

## Control of brain glutamine synthesis by nmda receptors

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## 1. ABSTRACT

Glutamine synthetase (GS) is involved in important processes in brain: modulation of the turnover of glutamate through the glutamate-glutamine cycle, detoxification of ammonia and, under certain circumstances, modulation of brain edema. Modulation of GS activity in brain is therefore important and its impairment or saturation may have pathological consequences. In this review we summarize the data showing that GS in brain is modulated by NMDA receptors and nitric oxide. Blocking NMDA receptors or nitric oxide synthase *in vivo* increases GS activity and glutamine content in brain, indicating that tonic activation of NMDA receptors and nitric oxide synthase maintain a tonic inhibition of GS. NMDA receptor-mediated activation of nitric oxide synthase is responsible only for part of the inhibition of GS. Other sources of nitric oxide also contribute to tonic inhibition. The inhibition is due to a covalent modification of GS, likely nitration of tyrosine residues. This modification would be reversible and it would be an enzyme that denitrosylate or denitrate GS. Moreover, GS would not be working at maximum rate and its activity may be increased pharmacologically by manipulating NMDA receptors or nitric oxide content. This may be useful for example to increase ammonia detoxification in brain in hyperammonemic situations.

## 2. INTRODUCTION

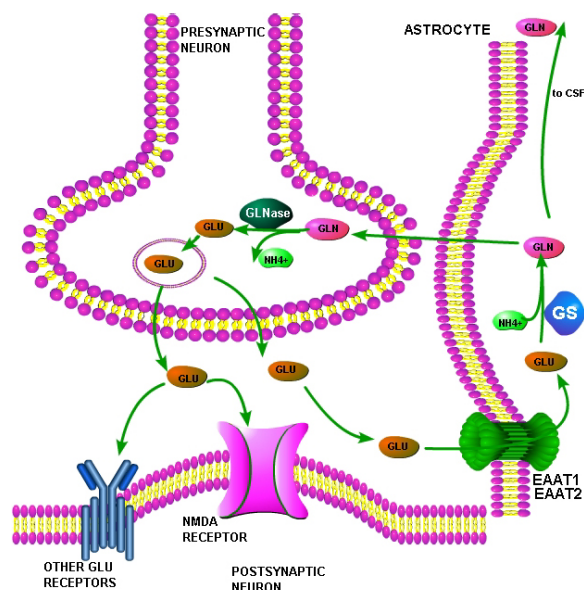
### 2.1. The glutamate - glutamine cycle

Glutamate is the main excitatory neurotransmitter in mammals and has two main types of receptors: metabotropic and ionotropic. Activation of ionotropic receptors leads to the opening of ion channels allowing the transport through them of Na<sup>+</sup>, K<sup>+</sup> and, in some cases, Ca<sup>2+</sup>. There are three main types of ionotropic glutamate receptors: NMDA, AMPA and kainate receptors.

Activation of NMDA receptors by its natural agonist glutamate leads to the opening of the ion channel allowing the entry of calcium and sodium in the post-synaptic neuron. The increase in intracellular calcium activates different enzymes and signal transduction pathways that are involved in the control of important cerebral processes such as neuronal plasticity, learning, memory, etc. However, excessive activation of NMDA receptors activates neurotoxic signal transduction pathways leading to neuronal degeneration and death (1-4).

To avoid excessive activation of its receptors, glutamate must be removed from the extracellular fluid and this is carried out by specific transporters that take up glutamate and transport it into the cells (5-6). Glutamate transporters are present both in neurons and in astrocytes,

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**Figure 1.** The glutamate-glutamine cycle in brain. Glutamate released into the synaptic cleft acts on postsynaptic receptors (NMDA and other types of glutamate receptors). Then glutamate is rapidly removed from synaptic cleft by glutamate transporters (e.g. EAAT1 and EAAT2) that are mainly located on surrounding astrocytes. Within the astrocytes, glutamate and ammonia are combined to form glutamine by glutamine synthetase (GS), an astrocyte-specific enzyme. To replenish the neurotransmitter pool of glutamate, glutamine is released from astrocytes and taken up by glutamatergic neurons. Once glutamine is taken up into the neuron phosphate-activated glutaminase (GLNase) splits it into glutamate and ammonia. Glutamate is then incorporated in synaptic vesicles that will release it to the synaptic cleft, starting a new cycle.

but the uptake from the synaptic cleft is mainly carried out by astrocytic transporters. In astrocytes, glutamate taken from the extracellular fluid is converted by glutamine synthetase to glutamine which is released to the extracellular fluid, taken up by neurons and hydrolyzed there by glutaminase to glutamate, thus replenishing the neurotransmitter pool (Figure 1).

