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Ammonia toxicity to the brain : Effects on creatine metabolism and transport and protective roles of creatine

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Abstract

Hyperammonemia can provoke irreversible damage to the developing brain, with the formation of cortical atrophy, ventricular enlargement, demyelination or gray and white matter hypodensities. Among the various pathogenic mechanisms involved, alterations in cerebral energy have been demonstrated. In particular, we could show that ammonia exposure generates a secondary deficiency in creatine in brain cells, by altering the brain expression and activity of the genes allowing creatine synthesis (AGAT and GAMT) and transport (SLC6A8). On the other hand, it is known that creatine administration can exert protective effects in various neurodegenerative processes. We could also show that creatine co-treatment under ammonia exposure can protect developing brain cells from some of the deleterious effects of ammonia, in particular axonal growth impairment. This article focuses on the effects of ammonia exposure on creatine metabolism and transport in developing brain cells, and on the potential neuroprotective properties of creatine in the brain exposed to ammonium.

Keywords

Ammonia, brain, hyperammonemia, toxicity, creatine, neuroprotection

Abbreviations

AGAT: arginine:glycine amidinotransferase; BBB: blood brain barrier; CK: creatine kinase; CNS: central nervous system; Cr: creatine; GAA: guanidinoacetate; GAMT: guanidinoacetate methyltransferase; MAP-2: microtubule associated protein 2; MCEC: microcapillary endothelial cell; NFM: medium weight neurofilament protein; NH_4^+: ammonium; PCr: phosphocreatine; SLC6A8: creatine transporter; UCD: urea cycle disease.

1: Introduction: ammonia toxicity to the brain

In pediatric patients, hyperammonemia can be caused by various acquired or inherited disorders such as urea cycle diseases (UCD) or organic acidemias [1]. The excess of circulating ammonia eventually reaches central nervous system (CNS), where the main toxic effects of ammonia occur. These are reversible or irreversible, depending on the age of onset as well as the duration and the level of ammonia (or more properly the ammonium ion NH_4^+) exposure. The brain is much more susceptible to the deleterious effects of NH_4^+ during development than in adulthood. Hyperammonemia can provoke irreversible damage to developing CNS, with presentation symptoms such as cognitive impairment, seizures and cerebral palsy [2,3]. Hyperammonemic neonates and infants develop cortical atrophy, ventricular enlargement, demyelination or gray and white matter hypodensities [3-5]. The extent of the irreversible damage depends on the maturation of the brain and on the magnitude and duration of NH_4^+ exposure [6-8].

The last few years have brought new data showing that ammonia exposure in CNS alters several amino acid pathways and neurotransmitter systems, cerebral energy, nitric oxide synthesis, axonal and dendritic growth, signal transduction pathways, as well as K^+ and water channels (see this same volume of Molecular Genetics and Metabolism: [9]). All these effects of ammonia on CNS may eventually lead to energy deficit, oxidative stress and cell death.

We and others have shown that NH_4^+ exposure impairs the metabolism and transport of arginine in developing brain cells [10-12]. Moreover, patients with UCD (except for deficiency in arginase I) can present shortage in arginine [13-15]. As arginine is precursor, among other pathways, of creatine (Cr) synthesis, NH_4^+ exposure of the brain can lead to

disturbances in cerebral energy and in particular in its Cr content. In particular, it was shown that NH_4^+ exposure generates a secondary deficiency in Cr in brain cells [16-18], which can be observed *in vivo* in UCD patients [19].

On the other hand, Cr administration has been postulated to exert protective effects in various neurodegenerative processes, including Huntington's and Parkinson's diseases, amyotrophic lateral sclerosis or cerebral ischemia (see discussion below) [20]. We could also show that Cr co-treatment under NH_4^+ exposure can protect developing brain cells from some of the deleterious effects of NH_4^+, in particular axonal growth impairment [16,21].

This article will focus on the effects of NH_4^+ exposure on Cr metabolism and transport in developing brain cells, and on the potential neuroprotective properties of Cr in the brain exposed to NH_4^+.

