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ORIGINAL PAPER

Neurotoxicity of Ammonia

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Abstract   Abnormal liver function has dramatic effects on brain functions. Hyperammonemia interferes profoundly with brain metabolism, astrocyte volume regulation, and in particular mitochondrial functions. Gene expression in the brain and excitatory and inhibitory neurotransmission circuits are also affected. Experiments with a number of pertinent animal models have revealed several potential mechanisms which could underlie the pathological phenomena occurring in hepatic encephalopathy.

Background

Ammonia is produced in the body by intermediary amino acid metabolism or arises from the actions of intestinal bacteria. In human adults approximately 1000 mmol (17 g) of ammonia is produced daily [1]. A part of this is reutilized in biosynthesis, while the remainder is waste and neurotoxic. Its normal concentration in the portal blood varies from 300 to 600 μM, but in the blood leaving the liver the concentration is reduced to 20–60 μM. The liver thus occupies a central position in the regulation of ammonia levels in the organism. The failure of liver functions in hepatic cirrhosis or for other reasons may thus result in an uncontrolled increase in levels of ammonia in the circulating blood. Ammonia at high concentrations penetrates from the blood into practically all organs. Although the brain is partially protected by the blood–brain barrier from toxic agents such as ammonia, excessive amounts of ammonia can pass into the brain, constituting a principal factor in the syndrome of hepatic encephalopathy. In patients, hepatic encephalopathy may result from acute liver failure or portal-systemic bypass with no intrinsic hepatocellular disease, or may be associated with cirrhosis and portal hypertension [2], all involving enormous costs to society. Ammonia concentrations can be experimentally elevated in a variety of ways, e.g., by administration of hepatotoxins as Professor Jan Albrecht has done in his many studies on ammonia toxicity, by peripheral administration of massive doses of ammonium chloride, or by directing the blood flow from the alimentary canal by end-to-side portacaval anastomosis, thus by-passing the liver. In addition to this, inborn errors in the urea cycle may lead to congenital hyperammonemia.

The brain is much more susceptible to the deleterious effects of ammonium during development than in adulthood. The concentration of ammonia in the blood is higher in newborns than in adults [3]. In the brain the normal ammonia content also diminishes during maturation [4]. Depending on the extent of hyperammonemia and its duration, more or less serious irreversible damage is caused to the brain, leading to mental retardation [5]. Hyperammonemia can provoke irreversible damage to the developing central nervous system, which leads to cortical atrophy, ventricular enlargement and demyelination, responsible for cognitive impairment, seizures and cerebral palsy [6]. An increased exposure to ammonia during the prenatal and lactation periods has been shown to cause long-lasting impairment of

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N-methyl-D-aspartate (NMDA) receptor functions [7]. The disruption of energy metabolism by ammonia and disturbances in axonal growth during development could also be factors contributing to mental retardation [8].

Increased accumulation of ammonia in the brain due to liver dysfunction is a major factor in the pathogenesis of hepatic encephalopathy [9]. Hyperammonemia apparently affects brain functions by several mechanisms. It blocks chloride efflux from postsynaptic neurons [10], causes depression of synaptic transmission [11], inhibits neuron-astrocyte trafficking of glutamate and affects postsynaptic glutamate receptors [12]. The firing of glutamatergic neurons in the CA1 region of the hippocampus evoked by applied glutamate is then abolished by ammonia. On the other hand, glutamate exocytosis is evoked by ammonia in cultured rat astrocytes [13]. Ammonia also affects other neurotransmitter systems in addition to the glutamatergic. The synthesis of histamine, serotonin, dopamine and noradrenaline in the brain is altered by hyperammonemia [14]. For example, ammonium chloride directly administered to the rat striatum via reversed microdialysis evokes a prompt accumulation of dopamine in the microdialysates [15]. Furthermore, ammonia influences the passage of different molecules across the blood–brain barrier. It modulates the transcellular passage of low- to medium-size molecules by affecting their carriers located at this barrier [16]. Ammonia also inhibits GABA uptake and enhances its release [17]. On the other hand, ammonia has been shown to stimulate glutamine uptake into non-synaptic mitochondria isolated from rat cerebral hemispheres [18]. We here briefly review these proposed mechanisms.

