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ORIGINAL PAPER

Multifactorial Effects on Different Types of Brain Cells Contribute to Ammonia Toxicity

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Abstract Effects of ammonia on astrocytes play a major role in hepatic encephalopathy, acute liver failure and other diseases caused by increased arterial ammonia concentrations (e.g., inborn errors of metabolism, drug or mushroom poisoning). There is a direct correlation between arterial ammonia concentration, brain ammonia level and disease severity. However, the pathophysiology of hyperammonemic diseases is disputed. One long recognized factor is that increased brain ammonia triggers its own detoxification by glutamine formation from glutamate. This is an astrocytic process due to the selective expression of the glutamine synthetase in astrocytes. A possible deleterious effect of the resulting increase in glutamine concentration has repeatedly been discussed and is supported by improvement of some pathologic effects by GS inhibition. However, this procedure also inhibits a large part of astrocytic energy metabolism and may prevent astrocytes from responding to pathogenic factors. A decrease of the already low glutamate concentration in astrocytes due to increased synthesis of glutamine inhibits the malate–aspartate shuttle and energy metabolism. A more recently described pathogenic factor is the resemblance between NH_4^+ and K^+ in their effects on the Na^+,K^+-ATPase and the Na^+,K^+, 2 Cl^- and water transporter NKCC1. Stimulation of the Na^+,K^+-ATPase driven NKCC1 in both astrocytes and endothelial cells is essential for the development of brain edema. Na^+,K^+-ATPase stimulation also activates production of endogenous ouabains. This leads to oxidative and nitrosative damage and sensitizes NKCC1. Administration of ouabain antagonists may accordingly have therapeutic potential in hyperammonemic diseases.

Keywords Acute liver failure · Brain edema · Endogenous ouabains · Glutamine synthetase · Hepatic encephalopathy

Introduction

Hyperammonemia causes diseases, among which hepatic encephalopathy and acute liver failure (ALF) are the quantitatively most important. Most studies of these diseases focus on a single pathological feature which is important for understanding the pathogenesis, symptomatology and treatment of the disease(s). This review takes a different approach by describing hyperammonemic diseases as multifactorial. It discusses several of these factors, as determined by a multitude of authors, including ourselves. It begins by describing the increases in magnitude of ammonia concentrations in blood and its fluxes from there to brain in hyperammonemic patients determined by Susanne Keiding and her co-workers. In this connection it points out that much larger amounts of ammonia turn over under physiological conditions in the glutamate–glutamine cycle, which is essential for supply of neurons with transmitter glutamate and GABA. These transmitters are synthesized in astrocytes because neurons are unable to do so. However, since the ammonia which is released from neurons in the glutamate–glutamine cycle (during glutamate

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synthesis from glutamine) is used in identical amounts in astrocytes (for synthesis of glutamine from glutamate) there is no net production of ammonia during the operation of this cycle. This is an important difference from hyperammonemic diseases, where excess ammonia is detoxified in astrocytes, almost exclusively by glutamine production from ammonia.

The possibility that glutamate conversion to glutamine during ammonia detoxification may have deleterious effects due to either an increase of glutamine or a decrease of glutamate is subsequently discussed. Conversion rates are described together with the increase in glutamine content and its possible adverse consequences. It is also discussed that reduction of glutamate has adverse functional consequences, although there probably is a small increase in glutamate synthesis.