This trafficking of glutamate and glutamine between astrocytes and neurons, usually called "the glutamate-glutamine cycle", is the major pathway by which the neurotransmitter pool of glutamate is recycled.

### 2.2. Glutamine metabolism in brain

After uptake into astrocytes, glutamate is converted to glutamine by glutamine synthetase (GS), which incorporates a molecule of ammonia in the molecule of glutamate and consumes one molecule of ATP:  
 $\text{Glutamate} + \text{NH}_4^+ + \text{ATP} \rightarrow \text{Glutamine} + \text{ADP} + \text{Pi}$

Ammonia is a normal product of degradation of proteins and other compounds, but at high concentrations ammonia is toxic and leads to functional disturbances of

the central nervous system and can lead to coma and death. To avoid the toxic effects of ammonia it is usually detoxified in the liver by incorporation into urea that is eliminated in urine. The enzymes of the urea cycle are not expressed in brain. The main mechanism for ammonia detoxification in brain is its incorporation in glutamine by glutamine synthetase.

Glutamine may also act as an osmolyte in brain and contributes to modulation of osmotic pressure and of brain edema (7-8). Glutamine synthetase plays therefore at least two very important roles in brain: Glutamine 1) modulates the turnover of the neurotransmitter pool of glutamate and 2) detoxifies ammonia and, under certain circumstances, it may modulate brain edema. Impairment or saturation of glutamine synthetase activity may lead therefore to important pathological consequences.

Glutamine synthetase expression in brain is limited almost exclusively to astrocytes (9), which also play therefore an essential role in the above processes: modulation of glutamate neurotransmitter pool and neurotransmission and detoxification of ammonia.

Glutamine synthesized in astrocytes is released through glutamine transporters to the extracellular fluid and it is taken up by other transporters in neurons (10). Within the neurons, glutamine is split again to glutamate and ammonia by the intra-mitochondrial phosphate-activated enzyme glutaminase. More details of the general aspects of glutamine metabolism in the brain are described by Jan Albrecht *et al.*, in other review in this issue.

### 3. MODULATION OF GLUTAMINE LEVELS IN BRAIN BY NMDA RECEPTORS AND NITRIC OXIDE.

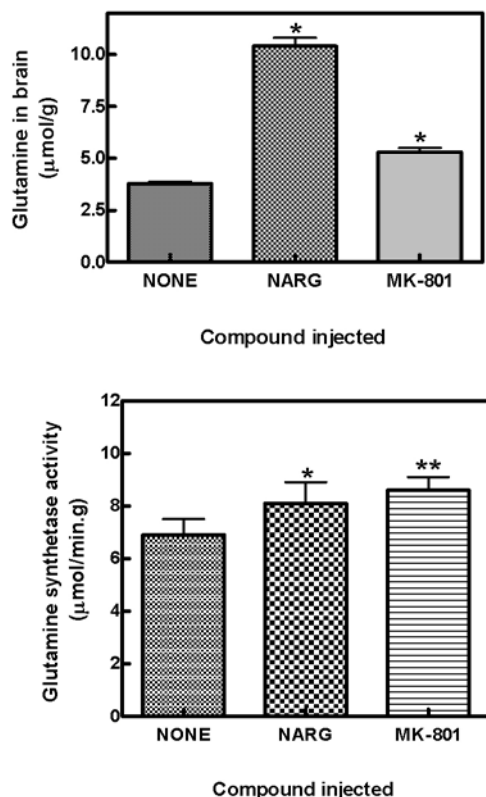
In this review we will summarize the data reported in the literature concerning the modulation of glutamine levels in brain by NMDA receptors.

