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Hyperammonemia-induced toxicity for the developing central nervous system

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Abstract

In pediatric patients, hyperammonemia can be caused by various acquired or inherited disorders such as urea cycle deficiencies or organic acidemias. The brain is much more susceptible to the deleterious effects of ammonium during development than in adulthood. Hyperammonemia can provoke irreversible damages to the developing central nervous system that lead to cortical atrophy, ventricular enlargement and demyelination, responsible for cognitive impairment, seizures and cerebral palsy. Until recently, the mechanisms leading to these irreversible cerebral damages were poorly understood. Using experimental models allowing the analysis of the neurotoxic effects of ammonium on the developing brain, these last years have seen the emergence of new clues showing that ammonium exposure alters several amino acid pathways and neurotransmitter systems, as well as cerebral energy metabolism, nitric oxide synthesis, oxidative stress, mitochondrial permeability transition and signal transduction pathways. Those alterations may explain neuronal loss and impairment of axonal and dendritic growth observed in the different models of congenital hyperammonemia. Some neuroprotective strategies such as the potential use of NMDA receptor antagonists, nitric oxide inhibitors, creatine and acetyl-L-carnitine have been suggested to counteract these toxic effects. Unraveling the molecular mechanisms involved in the chain of events leading to neuronal dysfunction under hyperammonemia may be useful to develop new potential strategies for neuroprotection.

Section

Disease-related neuroscience

Keywords

Hyperammonemia, ammonium, brain, development, neurotoxicity, neuroprotection

Abbreviations

ASL: argininosuccinate lyase; APV: 2-amino-5-phosphonovaleric acid; ASS: argininosuccinate synthetase; CATs: cationic amino acid transporters; ChAT: choline acetyltransferase; CNS: central nervous system; CPS-1: carbamoylphosphate synthetase 1; CSF: cerebrospinal fluid; GS: glutamine synthetase; HHH: hyperammonemia-hyperornithinaemia-homocitrullinuria syndrome; IQ: intellectual quotient; KO: knock-out; LTP: long term potentiation; MAP-2: microtubule associated protein 2; MAPK: mitogen-activated protein kinase; MPT: mitochondrial permeability transition; MRI: magnetic resonance imaging; NFM: medium weight neurofilament protein; NH_4^+ : ammonium; NMDA: N-methyl-D-aspartate; NO: nitric oxide; NOS: nitric oxide synthase; ORNT1: ornithine citrulline antiporter 1; OTC: ornithine transcarbamoylase; γ^+ LAT1: cationic amino acid γ^+ L-associated transporter 1 (system γ^+ L).

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Acknowledgments

References

1: Introduction

Ammonia is produced by amino acid metabolism and intestinal urease-positive bacteria, and is mostly present as ammonium (NH_4^+) at physiological pH in the circulation. NH_4^+ is maintained at low concentrations (50-150 μM in preterm neonates, 50-75 μM in term neonates, and <50 μM in adults; Donn and Banagale, 1984) by the urea cycle (Fig. 1), which converts NH_4^+ to urea in the liver prior to excretion of urea by the kidneys (Morris, 2002). The complete urea cycle is expressed only in the liver. In particular, the brain is unable to convert NH_4^+ to urea due to its lack of carbamoylphosphate synthetase 1 (CPS-1) and ornithine transcarbamoylase (OTC), despite expressing argininosuccinate synthetase (ASS), argininosuccinate lyase (ASL) and arginase 1. In the central nervous system (CNS), NH_4^+ is maintained however at relatively low concentrations by the action in astrocytes of glutamine synthetase (GS), which converts glutamate and NH_4^+ to glutamine (Felipo and Butterworth, 2002).

In adults, liver failure results in hyperammonemia responsible of a potentially severe neuropsychiatric disorder named hepatic encephalopathy, that progressively leads to altered mental status and coma. The symptoms of hepatic encephalopathy in adults can be reversed when NH_4^+ concentration returns to normal levels, provided that terminal cerebral edema is not reached. In pediatric patients, hyperammonemia can be caused by various inherited or acquired disorders (Table 1) (Donn and Banagale, 1984; Leonard and Morris, 2002), the most frequent being urea cycle disorders with an overall prevalence estimated at 1:8200 in the United States (Gropman and Batshaw, 2004). The developing brain is much more susceptible to the deleterious effects of NH_4^+ than is the brain of the adult. Hyperammonemia can provoke irreversible damages to the developing CNS, with presentation symptoms such as cognitive impairment, seizures and cerebral palsy (Flint Beal and Martin, 1998).

Hyperammonemic neonates and infants develop cortical atrophy, ventricular enlargement, demyelination or gray and white matter hypodensities (Msall et al., 1984; Harding et al., 1984; Filloux et al., 1986; Dolman et al., 1988; Wakamoto et al., 1999; Takeoka et al., 2001; Yamanouchi et al., 2002; Takanashi et al., 2003b; Majoie et al., 2004). The extent of the irreversible damages depends on the maturation of the brain and on the magnitude and duration of the exposure to NH_4^+ . Irreversibility mainly occurs in case of prolonged hyperammonemic crises and/or when blood NH_4^+ reaches levels between 200 and 500 μM , during the two first years of life (Msall et al., 1984; Uchino et al., 1998; Bachmann, 2002; Bachmann, 2003). This review will focus on the most recent advances in understanding the toxicity of hyperammonemia on the developing CNS.

2: Hyperammonemia in newborns and infants

2.1: Clinical presentation and treatments

Most patients with urea cycle disorders commonly present in the neonatal period with non-specific symptoms, which can be poor feeding, vomiting, somnolence, irritability and tachypnoea (Leonard and Morris, 2002). With the rise in plasma NH_4^+ , symptoms can progress rapidly towards hypothermia, lethargy and coma (Summar, 2001). In particular, hyperammonemia generates edema in CNS, due to astrocyte swelling, which leads to an increase in intracranial pressure and to brain herniation (Cordoba and Blei, 1996; Norenberg et al., 2005). Neurological problems such as seizures, abnormal posture and neuromuscular irritability are frequent and accentuated depending on the degree of the CNS swelling and of the rise of pressure on brain stem (Butterworth, 1998). Brain edema leads to hyperventilation and respiratory alkalosis, progressing to hypoventilation and apnoea (Brusilow and Maestri,

1996). Untreated, most babies will die, often with complications such as cerebral or pulmonary haemorrhage. Survivors from these episodes will eventually exhibit mental retardation, the degree of retardation correlating with the level and duration of neonatal hyperammonemia (Msall et al., 1984; Leonard and Morris, 2002), with no normal cognitive outcome, or a fatal issue, occurring if plasma NH_4^+ exceeds 300 μM at the presentation of the patients or reaches a peak above 500 μM (Bachmann, 2003; Enns et al., 2007).

In patients presenting with partial enzyme deficiencies of the urea cycle, the first recognized clinical episode may be delayed for months or years. NH_4^+ accumulation may be triggered by illness or catabolic stress resulting in multiple mild elevations of plasma NH_4^+ concentrations. The hyperammonemia is then less severe than in newborn patients and the symptoms milder (Takanashi et al., 2002). In most patients late-onset hyperammonemic episodes are marked by loss of appetite, cyclic vomiting, lethargy, and behavioral abnormalities (Smith et al., 2005; Harada et al., 2006). Protein avoidance may also be one of the symptoms found in patients with partial enzyme deficiencies, especially in female OTC deficient patients (Scaglia et al., 2002). Many of those patients also remain mentally retarded or have learning difficulties.

