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REVIEW

Ammonia toxicity to the brain

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Abstract Hyperammonemia can be caused by various acquired or inherited disorders such as urea cycle defects. The brain is much more susceptible to the deleterious effects of ammonium in childhood than in adulthood. Hyperammonemia provokes irreversible damage to the developing central nervous system: cortical atrophy, ventricular enlargement and demyelination lead to cognitive impairment, seizures and cerebral palsy. The mechanisms leading to these severe brain lesions are still not well understood, but recent studies show that ammonium exposure alters several amino acid pathways and neurotransmitter systems, cerebral energy metabolism, nitric oxide synthesis, oxidative stress and signal transduction pathways. All in all, at the cellular level, these are associated with alterations in neuronal differentiation and patterns of cell death. Recent advances in imaging techniques are increasing our understanding of these processes through detailed in vivo longitudinal analysis of neurobiochemical changes associated with hyperammonemia. Further, several potential neuroprotective strategies have been put forward recently, including the use of NMDA receptor antagonists, nitric oxide inhibitors, creatine, acetyl-L-carnitine, CNTF or inhibitors of MAPKs and glutamine synthetase. Magnetic resonance imaging and spectroscopy will ultimately be a powerful tool to measure the effects of these neuroprotective approaches.

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Introduction

Ammonia is produced by amino acid metabolism and intestinal urease-positive bacteria. In physiological conditions, it is mostly present as ammonium (NH_4^+) in serum. The urea cycle, which is fully expressed in the liver exclusively, serves to converts NH_4^+ to urea prior to renal excretion and to maintain low serum concentrations (50–150 μM in preterm neonates, 50–75 μM in term neonates, and <50 μM in adults). Although the brain cannot convert NH_4^+ to urea, NH_4^+ is also maintained at low levels in the central nervous system (CNS) by the astrocytic enzyme glutamine synthetase (GS), which synthesizes glutamine (Gln) from glutamate (Glu) and NH_4^+ (see Cagnon and Braissant 2007, and references therein).

Excessive NH_4^+ is toxic for the CNS. In adults, liver failure results in hyperammonemia which in turn leads to the potentially severe neuropsychiatric disorder hepatic encephalopathy (HE) characterized by altered mental status and coma. In the absence of irreversible cerebral edema, HE symptoms in adults are largely reversible when NH_4^+ returns to normal levels (Flint Beal and Martin 1998). In children, hyperammonemia can be caused by numerous inherited or acquired disorders (Leonard and Morris 2002), among which the best known are inherited urea cycle disorders (UCD) (Braissant 2010a; Gropman et al 2007; Tuchman et al 2008). The susceptibility of the developing brain to hyperammonemia leads to severe cognitive impairment, seizures and cerebral palsy (Enns 2008). Neonates and infants with important hyperammonemia develop cortical atrophy, ventricular enlargement, demyelination or gray and white matter hypodensities (Enns 2008; Gropman et al 2007; Tuchman et al 2008). The extent of irreversible brain damage depends on its maturational stage and on the

magnitude and duration of NH_4^+ exposure. Damage may become irreversible in case of prolonged hyperammonemia or when blood NH_4^+ reaches levels between 200 and 500 μM during the two first years of life (Bachmann 2003; Enns 2008; Msall et al 1984; Tuchman et al 2008; Uchino et al 1998).

This review focuses on the most recent advances in understanding NH_4^+ toxicity to the brain, with emphasis on novel tools, experimental models, therapies and neuroprotective strategies.

Hyperammonemia in humans

Hyperammonemia and its consequences on the brain develop secondary to various congenital or acquired causes (Cagnon and Braissant 2007). Examples include congenital portosystemic shunts (Kim et al 2012), extrahepatic portal vein obstruction (Pietrobattista et al 2010), and cirrhosis with portal hypertension. However, much of what is understood concerning ammonia neurotoxicity stems from patients with UCD.

Non-specific symptoms common to the immature and mature CNS

Non-specific symptoms are common in most UCD patients presenting in the neonatal period (poor feeding, vomiting, somnolence, irritability, tachypnoea) (Braissant 2010a). As NH_4^+ rises in serum, hypothermia, lethargy and coma progress rapidly (Summar 2001). In cases of partial UCD, clinical presentation can occur as late as months or years postnatally and are often triggered by illness or catabolic stress. In this case hyperammonemia is generally less severe and the symptoms are usually milder than in newborns (Takanashi et al 2002). Patients with late-onset hyperammonemia can present with loss of appetite, cyclic vomiting, lethargy or behavioral abnormalities (Harada et al 2006; Smith et al 2005). Patients with partial defects tend to sponteaneously avoid protein, especially female patients with ornithine transcarbamylase (OTC) deficiency (Scaglia et al 2002). Mental retardation and learning difficulties are frequent.

Cerebral edema: a common feature of the NH_4^+-exposed CNS

In response to elevated serum NH_4^+, the developing and the mature CNS respond similarly: Gln content in astrocytes rises through increased GS activity, and astrocytes swell. Under high NH_4^+ levels, osmoregulation is insufficient and cerebral edema develops, affecting all areas of the brain. In its most severe form, increased intracranial pressure eventually leads to brain herniation (Cordoba and Blei 1996; Norenberg et al 2005). In advanced cerebral edema, seizures, abnormal posture and neuromuscular irritability are frequent (Butterworth 1998). CNS edema first causes hyperventilation and respiratory alkalosis later progressing to hypoventilation and apnea (Brusilow and Maestri 1996). Without any treatment most infants will die. In survivors of infantile hyperammonemia, mental retardation is the norm (Bachmann 2003; Krivitzky et al 2009; Tuchman et al 2008).

Edema associated with HE can be followed by magnetic resonance spectroscopy (MRS) and magnetic resonance imaging (MRI) (Gropman 2010; Gropman et al 2010; Grover et al 2006; Oldham et al 2010). In the acute setting, serum NH_4^+ levels and cerebral edema are correlated with psychomotor performance (Foerster et al 2009; Yadav et al 2010).

Hyperammonemia in the adult brain does not provoke significant neuronal loss or structural damage to neurons, in contrast with what is observed in the developing CNS (Butterworth 2003).

Irreversible effects of ammonia on the developing brain

Irreversible damage to the developing brain results in mental retardation in most surviving children with UCD (Gropman et al 2007; Krivitzky et al 2009; Tuchman et al 2008). Neonatal onset leads to the most severe brain damage and the least IQ score, with significant volume loss of different parts of the developing brain as assessed by later MRI. Diffuse cortical atrophy, lesions in basal ganglia and thalamus, myelination delay and injury of the oligodendro-axonal unit are frequent (Majoie et al 2004; Takanashi et al 2003; Yamanouchi et al 2002). Cerebral MRI in UCD neonates suggest that some of these lesions might already be acquired in utero (Filloux et al 1986; Harding et al 1984; Majoie et al 2004; Takeoka et al 2001).

If hyperammonemia is diagnosed before irreversible cerebral insults, patients may have a normal neurodevelopment (Kurihara et al 2003). Many however remain mentally retarded or have learning difficulties (Smith et al 2005). Brain MRIs of late-onset UCD patients show cortical injury including acute ischemia, ventricular dilatation and myelination defects (Call et al 1984; Choi et al 2006; de Grauw et al 1990; Gropman et al 2010; Kim et al 2006; Kurihara et al 2003; Oldham et al 2010; Scaglia and Lee 2006). Similar lesions are found in patients with propionic acidemia or hyperammonemia-hyperornithinemia-homocitrullinuria syndrome who followed an unremarkable neonatal course (Harding et al 1991; Salvi et al 2001).

Only a few UCD cases have been analyzed by autopsy. Findings included microcephaly, shrinkage of hemispheres coupled to multiple cysts, ventricular dilatation, atrophy or necrosis of various brain nuclei or myelination defects (Dolman et al 1988; Takeoka et al 2001; Yamanouchi et al 2002). Microscopically, spongious brain tissue with extensive neuronal loss (in cortex and hippocampus particularly) was observed, together with gliosis and astrocytes with

water-clear, oval nuclei characteristic of Alzheimer's type II astrocytes.

