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GLUTAMINE IN THE PATHOGENESIS OF HEPATIC ENCEPHALOPATHY: THE TROJAN HORSE HYPOTHESIS REVISITED

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Abstract

Hepatic encephalopathy (HE) is major neuropsychiatric disorder occurring in patients with severe liver disease and ammonia is generally considered to represent the major toxin responsible for this condition. Ammonia in brain is chiefly metabolized ("detoxified") to glutamine in astrocytes due to predominant localization of glutamine synthetase in these cells. While glutamine has long been considered innocuous, a deleterious role more recently has been attributed to this amino acid. This article reviews the mechanisms by which glutamine contributes to the pathogenesis of HE, how glutamine is transported into mitochondria and subsequently hydrolyzed leading to high levels of ammonia, the latter triggering oxidative and nitrative stress, the mitochondrial permeability transition and mitochondrial injury, a sequence of events we have collectively termed as the Trojan horse hypothesis of hepatic encephalopathy.

Keywords

Ammonia; astrocytes; glutamine; glutaminase; hepatic encephalopathy; L-histidine; 6-diazo-5-oxo-norleueine (DON); mitochondrial permeability transition; oxidative stress

Introduction

Hepatic encephalopathy (HE) is the major neurological disorder associated with severe liver disease which presents in chronic and acute forms [1]. Complications of chronic HE (usually in the setting of alcohol-induced cirrhosis) are principally neuropsychiatric abnormalities characterized by personality disorders, altered mood, increased irritability, changes in sleep/wake cycles, decline in intellectual capacity and abnormal muscle tone [2].

The symptoms of acute HE (acute liver failure, ALF), on the other hand, progress more rapidly, wherein patients present with seizures, delirium, alterations in the level of

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consciousness and coma [3]. The major manifestation of ALF is brain edema (increased brain water content) resulting in increased intracranial pressure (ICP) and brain herniation. While acute HE has been associated with a high mortality (80–90%) [3,4], recent surveys have reported a somewhat lower mortality rate (approximately 60%) [5,6], likely due to better clinical management of this condition. ALF is usually a consequence of acetaminophen toxicity, viral-mediated hepatitis, or exposure to various hepatotoxins [7]. The major etiological factor in both chronic and acute HE is increased blood and brain levels of ammonia.

The pathogenetic manifestations of ALF primarily develop in astrocytes, as these are the cells that are most affected histopathologically [8]. This is likely due to the fact that ammonia is exclusively metabolized in these cells by glutamine synthetase, which converts ammonia and glutamate into glutamine [9]. Accordingly, high brain and CSF levels of glutamine are also characteristic features of HE [10–12].

Ammonia has been shown to result in oxidative/nitrative stress, mitochondrial dysfunction, and alterations in the activity of various metabolic signaling pathways, including activation of mitogen activated protein kinases and the transcriptional factors (NF-κB, p53) as well as cerebral inflammation, and all of these factors have been shown to contribute to the cerebral complications of ammonia toxicity and ALF [13–16]. Thus, increased production of reactive oxygen and nitrogen species, lipid peroxidation, oxidation of mRNA, oxidation/nitration of key astrocytic proteins, and induction of the mitochondrial permeability transition (mPT) have been reported in experimental models of ALF, as well as in cultured astrocytes exposed to a pathophysiological concentration of ammonia [13,14,17,18]. Additionally, strategies geared towards a reduction of the above abnormalities have been shown to exert beneficial effects in ALF [18, 19]. More recently, most of the above noted astrocytic abnormalities have been attributed to glutamine as a consequence of the ammonia “detoxification” process [20,21].

This article reviews the Trojan horse hypothesis in HE whereby glutamine is transported into mitochondria, where it is subsequently hydrolyzed to yield high levels of ammonia, ultimately resulting in the induction of the mPT, mitochondrial dysfunction and the generation of oxidative/nitrative stress (ONS).