2: Synthesis and transport of creatine in CNS

The Cr / phosphocreatine (PCr) / creatine kinase (CK) system is essential to buffer and regenerate ATP. In mammals, Cr is supplied by diet, or is synthesized from arginine and glycine by a two-step mechanism involving arginine:glycine amidinotransferase (AGAT), yielding guanidinoacetate (GAA), and guanidinoacetate methyltransferase (GAMT), which converts GAA to Cr; Cr is taken up by cells through a specific Cr transporter, SLC6A8 (also called CT1, CRT or CreaT), belonging to the Na^+-dependent neurotransmitter transporter family [22-24]. In adult mammals, the main pathway for Cr synthesis involves AGAT in kidney, the transfer of the intermediate GAA to liver and GAMT in hepatocytes [25,26].

However, both enzymes are also expressed in various other tissues. In particular, AGAT, GAMT and SLC6A8 are expressed in the brain [27,28], which is the main affected target of patients suffering of AGAT, GAMT or SLC6A8 deficiencies, manifesting in early infancy with severe neurodevelopmental delays, autism and epileptic seizures [29].

It has long been thought that most of Cr in CNS was of peripheral origin. However, the mammalian brain can synthesize its own Cr [30,31]. We and others have shown that AGAT and GAMT are expressed by all the main cellular types within the brain, namely neurons, astrocytes and oligodendrocytes [27,32-34]. This allows them to synthesize their own Cr [16,18]. The brain expression of SLC6A8 is different. *In vivo*, SLC6A8 is expressed by neurons and oligodendrocytes. It is also expressed by microcapillary endothelial cells (MCEC), but not by astrocytes, including in their feet lining blood brain barrier (BBB) [27,33,35-39]. This probably limits the permeability of BBB for Cr, and made us suggest that Cr most probably does not cross BBB efficiently and that CNS depends, at least for a part of it, on its own Cr synthesis [27,24,40].

The presence of SLC6A8 in neurons and oligodendrocytes suggests the transport of Cr between cells synthesizing Cr and cells with high energy requests. We also showed recently that in the brain, AGAT and GAMT seem rarely co-expressed within the same cell, suggesting that to allow Cr synthesis in CNS, GAA must be transported from AGAT- to GAMT-expressing cells through SLC6A8 [28,41].

AGAT, GAMT and SLC6A8 are also well expressed during vertebrate embryogenesis [42,43], and probably play essential roles in developing CNS as their deficiencies lead to neurological symptoms and severe neurodevelopmental delay in early infancy [29].

3: Functions of creatine in CNS

The main function of the Cr / PCr / CK system in vertebrate cells is the regeneration of ATP as well as the cell buffering of high energy phosphates [22-24]. In CNS specifically, the importance of Cr has been shown for the dendritic and axonal elongation (growth cone migration), the Na^{+}-K^{+}-ATPase activity, the release of various neurotransmitters, the maintenance of membrane potential, the Ca^{++} homeostasis and the restoration of ion gradients.

In the mammalian brain, total levels of Cr and CK activity are well correlated, their highest levels being reached in brain cells described with high and fluctuating energy demands, where AGAT, GAMT and SLC6A8 are expressed [22,40,44-46].

Beside its functions in buffering and transport of high energy phosphates, new roles for Cr have recently been suggested in CNS. Cr and GAA can affect GABA-ergic neurotransmission as partial agonists on post-synaptic GABA(A) receptors [47-50]. These data stimulated research showing that Cr is exocytotically released from central neurons in an action potential-dependent manner [51], and may thus be considered as neuromodulator or even true neurotransmitter. Cr was also suggested as an essential brain cell osmolyte, particularly in astrocytes which, when exposed to hypo-osmotic swelling conditions, stimulate the release of their osmotically active Cr [52,53]. In contrast, ammonium-exposed MCEC *in vitro* stimulate their Cr uptake [54], suggesting that cells composing BBB (e.g. MCEC and astrocytes lining them) behave differentially during swelling. Finally, Cr was proposed to regulate appetite and weight by acting on specific hypothalamic nuclei [55].