Not all encephalopathies in children are due to UCD or primary gene defects affecting the liver, and there is increasing understanding that behavioral and neurological changes are frequent in patients with chronic liver failure (CLF) or portocaval shunts (PCS) even in absence of any significant hyperammonemia (Caudle et al 2010, 2012; Yadav et al 2010). Clinical signs and electro-encephalogram findings in children with acute liver failure (ALF) are relatively well characterized. In contrast, while children affected with CLF display neurocognitive deficits at an early age, the subtleties of their CNS alterations are much less understood (Bajaj et al 2011; Caudle et al 2010; Yadav et al 2010). In adults, minimal hepatic encephalopathy (MHE) is often hidden and only unmasked by specialized tests (Bajaj et al 2012; Felipo et al 2012). Recent data show that adults with MHE present cortical thinning that parallels their cognitive impairment associated with early stages of liver disease (Montoliu et al 2012). Although children with liver disease or PCS can also display T1 hyperintensity of the pallidi by MR, the clinical, biological, and imaging subtleties of pediatric MHE still need to be defined.

Available treatment options

Neonatal hyperammonemia and UCDs

The rapid removal of NH_4^+ should be the immediate therapeutic goal in neonatal hyperammonemia (Walker 2009). Protein restriction is the cornerstone of therapy, particularly in severe cases. Dialysis (hemodialysis, hemodiafiltration or continuous veno-venous hemofiltration) is indicated for hyperammonemia which does not correct rapidly or which is refractory to conservative measures (Leonard et al 2008). Intravenous glucose (Glc) is essential to reverse catabolism, together with careful use of insulin to avoid fluctuations in serum Glc levels (Summar 2001). Intravenous administration of sodium benzoate and sodium phenylacetate, both nitrogen scavengers, is an alternative and frequently used approach to achieve sufficient nitrogen excretion in acute phases (including neonatal) (Enns 2010; Shih 2007; Tuchman et al 2008). Long term control of NH_4^+ makes use of the same compounds or oral sodium phenylbutyrate, in combination with a low-protein diet (Batshaw et al 2001; Berry and Steiner 2001; Cederbaum et al 2010; Enns et al 2007; Scaglia 2010). In UCD, large doses of arginine (Arg) for argininosuccinate synthetase (ASS) and argininosuccinate lyase (ASL) deficiencies, or citrulline for carbamylphosphate synthetase 1 (CPS-1) and OTC deficiencies, further promote nitrogen excretion (Brusilow et al 1979; Leonard and Morris 2002). Orthotopic liver transplantation is currently the only option for severe, uncontrollable UCD (Lee and Goss 2001). However, cell-based rather than organ-based gene therapy is the ultimate goal and is an area of intense research (Meyburg and Hoffmann 2010).

Early diagnosis and intensive treatment are often insufficient to prevent death, and neurological problems are frequent in survivors (Bachmann 2003). High resolved proton spectroscopy (^{1}H-MRS) may prove to be a useful tool in tailoring the care for patients with hyperammonemia regardless of the underlying cause. In patients with CLF for example, spectroscopic findings consistent with HE/MHE may be a new indication for liver transplant or for the prescription of the same nitrogen scavengers as those used in UCD.

Chronic hepatic encephalopathy

In adult patients with suspected or diagnosed chronic HE, the mainstay of management is the reduction in gut-derived ammonia. Historically, the drug of choice was lactulose which decreases luminal pH, thereby favoring the transformation of non-resorbable NH_4^+ produced by enteric commensals and thus decreasing NH_4^+ in portal venous blood and nitrogen load to the liver (Als-Nielsen et al 2004; Patil et al 1987). More recently, the oral non-absorbable antibiotic rifaximin has become the drug of choice. In a pivotal trial in patients with CLF, rifaximin prevented HE relapse more efficiently than placebo (Bass et al 2010). A recent meta-analysis suggests that it is at least as effective as other oral treatments such as disaccharides and other antibiotics. It further suggests that it may have fewer side effects than previously used agents, and that it may in fact improve performance on psychometric tests (Eltawil et al 2012).

Nitrogen scavengers are also used in HE patients. L-ornithine-L-aspartate (LOLA) is used to supply ornithine (Orn) to the urea cycle, thereby favoring NH_4^+ conversion to urea in residual periportal hepatocytes and Gln synthesis from Glu and NH_4^+ in skeletal muscle, which in liver failure is an important metabolic alternative for the breakdown of NH_4^+. In rodent models, LOLA significantly increased urea production and blood Gln levels, and decreased CNS NH_4^+ while slowing the rise in brain Gln (Rose et al 1998). Results from human trials are controversial however: both fasting and post-prandial serum NH_4^+ falls within 7-days of treatment initiation in parallel with improved psychomotor performance and overall well-being (Kircheis et al 1997). However, serum Gln rises contributing in turn to ammoniagenesis, rebound hyperammonemia and severe HE following LOLA withdrawal (Olde Damink et al 2002). No studies have been done in children, although anecdotal, unpublished reports suggest that daytime drowsiness and attention deficits may improve in LOLA-treated older ones. L-ornithine-phenylacetate (OP) is an elegant alternative as it

is thought to act both on NH_4^+ transformation and elimination. Its Orn moiety uses muscle GS activity to convert NH_4^+ into Gln, while phenylacetate combines with Gln as phenylacetylglutamine (Jalan et al 2007). OP showed a beneficial effect in rat models of CLF with acute HE decompensation. In lipopolysaccharide-induced HE exacerbation in BDL rats, OP intraperitoneal administration reduced brain edema and rate of progression to coma (Wright et al 2012). This study is auspicious of benefiting human subjects since OP was administered once the rats were diagnosed with HE. Conversely another study showed that pre-emptive OP limited HE severity in PCS rats in which HE exacerbation was triggered by administration of blood in the gastrointestinal tract (Oria et al 2012). Although it can be argued that patients at risk of severe gastrointestinal bleeds could benefit from primary OP prophylaxis, this study seems less clinically significant as the cost-effectiveness of such an approach might be prohibitive. However, at this time it is too early to formally conclude since human studies using this newer compound are forthcoming.

Experimental models to study NH_4^+ toxicity to the brain

In vivo: *Spf* mice, KO mice and rat models of hyperammonemia

Sparse-fur (*Spf*) mice have a single point substitution in the OTC gene, with X-linked transmission, mimicking the human disease (Veres et al 1987). Hepatic OTC activity is 5–13 % of that in normal mice. Adult *Spf/Y* mice show NH_4^+ blood and brain levels increased by 1.5- and five-fold respectively (Ratnakumari et al 1992). Neuropathologic studies in *Spf* mice show similar brain alterations as those observed in OTC patients: brain size reduction (including decreased striatum volume) and ventricular enlargement (Hopkins et al 1998). Several knock-out (KO) mice have been engineered to model UCDs (Deignan et al 2008): ASS, ASL, arginase I and arginase II KO mice, as well as double KO mice for arginases I+II. $ASS^{-/-}$ and $ASL^{-/-}$ mice die a few days after birth, with plasma NH_4^+ increased four-fold. Arginase I and arginases I+II KO mice die from hyperammonemia 14 days postnatally, with a ten-fold increase in plasma NH_4^+.

Different rat models exist to analyze the effects of hyperammonemia on the CNS. For example, pregnant rats can be fed a diet containing NH_4-acetate from day 1 of pregnancy until weaning, followed by feeding the pups after weaning with high NH_4^+-containing diet. NH_4^+ levels in the brain of these animals is 1.4 times higher as compared to control (Aguilar et al 2000). Alternatively, hyperammonemia can be induced in adult rats by intraperitoneal injections of NH_4^+-acetate, continuous iv infusion of NH_4Cl, iv urease infusion (Robinson et al 1992b), administration of a NH_4^+-acetate containing diet (Azorin et al 1989) or surgical PCS (Song et al 2002). The following are the most valid in vivo models of ALF: hepatic-devascularized rats, thioacetamide-treated rats, NH_4^+-treated portocaval-shunted rats and galactosamine-treated rats (see Butterworth et al 2009 for a review; Bosoi et al 2012; Cauli et al 2011; Chavarria et al 2010; Cudalbu et al 2012b; Kanamori et al 1993; Shen et al 1998; Zwingmann et al 2003).

In vitro: monotypic brain cell cultures and organotypic mixed-cell cultures

NH_4^+ toxicity has been studied in monotypic primary cultures of neurons or astrocytes (Jayakumar et al 2006; Klejman et al 2005) as well as in organotypic cultures of hippocampal rat brain slices (Chepkova et al 2006). These models provide several clues regarding the mechanisms of cellular NH_4^+ toxicity, but they do not allow for the analysis of the effects of hyperammonemia on the developing CNS, especially with respect to the relationships between developing neurons and glia.