Historical perspective on glutamine in the pathogenesis of HE

Early in the evolution of the role of ammonia in the pathogenesis of hepatic encephalopathy [22–24], a seminal finding by Warren and Schenker [25] documented that methionine sulfoximine (MSO), an inhibitor of glutamine synthetase, significantly protected mice from acute ammonia toxicity, including a lowering of the seizure threshold, prevention of coma and improvement of their survival. These investigators thus proposed that glutamine may be a harmful factor in the pathogenesis of HE. Subsequent studies by other groups disclosed that MSO normalized the decreased glucose utilization in a rat model of chronic HE [26], and restored altered vascular CO_2 responsiveness [27] in a rat model of chronic HE. MSO was also shown to prevent the cytotoxic edema in experimental models of HE and hyperammonemia [28–30], as well as the cell swelling in cultured astrocytes following

exposure to ammonia [31]. These critical studies highlighted the crucial role of glutamine in the pathogenesis of HE.

Mechanisms by which glutamine may contribute to brain edema/astrocyte swelling are not clear. A commonly held view is that the accumulation of glutamine leads to an osmotic shift of water into astrocytes [32]. While glutamine is indeed an osmolyte [33], it remains to be proven whether this amino acid is responsible for the osmotic shift of water into neural cells (principally astrocytes). Noteworthy, there is data showing a lack of temporal correlation of astrocyte glutamine concentration with the extent of cell swelling, as well as reports documenting the reduction of brain edema by various modalities, in the absence of a commensurate reduction in cerebral glutamine concentrations [34–36]. Furthermore, one study showed no temporal correlation between glutamine concentration and the extent of cell swelling in cultured astrocytes following treatment with ammonia [37]. Thus, while glutamine appears to play a crucial role in the mechanism of ALF, it is unlikely that it does so by an osmotic effect.

Recently, another view on the osmotic aspects of glutamine in the production of brain edema has been proposed. According to this hypothesis, glutamine is synthesized in astrocytes during the process of ammonia removal, released into the brain extracellular space via the small neutral amino acid transporter 5 (SNAT5), and then taken up by neurons to generate glutamate. A defect in the inter-cellular trafficking of glutamine between neurons and astrocytes has been postulated to occur which may contribute to the pathogenesis of ALF [38]. This proposal was based on the observation that mRNA levels of SNAT5 were found to be reduced in cerebral cortex of rats with ALF [38], a process postulated to result in the accumulation of glutamine in astrocytes, ultimately leading to cell swelling by an osmotic effect.

This view, however, is at variance with the well known fact that extracellular glutamine levels in brain are increased by over 5-fold in patients and in experimental models of ALF [11,39,40]. Accordingly, if high levels of glutamine were to be achieved in astrocytes, brain extracellular levels of glutamine would have shown a commensurate reduction (since astrocytes are the major source of extracellular glutamine in brain). This proposal, moreover, relied on a reduction in mRNA levels of SNAT5; yet, a comparable reduction in SNAT5 protein was never documented. In recent studies, we in fact found that protein levels of SNAT5 were unchanged in cerebral cortical sections of mice with ALF induced by hepatotoxin thioacetamide (TAA) (Figure 2). Likewise, cultured astrocytes treated with a pathophysiological concentration of ammonia (5 mM NH_4Cl) did not result in any change in SNAT5 protein expression (Figure 3).

The Trojan horse hypothesis

In 2006, an alternate mechanism was proposed whereby glutamine may result in harmful effects in brain, the so called Trojan horse hypothesis. Fundamental studies prior to formulating this hypothesis were carried out by Zieminska et al. [41] in isolated mitochondria from rat brain wherein, glutamine caused a marked Ca^{2+}-dependent mitochondria swelling (mPT), which was sensitive to cyclosporine A (CsA), an inhibitor of

the mPT [41]. Notably, the direct exposure of ammonia to mitochondria did not elicit the mPT [41]. These observations were subsequently extended with the use of cultured astrocytes. We found that glutamine also resulted in the induction of the mPT in these cultures [42]. Further studies on mechanisms by which glutamine, presumably an innocuous amino acid, induces the mPT, disclosed that glutamine is transported into mitochondria where it undergoes hydrolysis, thereby yielding high levels of ammonia; the latter triggers oxidative stress and mitochondrial dysfunction which ultimately leads to the mPT and astrocyte swelling [42], a process that was blocked by MSO [43].