4: Brain cell 3D cultures: a model for analyzing the effects of NH_4^+ on CNS

We have developed primary brain cell 3D cultures in aggregates as a valid experimental model to study the effects of NH_4^+ on developing CNS [10,16,18,56]. These cultures are prepared from the CNS of rat embryos, contain all types of brain cells (neurons, astrocytes, oligodendrocytes, microglia) and consist of even-sized spheres that are maintained in suspension by constant gyratory agitation in a serum-free, Cr-free, chemically-defined medium. They are classified as organotypic as their different cell types interact together and develop specialized structures (synapses, myelinated axons) as well as electrical activity in a manner resembling *in vivo* CNS [56]. Hyperammonemia is mimicked in this model by treating cultures with NH_4Cl [16]. Compared to classical monotypic cultures, 3D brain cell cultures present the advantage to allow the study of irreversible NH_4^+ toxicity in a model that mimic brain complexity at different stages of maturation. At the same time, these cultures allow to study the effects of hyperammonemia devoid of the confusing variables attributable to secondary effects of hyperammonemia found in animal models [57]. Brain cell 3D cultures synthesize their own Cr and express AGAT, GAMT and SLC6A8 as the *in vivo* CNS, showing in particular the absence of SLC6A8 in astrocytes under normal conditions [16,18].

5: NH_4^+ impairs axonal growth and generates a secondary Cr deficiency in brain cells

The developing brain of hyperammonemic neonates and infants can present cortical atrophy or gray and white matter hypodensities, which are reminiscent of neuronal fiber loss or defects of neurite outgrowth. A loss of medium spiny neurons and increased numbers of reactive oligodendroglia and microglia in striatum are also observed [58]. *Spf* mice show a decrease in both dendritic tree complexity and spine density in the layer V pyramidal cells of frontoparietal cortex [59]. Alteration of neurite outgrowth by hyperammonemia might be due to dysregulation of cytoskeletal elements. NH_4^+-acetate fed rats present a decreased phosphorylation of the dendritic protein microtubule associated protein 2 (MAP-2), in parallel with an increase of MAP-2 binding to microtubules [60]. We showed that NH_4^+ exposure in 3D brain cell cultures inhibits axonal growth, together with a decreased medium weight neurofilament (NFM) expression and phosphorylation (**Figure 1**; [16,61]). This occurs only in developing brain cells but not after neuronal maturation and synaptogenesis (**Figure 1**), in line with clinical data showing NH_4^+-induced irreversible damage to CNS in neonates and infants but not in adults [62].

As, during CNS development, Cr is particularly important during neurite elongation at the levels of growth cones, we hypothesized that developing brain cells under NH_4^+ exposure may undergo a decrease in their Cr pools.

We could thus show that axonal growth inhibition by NH_4^+ during brain cell development is indeed accompanied by a decrease of intracellular Cr in developing brain cells (**Figure 2**; [16,18]). To better understand how NH_4^+ alters Cr pools in CNS, we analyzed AGAT, GAMT and SLC6A8 gene regulation in 3D brain cell cultures under NH_4^+ exposure [18], and measured intracellular levels of Cr and GAA by tandem mass spectroscopy. We showed that the Cr/GAA ratio in NH_4^+-exposed brain cells was increased, suggesting an inhibition of

AGAT enzymatic activity under NH_4^+ exposure (**Table 1**). NH_4^+ exposure also differentially altered AGAT, GAMT and SLC6A8 gene expression and protein activity, in a cell type-specific manner. In particular, we found that NH_4^+ exposure induces SLC6A8 expression in astrocytes, while it represses it in oligodendrocytes (**Table 1**). We also showed that it increased GAMT expression in oligodendrocytes [18]. This probably alters the energy requirements of these respective glial cells. This might be related to the increased number of astrocytic mitochondria observed under hyperammonemia, in parallel with astrocyte swelling and Alzheimer's type II astrocytosis, while in contrast the number of oligodendrocytic mitochondria is decreased [63-65].