To this end, we have developed 3D primary reaggregated brain cell cultures as a valid experimental model to study the effects of NH_4^+ on the developing CNS (Braissant et al 2002, 2008). These cultures, which are classified as organotypic, are prepared from the brain of rat embryos, contain all types of brain cells (neurons, astrocytes, oligodendrocytes, microglia) and grow in a manner resembling in vivo CNS (Honegger and Monnet-Tschudi 2001). In this model hyperammonemia is mimicked by treating cultures with NH_4Cl. Compared to classical monotypic cultures, 3D brain cell cultures allow for the analysis of irreversible NH_4^+ toxicity in a model that mimics brain complexity at different maturational stages. These cultures are also a useful tool to examine the effects of hyperammonemia in isolation, devoid of the confounding variables found in animal models owing to the secondary effects of hyperammonemia.

Mechanisms of CNS ammonium toxicity

Amino acids disturbances

By synthesizing Gln from NH_4^+ and Glu, the astrocytic enzyme GS is the major CNS pathway of NH_4^+ removal. Accordingly, hyperammonemia with high NH_4^+ levels increase Gln in brain cells, as seen in OTC patients (Connelly et al 1993), *Spf* mice (Inoue et al 1987), organotypic brain cell cultures (Bachmann et al 2004) and in NH_4^+-infused rat (Figs. 1 and 3). Gln is osmotically active and its NH_4^+-induced increase leads to cytotoxic edema by astrocyte swelling. According to the "Trojan horse" hypothesis, astrocyte swelling under NH_4^+ exposure may be subsequent to Gln transport into mitochondria, Gln being cleaved back to ammonia upon

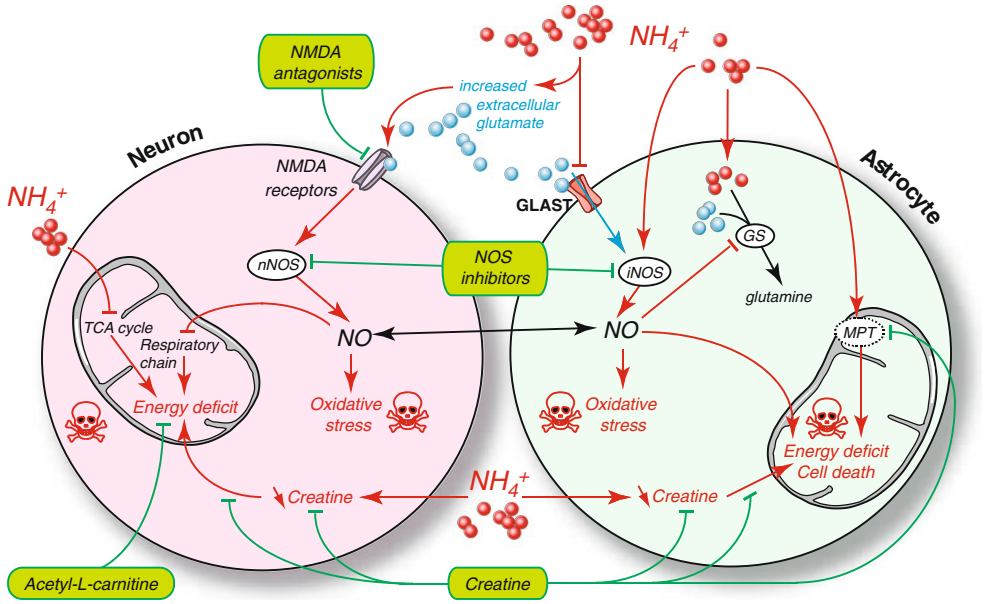


Fig. 1 NH_4^+ toxicity for the central nervous system. Toxic effects of NH_4^+ to neurons and astrocytes are shown in *red*. In particular, NH_4^+ exposure generates oxidative stress, energy deficit and cell death in CNS through disturbances of the NO pathway, inhibition of the TCA cycle, opening of the mitochondrial permeability transition and secondary creatine deficiency. Protective effects of creatine, acetyl-L-carnitine, NMDA antagonists and NOS inhibitors against NH_4^+ toxicity are shown in *green*. **GLAST**: NA^+-dependent Glu/Asp transporter; **GS**: glutamine synthase; **iNOS**: inductible nitric oxide synthase or NOS2; **MPT**: mitochondrial permeability transition; **NMDA**: N-methyl-D-aspartate; **nNOS**: neuronal nitric oxide synthase or NOS1; **NO**: nitric oxide

entry into mitochondria, thereby producing reactive oxygen species and inducing the mitochondrial permeability transition (MPT) (see below) (Albrecht and Norenberg 2006; Albrecht et al 2010; Rama Rao et al 2012). In ALF, Gln trapping in astrocytes affects adjacent glutamatergic neurons, decreasing excitatory transmission and increasing neuroinhibition (Desjardins et al 2012).

Furthermore, astrocyte swelling can cause a secondary release of Glu into the intercellular space which, coupled to the conversion of Glu and NH_4^+ to Gln, can decrease intracellular pools of Glu, and induce the death of glutamatergic neurons (Hertz and Kala 2007; Qureshi and Rao 1997). Indeed, Glu is significantly decreased in the cerebral cortex of *Spf* mice and NH_4^+-exposed brain cell 3D cultures (Bachmann et al 2004; Ratnakumari et al 1994a). NH_4^+ excess can also be detoxified by converting α-ketoglutarate to Glu by glutamate dehydrogenase (GDH), albeit its activity being much lower than that of GS in astrocytes. The consequence is depletion of α-ketoglutarate from the tricarboxylic acid (TCA) cycle.

Patients with UCD (except arginase I deficiency) present with decreased plasma Arg, hence the indication for Arg supplementation (Leonard and Morris 2002; Scaglia et al 2004; Scaglia and Lee 2006). Arg is the precursor for nitric oxide (NO) and creatine (Cr) synthesis. Consequently, decompensated UCD is associated with disturbances in the citrulline-NO cycle and in Cr metabolism both in the brain and peripherally (see below). *Spf* mice display deficient Arg synthesis, not unlike what is seen in the CNS of OTC patients (Ratnakumari et al 1996b). In contrast, intracellular Arg increases when brain cells with a normal Arg supply are exposed to NH_4^+. This has been shown repeatedly in brain cell organotypic cultures (Bachmann et al 2004), in rat cerebellar synaptosomes (Rao 2002) and in rat primary astrocytes (Zielinska et al 2012). The NH_4^+-induced expression of ASS and ASL in astrocytes may also contribute to this process (Braissant et al 1999b).

Finally, large neutral amino acids (tryptophan Trp, tyrosine, phenylalanine, methionine, histidine) accumulate in the CNS of *Spf* mice (Bachmann and Colombo 1984; Inoue et al 1987). Tryptophan accumulation may lead to disturbances in serotoninergic neurotransmission.

Alterations in neurotransmission systems

If amino acid metabolism is altered in hyperammonemia, it follows that neurotransmission should be affected. NH_4^+ exposure leads to astrocyte swelling, pH- and Ca^{++}-dependent Glu release from astrocytes, inhibition of Glu re-uptake by astrocytes (inhibition of GLAST transporter) and excess depolarization of glutamatergic neurons (Cagnon and Braissant 2007; Chan et al 2000; Rose 2006). These abnormalities, in turn, induce excess extracellular Glu accumulation. Increased Glu release by brain cells is observed in *Spf* mice, in rabbit models of acute hyperammonemia and in primary astrocytes exposed to NH_4^+ (de Knegt et al 1994; Rao and Qureshi 1999; Rose et al 2005). Excessive extracellular Glu is excitotoxic, essentially through N-methyl-D-

aspartate (NMDA) receptor activation. NMDA receptor activation in turn leads to an array of metabolic alterations affecting NO metabolism and Na^+/K^+-ATPase. ATP shortage, mitochondrial dysfunction, free radical accumulation and oxidative stress ultimately ensue (see below) and lead to cell death (Fig. 1) (Braissant 2010a; Rodrigo et al 2009). AMPA and mGluR receptors are also affected by NH_4^+ exposure. Finally, NH_4^+ can also alter other neurotransmission systems via its effect on glutamatergic excitotoxicity (e.g., activation of GABA or benzodiazepine receptors) (Cauli et al 2009).