Activation of NMDA receptors leads to the opening of the ion channel allowing the entry of calcium and sodium in the post-synaptic neuron. Calcium binds to calmodulin (CM) and activates several enzymes including neuronal nitric oxide synthase (nNOS). This leads to an increase in the formation of nitric oxide (NO) (11-13).

Nitric oxide is a radical that may react with different groups in the proteins. Moreover nitric oxide can also react with the superoxide ion to produce peroxynitrite that can also react with proteins. Nitric oxide can lead directly or indirectly (via peroxynitrite) to posttranslational protein modifications such as nitrosylation or nitration of the proteins (14-17). One of these reactions by which nitric oxide may modify proteins is nitration of tyrosine residues leading to formation of nitrotyrosine. When the proteins modified are enzymes these modifications may lead to changes (either activation or inhibition) in the activity of the enzyme (14-17).

Nitric oxide is a gas and therefore the nitric oxide formed in the post-synaptic neuron following activation of

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**Figure 2.** Blocking NMDA receptors with MK-801 or inhibiting nitric oxide synthase with nitroarginine increases glutamine synthetase activity and glutamine content in brain. Groups of 8 rats were injected i.p. with 45 mg/kg of nitroarginine, with 2 mg/kg of MK-801 or with saline (controls). Rats were killed by decapitation 25 min after injection. Values are significantly different from controls are indicated by asterisks (\*  $p < 0.01$ ; \*\*  $p < 0.001$ ). Data with permission from 24, 31.

NMDA receptors can diffuse to neighboring astrocytes, react with glutamine synthetase and modify its activity. In this review we summarize the reports that support that NMDA receptors and nitric oxide modulate glutamine synthetase activity and glutamine levels in brain.

### 3.1. *In vivo* studies

#### 3.1.1. Tonic activation of NMDA receptors maintains a tonic inhibition of glutamine synthetase in brain: blocking NMDA receptors in brain *in vivo* increases glutamine synthetase activity and glutamine content in brain

As mentioned above, high ammonia levels are toxic and may lead to death. It has been shown that acute intoxication with high ammonia levels leads to excessive activation of NMDA receptors in brain which is responsible for ammonia-induced death which may be prevented by blocking NMDA receptors with selective antagonists (18-20).

Acute ammonia toxicity leads to ATP depletion in brain which contributes to the toxic effects

of ammonia and to ammonia-induced death of animals. Acute ammonia toxicity provides large amounts of substrate for glutamine synthetase, leading to increased formation and content of glutamine and therefore to increased consumption of ATP. It has been suggested that increased glutamine synthesis play a main role in the mediation of ammonia-induced depletion of ATP and of ammonia-induced toxicity and death (21-23).

Kosenko *et al.* (24) performed experiments to discern the relative role of glutamine synthesis and of activation of NMDA receptors in the ammonia-induced depletion of brain ATP and death. In these studies rats were injected i.p. with: 1) saline (control group); 2) a large dose of ammonia (7 mmol/kg); 3) the same dose of ammonia after blocking NMDA receptors by injecting MK-801; 4) another control group of rats injected only with MK-801. This last control group is the more relevant for the present review and provided the first evidence for the modulation of glutamine levels in brain *in vivo* by NMDA receptors.

Kosenko *et al.* (24) studied the levels of ATP and glutamine in brain and the activity of glutamine synthetase in each group 15 min after ammonia (or saline) injection. The results clearly indicated that ammonia-induced depletion of ATP is not due to increased glutamine formation but to activation of NMDA receptors. What is more important for this review is that the content of glutamine in brain was significantly increased in the rats injected only with MK-801 (Figure 2). In control rats the content of glutamine was  $3.8 \pm 0.1 \mu\text{mol/g}$  brain; however in rats injected only with MK-801 glutamine content was  $5.3 \pm 0.2 \mu\text{mol/g}$ . The authors assessed whether this significant increase in glutamine content induced by blocking NMDA receptors was due to increased glutamine synthetase activity. The activity of this enzyme was determined *in vitro* in brain homogenates from control rats and from rats injected with MK-801. The activity in control rats was  $6.9 \pm 0.6 \mu\text{mol/min} \times \text{g}$ . In rats injected with MK-801 the activity was significantly increased to  $8.6 \pm 0.5 \mu\text{mol/min} \times \text{g}$  (24) (see Figure 2).