Another reason why hyperammonemia exerts deleterious effects is the similarity between the ammonia ion, NH_4^+, and K^+. This is the reason for the most dreaded consequence of ALF, cerebral edema, which is due to increased uptake of ions and water mediated by operation of both the Na^+,K^+-ATPase and NKCC1, a cotransporter of Na^+,K^+, 2 Cl^- and water. A similar edema occurs when the extracellular K^+ concentration is highly increased during brain ischemia, and in both cases concomitant effects on endothelial cells are crucial for cell swelling. An important difference between the two ions is that NH_4^+ is more potent than K^+. This difference is accentuated by inflammatory events. The Norenberg and Albrecht groups have thoroughly studied these events, which are accompanied by increases in compounds like reactive oxygen species (ROS), nuclear factor kappa B (NF-κB) and nitric oxide (NO). The inflammation is probably also a result of the similarity between NH_4^+ and K^+, since they both stimulate the Na^+,K^+-ATPase. The catalytic effect of this ATPase is essential for the ion fluxes creating the gradients driving NKCC1 and leading to the edema, but the stimulation of the Na^+,K^+-ATPase has also signaling effects. This is because it activates a pathway initiated by nanomolar concentrations of endogenous ouabains, as shown by Liang Peng and her coworkers. One branch of this pathway is essential for the catalytic function of the ATPase, and the other leads to the inflammation.

The similarity between NH_4^+ and K^+ is also the reason for stimulation of a specific glycolytic enzyme and thus of glycolysis, but it is unknown if the increased lactate production has any adverse effects. It is also not known whether the similarity between NH_4^+ and K^+ could be associated with a decrease in cyclic GMP not only in neurons but also in astrocytes, which is associated with impairment of memory in hepatic encephalopathy, as demonstrated by the groups of Vincente Felipo and Jan Albrecht.

Ammonia Fluxes in Hepatic Encephalopathy Compared to Those in the Glutamate–Glutamine Cycle

Hepatic Encephalopathy

Human ammonia toxicity is generally not secondary to exposure to exogenous ammonia but to failure of normally occurring hepatic detoxification of ammonia generated in the gut [1]. This leads to arterial hyperammonia and cerebral ammonia uptake. Most, but not all, patients with liver disease (fibrosis, cirrhosis) develop neurological abnormalities referred to as hepatic encephalopathy. Chronic hepatic encephalopathy is the most common form of this condition and is characterized by changes in personality, altered mood, declining intellectual capacity, and abnormal muscle tone [2]. Encephalopathy caused by ALF is generally a result of drug toxicity and presents with an abrupt decline in mental function, systemic inflammation and ultimately multi-organ failure and coma [3]. A frequent, although recently less common neuropathological finding is severe brain edema, which leads to increased intracranial pressure and is associated with a high mortality rate. However, using specialized MRI imaging some degree of cerebral edema is often detectable even in less fulminant hepatic encephalopathy [4].

Ammonia Concentrations and Fluxes in Hyperammonic Diseases

Arterial ammonia concentrations and blood brain fluxes of ammonia have been measured in patients with liver fibrosis with and without hepatic encephalopathy [5]. An arterial ammonia concentration above the normal level of <30 nM causes hepatic encephalopathy due to uptake in the brain from the systemic circulation, and both arterial concentrations and uptake rates are higher in patients with liver fibrosis who show sign of encephalopathy than in those who do not (Fig. 1). However, as shown in Fig. 1 even in the latter group the arterial concentration does not exceed 100 μM and the ammonia flux into brain is generally not above 15 nmol/min/ml(~g) brain [5].

Higher ammonia levels are found in ALF and in children (and some adults) suffering from inborn errors of metabolism for example in the urea cycle [6], where the arterial ammonia concentration can exceed 1000 μM [7]. High arterial ammonia concentrations are also seen in patients with Reye's syndrome [8] and in patients with ALF precipitated by intake of certain drugs or foods. These include normal doses of valproic acid in some patients, who may have suffered from undiagnosed inborn errors of metabolism [9–12]. Overdoses of acetaminophen [13, 14] or intake of certain mushrooms [15] can also lead to ALF. It is likely that the correlation between arterial ammonia concentration and