The immediate therapeutic goal for neonatal hyperammonemic conditions is the quick removal of ammonia. Proteins should be restrained from diet, in particular in case of severe neonatal hyperammonemia. When the metabolic alteration cannot be controlled rapidly, patients have to be dialyzed, preferentially through hemodialysis, hemodiafiltration or continuous veno-venous hemofiltration (Leonard et al., 2007). To reverse catabolism in this critical phase, patients are given glucose intravenously, together with spare insulin infusion to avoid swings in glucose levels (Summar, 2001). The metabolic control of NH_4^+ levels in this neonatal acute phase makes use of intravenous infusion of sodium benzoate and sodium

phenylacetate, that create alternative pathways for nitrogen excretion (Brusilow et al., 1979; Shih, 2007). Following these short term measures, the long term control of NH_4^+ uses oral treatments with the same compounds or sodium phenylbutyrate, in combination with a low-protein diet (Feillet and Leonard, 1998; Berry and Steiner, 2001; Batshaw et al., 2001; Enns et al., 2007). Acute intercurrent crisis of hyperammonemia are treated again with intravenous infusion of these compounds. Moreover, nitrogen excretion is promoted by large doses of arginine given to ASS and ASL deficient patients, while citrulline is preferred for the treatment of CPS-1 and OTC deficiencies (Brusilow et al., 1979; Leonard and Morris, 2002). Orthotopic liver transplantation has to be considered for the more severe urea cycle defects such as CPS-1 and OTC deficiencies (Lee and Goss, 2001). Even with early diagnosis and intensive treatment, mortality is high and neurological problems are frequent in survivors (Bachmann, 2003).

Very recently, new additional measures have been proposed, in combination with the conventional treatments described above, for the prospective treatment of babies at risk of neonatal hyperammonemia because of familial history (Leonard et al., 2007).

2.2: Irreversible effects on the developing brain

Mental retardation, as symptom of irreversible damage to the developing brain, is observed in the majority of children surviving with inborn errors of urea cycle. Important decrease in intellectual quotient (IQ), often combined with one or more neurological disabilities, is found in these patients, with strong correlations between the degree of IQ decrease, the level of developmental disabilities, the gravity of brain abnormalities as observed by computed tomography or magnetic resonance imaging (MRI), and the level, the duration and the age at

onset of hyperammonemia (Msall et al., 1984; Nagata et al., 1991; Bachmann, 2003). Neonatal onset of hyperammonemia leads to the most severe brain damages and the least IQ score.

Brain MRI of patients with neonatal onset of hyperammonemia resulting from different urea cycle disorders have shown important volume loss of specific parts of the developing CNS (Msall et al., 1988). In particular, diffuse cortical atrophy and lesions in basal ganglia have been observed in OTC and CPS-1 deficient patients, sometimes with cortical multicystic formations and appearance of myelination delay (Yamanouchi et al., 2002; Takanashi et al., 2003b). The same observations were also made in an ASS deficient neonate, with also lesions in thalamic nuclei, and a probable injury of the oligodendro-axonal unit (Majoie et al., 2004). MRI observation of brain lesions within the very few first days of life (0 to 8 days) in some patients with OTC, CPS-1 and ASS deficiencies suggests that some of these lesions or defects of development due to urea cycle diseases might be acquired already in utero (Harding et al., 1984; Filloux et al., 1986; Takeoka et al., 2001; Majoie et al., 2004). In particular, it was proposed that the hypomyelination found in OTC neonates might be due to prenatal damages to oligodendrocytes, and that the multiple bilateral cerebellar heterotopia observed in the same patients might be the consequence of an arrest of neuronal migration in the embryonic cerebellum (Harding et al., 1984). Since OTC deficiency is an X-linked disorder, it is conceivable that fetal metabolic insufficiencies may not be fully compensated by the maternal organism. Filloux et al. postulated that the brain lesions described in the first days of life represent the initial neuropathological manifestations that, in case of prolonged survival, lead in part to the atrophic and degenerative changes observed later (Filloux et al., 1986).

Patients with later onset of hyperammonemia may have a normal neurodevelopment, if the diagnosis is made before they sustain an irreversible cerebral insult (Kurihara et al., 2003); however, many remain mentally retarded or have learning difficulties. Brain MRI of patients with late-onset OTC deficiency have shown injury to the cortex including acute ischemia, ventricular dilatation and defects of myelination (de Grauw et al., 1990; Kurihara et al., 2003; Takanashi et al., 2003a). Similar lesions were found in late onset CPS-1, ASS and arginase 1 deficiencies (Call et al., 1984; Scaglia and Lee, 2006; Choi et al., 2006; Kim et al., 2006), as well as in patients with organic acidemias such as propionic acidemia, or with hyperammonemia-hyperornithinaemia-homocitrullinuria (HHH) syndrome, and presenting with a normal neonatal life (Harding et al., 1991; Salvi et al., 2001).

The rare analyzed autopsy cases of urea cycle deficiencies reveal, depending on the age of death and whether they developed neonatal or late onset hyperammonemia, microcephaly, shrinkage of hemispheres with appearance of multiple cysts, ventricular dilatation, atrophy or necrosis of various nuclei (particularly in caudate putamen, thalamus and hypothalamus) and defects of myelination (Dolman et al., 1988; Takeoka et al., 2001; Yamanouchi et al., 2002). Microscopically, the brain tissue often appears spongy with extensive neuronal loss (particularly in cortex and hippocampus), is markedly gliosed and presents astrocytes with water-clear, oval nuclei characteristic of Alzheimer's type II astrocytes. Recently, emperipolesis, i.e. the active penetration of one cell by another one which remains intact, has also been described in the cerebral white matter of a 46-years old patient with ASS deficiency, where oligodendrocytes have been shown engulfed within hypertrophic astrocytes (Nishie et al., 2006).

In contrast with the developing brain, hyperammonemia (generally associated with liver failure) does not result in significant neuronal loss or structural damage to neurons in the adult brain (Butterworth, 2003).

3: Experimental models for the study of the effects of hyperammonemia on the developing CNS

3.1: The sparse-fur mouse

The sparse-fur (*spf*) mouse is an ideal animal model to study the effects of hereditary hyperammonemia, as this mouse has a single point substitution in the OTC gene, with an X linked transmission (Qureshi 1979, Veres 1987). Two mouse strains with OTC mutations have been described: sparse-fur (*spf*) and sparse-fur with abnormal skin and hair (*spf^{ash}*) (Doolittle et al., 1974; DeMars et al., 1976). The hepatic OTC activity is 5 to 10% in the *spf^{ash}* and 13% in the *spf*, as compared to the normal mice (Qureshi and Rao, 1997). Adult *spf/Y* mice (12 weeks old) show plasma and brain NH_4^+ levels increased 1.5 and 5 fold respectively (Ratnakumari et al., 1992). In these mice, a 3-fold increase in blood NH_4^+ is observed immediately after weaning as compared to control litter mates, which is probably due to dietary change from mother's milk to the conventional laboratory chow and to the subsequent changes in the intestinal flora from non-urease producing to urease producing bacteria that increase blood NH_4^+ level (Gushiken et al., 1985; Inoue et al., 1987). Recent analysis of the *spf^{ash}* mice have shown that despite their reduced OTC activity, these mice are able to detoxify ammonia and keep normal ureagenesis when they are infused with a balanced, complete mixture of amino acids, while an unbalanced amino acid infusion (i.e. a glycine-alanine mixture) make them hyperammonemic and decreases their ureagenesis (Marini et al., 2006b). This ability of *spf* mice to detoxify ammonia and keep normal ureagenesis under

balanced amino acids is probably due to the presence of arginine in the complete mixture of amino acids. Neuropathologic studies in the *spf* mouse revealed similar brain alterations as those observed in patients with OTC deficiency. *Spf* mice exhibit an overall brain size reduction with a reduced volume of the striatum and ventricular enlargement (Hopkins et al., 1998).