6: Neuroprotection by creatine under NH_4^+ exposure

As NH_4^+ exposure inhibits axonal growth and generates a simultaneous secondary Cr deficiency in brain cells, the next experimental step was to investigate whether a Cr co-treatment under NH_4^+ exposure could be neuroprotective, in particular on axonal growth. Indeed, we could show that a Cr co-treatment is able to protect axonal growth under NH_4^+ exposure (**Figure 3 A-C**; [16]). However, this was possible only in the presence of glial cells. When neuron-enriched 3D cultures were used, in which glial cells have been wiped out by cytosine arabinoside, axonal growth was still impaired by NH_4^+ exposure, but Cr co-treatment did not exert any protection on axonal growth (**Figure 3 D-F**; [16]). Thus, the mechanism of axonal protection by Cr is glial cell-dependent. This protecting factor, be it glial or neuronal under glial dependence, is stimulated by Cr treatment and can be a contact or a soluble factor. To solve this, mixed-cell 3D cultures (i.e: containing glial cells) were treated with 1mM Cr, and their culture medium was used to condition neuron-enriched aggregates exposed to 5mM

NH_4Cl. These conditions allowed the protection of axonal growth in neuron-enriched cultures exposed to NH_4^+ (**Figure 3 G**). Thus, a soluble, glial-dependent factor appears implicated in the axonal growth protection by Cr under NH_4^+ exposure.

As NH_4^+ exposure inhibits axonal growth and decreases Cr, while Cr co-treatment under NH_4^+ protects axonal growth [16], methods to efficiently sustain Cr concentration in the developing hyperammonemic CNS should be assessed. Under physiological conditions, Cr can cross from blood to brain through BBB [35], but with a low permeability [66], partly because astrocytes lining BBB do not express SLC6A8 [18,27,28]. This explains why AGAT- and GAMT-deficient patients, who can benefit from Cr supplementation, have to be treated on months with very high doses of Cr to replenish their cerebral Cr [29,67]. MCEC at BBB express SLC6A8 [35]. NH_4^+ exposure increases both SLC6A8 and Cr uptake in MCEC [54]. As we demonstrate that SLC6A8 is induced in NH_4^+-exposed astrocytes, BBB of the hyperammonemic CNS might thus be more permeable to Cr than under physiological conditions, and supplying oral Cr to hyperammonemic neonates or infants might likely contribute to protect their brain development. The question of treating hyperammonemic patients with Cr should be put in perspective of the treatment of other brain pathologies with Cr. Indeed, Cr administration can exert protective effects in various neurodegenerative processes, including Huntington's and Parkinson's diseases [20], for which phase II (Huntington's and Parkinson's diseases) and phase III (Huntington's) clinical trials are already ongoing [68,69].

7: Dependence on the brain cell model: discrepancies on Cr metabolism and transport

As described above, it has been known for a long time that CNS can synthesize Cr [31], however until recently almost nothing was known, at the molecular level, on the way brain cells synthesize and / or take up Cr. 20 years ago, an important paper by Möller and Hamprecht showed a detailed description of the capacity of Cr uptake *in vitro* by numerous types of primary brain cells as well as immortalized cell lines [70]. They concluded that astrocytes have the highest activity of Cr transporter.

We showed that *in vivo* AGAT and GAMT can be found in all cell types of the brain, while, in contrast to what was demonstrated in primary cultures of astrocytes, SLC6A8 is not expressed in astrocytes [27]. We further demonstrated that organotypic cultures like brain 3D mixed-cell cultures synthesize their own Cr and express AGAT, GAMT and SLC6A8 as the *in vivo* CNS, including the absence of SLC6A8 from astrocytes [18].

In contrast, when we analyzed brain 3D neuron-enriched cultures, from which astrocytes and oligodendrocytes have been eliminated, we showed that in absence of glial cells, AGAT and GAMT are totally silent from neurons and from the very few remaining astrocytes, while both genes are well expressed in both cell types when glial cells are present [18]. This is also reflected by the intracellular Cr of neuron-enriched cultures, which is measured at levels 10 times lower than in mixed-cell cultures.

It appears thus that monotypic primary brain cells, be them in monolayers or in 3D cultures, do not behave as the *in vivo* CNS for Cr metabolism and transport. Thus, to analyze Cr in brain cells in conditions as near as possible of the *in vivo* brain, complex 3D, organotypic and mixed-cell culture systems should be used.

8: Conclusions

Hyperammonemia during development is associated with neuronal cell loss and cerebral atrophy that lead to mental retardation and cerebral palsy in pediatric patients. Among the various pathogenic mechanisms involved, alterations in axonal and dendritic growth and cerebral energy have been demonstrated.