These results show that blocking NMDA receptors in rat brain *in vivo* increases glutamine content in brain and the activity of glutamine synthetase and indicate that tonic activation of NMDA receptors maintains a tonic inhibition of glutamine synthetase in brain. Blocking NMDA receptors “releases” this tonic inhibition and results in increased activity of glutamine synthetase.

Moreover, the above results also show that the effect of blocking NMDA receptors *in vivo* by injecting MK-801 is maintained in the *in vitro* assay of glutamine synthetase activity after preparation of brain homogenates. This indicates that the effect is due to a covalent modification (see below) of the enzyme that remains after sample preparation.

### 3.1.2. Tonic activity of nitric oxide synthase maintains a tonic inhibition of glutamine synthetase in brain. Inhibition of nitric oxide synthase *in vivo* increases glutamine synthetase activity and glutamine content in brain

NMDA receptors are mainly located in neurons while glutamine synthetase is located in astrocytes (25). The modulation of glutamine synthetase activity by NMDA receptors would require therefore an intercellular messenger that can “sense” the activation state of NMDA receptors in neurons and “transmit” this information to glutamine synthetase in astrocytes. A good candidate to act as intercellular messenger is nitric oxide. Activation of NMDA receptors leads to increased intracellular  $\text{Ca}^{2+}$ , which binds to calmodulin and activates nitric oxide synthase, increasing the formation of nitric oxide. This compound is a gas and therefore can diffuse to neighboring astrocytes and react with glutamine synthetase. Nitric oxide is a radical and it has been reported that bacterial glutamine synthetase activity is inhibited by oxidants (26-30). We therefore proposed that nitric oxide formed in neurons following activation of NMDA receptors and nitric oxide synthase may reduce the activity of glutamine synthetase in astrocytes.

To assess this possibility we tested whether inhibition of nitric oxide synthase in rat brain *in vivo* (by i.p. injection of nitroarginine) is also able to increase the content of glutamine and the activity of glutamine synthetase in brain.

The content of glutamine in brain of control rats was 3.9  $\mu\text{mol/g}$  tissue and increased to 10.2  $\mu\text{mol/g}$  15 min after i.p. injection of nitroarginine (Figure 2). In the same experiment, the content of glutamate in control rats was 8.9  $\mu\text{mol/g}$  tissue and decreased to 5.9  $\mu\text{mol/g}$  in rats injected with nitroarginine (31). This clearly shows that inhibition of nitric oxide synthase increases remarkably glutamine content in brain.

We then assessed whether this is due to increased glutamine synthetase activity. The activity in brain homogenates from control rats was  $7.9 \pm 0.6 \mu\text{mol/ min} \times \text{g}$ . In brain homogenates from rats injected with nitroarginine the activity was significantly increased to  $9.1 \pm 0.4 \mu\text{mol/ min} \times \text{g}$  (31) (see Figure 2). These results show that inhibition of nitric oxide synthase in rat brain leads to increased glutamine synthetase activity, indicating that tonic activity of nitric oxide synthase maintains a tonic inhibition of glutamine synthetase. Inhibition of nitric oxide synthase “releases” this inhibition and results in increased activity of glutamine synthetase.

It should be noted that in rats injected with nitroarginine to inhibit nitric oxide synthase glutamine content in brain reaches 10.2  $\mu\text{mol/g}$  while in rats injected with MK-801 to block NMDA receptors glutamine only reaches 5.3  $\mu\text{mol/g}$  (Figure 2). The increase in glutamine is therefore larger when nitric oxide synthase is inhibited than when NMDA receptors are blocked. This suggests that NMDA-mediated activation of nitric oxide synthase provides only part of the nitric oxide that is tonically

inhibiting glutamine synthetase and that NO derived from other sources (e.g. other nitric oxide synthases) is also contributing to the nitric oxide-mediated inhibition of glutamine synthetase in brain.