HE is characterized by an imbalance in cholinergic system activity in humans, BDL rats and rats fed with NH_4^+ (Garcia-Ayllon et al 2008). A significant decrease of cholinergic neurons is observed in the forebrain of *Spf* mice (Ratnakumari et al 1994b) as well as in brain cell 3D cultures exposed to NH_4^+ (Braissant et al 2002). In the CNS of *Spf* mice choline acetyltransferase (ChAT) activity decreases immediately after weaning and reaches significantly lower levels in adult animals. Likewise, cholinergic muscarinic M1 (postsynaptic) and M2 (presynaptic) receptors are altered (Michalak and Butterworth 1997; Ratnakumari et al 1996a). These data suggest that hyperammonemia can severely impair cholinergic neurotransmission.

Trp and 5-hydroxyindoleacetic acid (respectively precursor and metabolite of serotonin) are increased in *Spf* mice brain and in the CSF of UCD children (Bachmann and Colombo 1984; Hyman et al 1987). Receptor binding studies of *Spf* mice revealed a significant loss of $5HT_2$ receptor and a concomitant increase in $5HT_{1A}$ receptor (Robinson et al 1992a). These hyperammonemia-induced alterations of the serotoninergic system may be involved in anorexia and sleep disturbance observed in UCD (Hyman et al 1986).

Cerebral energy deficit

Spf mice show decreased ATP in their brain (Ratnakumari et al 1992), together with a lower cytochrome C oxidase expression and activity, suggesting that ATP reduction might be due to a slowing of the electron transport chain enzymes (Fig. 1) (Rao et al 1997). The deficit in brain energy metabolites under hyperammonemia might also be due to TCA cycle inhibition via α-ketoglutarate dehydrogenase (see above) (Hertz and Kala 2007; Lai and Cooper 1986). However ATP depletion alone is not enough to induce cell death in CNS (Marcaida et al 1995).

The Cr/phosphocreatine/creatine kinase system is essential for cellular energy through buffering and regeneration of ATP, both systemically and in the brain (Béard and Braissant 2010; Braissant 2012; Braissant et al 2011; Brosnan and Brosnan 2010; Brosnan et al 2007). *Spf* mice show decreased brain Cr (Ratnakumari et al 1996b), and NH_4^+ exposure generates a secondary Cr deficiency in brain cells (Braissant et al 2008, 2002).

Primary astrocyte cultures, but not neuronal cultures, exposed to NH_4^+ show opening of MPT (Alvarez et al 2011; Bai et al 2001), leading to altered oxidative phosphorylation, cessation of ATP synthesis, production of reactive oxygen species and cell death (Fig. 1). Increased superoxide production and decreased antioxidant enzyme activity were also observed in the brain of NH_4^+-infused rats (Kosenko et al 1997). This was prevented both by nitroarginine-mediated NOS inhibition and by NMDA receptor antagonists, suggesting that NH_4^+-induced oxidative stress is at least in part due to increased NO formation through excessive NMDA receptor activation (Kosenko et al 1999, 1998). In keeping with this hypothesis, free radical production is enhanced in primary cultures of astrocytes exposed to NH_4^+ (Murthy et al 2001; Reinehr et al 2007).

Alteration of nitric oxide synthesis

NO is produced from Arg by nitric oxide synthase (NOS) which constitutes the citrulline-NO cycle in concert with ASS and ASL. The citrulline-NO cycle is well expressed in CNS, together with cationic amino acid transporters (CATs, y^+LATs) allowing for a steady Arg supply to brain cells (Braissant et al 1999a, 2001; Wiesinger 2001).

In neurons under Arg normal supply, the activation of NMDA receptors by NH_4^+ exposure activates neuronal NOS (nNOS or NOS1) and stimulates NO synthesis. This finding has been demonstrated in PCS rats, in the CNS of rats infused with NH_4^+-acetate, and in primary cultures of cortical neurons (Kosenko et al 1998; Rao 2002; Rodrigo et al 2005). Likewise, in glial cells, inducible NOS (iNOS or NOS2) activity can generate high concentrations of NO. NH_4^+ exposure induces iNOS expression and enhances NO synthesis in primary astrocyte culture (Schliess et al 2002). This is also coherent with the observation that brain cell cultures exposed to NH_4^+ increase their Arg content and induce expression of ASS and ASL in astrocytes, thereby stimulating the citrulline-NO cycle (Bachmann et al 2004; Braissant et al 1999b; Zielinska et al 2011).

NH_4^+ exposure leads to excessive formation of NO which can impair mitochondrial respiration by interacting with superoxide anions leading to formation of highly toxic peroxynitrites. It therefore follows that neuronal and glial death ensues from secondary ATP depletion, increased free radicals and oxidative stress (Rodrigo et al 2009). Moreover, NH_4^+-induced production of NO can inhibit GS thus potentiating the consequences of hyperammonemia on the CNS (Rose and Felipo 2005).

Recent data also suggest that ammonia may alter blood–brain-barrier (BBB) permeability through a mechanism involving increased NO and oxidative stress in the brain microcapillary endothelium, thus contributing to vasogenic edema

induced in ALF conditions (Jayakumar et al 2012; Skowronska et al 2012).

NH_4^+ effects on NO are different in UCD. Except for arginase I deficiency, other UCDs are associated with Arg shortage (see above), thus impairing the citrulline-NO cycle. Accordingly, NOS activity and NO synthesis are decreased in CNS of *Spf* mice (Ratnakumari et al 1996b). In OTC deficiency, plasma and urinary NO metabolites (as markers of NO synthesis) are below the normal range, suggesting decreased NO synthesis (Nagasaka et al 2004). Arginase I deficiency presents with elevated plasma Arg levels, thereby inducing an upregulation of NO metabolism (Scaglia et al 2004; Scaglia and Lee 2006).

In summary, brain NO metabolism is affected in a number of ways by NH_4^+ exposure. Effects vary depending on whether the exposure is acute or chronic, on brain cell type, and whether Arg supply is normal or decreased.

Impairment of axonal and dendritic growth

Cortical atrophy, ventricular enlargement, and gray and white matter hypodensities are characteristic neuroimaging findings in children having suffered from hyperammonemia, suggesting neuronal fiber loss or defects in neurite outgrowth. In layer V neurons of frontoparietal cortex, *Spf* mice show a decreased complexity of dendritic arborescence as well as dendritic spine density (Hopkins et al 1998). Alteration of neurite outgrowth by hyperammonemia might be triggered by dysregulation of cytoskeletal elements. Rats fed with NH_4^+-acetate develop decreased phosphorylation of the dendritic protein microtubule associated protein 2 (MAP-2), together with an increase of MAP-2 binding to microtubules (Felipo et al 1993). NH_4^+ exposure of 3D developing brain mixed-cell primary cultures decreases medium weight neurofilament (NFM) expression and phosphorylation and inhibits axonal growth (Braissant et al 2002). This occurs only in developing brain cells but not after neuronal differentiation, in line with the clinical differences between pediatric and adult patients (see above).

Cell death and signaling transduction pathways

Irreversible damage caused by NH_4^+ exposure on the developing CNS is consistent with brain cell death, which we showed in neurons and oligodendrocytes of NH_4^+-exposed organotypic brain cell cultures (Braissant 2010a; Cagnon and Braissant 2007, 2008). In particular, NH_4^+ induces neuronal apoptosis through activation of caspases and calpain. We further showed that NH_4^+-induced calpain activation cleaves the cdk5 activator p35 to p25, which induces neurodegeneration.

NH_4^+ exposure may also trigger endogenous protective mechanisms to prevent or limit brain damage. We showed that ciliary neurotrophic factor (CNTF), an injury-associated survival factor expressed by astrocytes, is up-regulated by NH_4^+ through p38 mitogen-activated protein kinase (MAPK) activation (Cagnon and Braissant 2009), with secondary roles of the two other MAPKs, SAPK/JNK and Erk1/2 in oligodendrocytes and neurons respectively. Erk1/2, SAPK/JNK and p38 are activated in primary astrocytes by NH_4^+, and phosphorylation of Erk1/2 and p38 appears responsible for NH_4^+-induced astrocyte swelling, while phosphorylation of SAPK/JNK and p38 is involved in NH_4^+-induced inhibition of Glu uptake by astrocytes (Jayakumar et al 2006; Moriyama et al 2010; Schliess et al 2002). P53, a downstream target of MAPKs, is activated in NH_4^+-exposed astrocytes, contributing to astrocyte swelling and Glu uptake inhibition (Panickar et al 2009).