3.2: Knock-out mice

Knock-out (KO) mice as models of other urea cycle deficiencies have been developed recently: ASL, arginase 1 and arginase 2 deficient mice, as well as double KO mice for arginases 1 and 2 (Iyer et al., 2002; Reid Sutton et al., 2003; Deignan et al., 2006). Homozygous ASL^{-/-} null mice die within 48h after birth with plasma NH₄⁺ increased 4 fold. Arginase 1 KO mice and arginases 1/2 double KO mice die from hyperammonemia at 14 days postnatally, with a 10-fold increase in plasma NH₄⁺ level, while arginase 2 KO mice have no obvious phenotype (Iyer et al., 2002; Reid Sutton et al., 2003; Deignan et al., 2006). Neuropathological studies have not yet been published for these KO mice. Anyhow, as these enzymes are normally expressed in brain cells, ASL, arginase 1 and arginase 2 deficiencies may alter normal brain development independently of hyperammonemia (Braissant et al., 1999a; Yu et al., 2001), hampering thus the study of the specific effects of NH₄⁺ toxicity on the developing CNS in these models.

3.3: Rats models of congenital hyperammonemia

In vivo, long-term effects of chronic hyperammonemia have also been studied in rat models of congenital hyperammonemia. Authors exposed rats to NH₄⁺ during prenatal and lactation periods by feeding the pregnant rats with an NH₄⁺ acetate-containing diet from day 1 of pregnancy until weaning (at postnatal day 21). After weaning, pups were also fed the NH₄⁺

containing diet (Aguilar et al., 2000; Munoz et al., 2000). The amount of NH_4^+ in the brain of these hyperammonemic rats increased 1.4 times compared to control rats (Munoz et al., 2000).

3.4: Reaggregating brain cell 3D cultures treated with NH_4Cl

Our research team has developed reaggregating brain cell 3D cultures as a valid experimental model to study the specific effects of NH_4^+ toxicity on the developing CNS (Braissant et al., 1999b; Honegger and Monnet-Tschudi, 2001; Braissant et al., 2002; Braissant et al., 2006). These are primary cell cultures prepared from the CNS of rat embryos, containing all types of brain cells (neurons, astrocytes, oligodendrocytes and microglia) and consisting of even-sized, spherical structures that are maintained in suspension by constant gyratory agitation in a serum-free, chemically defined medium. Cells are able to migrate within the formed reaggregated structures, and to interact with each other by direct cell-cell contacts, as well as through exchange of nutritional and signaling factors. This tissue-specific environment enables aggregating neural cells to differentiate, and to develop specialized structures (synapses, myelinated axons) resembling those of the brain tissue (Honegger and Monnet-Tschudi, 2001). Aggregating cell cultures are therefore classified as organotypic cultures (Doyle et al., 1994). Chronic hyperammonemia is mimicked in this model by treating the cultures with 5 mM NH_4Cl for 8 days (Braissant et al., 1999b; Braissant et al., 2002). We have thus shown that axonal growth, as well as medium weight neurofilament (NFM) expression and phosphorylation, are altered by NH_4Cl exposure (Braissant et al., 2002). This occurs only in developing brain cell aggregates (treated from days 5 to 13: a period at which neurons progressively develop their processes) but not in more mature cultures (treated from day 20 to 28: a period at which neurons progressively undergo synaptogenesis and myelination) (Honegger and Monnet-Tschudi, 2001; Braissant et al., 2002). This difference in

vulnerability of the brain depending on its maturation level is in line with clinical data showing that hyperammonemia causes irreversible CNS damages such as neuronal cell loss in neonates and infants but not in adults (Brusilow and Horwich, 2001). Compared to classical brain cell cultures, brain cell reaggregated 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. In the same time, these cultures allow to study the effects of hyperammonemia devoid of confusing variables attributable to secondary effects of hyperammonemia found in animal models (Bachmann, 1992).

3.5: Other models of hyperammonemia

Hyperammonemia has been experimentally induced in vivo on adult rats by several other methods, including the intraperitoneal injection with NH_4^+ -acetate or infusion with urease (Robinson et al., 1992b), NH_4^+ -acetate containing diet (20% weight) (Azorin et al., 1989) or portacaval anastomosis (Song et al., 2002). In vitro, toxic effects of NH_4^+ have been studied on primary cultures of neurons or astrocytes (Chan et al., 2000; Schliess et al., 2002; Jayakumar et al., 2006) and on hippocampal rat brain slices (Chepkova et al., 2006). These models have provided several clues for the mechanisms of NH_4^+ toxicity on brain cells, but they do not allow the analysis of the effects of hyperammonemia on the developing CNS, in particular in its cellular complexity and the relationships between developing neurons and glial cells.

4: Toxicity of ammonium on experimental models of the developing CNS

4.1: Amino acids disturbances

4.1.1: Glutamine

As glutamine synthesis is the major brain pathway for the removal of NH_4^+ , hyperammonemic conditions with high levels of NH_4^+ in CNS should increase the synthesis of brain glutamine. As a result, a 2 to 3 fold increase in brain glutamine was reported by proton spectroscopy in two females with OTC deficiency (Connelly et al., 1993). A 2-fold increase in glutamine level was also reported in the brain of *spf* mice (Inoue et al., 1987). These results are confirmed by in vitro studies showing that NH_4Cl exposure of aggregating brain cell cultures increases their intracellular concentration of glutamine (Bachmann et al., 2004).

4.1.2: Glutamate

Glutamate concentrations are significantly reduced in cerebral cortex (but not in other brain regions) of *spf* mice as well as in NH_4Cl -exposed brain cell 3D cultures prepared from embryonic rat telencephalons (Ratnakumari et al., 1994a; Bachmann et al., 2004). This could be the result of increased glutamine formation from NH_4^+ and glutamate or of a loss of glutamatergic neurons (Qureshi and Rao, 1997).

4.1.3: Arginine

Arginine is an essential amino acid for the foetus and the neonate, who however synthesize parts of their arginine needs through the combined expression of CPS-1, OTC, ASS and ASL in the intestine (Husson et al., 2003; Curis et al., 2005). In the adult, arginine is considered a conditionally essential amino acid, and its endogenous synthesis pathway is decoupled between CPS-1 and OTC expressed in the intestine (while ASS and ASL are repressed) and synthesizing citrulline, and ASS and ASL being induced in proximal tubule cells of the kidney, which synthesizes arginine from the incoming citrulline produced by intestine (Wu and Morris, 1998; Husson et al., 2003). As a consequence, patients with urea cycle disorders

(except those with arginase 1 deficiency) have decreased plasma arginine concentrations and need a supplement of arginine to replace that which is not synthesized (Leonard and Morris, 2002; Scaglia et al., 2004; Scaglia and Lee, 2006). Severely affected CPS-1 and OTC deficient patients are better treated with citrulline rather than arginine (Leonard and Morris, 2002). As arginine is precursor for nitric oxide (NO) and creatine synthesis, urea cycle deficiencies can lead to disturbances of the citrulline-NO cycle (Fig. 2) (see 4.4: alteration of NO synthesis) and an alteration of creatine metabolism (Fig. 3) (see 4.3: cerebral energy deficit), including in brain cells (Braissant, 2007). *Spf* mice, which are deficient in their own arginine synthesis as are OTC-deficient patients, present decreased levels of arginine in their brain (Ratnakumari et al., 1996b). In contrast, experimental models with no evidence of arginine depletion show increased levels of arginine in brain cells placed under NH_4^+ exposure, as it was shown in aggregating brain cell 3D cultures exposed to NH_4Cl (Bachmann et al., 2004) and in rat cerebellar synaptosomes exposed to NH_4^+ -acetate (Rao, 2002). This might be due to the NH_4^+ -induced expression of ASS and ASL in astrocytes (Braissant et al., 1999b).