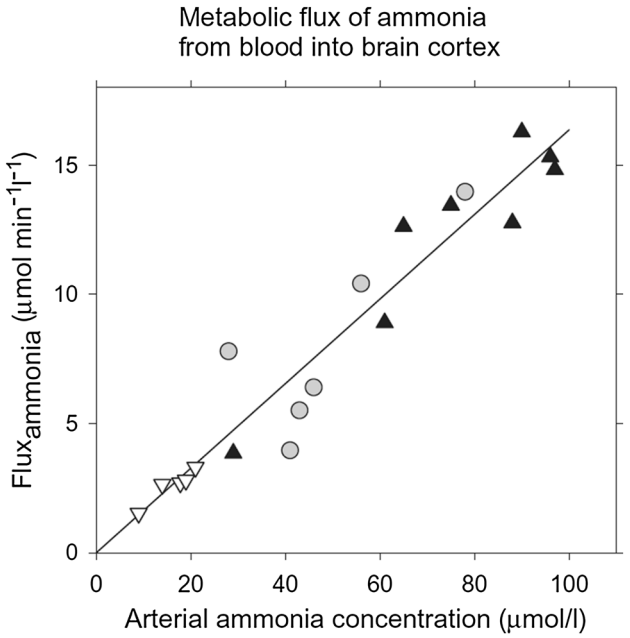

Fig. 1 Net metabolic flux of ammonia from blood to brain cortex as a function of arterial blood ammonia concentration in patients with cirrhosis with hepatic encephalopathy (*closed triangles*), patients with liver cirrhosis without hepatic encephalopathy (*open circles*), and healthy controls (*open triangles*). From Sørensen and Keiding [5]

brain uptake shown in Fig. 1 can be extrapolated to these levels.

An exciting and potentially quantitatively very important reason for ammonia-induced encephalopathy is the recently suggested possibility [16] that cerebral malaria could be due to elevated ammonia content specifically in blood vessels in brain. Plasmodium falciparum generates substantial amounts of ammonia but lacks detoxification mechanisms. It can therefore cause localized brain ammonia elevation and subsequent neurotoxic effects [16], including severe brain swelling [17]. Moreover, during *Plasmodium yoelii* infection in mice there is an increase in cerebral ammonia and lactate contents, and in glutamine synthetase (GS), phosphofructokinase and monoamine oxidase activities [18]. Many of these changes resemble hepatic encephalopathy as will become evident later in the review. Confirmation that cerebral malaria is a hyperammonemic brain disease would add a large number of cases without liver disease to the group of diseases presently known as hepatic encephalopathy and ALF.

In rats, which frequently are used to study mechanisms of hyperammonemic diseases, the normal arterial level of ammonia is higher (~170 nM) than in humans, and it rises to about 500 nM after portocaval anastomosis [19] and to >4 mM in animals given a large amount of ammonia i.p [20]. In both of these studies almost identical ammonia concentrations were reported in arteries and in brain. However, because ammonia crosses the blood–brain barrier mainly as NH_3 and pH is lower in brain (7.1) than in blood (7.4), brain ammonia level at equilibrium is normally 1.5–3 times higher in brain than in blood [21, 22].

In animal studies of hepatic encephalopathy it is important how the human disease can best be mimicked. The International Society for Hepatic Encephalopathy and Nitrogen Metabolism recommends portocaval anastomosis or bile duct ligation as animal models for hepatic encephalopathy and hepatic devascularization or thioacetamide treatment for simulation of ALF [23]. Exposure of brain slices, cell cultures, astrocytic-neuronal co-cultures or models of the neurovascular unit to different toxins are endorsed for in vitro studies. Consideration of the neurovascular unit may be especially important due to the fact that brain edema cannot develop on the basis of effects on neural cells alone but also requires an enhanced influx of water across the blood-brain barrier. This will be discussed in more detail together with brain swelling.