Astrocyte Swelling and Shrinking

Ammonia induces astrocytic swelling [19]. Astrocyte swelling is believed to be a key component in the cytotoxic brain edema [20] associated with acute liver failure and the increase in intracranial pressure and eventually brain herniation which is often the cause of death in patients with hepatic encephalopathy [21]. Elevated ammonia has also been shown to produce astrocytic swelling, tissue swelling and neuronal toxicity in organotypic slice cultures of cerebral tissue [22]. Astrocyte swelling has thus been generally assumed to be the key factor in the generation of ammonia toxicity and the increase in intracranial pressure leading to brain herniation and death [19, 23].

Glutamine has been thought to be the principal factor in ammonia detoxification. More recently, however, glutamine has been considered to mediate ammonia toxicity when in excess [24, 25]. In keeping with this assumption the cerebral glutamine content has been shown to correlate positively with the grade of hepatic encephalopathy [26]. Glutamine

may impair the mitochondrial function in astrocytes secondarily to its excessive accumulation in them. Glutamine has been shown to increase mitochondrial permeability [27]. Ammonia itself does not induce mitochondrial swelling, even though it increases glutamate uptake in mitochondria [18].

It has been demonstrated that activation of the neuronal NKCC1 (Na$^+$ K$^+$ 2Cl$^-$-cotransporter, encoded by the SLC12A2 gene) is involved in the astrocyte swelling induced by ammonia and in brain edema [28]. Preclinical results in non-anesthetized mouse model of childhood epilepsy (ornithine transcarbamylase-deficient mice), which are contradictory to earlier conceptions as to the critical role of astrocyte swelling, have recently been published. According to them an acute increase in extracellular ammonia does not lead primarily to astrocyte swelling but rather to unbalanced astrocyte buffering of potassium ions, with increases extracellular potassium overactivating the NKCC1, which in turn compromises inhibitory neurotransmission in the cerebral cortex and depolarizes the neuronal GABA reversal potential (E_{GABA}) [29]. Consequently, intracellular chloride is increased in neurons and the main fast-acting inhibitory system, GABA$_A$ receptor-mediated inhibition, is blunted, leading to a myoclonic seizure phenotype in this mouse model. This abnormal GABAergic excitation might be exacerbated by the known increase in extracellular GABA due to ammonia-induced inhibition of GABA uptake and enhancement of GABA release [17], and by increased synthesis of GABA by glutamate decarboxylase due to increased glutamate levels [30]. The potassium uptake mechanisms by Na$^+$/K$^+$-ATPase are then saturated by increased ammonium ion levels. According to these studies increased ammonia would thus seem not to lead to astrocyte swelling but rather to transient astrocyte shrinking. Astrocyte swelling or brain edema only occurs in the terminal stages of ammonia toxicity [31]. Importantly, the clinically used diuretic inhibitor of NKCC1, bumetanide, has been seen to block the effects of acute ammonia on GABAergic neurotransmission and prevent seizures in the mouse model [29]. It remains to be established whether these acute mechanisms show adaptation during more modest and prolonged hyperammonemia.

Molecular Mechanisms Involved

At present, several factors and pathways have been surmised to be associated in ammonia toxicity [32]. To date, these include oxidative and nitrosative stress [9, 33], adverse alterations in the glutamate-glutamine cycle, changes in mitochondrial permeability transition (MPT) [34], effects on neural transmission, activation of mitogen-activated protein kinases (MAPKs) and effects on the transcription factor nuclear factor-kappa B (NF-κB) [35]. Such effects

strongly suggest the involvement of phosphorylation of MAPKs in the mechanism of ammonia-induced astrocyte dysfunction associated with ammonia neurotoxicity [36]. Blockade of the MPT, MAPKs and NF-κB has been shown to reduce the extent of astrocyte swelling [35]. The cytoplasmic level of tumor suppressor phosphoprotein p53 is also increased in acute toxicity with large ammonium doses [37, 38]. These above effectors generate additional reactive oxygen–nitrogen species, phosphorylate various proteins and transcription factors, and cause mitochondrial dysfunction. Astrocytes exposed to ammonia also exhibit a reduction in intra- and extracellular levels of thrombospondin-1, an astrocytic factor involved in the maintenance of synaptic integrity. A defective release of thrombospondin-1 may impair synaptic integrity in chronic hepatic encephalopathy [39].