4.1.4: Other amino acids

Plasma ornithine and citrulline levels are low in *spf* mice (Qureshi and Rao, 1997). Interestingly, ornithine supplementation to hyperammonemic *spf^{ash}* mice can decrease their hyperammonemia and restore their ureagenesis, suggesting that ornithine has the potential to correct OTC deficiency in *spf^{ash}* mice (Marini et al., 2006a). Finally, large neutral amino acids (tyrosine, phenylalanine, methionine, tryptophan and histidine) accumulates in the brain of *spf* mice (Bachmann and Colombo, 1984; Inoue et al., 1987).

4.2: Alterations in the neurotransmitter systems

4.2.1: Glutamatergic system

An hypothesis to explain the neuronal loss observed under hyperammonemia involves excitotoxicity injury through N-methyl-D-aspartate (NMDA) receptors. Although NH_4^+ is presumed to be the principal neurotoxin, there is evidence that other neurochemical alterations may also be involved. Tryptophan, which is increased under hyperammonemia (Bachmann and Colombo, 1984), can be oxidized to quinolinic acid, an excitotoxin acting on NMDA receptors. As a result of tryptophan increase, quinolinic acid is increased in the cerebrospinal fluid (CSF) of children with congenital hyperammonemia as well as in various regions of *spf* mice brain (Batshaw et al., 1993; Robinson et al., 1995). Infusion of quinolinic acid into the brain is associated with selective death of spiny neurons in the striatum (Schwarcz and Kohler, 1983; Robinson et al., 1995). This result is consistent with the loss of spiny neurons observed in the striatum in *spf/Y* mice (Robinson et al., 1995; Ratnakumari et al., 1995b) and suggests that NH_4^+ neurotoxicity may be mediated by quinolinic acid in patients with congenital hyperammonemia (Fig. 2).

The release of endogenous glutamate, induced by depolarization, from synaptosomes isolated from *spf* mice at 35 days is significantly elevated, while the uptake of glutamate into synaptosomes is significantly reduced (Rao and Qureshi, 1999). These results suggest that the level of extracellular glutamate is increased in the brain of *spf* mice. This is in accordance with the increased level of extracellular glutamate observed by microdialysis in rabbit with acute hyperammonemia as well as with experiments showing that NH_4^+ leads to glutamate release from cultured astrocytes (de Knecht et al., 1994; Rose et al., 2005). Moreover, the astrocytic glutamate transporter GLAST is decreased under NH_4^+ exposure, leading to a decreased re-uptake of extracellular glutamate (Fig. 2) (Chan et al., 2000). As a consequence of a persistent release of excess glutamate, a significant reduction in NMDA receptors has been shown in the cerebral cortex of *spf* mice (Hopkins and Oster-Granite, 1998; Rao and

Qureshi, 1999). This down-regulation might be a mechanism of protection against excess stimulation of the NMDA receptors by glutamate and/or quinolinic acid, which induces excitotoxicity by the activation of an intracellular cascade responsible for neuronal dysfunctions and death (Ratnakumari et al., 1995a). Although NMDA receptors may be down-regulated, a certain level of persistent excitotoxicity may continue and lead to the neuronal loss observed in patients with congenital hyperammonemia (Rao and Qureshi, 1999). Interestingly, NMDA receptor antagonists MK-801 and 2-amino-5-phosphonovaleric acid (APV) improve neuronal survival in primary cortical neurons from newborn rats treated with NH_4Cl (Fig. 2) (Klejman et al., 2005). Moreover, APV diminishes the impairment of long term potentiation (LTP) by NH_4^+ exposure in rat hippocampal slices (Izumi et al., 2005).

4.2.2: Cholinergic system

A significant loss of forebrain cholinergic neurons was observed in *spf* mice (Ratnakumari et al., 1994b). Similarly in aggregating brain cell 3D cultures, data showed that early in development cholinergic neurons were impaired by NH_4Cl treatment (Braissant et al., 2002). A developmental study in *spf* mice showed that choline acetyltransferase (ChAT) activity level starts to decrease immediately after weaning and reaches significantly lower level in adult mice (Ratnakumari et al., 1995b). Moreover, subsequent studies of muscarinic cholinergic M1 (postsynaptic) and M2 (presynaptic) receptors revealed an up to 54% increase of the M1 receptor and a concomitant decrease by up to 60% of the M2 receptor in *spf* mice (Ratnakumari et al., 1996a). Increase in the M1 post-synaptic receptors could be due to an up-regulation in response to the loss of cholinergic presynaptic neurons (Michalak and Butterworth, 1997). Together, these results points towards a severe impairment of cholinergic system by hyperammonemia. As cholinergic system plays a crucial role in memory and other cognitive functions, this impairment under hyperammonemia may be responsible for

cognitive dysfunctions observed in patients with urea cycles disorders and in *spf* mice (Russell, 1996).

4.2.3: Serotonergic system

Tryptophan (precursor for serotonin) and 5-hydroxyindoleacetic acid (metabolite of serotonin) are enhanced in *spf* mice brain and in CSF of children with congenital hyperammonemia (Bachmann and Colombo, 1984; Hyman et al., 1987). Moreover, receptor binding studies in *spf* mice reveal a significant loss of 5HT₂ receptor and a concomitant increase in 5HT_{1A} receptor (Robinson et al., 1992a). These data demonstrate alterations of the serotonergic system in congenital hyperammonemia that may be involved in anorexia and sleep disturbance observed in children with urea cycle disorders (Hyman et al., 1986).

4.3: Cerebral energy deficit

In the brain of *spf* mice, the ATP concentration is decreased (Ratnakumari et al., 1992), while a significant decrease in cytochrome C oxidase expression and activity is observed, suggesting that reduction in ATP levels could be due to a reduction of the activity of the electron transport chain enzymes (Fig. 2) (Rao et al., 1997). It has also been suggested that the deficit in brain energy metabolites in hyperammonemia may be due to inhibition of the TCA cycle, especially at the level of α -ketoglutarate dehydrogenase (Fig. 2) (Lai and Cooper, 1986). However, it has been shown that ATP depletion alone is not enough to induce neuronal death (Marcaida et al., 1995).

