVA with L-glutamine dose as the factor). The effect of 1.0 g/kg glutamine (8%) on striatal GABA was not significant, but the effect of 2.0 g/kg (14%) was.

Effects of oral L-glutamine on plasma glutamate and glutamine levels

The effects of oral L-glutamine on plasma glutamate and glutamine concentrations are shown in **Table 1**. In vehicle-treated animals, plasma glutamate and glutamine concentrations were 116.9 ± 5.4 μM and 722.2 ± 38.5 μM . Oral L-glutamine administration significantly increased dose-dependently plasma glutamine concentrations obtained after 2.5 h ($F_{2,21}=4.6$, $P<0.05$) without affecting those of glutamate ($F_{2,21}=1.1$, $P>0.05$) (1-way ANOVA with L-glutamine dose as the factor).

DISCUSSION

These data demonstrate that giving animals L-glutamine, an amino acid precursor for brain glutamate and GABA synthesis, by gavage increases their striatal tissue levels and ECF concentrations of GABA (**Table 1**; **Fig. 1**), as measured by postmortem assays and *in vivo* microdialysis. The enhancement of ECF GABA occurs

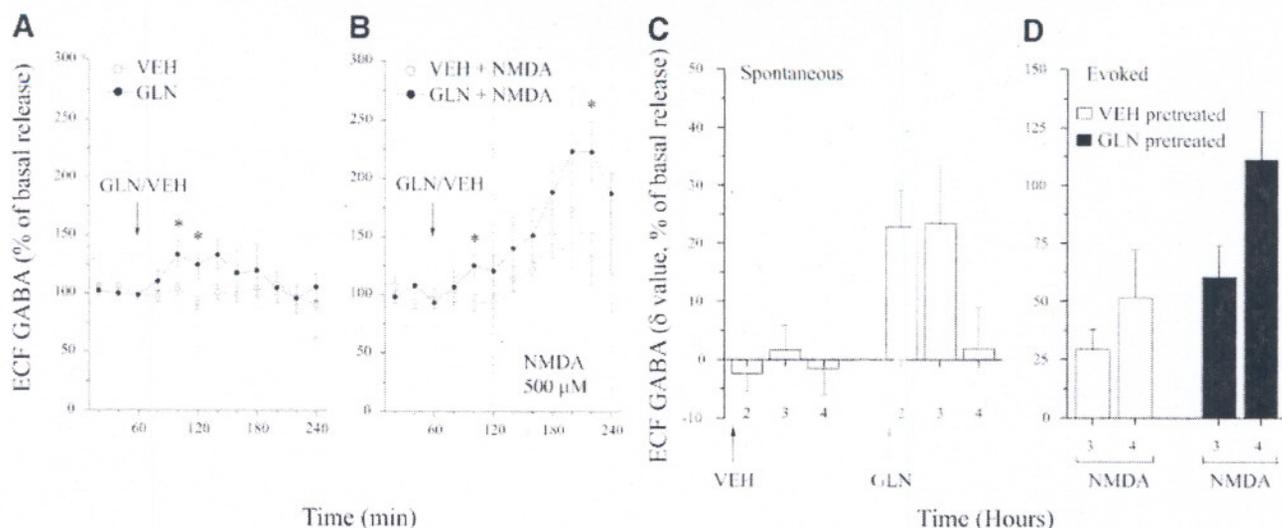


Figure 1. Effect of oral glutamine on ECF GABA concentrations. The effects of L-glutamine (0.5 g/kg) on striatal ECF GABA concentrations estimated by its concentrations in dialysates are shown in panel A (spontaneous release condition) and panel B (spontaneous release, followed by evoked release with NMDA). Each data point represents the sample collected during the preceding 20 min. During the first hour of collection, GABA baseline concentrations in each rat were established, pooled, and set as 100%. At the start of the second hour, glutamine or vehicle was administered orally. Glutamine increased ECF GABA concentrations during the next 2 h compared with those after vehicle (A). The maximal increase (by 30%, $P < 0.05$) occurred around the start of the 3rd hour (i.e., 1 h after rats received glutamine; panel A). NMDA alone (500 μ M in aCSF, perfused continuously during the 3rd and 4th hours) significantly increased ECF GABA concentrations, causing a maximal 70% rise ($P < 0.01$, panel B). Among animals that had also received glutamine, the NMDA dose increased ECF GABA concentrations, causing a maximal 120% rise ($P < 0.01$, panel B). Changes in ECF GABA concentrations (δ value) between baseline samples during the first hour of collection and those thereafter are presented in panel C (spontaneous conditions; data collected during the first hour were omitted) and panel D (evoked conditions; data collected during the first 2 h were omitted). Horizontal axes indicate time (hours) of the microdialysis experiments. Data represent the average δ values per hour obtained by pooling the three 20 min interval samples; thus each data point represents the sample collected during the preceding hour. At the start of the second hour, vehicle or glutamine were administered (C); NMDA was perfused during the 3rd and 4th hours to rats that had or had not received glutamine (D). Two-way ANOVA using time (hours) and groups (glutamine or vehicle) as the factors revealed a significant effect of glutamine on ECF GABA concentrations under both spontaneous and evoked conditions. Data are given as means \pm SE. * $P < 0.05$ vs. the groups of rats received vehicle with/without NMDA (unpaired Student's t test, 2-tailed). GLN: L-glutamine; VEH: vehicle.

under both spontaneous and evoked (by NMDA) conditions. Oral L-glutamine also dose-dependently increases plasma and striatal glutamine levels without affecting those of glutamate.