### 3.2. *In vitro* studies

#### 3.2.1. NMDA inhibits glutamine synthetase activity in brain slices *in vitro* by a mechanism involving nitric oxide synthase

McBean *et al.* (32) showed that incubation of coronal slices of rat brain with neurotoxic concentrations of NMDA (500  $\mu\text{M}$ ) for 40 min reduces the activity of glutamine synthetase by 21%. NMDA did not directly inhibit the activity of glutamine synthetase. Pre-incubation of the slices with nitroarginine, a competitive inhibitor of nitric oxide synthase effectively prevented the NMDA-induced reduction in glutamine synthetase activity (32). These results are in complete agreement with the results of Kosenko *et al.* (24, 31) summarized above.

#### 3.2.2. Glutamine synthetase activity is reduced by nitric oxide due to a (likely reversible) covalent modification of the enzyme

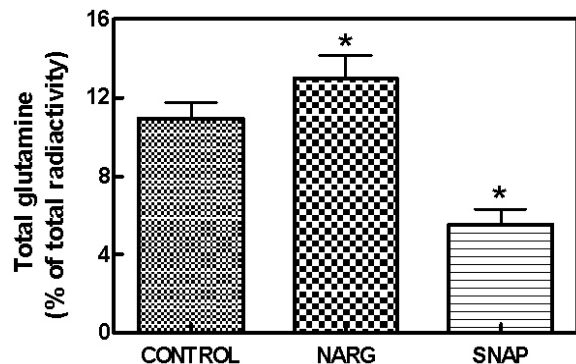
In the studies of Kosenko *et al.* (24, 31) summarized above, the effects of i.p. injection of MK-801 or of nitroarginine injection on glutamine synthetase activity can be observed in the *in vitro* assay after preparation of brain homogenates, indicating that the effects are due to a covalent modification of the enzyme.

This suggests that nitric oxide induces a nitration or a nitrosylation of glutamine synthetase that reduces its activity. Moreover, the results also indicate that the effect is reversible and that inhibition of nitric oxide synthase (or blocking of NMDA receptors) results in increased activity (reduced inhibition) of glutamine synthetase. This suggests that there must be an enzyme that denitrosylates or denitrates glutamine synthetase, resulting in increased activity of the enzyme.

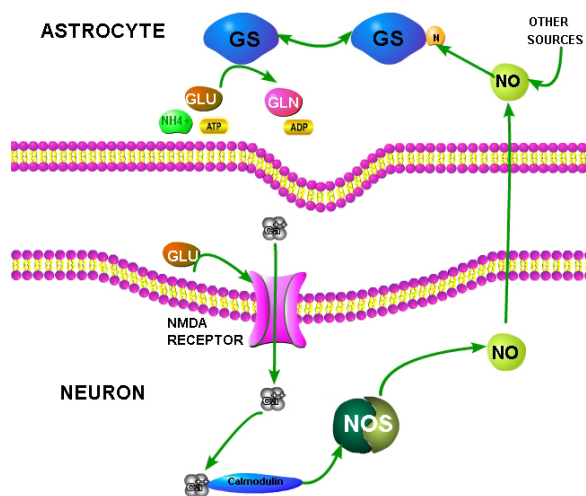
To further confirm that nitric oxide inhibits glutamine synthetase we assessed in primary cultures of astrocytes whether addition of a nitric oxide generating agent (SNAP) reduces the activity of glutamine synthetase in the intact astrocytes in culture. We also assessed whether inhibition of endogenous nitric oxide synthase activity with nitroarginine increases glutamine synthetase activity.

The activity of glutamine synthetase in intact astrocytes was determined by adding radioactive glutamate to the culture medium. This glutamate is taken up by the astrocytes and is used by glutamine synthetase to form radioactive glutamine. Part of the glutamine formed remains into the astrocytes and part is released to the extracellular medium. We measured the time-course of the changes in radioactive glutamate and glutamine in the culture medium and into astrocytes (33). Formation of radioactive glutamine is a direct measure of the activity of glutamine synthetase in the astrocytes. The total formation of glutamine (intracellular + extracellular) is shown in Figure 3. In control astrocytes there is a linear continuous formation of radioactive glutamine by glutamine synthetase

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**Figure 3.** The nitric oxide generating agent SNAP reduces glutamine synthetase activity in intact astrocytes in culture. Primary cultures of rat astrocytes were treated with 1 mM SNAP or 200  $\mu$ M nitroarginine (NARG, inhibitor of nitric oxide synthase) and [ $^{14}$ C]glutamate. [ $^{14}$ C]glutamine formed by glutamine synthetase was measured at 60 minutes. Values that are significantly different from controls are indicated by asterisks (\*  $p < 0.001$ ). Data with permission from 33.