The creatine / phosphocreatine / creatine kinase system is essential for the buffering and transport of high-energy phosphates (Wyss and Kaddurah-Daouk, 2000), and the mammalian brain is able of its own creatine synthesis both in adulthood and during development (Braissant et al., 2001b; Braissant et al., 2005; Braissant et al., 2007). Creatine is decreased in

the brain of *spf* mice (Ratnakumari et al., 1996b). In brain cell 3D primary cultures exposed to NH_4Cl , we have shown that axonal growth is impaired and that creatine and phosphocreatine are decreased (Fig. 3) (Braissant et al., 2002; Braissant et al., 2006). These studies support the hypothesis of an energy deficit induced by NH_4^+ exposure. Recent data demonstrate that NH_4^+ exposure generates a secondary creatine deficiency in brain cells, through disturbances of creatine transport and synthesis pathway (Braissant, Cagnon, Monnet-Tschudi, Speer, Wallimann, Honegger and Henry, submitted for publication). Interestingly, axonal growth is protected under NH_4^+ exposure by creatine co-treatment, in a glial cell-dependent manner (Braissant et al., 2002) (see also below 4.7: impairment of axonal and dendritic growth). The protective potential of creatine has been illustrated in numerous models of neurodegeneration (Fig. 2 and 3) (Brewer and Wallimann, 2000; Wilken et al., 2000).

Another agent, acetyl-L-carnitine, has also been proposed for the recovery of cerebral energy deficits induced by NH_4^+ . The main role of carnitine is to shuttle acyl-CoA derived from fatty acids across the inner mitochondrial membrane. Once inside the mitochondria, the acyl-CoA molecule undergoes beta-oxidation producing acetyl-CoA that can enter the Krebs cycle leading to the production of ATP. It has been shown that acetyl-L-carnitine is able to enhance the restoration of ATP and phosphocreatine levels in a model of ischemia (Aureli et al., 1994). A treatment of pregnant *spf* mice with acetyl-L-carnitine, starting from day 1 of conception, resulted in a significant restoration of ChAT activity levels in some brain regions of the *spf/Y* offspring, suggesting that acetyl-L-carnitine may be neuroprotective in NH_4^+ -induced toxicity (Ratnakumari et al., 1995b). It was suggested that this protective effect may be due to the restoration of the cytochrome C oxidase activity in *spf* mice (Rao et al., 1997). Aside from being an essential component of fatty acid metabolism, acetyl-L-carnitine is also a free-radical scavenger and may contribute to the protection of cells against oxidative stress (Zanelli et al., 2005).

4.4: Alteration of nitric oxide synthesis

NO is synthesized by nitric oxide synthase (NOS) from the unique precursor L-arginine. NOS, when expressed in concert with ASS and ASL, constitutes the citrulline-NO cycle, which allows the recycling of the by-product of NO synthesis, citrulline, to arginine (Fig. 2) (Morris, 2004). ASS and ASL can be found in almost every cell, including in the CNS, albeit most of the time at lower levels than when expressed for the urea cycle in hepatocytes (Wakabayashi, 1998; Braissant et al., 1999b; Husson et al., 2003). It has been suggested that to assure their specific needs in NO synthesis, brain cells depend on (i) arginine supply from periphery and between brain cells with the implication of the various cationic amino acid transporters (CATs) and other cationic amino acid carriers such as γ^+ L-associated transporters (γ^+ LATs), (ii) the regulation of arginine levels by arginase 2, and (iii) the recycling of citrulline to arginine by ASS and ASL, sometimes in cells different from the ones synthesizing NO (Braissant et al., 1999a; Wiesinger, 2001; Braissant et al., 2001a; Braissant, 2007).

In postsynaptic neurons, NO is formed following activation of glutamate receptors, mainly of the NMDA subtype. After this activation, Ca^{2+} is transiently increased in the cytosol and forms a complex with calmodulin that binds to and activates constitutive neuronal NOS (nNOS or NOS1). This leads to an increased production of NO, which in turn activates soluble guanylate cyclase resulting in increased concentration of cGMP. This glutamate-NO-cGMP pathway modulates important cerebral processes such as intercellular communication and LTP (Rodrigo and Felipo, 2006). As NMDA receptors are activated by NH_4^+ exposure, it suggests that hyperammonemia can activate nNOS and stimulate NO synthesis in neurons (Fig. 2). It has been shown that treatment of primary cultures of cortical neurons with NH_4^+

results in an increase of the cGMP level in response to SNAP, a NO-generating agent (Rodrigo et al., 2005). In contrast, in primary cultures of cerebellar neurons exposed to NH_4^+ , the formation of cGMP is reduced following SNAP addition (Rodrigo et al., 2005). The mechanism by which NH_4^+ can lead to opposite effects in cerebellum and cerebral cortex is not understood by now.

In glial cells, NO is synthesized after the transcriptional induction of a Ca^{2+} -independent inducible NOS isoform (iNOS or NOS2). In contrast to nNOS-derived NO formation which occurs transiently, iNOS activity can generate high concentrations of NO (for review see Moncada and Bolanos, 2006). In primary cultures of astrocytes, NH_4^+ induces iNOS expression and enhances NO synthesis (Schliess et al., 2002). It is known that elevated NO concentrations can interact with superoxide anion, leading to the formation of the highly toxic peroxynitrites. Thus, the excessive formation of NO by astrocytes induced by NH_4^+ may induce peroxynitrites in the neighboring neurons, leading to neuronal death (Fig. 2). Moreover, it has been suggested that NH_4^+ -induced production of NO could inhibit GS, the only NH_4^+ detoxification pathway in the brain, which might thus even worsen the consequences of hyperammonemia on CNS (Fig. 2) (Rose and Felipe, 2005). This increase in NOS activity and NO synthesis under NH_4^+ exposure is coherent with our observation in brain cell 3D cultures exposed to NH_4Cl , which increase their arginine content and induce the astrocytic expression of ASS and ASL, thus stimulating the citrulline-NO cycle (Braissant et al., 1999b; Bachmann et al., 2004).

NO synthesis has also been studied in vivo. The NOS activity and NO synthesis have been shown decreased in the brain of *spf* mice (Ratnakumari et al., 1996b). Similarly, in patients with OTC deficiency, plasmatic and urinary NO metabolites (as markers for NO synthesis) were shown below the normal range showing a decrease of NO synthesis (Nagasaka et al.,

2004). This is in accordance with the decrease in arginine (precursor for NO) content observed under urea cycle deficiencies. In contrast, experimental models with adequate arginine supplementation show opposite effects on NO synthesis under hyperammonemia. For instance, nNOS expression is increased, both at mRNA and protein levels, in the hyperammonemic brain of portacaval shunted rats (Rao, 2002), and NO production is increased in the brain of rats exposed to NH_4^+ -acetate (Kosenko et al., 1998).

4.5: Oxidative stress

Oxidative stress results from an imbalance between formation of free radicals and their neutralization by antioxidants. Free radicals are highly reactive, unstable molecules that have an unpaired electron in their outer shell. Free radicals are generally reactive oxygen or nitrogen species such as hydrogen peroxide, hydroxyl radical, nitric oxide, peroxynitrite and superoxide anion. They react with various cellular components leading to DNA damage, mitochondrial malfunction, cell membrane damage and eventually cell death.

Increased superoxide production and decreased activities of antioxidant enzymes such as glutathione peroxidase, superoxide dismutase and catalase, were observed in brain rats injected with NH_4^+ -acetate (Kosenko et al., 1997; Kosenko et al., 1998). In this model, inhibition of NOS by nitroarginine, or the use of NMDA receptor antagonists, prevent those changes suggesting that NH_4^+ -induced oxidative stress is due to the increased formation of NO as a consequence of excessive activation of NMDA receptors (Fig. 2) (Kosenko et al., 1998; Kosenko et al., 1999). Similarly, the production of free radicals is enhanced in a dose-dependent manner in primary cultures of astrocytes exposed to NH_4^+ (Murthy et al., 2001; Reinehr et al., 2007).