Much larger amounts of ammonia are generated in the brain during the operation of the glutamate–glutamine cycle. This is a physiological metabolic pathway transporting glutamate via glutamine to neurons from astrocytes where it is generated (and eventually degraded in similar amounts) or accumulated after its neuronal release [24, 25]. Flux in the glutamate–glutamine cycle corresponds to the total neuronal rate of glucose uptake [26] or ~75 % of the total glucose uptake rate, which amounts to 0.7 µmol/min per g wet wt. in rat brain [27] and to 0.3 µmol/min per g wet wt. in human brain [28]. In human brain the rate of ammonia formation and degradation in the glutamate–glutamine cycle accordingly corresponds to 0.2–0.25 µmol/min per g wet wt. It is thus normally at least ten times larger than the rate of glutamine production due to ammonia uptake and subsequent detoxification even during hepatic encephalopathy (Fig. 1). In contrast to excess ammonia entering the brain due to an increased arterial concentration of ammonia, that generated during conversion of glutamine to glutamate in the glutamate–glutamine cycle is re-utilized in astrocytes during formation of glutamine from glutamate. There is accordingly no increase in total brain ammonia or glutamine concentrations. This is a very important difference from hyperammonemic brain diseases.

Effects of Increased Ammonia Detoxification via Glutamine Synthesis

Glutamate and Glutamine

Berl et al. [29] studied metabolism of excess ammonia entering the brain from the circulation during a 25-min period. They concluded that (a) glutamine was the only cerebral amino acid that showed a considerable increase; (b) this increase occurred without a corresponding decrease in

glutamate (which accordingly must have been synthesized in corresponding amounts intracerebrally); and (c) the synthesized glutamine was formed from a small pool of glutamate that was both rapidly turning over and distinct from a larger tissue glutamate pool (which must have been the glutamate associated with the glutamate–glutamine cycle). Cooper et al. [30] expanded this information by demonstrating that infusion of physiological concentrations of [^{13}N] ammonia led to a rapid increase in the specific activity of the amide nitrogen in glutamine. The simultaneous demonstration by Norenberg and Martinez-Hernandez [31] that GS is an astrocyte-specific enzyme in brain established hepatic encephalopathy as a primarily astrocytic disease. Other authors have presented evidence that GS is also expressed in oligodendrocytes, but the original astrocyte-specific localization has been confirmed by Anlauf and Derouiche [32].

Cudalbu et al. [22] simultaneously measured [5-^{15}N] glutamine and [2-^{15}N]glutamine, glutamate content and net glutamine accumulation in the brains of rats exposed to an arterial ammonia concentration of 1 mM for 7 h. Mathematical modeling of the data provided reliable determination of both glutamate–glutamine cycle flux (0.26 µmol/g per min), and net glutamine accumulation (0.033 µmol/g per min). The results show an increase in glutamine accumulation under hyperammonemia, which amounted to 70 nmol/g per minute or about one quarter of the glutamine formation rate in the glutamate–glutamine cycle. This is consistent with the conclusion above based on ammonia fluxes that the rate of ammonia detoxification in hepatic encephalopathy becomes closer to that of ammonia turnover in the glutamate–glutamine cycle. That the demonstrated glutamate–glutamine cycle flux is similar to that described above in human brain in spite of a higher respiratory rate in the rat brain is probably because the experiments were performed in brain slices.

Huang et al. [33] had previously shown in cultured astrocytes that chronic (3 days) exposure to 3 mM ammonia (which is similar to the final brain concentration measured by Cudalbu et al. [22]) significantly increased glutamine formation from glutamate from 2.1 to 3.35 nmol/min per mg protein. With 200 mg protein per g wet wt. [34]. this increase corresponds to 250 nmol/min per g astrocytic wet wt., which with astrocytes accounting for ~25 % of cortical volume [25] equals 60–70 nmol/min per g brain wet wt., i.e., a similar effect of ammonia as that found in brain slices [23]. This similarity supports the validity of the use of well differentiated cultured astrocytes for study of ammonia toxicity.