**Figure 4.** Proposed mechanism of modulation of glutamine synthetase activity by NMDA receptors and nitric oxide. Activation of NMDA receptors leads to activation of neuronal nitric oxide synthase (NOS) and increases the formation of nitric oxide (NO). NO is a gas that can diffuse to neighbouring astrocytes and modify covalently glutamine synthetase (GS) (likely by tyrosine nitration). This modification reduces GS activity. In addition of the NO generated as a consequence of activation of NMDA receptors, other sources of NO also contribute to modulation of glutamine synthetase activity in rat brain *in vivo*. The covalent modification of glutamine synthetase induced by NO would be reversible, likely by a not yet identified enzyme. Reducing the activation of NMDA receptors or of nitric oxide synthase reduces the tonic inhibition of glutamine synthetase by NO resulting in enhanced activity of the enzyme. Modified with permission from 33.

and addition of the nitric oxide-generating agent SNAP reduces glutamine formation by more than 50%. This confirms that in intact astrocytes in culture nitric oxide inhibits glutamine synthetase activity (Figure 3).

Moreover, nitroarginine added to the culture medium increased slightly but significantly the formation of glutamine, indicating that endogenous nitric oxide synthase maintains a mild tonic inhibition of glutamine synthetase in astrocytes in culture. These results show that nitric oxide inhibits glutamine synthetase activity in a reversible manner.

A possible mechanism to explain the results of the above studies is summarized in the scheme shown in Figure 4. Activation of NMDA receptors leads to activation of neuronal nitric oxide synthase and increases the formation of nitric oxide. This radical can diffuse to neighboring astrocytes and modify covalently glutamine synthetase (likely by tyrosine nitration or nitrosylation). This modification reduces the activity of glutamine synthetase. In addition to the nitric oxide generated as consequence of activation of NMDA receptors, other sources of nitric oxide also contribute to modulation of glutamine synthetase activity in rat brain *in vivo*. The covalent modification of glutamine synthetase induced by nitric oxide is reversible, likely by a not yet identified enzyme. Reducing the activation of NMDA receptors or of nitric oxide synthase reduces the tonic inhibition of glutamine synthetase by nitric oxide resulting in enhanced activity of the enzyme (Figure 4).

### 3.2.3. In cultured astrocytes, glutamine synthetase can be nitrated in tyrosine by activation of NMDA receptors

Schliess *et al.* (34) studied the effects of addition of ammonia to primary cultures of astrocytes on protein tyrosine nitration. They showed that ammonia induces nitration of tyrosines in several proteins, including glutamine synthetase. Ammonia-induced nitration of most proteins is prevented by previous blocking of NMDA receptors with MK-801 and addition of NMDA induces a pattern of protein nitration similar to that induced by ammonia. Ammonia-induced protein nitration was mediated by induction of iNOS. Although it was not studied specifically for glutamine synthetase, the authors assume that nitration of glutamine synthetase is also mediated by activation of NMDA receptors and subsequent increase in nitric oxide. The increase in glutamine synthetase nitration was associated with a significant decrease of its activity by ca. 30% in cultured astrocytes (34). The same group also showed later that tyrosine nitration of glutamine synthetase is also increased in liver of rats treated with lipopolysaccharide and that this was also associated with a reduction of the activity of the enzyme, indicating that modulation of glutamine synthetase by nitric oxide may occur in most tissues (35). Modulation of glutamine synthetase by NMDA receptors has been only shown for the moment in brain.

Schliess *et al.* (34) also showed that nitration of glutamine synthetase is increased in brain of rats injected with ammonia as well as in rats with porta-caval

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anastomosis, an animal model of chronic liver failure and hyperammonemia.