4.6: Induction of mitochondrial permeability transition

The mitochondrial permeability transition (MPT) is a sudden increase of the inner mitochondrial membrane permeability to ions and solutes, which causes disruption of mitochondrial inner membrane potential (maintained by the pumping out of protons by the electron transport chain) and diffusion of solutes down their concentration gradient. This leads to defective oxidative phosphorylation, cessation of ATP synthesis and the generation of reactive oxygen species. MPT is one of the major causes of cell death in a variety of conditions (Kroemer et al., 2007).

In primary cultures of astrocytes, NH_4^+ induces a collapse of the mitochondrial inner membrane potential that is associated with enhanced mitochondrial permeability (Fig. 2) (Bai et al., 2001). Similar treatments of cultured neurons failed to produce a dissipation of the mitochondrial inner membrane potential, suggesting that NH_4^+ -induced neuronal death may not be mediated by MPT (Bai et al., 2001). It has been shown that creatine is able to inhibit MPT pore opening (O'Gorman et al., 1997; Dolder et al., 2003). As MPT is causally related to cell death and is induced by NH_4^+ treatment in glial cells, creatine may represent a potential strategy for the CNS protection against NH_4^+ toxicity (Fig. 2 and 3).

4.7: Impairment of axonal and dendritic growth

Cortical atrophy, ventricular enlargement, or gray and white matter hypodensities are found in the developing brain of hyperammonemic neonates and infants (Msall et al., 1984; Harding et al., 1984; Call et al., 1984; Filloux et al., 1986; Dolman et al., 1988; Uchino et al., 1998; Wakamoto et al., 1999; Majoie et al., 2004). These clinical findings are reminiscent of neuronal fiber loss or defects of neurite outgrowth (Fig. 3). A significant loss of medium spiny neurons and increased numbers of reactive oligodendroglia and microglia in the striatum are also observed (Robinson et al., 1995). Moreover in *spf* mice, analysis of the

dendritic tree of layer V pyramidal cells in frontoparietal cortex revealed a significant decrease in both the complexity of the dendritic arbor and in dendritic terminal spine density (Hopkins et al., 1998). The alteration of dendritic arbor development under hyperammonemia might be triggered by dysregulations of dendritic cytoskeletal elements. Felipo et al. showed for instance that hyperammonemic 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 (Felipo et al., 1993). Using aggregating brain cell 3D primary cultures prepared from fetal rat telencephalon, we have shown that NH_4^+ exposure inhibits axonal growth, together with a decreased of NFM expression and phosphorylation (Braissant et al., 2002; Braissant, 2006). We further showed that the inhibition of axonal growth is accompanied by a decrease of creatine in the developing brain cells, and that creatine co-treatment under NH_4^+ exposure can protect axonal growth and restore NFM expression and phosphorylation in a glial cell-dependent manner (Fig. 3) (Braissant et al., 2002).

4.8: Disturbances in signaling transduction pathways

Recent works suggest that disturbances in upstream cell-signaling pathways may be responsible for numerous downstream mechanisms of developmental defaults or cell death in the brain (Mielke and Herdegen, 2000; Stanciu et al., 2000). Among these, mitogen-activated protein kinases (MAPKs) are good candidates for mediating upstream signals that lead to hyperammonemia-induced brain cell injury. MAPKs are part of an evolutionarily conserved mechanism that transduces extracellular signals, stress and injury to intracellular responses that balance survival, differentiation and death of the cell (Xia et al., 1995). Recent studies have shown that Erk1/2, JNK1/2/3 and p38^{MAPK} are activated in astrocytes by NH_4^+ (Schliess et al., 2002; Jayakumar et al., 2006). Early phosphorylation of Erk1/2 and p38^{MAPK} seems

responsible for NH_4^+ -induced astrocyte swelling whereas early phosphorylation of JNK1/2/3 and p38^{MAPK} is involved in NH_4^+ -induced inhibition of glutamate uptake in astrocytes (Schliess et al., 2002; Jayakumar et al., 2006). During CNS development, MAPKs are also involved in neuronal maturation and differentiation. In particular, Erk1/2 is involved in axonal and dendritic outgrowth, by regulating the phosphorylation state of cytoskeletal proteins such as neurofilaments (axons) and MAP-2 (dendrites), either directly or through activation of Cdk5 (Fig. 3) (Veeranna et al., 1998; Harada et al., 2001). MAPKs are dysregulated in NMDA excitotoxicity and cerebral ischemia (Borsello et al., 2003a; Borsello et al., 2003b), and have been proposed as targets in therapeutic approaches to counteract these CNS pathophysiological conditions (Mehta et al., 2007). Although activation levels of MAPKs have not been studied yet in neurons under NH_4^+ -exposure, the manipulation of MAPK pathways may represent a novel therapeutic target for protecting the CNS development of pediatric patients with hyperammonemia from the deleterious effects of NH_4^+ .

4.9: Impairment of cognitive performance

In line with clinical data showing that patients with urea cycle deficits often show mental retardation, several studies demonstrate that hyperammonemia induces impairment of cognitive performance in animal models. Using two learning tasks (passive avoidance test and Morris-type water maze), D'Hooge et al. have demonstrated deficits in cognitive functions in *spf* mice during episodes of hyperammonemia (D'Hooge et al., 2000). Aguilar et al. compared the cognitive performance of rats prenatally and neonatally exposed to NH_4^+ , with adult rats exposed for 15 days (Aguilar et al., 2000). With respect to passive avoidance behavior, adult hyperammonemia had no effect whereas the pre/neonatal hyperammonemia produced a clear increase in the step-through latency, suggesting that NH_4^+ exposure during prenatal and neonatal period impairs memory or conditioned learning of the animals. Moreover, LTP, a

phenomenon that is considered to be the molecular basis for some forms of memory and learning, is markedly reduced in hippocampal slices from rats prenatally and neonatally exposed to ammonia (Munoz et al., 2000). The impairment of LTP in hyperammonemia could be responsible for at least some of the cognitive alterations found in hyperammonemic rats and *spf* mice and could be involved in the origin of the mental retardation in infants and children with congenital hyperammonemia.

5: Conclusion and perspectives for neuroprotection against ammonium toxicity on the developing CNS

In conclusion, hyperammonemia is associated with neuronal cell loss and cerebral atrophy that lead to mental retardation and cerebral palsy in pediatric patients. These toxic effects of NH_4^+ are specific to the developing brain, as neuronal damages are not observed in the brain of adult patients with hyperammonemia due to liver failure. While, for a long time, the mechanisms of toxic effects of NH_4^+ on the developing brain were poorly understood, several recent clues have been provided by different models of hyperammonemia such as *spf* mice, rats treated prenatally and neonatally with NH_4^+ -acetate, as well as with brain cell 3D primary cultures treated with NH_4Cl during the development phase. Alterations in amino acids synthesis, in neurotransmitter functions, in nitric oxide metabolism as well as in cerebral energy, induction of oxidative stress and MPT have been observed, and may be responsible for neuronal cell death and axonal growth inhibition. Recent studies suggest also disturbances in signaling transduction pathways. Some neuroprotective strategies have been suggested by experiments in the different models, including the use of NMDA receptor antagonists, NOS inhibitors, creatine and acetyl-L-carnitine (Fig. 2 and 3).