While the results documenting the mitigation of the mPT and oxidative stress by MSO implied the involvement of glutamine in this process, the precise mechanisms by which such protection occurred was not apparent. In order for glutamine to exert mitochondrial abnormalities, it must first be carried into mitochondria, where it undergoes hydrolysis by phosphate-activated glutaminase (PAG) to generate ammonia and glutamate. Accordingly, studies employing L-histidine, an inhibitor of glutamine transport into mitochondria, showed a significant attenuation in the ammonia-induced mPT and oxidative stress. Likewise, 6-diazo-5-oxo-L-norleucine (DON), an inhibitor of phosphate-activated glutaminase (PAG), blocked the mPT and free radical production by ammonia. This clearly established a Trojan horse role for glutamine in the mechanism of ammonia neurotoxicity, whereby glutamine enters mitochondria, followed by its hydrolysis that yields toxic levels of ammonia in the organelle. For further details on the Trojan horse hypothesis, see references [20,21,44].

Studies showing inhibition by DON and L-histidine in the activation of mitogen-activated protein kinases (MAPKs) also support this hypothesis [21]. Likewise, some of the astrocytic abnormalities caused by ammonia, including activation of the transcriptional factors NF-κB, p53, as well as the decreased uptake of glutamate were rectified by treatment with either DON or L-histidine, all of which support the Trojan horse hypothesis.

This hypothesis is also in keeping with *in vivo* conditions in ALF induced by hepatotoxin TAA. L-histidine treatment of rats with ALF significantly blocked the induction of oxidative stress, the mPT and the development of brain edema [45]. A recent study also disclosed that reduction of mitochondrial glutathione levels in brains of rats with ALF contribute to the induction of oxidative stress [46], a phenomenon that was attenuated by treatment with L-histidine.

While the Trojan horse hypothesis represents a major mechanism by which glutamine mediates the deleterious effects of ammonia in HE, concerns have been raised as to its validity [47]. The presence of glutaminase in astrocytes, a key factor implicated in the Trojan horse hypothesis, was questioned, since glutaminase was generally considered to be exclusively present in neurons and brain levels of PAG were reported to be relatively low [48]. However, a study by Wurdig and Kugler [49] clearly identified PAG rat brain astrocytes by enzyme histochemistry. Likewise, cultured astrocytes were shown to express abundant quantities of PAG [50–52], which subsequently was shown to be localized in mitochondria isolated from cultured astrocytes [53]. A subsequent study unequivocally confirmed the presence of L-type glutaminase (LGA) in astrocytes in rat brain [54]. Employing cortical sections of mouse brain, we also localized LGA in astrocytic

mitochondria as observed by triple immunocytochemistry (Figure 1). Together, these reports clearly document the presence of LGA in astrocytic mitochondria, a finding that strongly is in keeping with the Trojan horse mechanism.

Concluding remarks

The Trojan horse hypothesis continues to represent a major mechanism by which glutamine contributes to the pathogenesis of HE. It postulates that glutamine is transported into mitochondria, where it undergoes hydrolysis to yield high levels of ammonia, resulting in deleterious effects, including induction of the mitochondrial permeability transition and oxidative/nitrative stress. Accordingly, inhibition of glutamine synthesis, inhibition of glutamine transport into mitochondria, or reducing the activity of phosphate-activated glutaminase were all found to exert beneficial effects in ALF, as shown by a reduction in astrocyte swelling/brain edema, improvement of mitochondrial function, enhanced cerebral energy metabolism, increased astrocytic glutamate uptake, as well as inhibition of the activity of detrimental signaling mechanisms. We propose that targeting astrocytic glutamine transport and/or its hydrolysis in mitochondria remains an attractive strategy for the treatment of HE and other hyperammonemic disorders.