Ammonia markedly enhances the generation of reactive oxygen and nitrogen species (ROS and RNS), including the highly toxic peroxynitrite, in astrocytes [40, 41]. The production of hydroxyl radicals is also increased in vivo in the rat striatum upon microdialysis of ammonium chloride [42]. The generation of reactive oxygen and nitrogen species, in turn, induces protein tyrosine nitration [43], lipid peroxidation [44], S-nitrosylation of cysteine residues in proteins, and nucleic acid oxidation [45]. Glutathione is the major antioxidant in the brain and could counteract the harmful effects of reactive oxygen and nitrogen species. The synthesis of glutathione in cultured astrocytes [46] and the uptake of its precursor cysteine [47] are fomented by ammonia. In line with these observations, the glutathione content is increased in the brain extracellular spaces after administration of ammonium chloride [48].

Elevated concentrations of ammonia induce the formation of free radicals in astrocytes, which is associated with the synthesis of glutamine [40]. When the glutamine transport into cultured astrocytes is prevented, the generation of ammonia-induced reactive oxygen species production, cell swelling, MPT, and loss of ATP are completely blocked or significantly attenuated [49]. These findings clearly implicate mitochondrial glutamine transport in the mechanism of ammonia neurotoxicity. It has been proposed that the glutamine-derived ammonia within mitochondria interferes with mitochondrial functions, giving rise to excessive production of free radicals and induction of MPT, two phenomena which bring about astrocyte dysfunction, including cell swelling [50]. Glutamine thus induces oxidative stress and MPT, being critical in the development of astrocyte swelling in hyperammonemia [23].

There is strong evidence to indicate that oxidative stress is involved in the induction of MPT by ammonia, and that oxidative stress and the subsequent induction of MPT contribute to the pathogenesis of hepatic encephalopathy and other hyperammonemic disorders [51, 52]. Altered

bioenergetics and oxidative stress appear to be critical factors in this pathogenesis [52]. MPT seems thus to represent an important component in the pathogenesis of hepatic encephalopathy and other hyperammonemic states [53]. In line with these speculations, direct application of glutamine to cultured astrocytes increases free radical production and induces MPT [54]. Only astrocytes, but not neurons, generate free radicals following glutamine exposure [55].

Brain glucose consumption is diminished in portacaval shunt-induced [56] and thioacetamide-induced [57] hyperammonemic states. Hyperammonemia can induce cerebral energy failure by several mechanisms [58, 59]. The high concentration of ammonia interferes with oxidative metabolism in the brain through an inhibitory effect on the tricarboxylic acid cycle [60]. Ammonia also induces ATP depletion due to activation of Na^+/K^+-ATPase, which, in turn, is a consequence of decreased phosphorylation by protein kinase C (PKC) [61]. Ammonium chloride also affects energy metabolism by increasing the neuronal tricarboxylic acid cycle activity and switching it in astrocytes towards glutamine synthesis [62]. At variance with this assumption the excess ammonia has been reported to interfere with brain energy metabolism by inhibiting the tricarboxylic acid cycle and this inhibition may result in depletion of ATP in the brain cells [63]. Moreover, there are also other controversial findings on this topic. It has been consistently reported that hepatic encephalopathy and concomitant hyperammonemia lead to reduced cerebral oxygen consumption. However, this may not be directly linked to an effect of ammonia but related to the fact that hepatic encephalopathy is always associated with reduced brain activity [64]. The whole-brain oxidative metabolism in patients with hepatic encephalopathy may not be due to malfunction of oxidative metabolism in astrocytes. The observed decline of brain oxidative metabolism may result from changes in neurons and their energy turnover [65].

One of the primary roles of astrocytes is to protect neurons against excitotoxicity by taking up excess ammonia and glutamate and converting them into glutamine via glutamine synthetase, which is located almost exclusively in astrocytes [66, 67]. Changes in the expression of this enzyme reflect changes in astroglial functions, hence also affecting neuronal functions [68]. Newly synthesized glutamine is transferred to neurons and hydrolyzed by glutaminase to glutamate [30]. In hepatic encephalopathy the expression of glutamate transporter (EEAT-2) is decreased, which impairs the cycling of glutamate-glutamine between astrocytes and neurons. Consequently, extracellular level of the main fast-acting excitatory neurotransmitter glutamate is increased, the NMDA receptor-mediated signaling activated, including RNS production, and tyrosine residues are nitrated. This sequence of events has been considered a cornerstone in the pathogenesis of hepatic encephalopathy [69].