Channels and transporters

Brain edema due to hyperammonemia is thought to occur essentially through astrocyte swelling. In recent studies both in primary astrocyte cultures exposed to NH_4^+ and in thioacetamide-treated rats (an in vivo model of ALF), the Na^+-K^+-Cl^- cotransporter-1 (NKCC1) was activated in response to NH_4^+ exposure, thus increasing water entry in astrocytes (Jayakumar and Norenberg 2010; Jayakumar et al 2011). It was shown that connexin 43 (Cx43), aquaporin 4 (Aqp4) and the astrocytic inward-rectifying K^+ channels Kir4.1 and Kir5.1 are decreased in astrocytes of *Spf*/GFAP-EGFP mice (Lichter-Konecki et al 2008). Kir4.1 is also down-regulated in the cortex of rats with liver failure (Obara-Michlewska et al 2011). In a rat model of ALF, Aqp4 is increased in the astrocytic feet lining BBB (Rama Rao et al 2010), where these channels are co-localized and regulate K^+ and water transport. NH_4^+ is known to cross some aquaporins, which might link cerebral metabolism to volume control (Holm et al 2005). Astrocytes may respond to elevated blood NH_4^+ by inducing a protective down-regulation of Cx43, Aqp4, and Kir4.1/Kir5.1, thus slowing NH_4^+ influx and decreasing water and K^+ efflux. Increased brain extracellular K^+ and water may come with a price, however: brain edema, a phenomenon which has been observed in UCD patients (Lichter-Konecki et al 2008).

Impairment of cognitive performance

Consistent with what is seen in patients, several animal models with hyperammonemia show impaired cognitive performance. *Spf* mice show deficits in cognition during hyperammonemic episodes (D'Hooge et al 2000). Prenatal and neonatal exposure to NH_4^+ in rats appears to impair memory or conditioned learning, while no such effect is observed when NH_4^+ exposure occurs in adults (Aguilar et al 2000). Long term potentiation (LTP), which is considered as the molecular basis of some forms of memory and learning, is significantly decreased in hippocampal slices from rats prenatally and neonatally exposed to ammonia (Munoz et al 2000). LTP impairment in hyperammonemia might be responsible for at least some of the

cognitive alterations found in hyperammonemic rats and *Spf* mice, and could be involved in some aspects of mental retardation in pediatric patients exposed to NH_4^+.

Neuroprotection strategies

The progressive discovery of the various toxic effects of NH_4^+ on the CNS, as described above, has led in the last years to the proposal of various neuroprotective strategies. They all have the following aims: 1) restoring CNS energy status 2) allowing normal brain cell development and 3) protecting them from cell death. These different approaches were developed both in in vitro and in vivo models, but have yet to be tested in human subjects. Their potential lies in their association with treatments aimed at decreasing NH_4^+ levels, the idea being to protect neurons all the while reducing the insult.

Preventing NH_4^+-induced excitotoxicity through NMDA receptors and excessive production of NO was proposed by using NMDA receptor antagonists such as MK-801 and 2-amino-5-phosphonovaleric acid (APV), which appear to improve neuron survival in primary cortical neurons of newborn rats exposed to NH_4^+ (Klejman et al 2005). NMDA receptor antagonists have also demonstrated their neuroprotective properties in a galactosamine-injected rat model of ALF (Rodrigo et al 2009). Moreover, APV diminishes the $NH4^+$-induced impairment of LTP in rat hippocampal slices (Izumi et al 2005). NMDA antagonists and NOS inhibitors such as nitroarginine are potential candidates to counter the deleterious effects of NH_4^+-induced NO upregulation (Klejman et al 2005; Kosenko et al 1998, 1999), as they can prevent increased superoxide production and decreased antioxidant enzyme activity in the brain of NH_4^+-infused rats (Kosenko et al 1997).

Cr and acetyl-L-carnitine have been proposed to protect from NH_4^+-induced cerebral energy deficits. NH_4^+ exposure can lead to a secondary Cr deficiency (Braissant et al 2008). However, Cr co-treatment under NH_4^+ exposure is neuroprotective. Cr appears to protect axonal growth in NH_4^+-exposed organotypic cultures of rat brain cells, where it also restores NFM expression and phosphorylation in a glial cell-dependent manner (Fig. 2) (Braissant et al 2002; Braissant 2010b). In the same model, Cr also prevent the loss of cholinergic neurons. Cr is also neuroprotective by inhibiting MPT opening (Dolder et al 2003). Using Cr as a neuroprotective agent may be facilitated by the NH_4^+-induced activity of the Cr transporter SLC6A8 both at BBB and in surrounding astrocytes (Bélanger et al 2007; Braissant et al 2008; Braissant 2012). Offspring of *Spf* mice treated with acetyl-L-carnitine from day 1 of conception displayed a significant restoration of ChAT activity when exposed to NH_4^+, suggesting a neuroprotective role for acetyl-L-carnitine (Ratnakumari et al 1995). Its neuroprotective mechanisms may include restoration of cytochrome C oxidase activity (Rao et al 1997) or free-radical scavenger action to protect cells against oxidative stress (Zanelli et al 2005).

Protecting NH_4^+-exposed brain cells by modulating intra- and extracellular signaling pathways may also be effective. We have shown that roscovitine, a cdk5 inhibitor, protects neurons from NH_4^+-induced death. However, as roscovitine also impairs axonal growth probably through inhibition of the remaining cdk5/p35 activity, cdk5 appears as a promising therapeutic target to treat hyperammonemic newborns or infants provided that one can selectively inhibit cdk5/p25 (Cagnon and Braissant 2008). Specifically inhibiting the p38 MAPK pathway, which is activated under NH_4^+-exposure (Cagnon and Braissant 2009), appears to be neuroprotective in portacaval shunted rats (Agusti et al 2011). We also demonstrated that co-treatment with exogenous CNTF protects oligodendrocytes from NH_4^+ toxicity through SAPK/JNK (Cagnon and Braissant 2009), suggesting that CNTF may have therapeutic implications to counteract demyelination in hyperammonemic patients.

Finally, attenuating NH_4^+-induced edema in the CNS by inhibiting GS activity through the use of methionine sulfoximine (MSO) has long been proposed. MSO was demonstrated to decrease NH_4^+-induced astrocyte swelling and brain edema in various in vivo rat models (Tanigami et al 2005; Willard-Mack et al 1996), and was recently shown to divert NH_4^+ detoxication from Gln synthesis (GS activity) to alanine (Ala) through GDH activity in co-cultures of astrocytes and neurons (Dadsetan et al 2011). MSO also appeared beneficial in mice under ALF by promoting their survival, however in that case through CNS-independent mechanisms (Jambekar et al 2011). As MSO is not a specific inhibitor of GS, but also affects other targets such as γ-glutamyl cysteine synthetase, further work is needed in search of more specific GS inhibitors to evaluate the potential of brain GS inhibition in NH_4^+ exposure.

In conclusion, much more work is needed to establish the best neuroprotective strategies, which may use the above-mentioned molecules alone or in combination, and coupled to NH_4-lowering agents.