These results agree with the previous reports showing that activation of NMDA receptors (either tonic or induced by ammonia) reduces the activity of glutamine synthetase by ca. 20-30% in brain and that this is due to a nitric oxide-mediated covalent modification of glutamine synthetase (24, 31, 36). Moreover, the report of Schliess *et al.* (34) shows that this covalent modification would be nitration of tyrosine residues.

Schliess *et al.* (34) propose that activation of NMDA receptors in astrocytes would mediate the effects of ammonia on glutamine synthetase nitration. It is considered that NMDA receptors are located essentially in neurons. The presence of NMDA receptors in astrocytes *in vivo* remains controversial. Some authors believe that maybe NMDA receptors could be expressed in astrocytes in culture but not in brain *in vivo*. Other authors suggest that NMDA receptors are expressed only in reactive astrocytes or microglia but not in normal astrocytes (37, 38). It is likely that in brain *in vivo* modulation of glutamine synthetase by NMDA receptors should be attributed to neuronal receptors although the contribution of NMDA receptors in astrocytes should not be disregarded until specific studies are performed to clarify this possibility.

In any case, the reports summarized above clearly show that NMDA receptors modulate glutamine synthetase activity and glutamine content in brain.

## 4. PERSPECTIVE

The studies summarized above show that:

1) Blocking NMDA receptors *in vivo* increases glutamine synthetase activity and glutamine concentration in brain, indicating that tonic activation of NMDA receptors maintains a tonic inhibition of glutamine synthetase in brain. Blocking NMDA receptors "releases" this inhibition and results in increased activity of glutamine synthetase.

2) Blocking NMDA receptors *in vivo* increases the activity of glutamine synthetase assayed *in vitro* in brain homogenates. This indicates that the increase in activity is due to a covalent modification of the enzyme that remains in the *in vitro* assay after preparation of the homogenates.

3) Nitric oxide induces tyrosine nitration of glutamine synthetase and inhibits its activity in astrocytes in culture. This indicates that the covalent modification that inhibits glutamine synthetase would be a tyrosine nitration.

4) Inhibition of nitric oxide synthase with nitroarginine increases the activity of glutamine synthetase and glutamine content in brain. This indicates that the covalent modification induced by nitric oxide that inhibits glutamine synthetase must be reversible. This suggests the possibility of the existence of some enzyme that "denitrate"

glutamine synthetase and supports the possibility that tyrosine nitration-denitration of glutamine synthetase could be a "physiological" mechanism to modulate glutamine synthetase activity and glutamine levels in brain.

5) The increase in glutamine content in brain is higher when nitric oxide synthase is inhibited with nitroarginine that when NMDA receptors are blocked with MK-801. This suggests that, in addition to NMDA mediated activation of nitric oxide synthase, other sources of nitric oxide are also contributing to the tonic inhibition of glutamine synthetase in brain.

The modulation of glutamine synthetase activity in astrocytes following activation of NMDA receptors in neurons would provide a new mechanism to "coordinate" the activity of neurons and astrocytes. Part of the nitric oxide formed in the neurons containing NMDA receptors could reach neighboring astrocytes allowing them to "know" the state of activation of the neurons and adapting the activity of glutamine synthetase (and maybe also of other enzymes) to the changing requirements associated to cerebral activity.

The above results also show that, in contrast to some reports in the literature, glutamine synthetase is not working at maximum rate in brain and its activity may be increased pharmacologically by manipulating the activity of NMDA receptors or the levels of nitric oxide. This may be useful for example to increase ammonia detoxification in brain in hyperammonemic situations.

## 5. ACKNOWLEDGEMENTS

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**Abbreviations:** ADP: adenosine di-phosphate; ATP: Adenosine tri-phosphate; CM: calmodulin; GS: glutamine synthetase; iNOS: inducible nitric oxide synthase; MK-801: (+)-5-methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine maleate; NMDA: N-methyl-D-aspartate; nNOS: neuronal nitric oxide synthase; NO: nitric oxide; SNAP: S-nitroso-N-acetyl-penicillamine

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