As illustrated in this article, NH_4^+ exposure impairs axonal growth in developing brain cells and simultaneously generates a secondary Cr deficiency, likely through inhibition of AGAT activity. A Cr co-treatment can protect axonal growth under NH_4^+ exposure, in dependency of glial cells.

Finally, the Cr transporter, SLC6A8, which is normally silent in astrocytes *in vivo*, is induced in astrocytes by NH_4^+ exposure, which also increases SLC6A8 expression and Cr uptake in MCEC. This might facilitate the entry of Cr into the brain, and raises the question of the potential treatment of hyperammonemic patient with Cr to protect their developing CNS.

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Table 1 : Main effects of NH_4^+ on AGAT, GAMT and SLC6A8 expression and activity in brain cells (data taken, and figure modified, from [18]) [a].

Effects of NH_4^+ on:		**in** (cells involved) **:**
AGAT activity :	⇓	ND
GAMT expression :	⇑	oligodendrocytes
SLC6A8 expression :	⇑	astrocytes
	⇓	oligodendrocytes

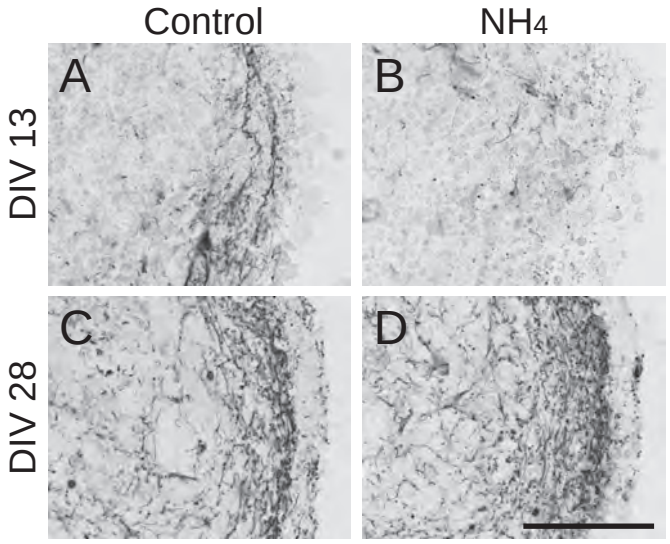
[a] ⇑ : increase or induction; ⇓ : decrease or repression; ND: not determined.

Figure legends

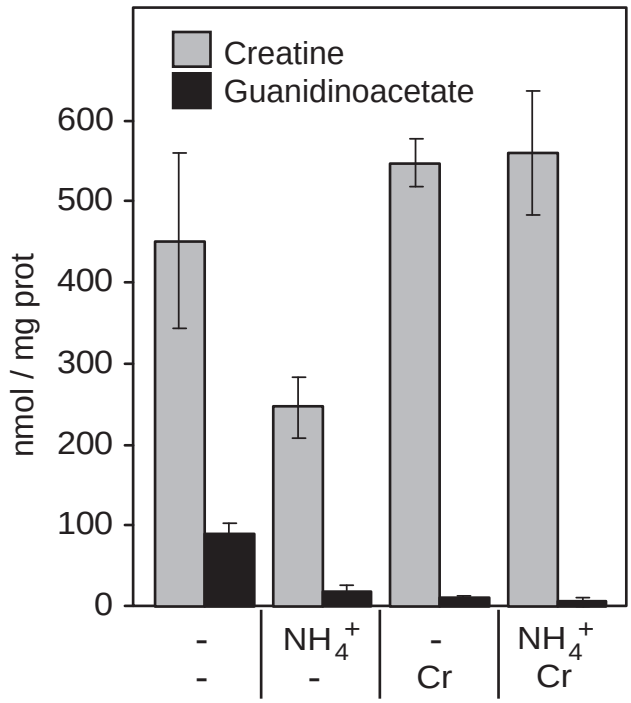
Figure 1: NH_4^+ exposure impairs axonal growth during development, but does not affect axons after synaptogenesis. Immunohistochemistry against phosphorylated NFM. Day in vitro (DIV) 13 (**A**,**B**; development) or 28 (**C**,**D**; post-synaptogenesis) brain mixed-cell 3D cultures. **A**,**C**: control cultures; **B**,**D**: cultures exposed to 5 mM NH_4Cl from DIV 5 to 13 (**B**) or 20 to 28 (**D**). Bar: 100 μm. Data taken, and figure modified, from [16].