In vivo investigation of the brain during HE

Understanding the pathophysiology of HE and NH_4^+ toxicity to the brain requires experimental approaches focusing on the CNS in its cellular and molecular complexity (Braissant 2010a; Butterworth 2012; Cagnon and Braissant 2007). Although there is extensive research examining the biochemistry of ammonia-induced neurotoxicity in various in vitro systems varying from primary monolayer cultures (neuronal or glial) to organotypic cultures (including mixed ones neuronal + glial) (see above), in vivo data are often missing. In the last decades, MRI and MRS have become powerful and reliable diagnostic tools with the unique advantages of being applicable in vivo,

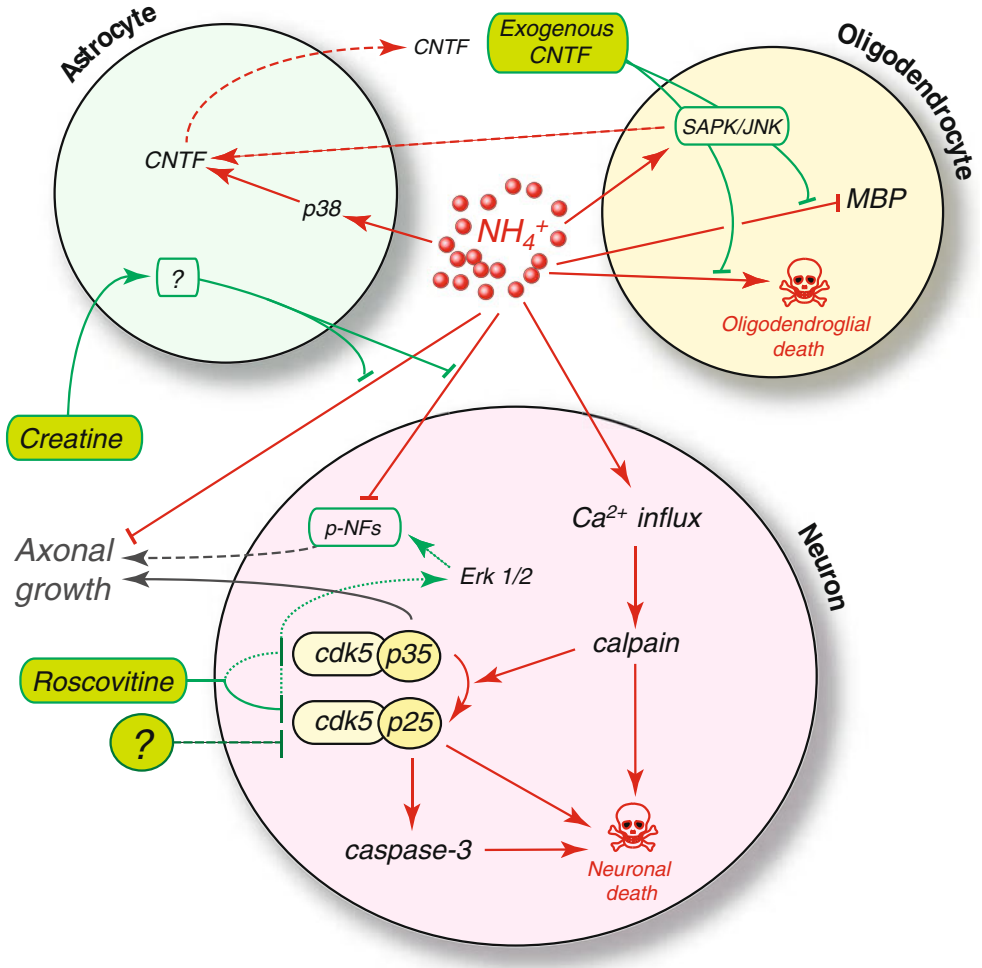


Fig. 2 Proposed mechanisms leading to brain cell death under NH_4^+ exposure, and effects of NH_4^+ exposure on CNS intracellular and extracellular signaling pathways. Toxic effects of NH_4^+ are shown in *red*, while protective effects of creatine, roscovitine and exogenous CNTF are shown in *green*. NH_4^+ activates calpain, which can induce neuronal death directly. Activated calpain also cleaves p35 to p25 and activates caspase-3, causing neuronal death. Roscovitine decreases neuronal death by inhibiting cdk5/p25 and the subsequent caspase-3 activation. By inhibiting cdk5, roscovitine activates the Erk1/2 pathway, which stimulates the phosphorylation of neurofilaments. However, roscovitine also inhibits axonal growth through inhibition of cdk5/p35. Targeting cdk5 to inhibit NH_4^+-induced neuronal death should thus be focused on the specific inhibition of cdk5/p25. Creatine protects axonal growth under NH_4^+ exposure in a glial cell-dependent way. NH_4^+ activates MAPKs in brain cells, and particularly p38 in astrocytes, which increases their release of CNTF. Exogenous CNTF exerts a protective effect on oligodendrocytes, through SAPK/JNK. **CNTF**: ciliary neurotrophic factor; **Erk1/2**: extracellular signal regulated kinases 1/2; **MAPKs**: mitogen-activated protein kinases; **p38**: p38 kinase; **SAPK/JNK**: stress-activated protein kinase or c-Jun NH_2-terminal kinase

non-invasively and longitudinally to monitor disease progression or effect of treatments (Cudalbu et al 2012a).

In vivo ^{1}H magnetic resonance spectroscopy and spectroscopic imaging (SI) to study brain osmolytes, energy metabolism and neurotransmission in HE

Proton MRS (^{1}H MRS) is a powerful tool to non-invasively investigate in vivo brain metabolism of rodents and humans. Very high magnetic field strengths (≥7T) combined with the possibility of acquiring spectra at very short echo time (TE) (< 10 ms) have dramatically increased the number of brain metabolites detectable in vivo. At present, this neurochemical profile comprises about 20 metabolites and neurotransmitters (Mekle et al 2009; Mlynarik et al 2008a; Pfeuffer et al 1999; Tkác et al 1999, 2009). They are involved in: myelination/cell proliferation (phosphocholine, glycerophosphocholine, phosphoethanolamine, N-acetylaspartate NAA, N-acetylaspartylglutamate NAAG), energy metabolism (Glc, lactate Lac, Cr, phosphocreatine, Ala), osmoregulation (taurine, myo-inositol), neurotransmission (Glu, Gln, aspartate, γ-aminobutyrate GABA, glycine) and antioxidants (ascorbate, glutathione). While ^{1}H MRS allows signal detection from a well-defined single volume in the brain (Fig. 3a), proton spectroscopic imaging (^{1}H SI) allows the simultaneous detection of multiple spectra from different brain regions and thus the study of spatial metabolite

distribution in various regions of the brain (Fig. 3b) (Cudalbu et al 2010; Mlynarik et al 2008b). In vivo ^{1}H MRS and ^{1}H SI were used to study brain metabolism in animal models of hyperammonemia without liver failure (continuous infusion or single i.p. injection of ammonia) (Cauli et al 2007; Cudalbu et al 2012b; Fitzpatrick et al 1989) or ALF (galactosamine injection, portacaval anastomosis followed by hepatic artery ligation) (Cauli et al 2011; Chavarria et al 2010; Nyberg et al 1998) and in human studies of ALF and CLF (Chavarria et al 2011; Rovira et al 2008; Spahr et al 2002). The main finding in all of these studies is the increase in brain Gln concentration as shown in Fig. 3a and b.

In a recent in vivo study using continuous infusion of NH_4Cl we showed that Gln increased immediately after the initiation of NH_4^+ exposure and continued to increase linearly over time (2.3 ± 0.4 μmol/g before infusion, reaching 17.7 ± 4.0 μmol/g at the end of infusion) (Fig. 3a), suggesting that no delay in Gln accumulation occurred (Cudalbu et al 2012b). No significant differences in the total concentrations of all other metabolites were observed. The linear and continuous increase of total Gln under NH_4Cl infusion observed in our in vivo ^{1}H MRS data implies an increased anaplerosis coupled to the NH_4^+ detoxification pathway (Berl et al 1962; Shen et al 1998; Zwingmann 2007). Furthermore, we mapped regional brain metabolism using ^{1}H SI (Cudalbu et al 2010) in the same rat model of hyperammonemia. Figure 3b illustrates the metabolic maps of Gln, Glu, Ins, NAA+NAAG and Lac superimposed on the anatomical T_{2w} images and acquired at different time points during NH_4^+ infusion. As for ^{1}H MRS data, the Gln increase at different time points was apparent from the maps with no significant differences for the concentration of other brain metabolites. Additionally, the Gln increase was higher in the cortex than in the hippocampus (16.2±2.7 mmol/kgww in the cortex and 11.5±1.2 mmol/kgww in the hippocampus after 5.5 h of NH_4^+ infusion, p=0.03). Consequently, these results showed a higher net Gln synthesis flux in cortex than in hippocampus.