Due to the increased flux from glutamate to glutamine during hyperammonemia brain glutamine is increased in hyperammonemic states [21, 22, 35–39]. This applies also to patients with hepatic encephalopathy [40, 41]. It has been suggested that the increased glutamine content might contribute to ammonia toxicity and ammonia-induced brain swelling [42–45], which in hyperammonemic rats is prevented by inhibition of GS [46]. The content of glutamine in normal human brain cortex is ~3 mM [47, 48], but a slightly higher value was found in rats [49]. Most of this glutamine must be astrocytic, since the glutamine content is higher in astrocytes than in neurons [50]. The L system-dependent exchange of systemic tryptophan with brain glutamine is increased in cerebral capillary endothelial cells treated with ammonia, or isolated from rats with hepatic encephalopathy [51]. This prevents a continuous rise in cerebral glutamine. The change in osmolarity induced by glutamine accumulation can be calculated to be about 3 % from the culture data by Huang et al. [33]. It can therefore not be expected to cause significant swelling. Moreover, myo-inositol and other osmolytes are decreased [39], and mild hypothermia delays the development of brain edema in portacaval-shunted rats without any effect on cerebral glutamine level [52].

A different possible reason for a deleterious role of glutamine accumulation in astrocytic edema is that glutamine uptake in mitochondria might function as a “Trojan horse” introducing ammonia into mitochondria with subsequent free radical production and mitochondrial damage in astrocytes [44], but not in neurons [53, 54]. Intramitochondrial release of ammonia causes mitochondrial permeability transition and free radical production [53–57]. The “Trojan horse” theory is supported by the finding that histidine, which inhibits glutamine uptake in mitochondria, prevents swelling in ammonia-exposed astrocytes [57]. However, this observation must be interpreted with caution because histidine also exerts direct effects against oxidative stress [58–60]. Prevention of glutamine formation also inhibits the glutamate–glutamine cycle, and since most ATP produced in astrocytes during and after formation of glutamine is derived when glutamate subsequently is oxidized in astrocytes [61–63], it will severely reduce astrocytic energy metabolism. Energy metabolism is required for brain swelling, which during complete medial cerebral artery occlusion in rats (MCAO) does not become significant until after reperfusion [64].

Glutathione, Malate–Aspartate Shuttle, Tricarboxylic Acid (TCA) Cycle, Pyruvate Carboxylase and Metabolic Rate

A drastic reduction in glutathione content in the thioacetamide model of hepatic encephalopathy might be a result of reduced glutamate content due to conversion of glutamate to glutamine, since glutamate is an essential precursor for glutathione [65, 66]. In rats made chronically hyperammonemic by portal-systemic shunting intraperitoneal injection of ammonium acetate induces a brief period of pre-coma (10–15 min), associated with a decreased glutamate content, and followed by a deep coma and high mortality [67]. In

brain tissue from cirrhotic patients with hepatic encephalopathy cerebral glutamate content is similarly reduced by about 20 % [68].

Another consequence of decreased glutamate content might be interference with the function of the malate–aspartate shuttle (MAS) (Fig. 2), which is needed for normal glucose metabolism in order to transfer reducing equivalents from the cytoplasm to mitochondria. This function is essential because one oxidative reaction occurs during glycolysis and the generated NADH is not able to traverse the mitochondrial membrane (e.g. [69]). Kosenko et al. [70] found a 20 % decrease in MAS activity in non-synaptic rat brain mitochondria after injection of ammonia acetate in rats. Lai et al. [71], studying glutamate metabolism and contents of glutamate, aspartate and glutamine in astrocyte cultures with or without 3 mM ammonium chloride similarly concluded that ammonia inhibits MAS in astrocytes. Based on levels of metabolic enzymes in mitochondria, synaptosomes and cytosol isolated from brains of normal rats and rats injected with ammonium acetate Ratnakumari and Murthy [72] reached an identical conclusion.