Involvement of *N*-methyl-D-aspartate Receptors

Ionotropic NMDA receptors are involved in many functions in the central nervous system. The severity of the symptoms caused by hyperammonemia is positively correlated with the activation of NMDA receptors [70]. The acute neurotoxic effects of ammonia may thus be due mainly to overactivation of NMDA receptors, possibly potentiated by impaired control of their function by metabotropic glutamate receptors [71]. The sequence of events consists of increased extracellular glutamate stimulating NMDA receptors, which leads to increased intracellular Ca^{2+} and subsequent activation of NADPH oxidase (superoxide production, ROS) and NO synthase (NO production, RNS). Superoxide and NO can then promote the formation of peroxynitrite and protein tyrosine nitration. On the other hand, long-term exposure to ammonia of cultured cerebellar neurons impairs the glutamate-NO pathway in a dose- and time-dependent manner. The glutamate-induced formation of cGMP is reduced without effects on NO synthase [72].

The ammonia-induced swelling of rat cerebral cortical slices is significantly attenuated by NMDA receptor antagonists, inhibitors of the NO synthase, and taurine [73]. Ammonia treatment in vivo reduces synthesis of kynurenic acid, which is an endogenous, broad-spectrum antagonist of ionotropic glutamate receptors [74]. Inhibition of excitatory synaptic transmission by elevated brain ammonia has been assumed to underlie the central nervous system depression in hepatic encephalopathy [75]. Ammonia may stimulate the expression of inducible NO synthase in astrocytes, leading to excessive formation of NO, which in turn could trigger the formation of peroxynitrite in adjacent neurons, inducing their death [76]. The NMDA receptor antagonist dizocilpine (MK-801) blocks the ammonia-induced generation of reactive oxygen and nitrogen species in astrocytes [77]. On the other hand, administration of ammonium chloride has been reported to reduce the expression of two NMDA receptor subunits (GluN2A and GluN2B) in the rat hippocampus [78]. Ammonium chloride infusion into the rat striatum in vivo via a microdialysis probe increases glutamine, NO oxidation products and cGMP in the microdialysate [79]. Likewise, it activates NMDA receptors and foments the generation of hydroxyl radicals [42]. Ammonia also induces apoptosis as a result of a complex interplay of at least three signalling molecules: NO, PKC and NF-κB. The NF-κB is possibly involved in the induction of iNOS and the generation of toxic levels of NO in C6 glioma cells [80]. Figure 1 summarizes the main sequelae of ammonia neurotoxicity.

More recently, Cauli et al. [81, see for refs] have used cerebellar in vivo microdialysis to assess the mechanisms of ammonium-induced impairment of the glutamate-NMDAR-NO-cGMP pathway. NMDA-triggered citrulline and cGMP production was monitored in dialysates. The cGMP and
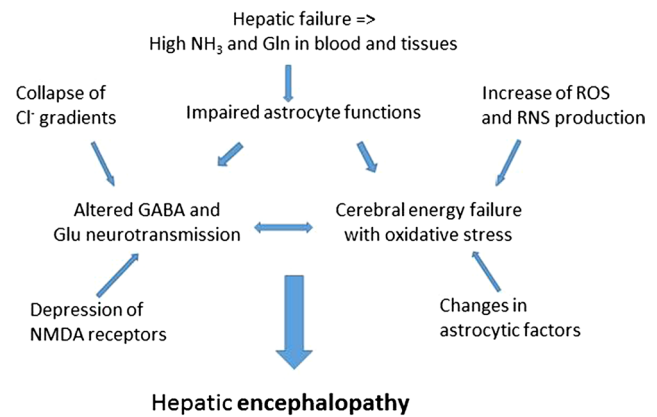