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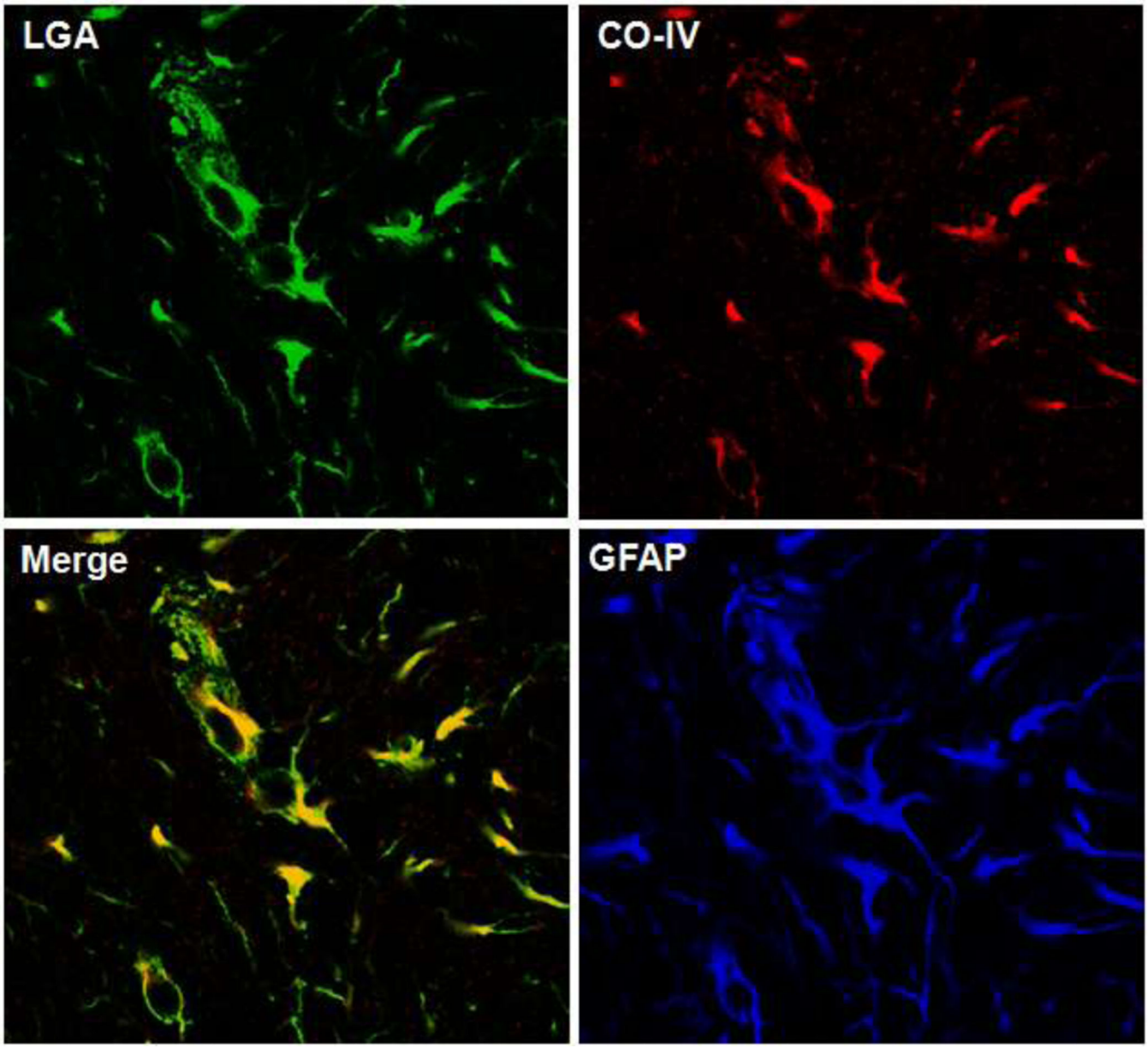

Figure 1.

Immunohistochemistry (IHC) of L-type glutaminase (LGA) (green fluorescence) in normal mouse brain cortical sections. Frozen brain sections were performed as described previously [45]. Briefly, sections were fixed in ice-cold methanol and incubated overnight at 4°C with antibodies to LGA (goat polyclonal, 1:100), cytochrome oxidase subunit IV (red fluorescence, CO-IV, mouse monoclonal, 1:100) and GFAP (blue fluorescence, rabbit polyclonal, 1:400); washed 3-times with phosphate-buffered saline containing 0.1% Triton X 100; incubated with fluorescent secondary antibodies with different excitation wavelengths; and fluorescence was visualized with a confocal microscope. Note the merged image showing marked co-localization of LGA with CO-IV consistent with the mictochondrial localization of LGA.