As illustrated in this review, unraveling new therapeutic targets to protect the brain of hyperammonemic neonates and infants will require experimental approaches focusing on the developing CNS in its cellular complexity, with neurons and glial cells interacting together. Thus, future studies may focus not only on the intracellular (e.g. MAPKs or others), but also on the extracellular signaling pathways disturbed between developing brain cells under NH_4^+ exposure.

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Tables

Table 1 : Causes of hyperammonemia in neonates and infants.

Inherited disorders :

Urea cycle enzymes defects

- Carbamoylphosphate synthetase 1 (CPS-1) deficiency
- Ornithine transcarbamoylase (OTC) deficiency
- Arginosuccinate synthetase (ASS) deficiency (citrullinemia)
- Arginosuccinate lyase (ASL) deficiency (arginosuccinic aciduria)
- Arginase 1 deficiency
- N-acetylglutamate synthetase deficiency

Defects in transporters of urea cycle intermediates

- Lysinuric protein intolerance (γ^+ -LAT1 deficiency)
- Hyperammonemia-hyperornithinaemia-homocitrullinuria syndrome (ORNT1 deficiency)
- Citrin (mitochondrial aspartate glutamate carrier) deficiency

Organic acidemias

- Propionic acidemia
- Methylmalonic acidemia
- B-ketothiolase deficiency
- Isovaleric acidemia

Fatty acid oxidation disorders

- Medium chain acyl-CoA dehydrogenase deficiency
- Systemic carnitine deficiency
- Long chain fatty acid oxidation defects

Other inborn errors of metabolism

- Pyruvate carboxylase deficiency
- Ornithine aminotransferase deficiency
- Δ^1 -pyrroline-5-carboxylate synthase (P5CS) deficiency

Acquired disorders :

- Transient hyperammonemia of the newborn
- Herpes simplex systemic infection
- Liver failure
- Infection with urease positive bacteria
- Portacaval shunt
- Perinatal asphyxia

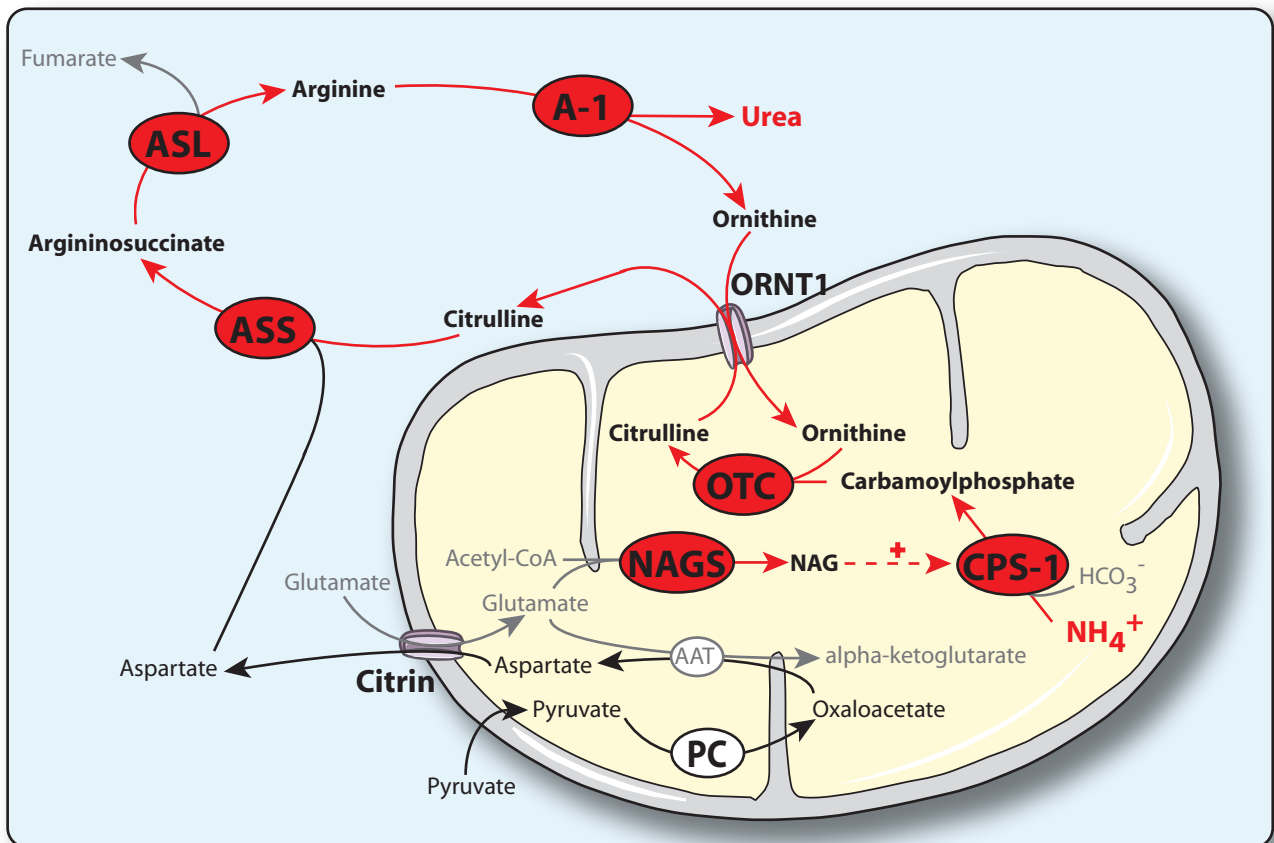
Figure legends

Figure 1: Expression of the whole urea cycle in hepatocytes. Hyperammonemia occurs in case of deficiency in one of the six enzymes of the urea cycle (CPS-1 OTC, ASL, ASS, A-1 and NAGS; illustrated in red), or in ORNT1, citrin or pyruvate carboxylase. Other inborn errors of metabolism or acquired disorders generating hyperammonemia are listed in Table 1. A-1: arginase 1; AAT: aspartate aminotransferase; ASL: argininosuccinate lyase; ASS: argininosuccinate synthetase; CPS-1: carbamoylphosphate synthetase 1; NAG: N-acetylglutamate; NAGS: N-acetylglutamate synthetase; ORNT1: ornithine citrulline antiporter 1; OTC: ornithine transcarbamoylase; PC: pyruvate carboxylase.