Fig. 1 Main factors responsible for hepatic encephalopathy, the most severe form of neurotoxicity induced by hyperammonemia

citrulline levels could be regulated by both NMDAR and GABA$_A$R activities, and importantly, the neurosteroid sensitivities of both receptor systems were altered depending on the increased ammonium levels. Several neurosteroids, for example allopregnanolone, tetrahydrodeoxycorticosterone and dehydroepiandrosterone sulphate, reduced the pathway, whereas pregnenolone sulphate enhanced it. The results were taken to indicate that certain neurosteroids might contribute to cognitive symptoms of hyperammonemia while others alleviate them [82, 83]. In line, Zorumski et al. [84, 85] have reported that 100 μM ammonium in vitro inhibits the induction of NMDAR-dependent long-term potentiation (LTP) via increased neurosteroid synthesis in hippocampal slices. The effect of ammonium could be blocked by finasteride, a selective inhibitor of 5α-reductase needed for neurosteroid synthesis, and by GABA$_A$R blocker picrotoxin, as well as by L-carnitine. The key step in the neurosteroid synthesis is mediated by the mitochondrial 18 kDa translocator protein (TSPO, formerly known as peripheral benzodiazepine receptor), which enhances the uptake of the precursor cholesterol for the synthesis [86]. The TSPO is strongly upregulated in animal models of hyperammonemia and in patients with hepatic encephalopathy [87–91], which then increases neurosteroid levels [92, 93]. These interesting observations await for translation to clinical trials, particularly as finasteride has alleviated various symptoms in a model of thioacetamide-induced hepatic encephalopathy in rats [94].

How to Alleviate Ammonia Toxicity? Preclinical vs. Clinical?

The severity of symptoms in ammonia toxicology has been the impetus in the search for methods and compounds which could alleviate them, in addition to possible neurosteroid-related mechanisms (see above). Some

neuroprotective strategies such as the potential use of NMDA receptor antagonists, NO inhibitors, creatine and acetyl-L-carnitine have been suggested to counteract the toxic effects of ammonia [6]. L-Carnitine has been found to suppress ammonia-induced seizures and biochemical alterations in the brain in mice [95, 96]. L-Carnitine and its analogues thus have a potential to suppress ammonia neurotoxicity [97]. For example, treatment with acetyl-L-carnitine has preserved ATP in the brain, while lowering ammonia in the blood and brain less markedly [98]. By assuming that acute ammonia toxicity is mediated by activation of the NMDA receptors, the protective effect of L-carnitine against glutamate toxicity may result from its ability to increase the affinity of glutamate for the metabotropic receptors [96, 99]. Especially the mGluR5 receptors have been implicated [100]. In human patients in whom valproate has caused hepatic encephalopathy as a serious adverse effect, treatment with L-carnitine speeds up the decrease in ammonemia [101]. The inhibitor of NO synthetase nitroarginine also attenuates acute ammonia toxicity and ammonia-induced alterations in brain energy metabolites by a mechanism which does not involve the activation of NMDA receptors [102].

Increased accumulation of cGMP in the rat striatum by intrastriatal infusions of ammonium chloride or NMDA has been virtually abolished by co-infusion of taurine [42]. This inhibitory compound has also attenuated the simultaneous accumulation of hydroxyl radicals. Similar co-infusion of the potent glycine site-specific NMDA receptor antagonist CGP 78608 ((1S)-1-[[(7-bromo-1,2,3,4-tetrahydro-2,3-dioxo-5-quinoxalinyl)methyl]amino]ethylphosphonate) abolished ammonia-induced cGMP synthesis [103]. Melatonin and dimethylsulfoxide have also been found to lower the thioacetamide-induced increase in brain ammonia [104]. Dimethylsulfoxide has also significantly reduced the basal glutamine concentration in the rat striatum and attenuated the basal concentration of cGMP in microdialysates [80]. Kynurenic acid, an endogenous NMDA receptor antagonist with a high affinity towards its glycine site, may counter the over-activation or depression of glutaminergic transmission observed at the different stages of hyperammonemia [105]. Glutathione can also counteract the generation of oxygen radicals provoked by ammonia. Upregulation of cystine uptake may contribute to this response [48]. Resveratrol, a polyphenol found in grapes and red wines, has also prevented ammonia toxicity by modulating oxidative stress and glial and inflammatory responses in astrocytes [106]. Inhibition of NKCC1 with bumetadine or more specific drugs, which also affect potassium regulation by astrocytes, may be a new and promising approach in the treatment of ammonia neurotoxicity [31].

Concluding Remark

Rapid advances in knowledge of the mechanisms involved in hyperammonemia-induced alterations in the brain are the basis for an understanding of the neurochemical, cellular, functional and structural effects caused by ammonia, hopefully leading to the invention of novel strategies in the treatment of hepatic encephalopathy.

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