Depletion of the very small glutamate pool in astrocytes [73] during ammonia-induced glutamine formation must in the long run lead to an increase in glutamate synthesis in order to maintain glutamine synthesis. However, in non-synaptic mitochondria from thioacetamide-treated rats and normal non-synaptic mitochondria treated with 3 mM ammonia there is a reduction of the activity of pyruvate carboxylase

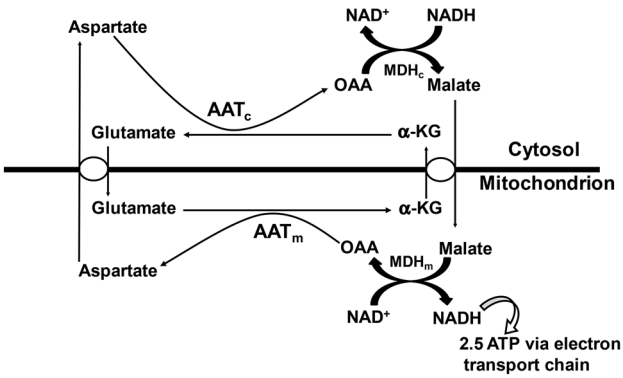

Fig. 2 In the malate–aspartate shuttle (*MAS*) cytosolic malate dehydrogenase (*MDHc*) oxidizes NADH and converts oxaloacetate (*OAA*) to malate (*top right* of figure), which enters the mitochondria in exchange with α-ketoglutarate (*α-KG*). The mitochondrial malate dehydrogenase (*MDHm*) re-oxidizes malate to OAA, which is transaminated to aspartate by the mitochondrial aspartate aminotransferase (*AATm*). Aspartate leaves the mitochondria in exchange with glutamate. In the mitochondria glutamate conversion to α-KG is essential for AATm activity forming aspartate from OAA and delivering α-KG for mitochondrial export. The glutamate imported into the mitochondria had been formed by cytosolic aspartate aminotransferase (*AATc*) from α-KG after its entry into the cytosol. Without MAS activity NADH formed in the cytosol during glycolysis would have been unable to enter the mitochondria for oxidation. From Hertz and Dienel [69]

[74], the enzyme necessary for increased production of TCA cycle intermediates and glutamate. As could be expected, the activity was negligible in synaptic mitochondria where an elevated ammonia concentration had no effect. However, Leke et al. [75, 76] found a significant increase in GABA formation from labeled glucose during hyperammonemic conditions in both co-cultures of cerebral neurons and astrocytes and in rats with liver cirrhosis. Increased anaplerosis has also been observed in cultured astrocytes in the presence of ammonia by Lapidot and Gopher [77] and in additional studies using cultured cells or animal models of hyperammonemia [78–80]. Zwingmann [81] has pointed out that the activity of an enzyme measured in tissue homogenates does not necessarily reflect the actual metabolic flux through the enzyme and reviewed additional studies indicating that ammonia increases anaplerosis.

A stimulatory effect of aspartate on glutamate and glutamine synthesis in astrocytes, probably by serving as an amino group donor, has been shown by Pardo et al. [82]. It revived the concept that α-ketoglutarate formation from glutamate during its degradation in the brain in vivo mainly is catalyzed by amino aspartate transferase [83, 84]. It also led to the concept that glutamate formation and eventual oxidation via the glutamate–glutamine cycle might be metabolically coupled [24, 25]. A potential reduction of glutamate oxidation by its enhanced use for glutamine formation under hyperammonemic conditions might accordingly also impair glutamate synthesis in the glutamate–glutamine cycle. This could explain an ammonia-induced inhibition of oxidation of both [2-^{14}C]pyruvate [85] and [1-^{14}C]pyruvate [86] and the prevention of this inhibition by addition of glutamate to the medium [86]. Reduced pyruvate-supported oxygen consumption has also been found in astrocytes obtained from rats with acute toxic liver damage [87]. Oxidative metabolism of glutamate in cultured astrocytes is also potently decreased by ammonia (Fig. 3) [71, 88].