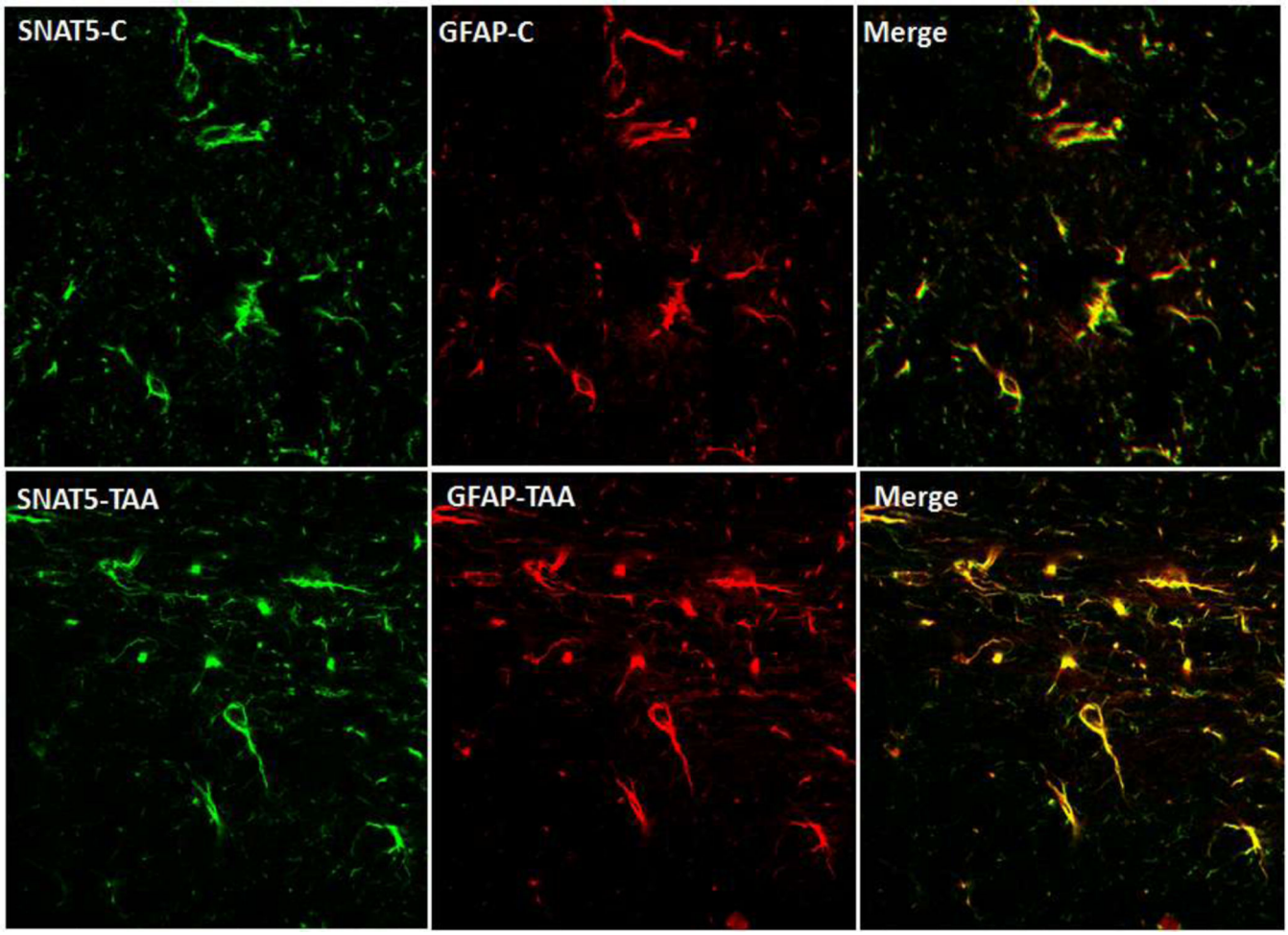

Figure 2.

Immunohistochemistry (IHC) of the glutamine transporter SNAT5 (green fluorescence) in brain cortical sections from control and mice treated with hepatotoxin thioacetamide (TAA) to induce ALF . Immunohistochemistry of frozen brain sections were performed as described in Figure 1. The antibodies used included SNAT5 (goat polyclonal, 1:100) and GFAP (red fluorescence, rabbit polyclonal, 1:400). Note that SNAT5 expression did not change in mice with TAA-induced ALF as compared to control animals (SNAT5-C).