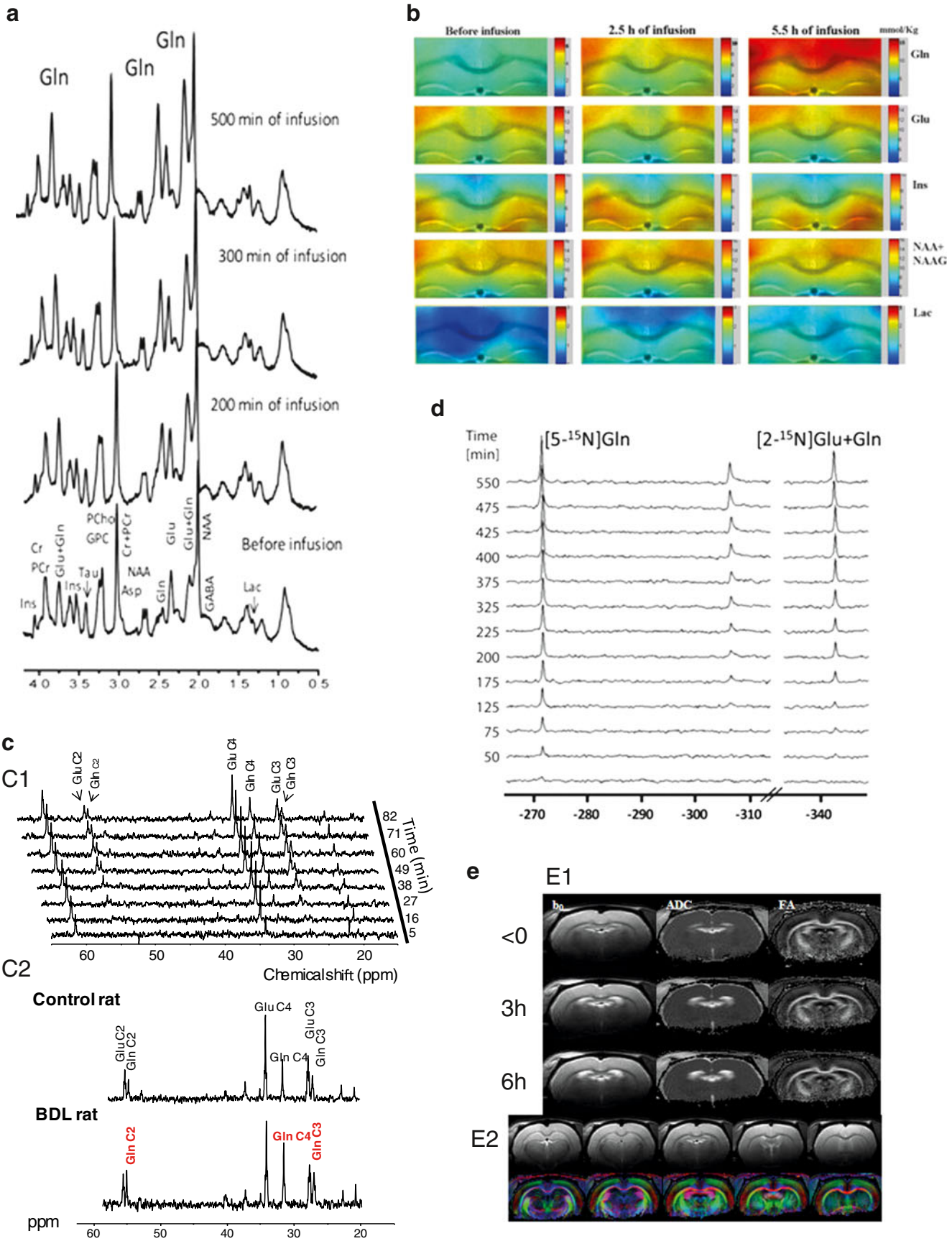
Studies performed on animal models of ALF (e.g., galactosamine injection, portocaval anastomosis followed by hepatic artery ligation) reported additional alterations in brain Lac concentration at later stages and the presence of brain edema. The mechanisms leading to Lac increase are not clear, but may indicate brain energy impairment secondary to ammonia metabolism and brain edema (Chavarria et al 2010). CLF is associated with an additional drop in brain osmolytes (Ins, tCho and Tau) (Chavarria et al 2011; Rovira et al 2008; Spahr et al 2002) probably reflecting an osmoregulatory response to Gln increase. The differences in brain osmolytes may partially explain the differential frequency of brain edema between ALF and CLF (Cordoba 1996). We recently characterized for the first time the in vivo and longitudinal progression of HE in a rat model of CLF by BDL by using ^{1}H SI and diffusion tensor imaging (McLin et al 2012). Gln was increased at all time points after BDL.

Fig. 3 **a** Representative in vivo ^{1}H MR spectra acquired at 9.4T in the rat brain during ammonium infusion, adapted from Cudalbu et al (2012b) (echo time 2.8 ms, 160 averages, voxel size of 5×7×7 mm^3; continuous infusion of NH_4Cl at 4.5 mmol/h per kilogram). From bottom to top: spectrum acquired before infusion and spectra acquired during infusion (200, 300 and 500 min of infusion); only Gln is marked in the last spectrum since it is the only metabolite changing its concentration, hereby increasing under NH_4^+ infusion. **Asp**: aspartate; **Cr**: creatine; **GABA**: γ-aminobutyrate; **Gln**: glutamine; **Glu**: glutamate; **GPC**: glycerophosphocholine; **Ins**: myo-inositol; **Lac**: lactate; **NAA**: N-acetylaspartate; **PCho**: phosphocholine; **PCr**: phosphocreatine; **Tau**: taurine. **b** In vivo metabolic maps of Gln, Glu, Ins, NAA+ NAAG (N-acetyl-aspartyl-glutamate) and Lac, superimposed on the anatomical T_{2w} images and acquired at different time points during NH_4^+ infusion at 9.4T, using ^{1}H SI (echo time 2.8 ms). The increase in the Gln pool at different time points during NH_4^+ infusion is apparent from the maps. **c** In vivo ^{13}C MRS spectra acquired in the rat brain during continuous infusion of [1,6-^{13}C]-glucose: c1) Time course showing the incorporation of ^{13}C into different carbon positions of Glu and Gln (only the first 90 min are shown) in a bile duct-ligated (BDL) rat; **c2)** In vivo brain ^{13}C MRS spectra acquired in control and BDL rats during the last hour (5th hour) of [1,6-^{13}C]-glucose infusion. Only the metabolites used for modeling are marked in the figure (Glu and Gln labeled at positions C4, 3 and 2). In red are shown the metabolites which are changing in the BDL rat as compared with control (Gln at positions C4, 3 and 2). **d** Representative series of in vivo ^{15}N spectra acquired under infusion of ^{15}N-enriched ammonium at 9.4T in a rat brain at different time points during infusion, adapted from Cudalbu et al (2012b). The ^{15}N labeling incorporation of NH_4^+ at position 5N of Gln ([5-^{15}N]Gln) allows the direct measurement of glutamine synthetase flux, whereas the incorporation of ^{15}N into [2-^{15}N]Gln+Glu provides further insight into the net flux through glutamate dehydrogenase. **e** In vivo diffusion tensor images (DTI) acquired at 9.4T in an animal model of hyperammonemia (continuous infusion of NH_4Cl)**: e1)** $\mathbf{b_0}$ image (b = 0 mm^2/s), diffusion tensor trace (**ADC**) and fractional anistropy (**FA**) maps acquired before and during NH_4^+ infusion; **e2)** images acquired before infusion, with the diffusion coefficient maps. $\mathbf{b_0}$ image (b = 0 mm^2/s) and color encoded map

Among other brain osmolytes, Ins, tCho and Tau decreased significantly over time. We concluded that prior to the appearance of severe neurological signs in CLF, the osmotic imbalance created by continuous increase of Gln is likely to be compensated by a concomitant decrease of other idiogenic osmolytes resulting in minimal brain edema.