Figure 2: Intracellular levels of creatine (Cr) and guanidinoacetate (GAA) in 3D brain cell cultures under continued exposure of NH_4Cl and/or Cr. Cr and GAA intracellular levels measured by tandem mass spectrometry; day in vitro (DIV) 13 control cultures, as well as cultures exposed from DIV 5 to 13 to 5 mM NH_4Cl, 1 mM Cr, or both, and harvested at DIV 13. Data taken, and figure modified, from [18].

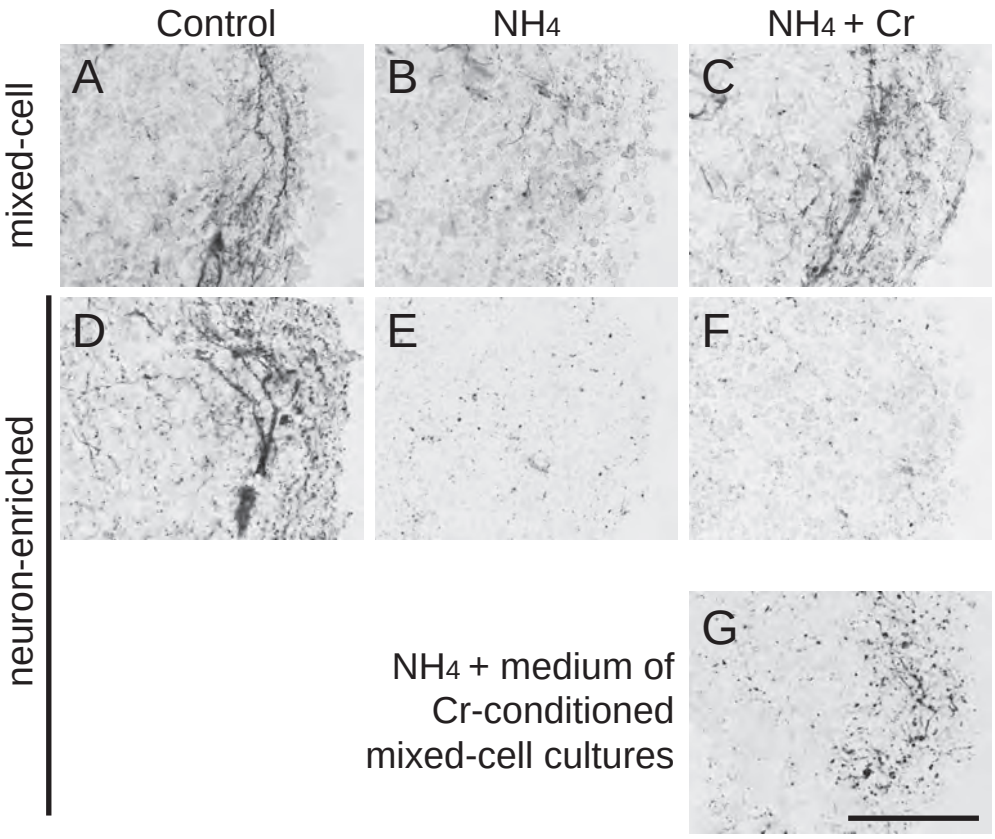
Figure 3: Axonal protection under NH_4^+ exposure depends on a glial-dependent soluble factor. Immunohistochemistry against phosphorylated NFM, as in [16]. Mixed-cell (**A-C**) or neuron-enriched (**D-G**) 3D cultures grown from days in vitro 5 to 13 as in [16], with (**B**,**C**,**E**,**F**) or without (**A**,**D**) 5mM NH_4Cl, and with (**G**) or without (**A-F**) addition of medium from mixed-cell 3D cultures conditioned by 1mM Cr. Bar: 100 μm.



Braissant, Figure 1



Braissant, Figure 2


Control
NH4
NH4 + Cr
mixed-cell
neuron-enriched
A
B
C
D
E
F
G
NH4 + medium of
Cr-conditioned
mixed-cell cultures