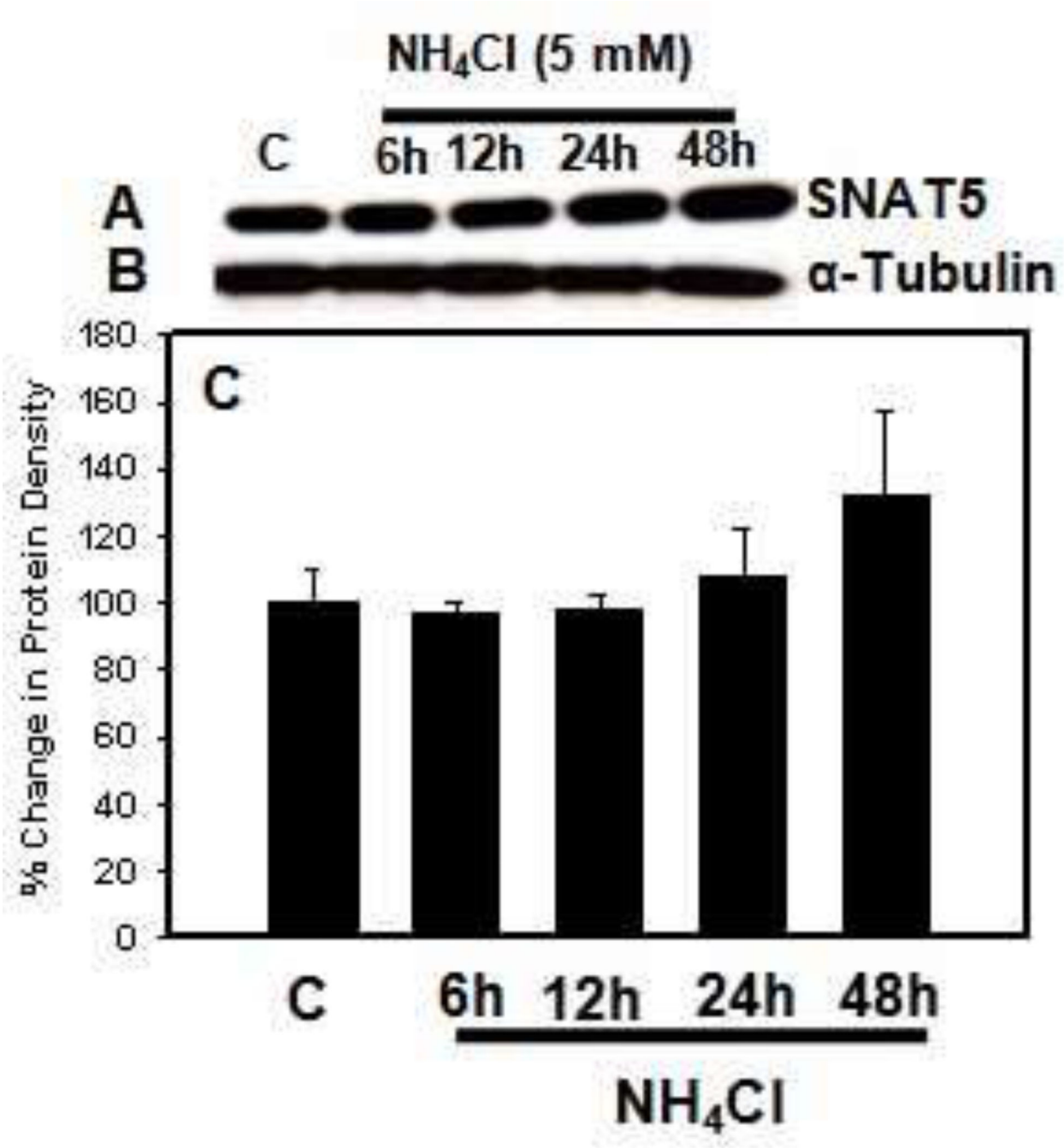

Figure 3.

Effect of ammonia (5 mM NH_4Cl) treatment to cultured astrocytes on SNAT5 protein expression. **A**. Representative immunoblot of SNAT5 protein density. **B**. Immunoblot of tubulin protein density (loading control) corresponding to the immunoblot of SNAT5. **C**. Quantification of SNAT5 protein densities. Note that ammonia treatment of astrocytes had no significant effect on SNAT5 expression at any time point. Values in each group are mean ± S.E.M of 2 individual culture plates taken from 2 separate seeding batches (n=4).