In vivo ^{13}C MRS to study neuroglial energy metabolism

Cerebral metabolism is compartmentalized between neurons and glia (Gruetter 2002). Glc is the primary substrate for cerebral energy production, while Lac exchange between astrocytes and neurons is not excluded under specific conditions (Magistretti et al 1999). In vivo ^{13}C MRS together with administration of [1,6-^{13}C]-Glc and an appropriate mathematical model of neuronal-glial metabolism is a unique technique to non-invasively investigate compartmentalized cerebral energy metabolism (Gruetter 2002). In particular, we measured ^{13}C incorporation into different carbon positions of Glu and Gln to determine fluxes through


a
Gln
Gln
Gln
500 min of infusion
300 min of infusion
200 min of infusion
Before infusion
Cr
PCr
Glu+Gln
Ins
Tau
PCho
GPC
Cr+PCr
NAA
Asp
Glu
Gln
Glu+Gln
NAA
GABA
Lac
40 35 30 25 20 15 10 05
b
Before infusion
2.5 h of infusion
5.5 h of infusion
mmol/Kg
Gln
Glu
Ins
NAA+ NAAG
Lac
c
C1
Glu C2
Gln C2
Glu C4
Gln C4
Glu C3
Gln C3
82
71
60
49
38
27
16
5
Time (min)
60 50 40 30 20
Chemical shift (ppm)
C2
Control rat
Glu C4
Glu C3
Glu C2
Gln C2
Gln C4
Gln C3
BDL rat
Gln C2
Gln C4
Gln C3
ppm
60 50 40 30 20
d
Time [min]
[5-15N]Gln
[2-15N]Glu+Gln
550
475
425
400
375
325
225
200
175
125
75
50
-270 -280 -290 -300 -310 -340
e
E1
b0
ADC
FA
<0
3h
6h
E2

important pathways involved in energy metabolism including: glycolysis, neuronal and astrocytic TCA cycle, malate-aspartate shuttle activity and glial anaplerotic pyruvate carboxylation (Fig. 3c). In addition, the Glu/Gln neurotransmitter cycle within the neuron-astrocyte functional unit can be measured. The rate of ^{13}C label incorporated as a function of time is related to metabolic rate thereby permitting the measurement of absolute metabolic fluxes. For example, the accumulation of ^{13}C label in Glu at the position C4 is indicative of both the neuronal and glial TCA cycle fluxes, whereas the labeling on Gln at the same position reveals the Glu-Gln neurotransmission flux. Further separation of the glial and neuronal TCA cycle activities is possible when measuring the C3 and C2 positions of Glu and Gln, due to the glial-specific activity of pyruvate carboxylase, diluting the carbon position 3 and labeling the position 2 of glial Glu.

In rat models of hyperammonemia by continuous infusion of NH_4^+ without liver failure, in vivo ^{13}C MRS has been used to measure neuroglial metabolism in conjunction with Glc infusion labeled at different positions, i.e., [1,6-^{13}C], [1-^{13}C] or [2-^{13}C]-Glc. Lanz et al (2011) and Sibson et al (1997, 2001) reported that anaplerosis appears to be the major NH_4^+ detoxification pathway, as measured in our ^1H MRS and ^{15}N NH_4^+ studies (Cudalbu et al 2010, 2012b), emphasizing the contribution of astrocytes in cerebral NH_4^+ processing. Neuronal metabolism appears less affected, as reflected by GluC4 and GluC3 fractional enrichment time courses.

Only a few studies have tried to measure energy metabolism in animal models of ALF. These were performed ex vivo using brain extracts, and reported increased Lac and alanine synthesis as well as stimulated pyruvate carboxylation. These findings suggest that a deficit in brain Glc metabolism rather than Gln accumulation is the major cause of cerebral complications in this model of ALF (Chatauret et al 2003; Zwingmann 2007).

We have recently characterized in vivo brain energy metabolism of rats with CLF (BDL) by using ^{13}C MRS together with administration of [1,6-^{13}C]-Glc and by following the kinetics of ^{13}C incorporation in Glu and Gln at positions C4, C3 and C2 over 5 h of [1,6-^{13}C]-Glc infusion (Fig. 3c1). Continuous acquisition in live animals showed that ^{13}C incorporation in Gln at positions C4, C3 and C2 was higher in BDL rats than in controls (Fig. 3c), suggesting an increase in glial TCA cycle activity. In addition, ^{13}C incorporation in position C2 of Gln was higher than in positions C4 or C3, indicating increased activity of glial-specific pyruvate carboxylase flux as compared with controls. Additional data from these dynamic studies are forthcoming and promise to shed important mechanistic information on metabolic fluxes during HE.

^{15}N MRS to study glutamate-glutamine metabolism

^{15}N MRS is an alternative approach to ^{13}C MRS to study Glu-Gln metabolism under hyperammonemia, which provides a more direct interpretation. In vivo ^{15}N MRS using ^{15}N-labeled NH_4^+ infusion was first used to analyze the incorporation of ^{15}N-NH_4^+ into [5-^{15}N]Gln and measure the GS flux (Kanamori et al 1993; Shen et al 1998). The incorporation of ^{15}N into [2-^{15}N]Gln+Glu was further use to study the net flux through GDH (Kanamori and Ross 1995). We recently developed a new ^{15}N pulse sequence to simultaneously detect [5-^{15}N]Gln and [2-^{15}N]Gln+Glu in vivo (Fig. 3d) (Cudalbu et al 2012b). Mathematical modeling of in vivo ^1H and ^{15}N MRS data, acquired interleaved on the same animal, allowed to reduce the number of assumptions and provided reliable determination of GS (0.30 ± 0.050 μmol/g/min), apparent neurotransmission (0.26 ± 0.030 μmol/g/min), GDH (0.029 ± 0.002 μmol/g/min) and net Gln accumulation (0.033 ± 0.001 μmol/g/min). Our in vivo measurements allowed to clearly show the increase of brain GS activity and net Gln accumulation under hyperammonemia conditions, supporting the concept of their implication in cerebral NH_4^+ detoxification.

In vivo MR diffusion to study brain edema

MR diffusion techniques (diffusion weighted or tensor imaging) (Fig. 3e) are used to investigate brain edema by measuring the relative translational motion of water molecules which is expressed as the apparent diffusion coefficient (ADC) (Le Bihan 1995). Changes in ADC reflect the presence of edema, which can be divided into cytotoxic (intracellular) and vasogenic (extracellular) edema. Most human and animal models have shown cytotoxic edema in ALF (Cauli et al 2011; Chavarria et al 2011, 2010; Ranjan et al 2005), while some studies proposed the coexistence of cytotoxic and vasogenic edema (Cauli et al 2011). A limited number of human studies on brain edema in CLF speak in support of the presence of mild vasogenic edema (Kale et al 2006).

As shown in the present review, several pathogenic mechanisms involved in HE can be explored in vivo using MRS and MRI. However, we need to emphasize that additional in vivo MRS and MRI studies are needed to assess the relationship between plasma NH_4^+ concentrations, brain Gln accumulation, osmoregulation, brain energy metabolism and brain edema in HE.

Conclusion and future directions

Hyperammonemia during brain development is associated with neuronal cell loss and cerebral atrophy leading to mental retardation and cerebral palsy in pediatric patients. In survivors, the pathogenic mechanisms of NH_4^+ toxicity to the brain involve alterations in amino acids pathways, neurotransmission systems, cerebral energy, NO synthesis, axonal and dendritic growth or signal transduction pathways (Figs. 1 and 2).

These disturbances can lead to cytotoxic brain edema, cell death, impairment of neurite outgrowth, defects in nerve cell migration, or hypomyelination, in turn leading to brain tissue atrophy, ventricular enlargement, gray or white matter hypodensities and demyelination. These toxic effects of NH_4^+ are specific to the developing brain, as neuronal damage is not observed in CNS of adult patients with hyperammonemia due to liver failure. In the mature brain, the main effect of NH_4^+ toxicity is the rise of Gln in astrocytes while osmoregulation is insufficient and cerebral edema develops, affecting CNS areas. Why the developing brain is so vulnerable to fluctuations in serum NH_4^+ levels remains to be elucidated. MRS promises to be a powerful tool both to characterize the molecular modifications characterizing the pathobiology in the developing brain and to monitor the effects of potential neuroprotective therapies.

Apart from the use of NH_4^+ scavengers such as Na^+-benzoate, Na^+-phenylacetate, Na^+-phenylbutyrate, OP or LOLA, new neuroprotective strategies have been proposed, making use of NMDA receptor antagonists, NOS inhibitors, Cr, acetyl-L-carnitine, inhibition of CDK5/p25, CNTF or inhibitors of MAPKs and GS (Figs. 1 and 2).

Understanding the pathophysiology of ammonia toxicity to the CNS, or unraveling new therapeutic targets to protect CNS from hyperammonemia, requires experimental approaches focusing on the brain in its cellular complexity, examining neurons and glia together (in vivo mouse and rat models; ex vivo CNS organotypic cultures; in vitro primary 3D brain cell cultures in aggregates). The extraordinary development of in vivo CNS imaging technologies (MRI, MRS, Fig. 3) should contribute significantly to directing future investigations, in particular by focusing on intra- and extra-cellular metabolic and signaling pathways disturbed in the brain during NH_4^+ exposure.

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Conflict of interest None.

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