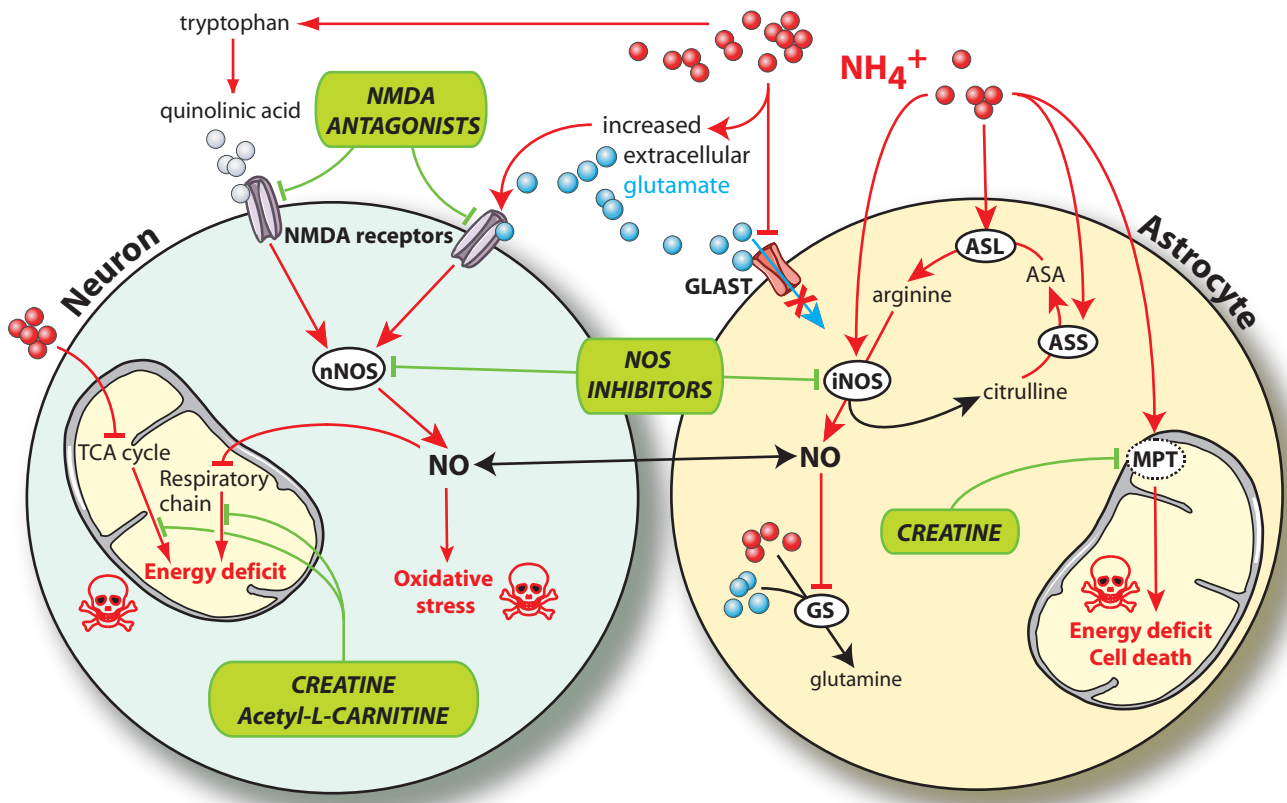
Figure 2: Neurotoxicity of hyperammonemia. The toxic effects of NH_4^+ exposure to neurons and astrocytes are illustrated in red. In particular, NH_4^+ exposure generates oxidative stress, energy deficit and cell death in the central nervous system through disturbances of the nitric oxide pathways, inhibition of the TCA cycle, and opening of the mitochondrial permeability transition. The protective effects of creatine, acetyl-L-carnitine, NMDA antagonists and NOS inhibitors against NH_4^+ toxicity to brain cells are illustrated in green. ASA: argininosuccinate; ASL: argininosuccinate lyase; ASS: argininosuccinate synthetase; GLAST: NA^+ -dependent glutamate / aspartate transporter; GS: glutamine synthase; iNOS: inducible nitric oxide synthase or NOS2; MPT: mitochondrial permeability transition; NMDA: N-methyl-D-aspartate; nNOS: neuronal nitric oxide synthase or NOS1; NO: nitric oxide;

Figure 3: Impairment of axonal growth by NH_4^+ exposure. Irreversible effects of NH_4^+ exposure on the developing brain can occur through inhibition of axonal growth. The toxic

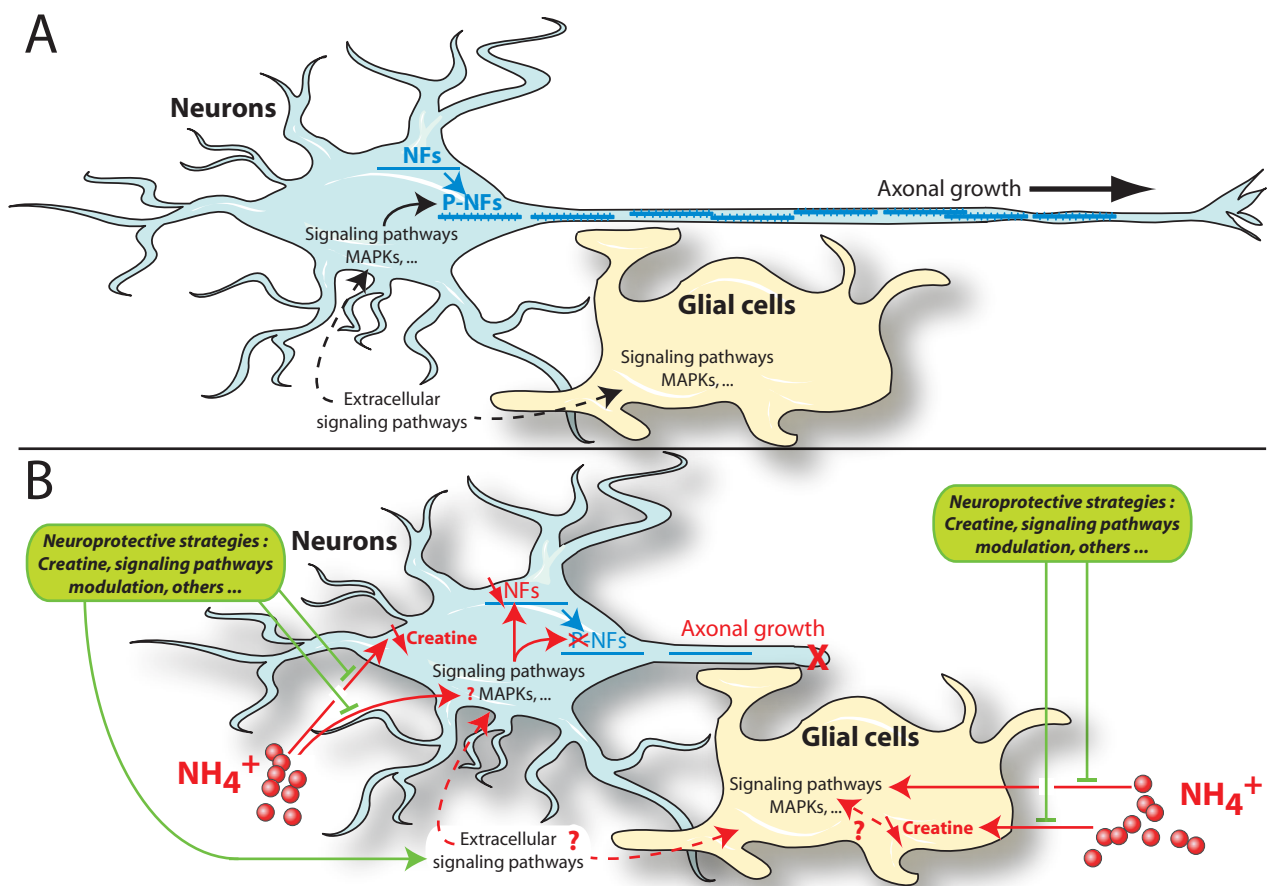
effects of NH_4^+ exposure on neurofilament protein expression and phosphorylation, on axonal growth, on signaling pathways and on the creatine content of brain cells are illustrated in red. Neuroprotection strategies to counteract NH_4^+ neurotoxicity are illustrated in green. **A:** Normal conditions of brain development. **B:** Hyperammonemia during brain development. MAPKs: mitogen activated protein kinase pathways; NFs: neurofilament proteins; P-NFs: phosphorylated neurofilaments.



Cagnon and Braissant, Figure 1.



Cagnon and Braissant, Figure 2.



Cagnon and Braissant, Figure 3.