Measuring GABA is challenging given its low concentration in brain microdialysates. The common HPLC-based method for amino acid analysis, which utilizes a precolumn derivatization of OPA and β -mercaptoethanol, forms an unstable derivative with GABA, with a half-life of only several minutes (23). Hence, we used the OPA/sulfite derivatization procedure, which pro-

vides a sensitive and robust method to measure GABA concentrations in both brain tissue and microdialysis samples.

Oral L-glutamine administration most likely increased the synthesis and level of GABA by increasing the saturation of GAD, in GABAergic neurons, with glutamate. We hypothesize that some of the circulating glutamine crossed the BBB, and entered GABAergic neurons, where it was deaminated by phosphate-activated glutaminase (PAG) to glutamate, then decarboxylated by GAD to GABA.

TABLE 1. Effect of L-glutamine on brain and plasma levels of glutamate, glutamine and GABA^a

Treatment	Brain (μ mol/g wet weight of tissue)			Plasma (μ M)	
	Glutamate	Glutamine	GABA	Glutamate	Glutamine
vehicle	11.25 \pm 0.19 (11)	5.60 \pm 0.11 (11)	1.76 \pm 0.04 (11)	116.9 \pm 5.4 (9)	722.2 \pm 38.5 (9)
GLN (0.5 g/kg)	11.20 \pm 0.23 (9)	5.88 \pm 0.11 (9)*	1.96 \pm 0.05 (9)*	115.0 \pm 6.2 (8)	865.3 \pm 46.1 (6)*
GLN (1.0 g/kg)	10.81 \pm 0.20 (10)	6.09 \pm 0.08 (10) [‡]	1.91 \pm 0.07 (8)	105.6 \pm 3.9 (7)	887.0 \pm 44.9 (9)*
GLN (2.0 g/kg)			2.00 \pm 0.15 (4)*		

^aRats (250 g) were orally administered various doses of L-glutamine or vehicle. All drugs were given 2.5 h before sacrifice. Data are given as means \pm SE. GLN: L-glutamine. Number of determinations is shown in parentheses. * $P < 0.05$, differs from vehicle group. [‡] $P < 0.01$, differs from vehicle group.

Previous studies and our current data confirm that exogenous L-glutamine can increase glutamine levels in the brain (ref. 20 and Table 1). ^{13}C -Labeled tracer studies have shown that dietary glutamine can be absorbed unchanged into blood (24). L-[^{14}C] Glutamine perfusion studies in rats showed that plasma glutamine can be taken up into the brain, mainly by the Na^+ -dependent N-system transport (9, 10). Under normal conditions, the glutamine concentration in plasma is 0.5–0.8 mM (ref. 25 and Table 1), which is much lower than the K_m (3.3 mM) of the N-system transporter (9); hence, this transporter in BBB is unsaturated by circulating glutamine. Since ECF concentrations of glutamine, measured by microdialysis with a zero-net-flux technique, are in the submillimolar range (0.38 mM) (26), most of the increase in brain glutamine is intracellular, probably including GABA-forming neurons.

There appears to be a limited supply of glutamine available in GABAergic neurons to form glutamate. Brain contains mainly a kidney-type PAG (25), which exhibits a K_m for glutamine in the range of 2–5 mM (25). Glutamine concentration in GABAergic neurons is estimated to be ~1.6 mM [20% of the whole brain glutamine concentrations (12)], which is lower than the K_m of PAG. Moreover, given that glutamate levels in GABAergic neurons are much lower than that of glutamatergic neurons (discussed below) and PAG has essentially the same activity in cultured GABAergic and glutamatergic neurons (27, 28), PAG probably does not reach its maximal activity in GABAergic neurons, since apparently more glutamate can be formed in glutamatergic neurons.

Increased glutamate availability in GABAergic neurons may regulate GABA formation and release. Brain glutamate levels are ~10 $\mu\text{mol/g}$ tissue (20), but the distribution of glutamate is highly compartmentalized (29) and GABAergic neurons utilize a small precursor pool of glutamate (<5% of the total amount; ref. 30) in which the glutamate concentration (~0.6 mM) appears to be lower than the K_m of GAD (0.2–1.2 mM) (13). Moreover, the rate of GABA synthesis in brain is a small fraction (3–17%) of total GAD activity (13), indicating that GAD also operates at only a small fraction of its maximal catalytic capacity. GABA, like glutamate, is unable to cross the BBB (31); hence, glutamine-induced increase in brain GABA levels and release are unlikely to be derived from circulating GABA.

Our data suggest that increased circulating (32, 33) and brain (33–36) glutamine levels leading to increased concentrations and release of brain GABA may be involved in the hepatic encephalopathy of liver failure and ammonia toxicity (37). GABAergic transmission has been implicated in the pathogenesis of hepatic encephalopathy (38, 39), and antagonists of GABA receptors can ameliorate manifestations of this disorder. Decreased glutamate levels have consistently been observed in brain homogenates from rats with acute liver failure (40) and from patients who died of hepatic coma (41).

Deficient GABA-mediated brain neurotransmission may be a factor in various diseases, including anxiety disorders (42), epilepsy (43), and schizophrenia (44). The ability of oral L-glutamine administration to increase brain GABA level and release in rats raises the possibility that this compound might possess some therapeutic benefits in these disorders. [F]

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