^{15}O-oxygen positron emission tomography (PET) studies by Iversen et al. [89] established a decrease in cerebral oxygen consumption in patients with cirrhosis and an acute episode of hepatic encephalopathy. Dam et al. [90], analogously showed a decrease in oxidative metabolism in patients with hepatic encephalopathy and a clear increase after recovery to control values. Keiding and Pavese [91] reviewed studies on brain metabolism in human patients with different degrees of encephalopathy and concluded that cerebral oxygen uptake is reduced to 2/3 in cirrhotic patients with clinically overt hepatic encephalopathy. Iversen et al. [92] examined specifically astrocytic metabolism with [^{11}C]acetate PET in patients with liver cirrhosis with and without hepatic encephalopathy. Although ammonia evoked no significant decrease it seemed to reduce astrocytic metabolism by between 10 and 15 % in both types of patients.

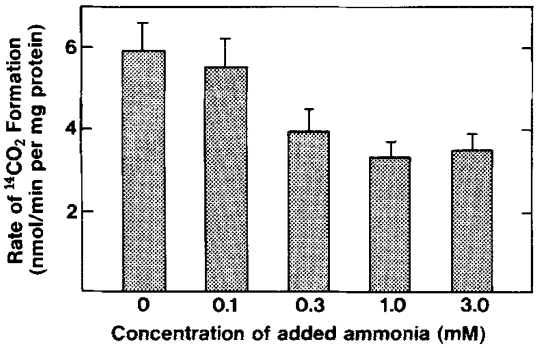

Fig. 3 Rate of $^{14}CO_2$ formation from [1-^{14}C]glutamate in primary cultures of astrocytes grown in tissue culture medium supplemented with 0.25% dBcAMP from the age of 2 weeks and acutely exposed to 0, 0.1, 0.3, 1.0, or 3.0 mM ammonia when they were at least 3 weeks old. CO_2 production rates were determined in an air-tight chamber during a 30-min period, at the end of which injections were made of perchloric acid to acidify the medium and of hyamine hydroxide into a suspended beaker for quantitative trapping of $^{14}CO_2$ within the chamber. From the measured activity in the trapped CO_2, the specific activity of [1-^{14}C] glutamate in the medium and the protein content of the culture rates of $^{14}CO_2$ formation were determined. Results are means of six to nine experiments and SEM values are shown by *vertical bars*. From Yu et al. [88]

Branched Chain Amino Acids and Glutamate Dehydrogenase

Other processes than pyruvate carboxylation might also contribute to glutamate formation in hyperammonemic brain. Some branched-chain amino acids (BCAAs) are transported rapidly across the blood–brain barrier [93], and valine and isoleucine are metabolized in the TCA cycle (via respectively acetyl CoA and succinyl CoA). A role of BCAAs, especially isoleucine, in production of glutamate and glutamine in brain and muscle was therefore suggested by Bak et al. [94]. Such a production might reduce the formation of CO_2 from these amino acids and their incorporation into protein. It may therefore be of interest that both of these processes were strongly inhibited by acute exposure to 3 mM ammonia in cultured astrocytes, although they were less affected by chronic exposure [95, 96]. There is also clinical evidence that a diet rich in BCAA increases event-free survival in cirrhotic patients, but this might be on account of increased ammonia detoxification in muscle [97].

The enzyme glutamate dehydrogenase (GDH) produces glutamate from α-ketoglutarate plus ammonia with concomitant oxidation of NAD(P)H. This process is stimulated by an increase in the ammonia concentration [98], and it might catalyze the increased glutamate production required for ammonia detoxification by glutamine production. The thermodynamic equilibrium constant of GDH favors glutamate formation, but in normal brain a high NAD^+/NADH ratio and a low ammonia concentration enables glutamate oxidation [99]. The involvement of GDH in glutamate oxidation is obvious in cultured astrocytes [100, 101]. However, it was mentioned above that in normal, non-hyperammonemic brain astrocytic glutamate formation and degradation might be metabolically coupled and to a large extent catalyzed by aspartate aminotransferase [24, 25]. Even if that is the case GDH might also play a role, possibly by supporting glutamate's cellular uptake, mediated by ion gradient-driven cotransport with Na^+, which in the long run requires energy-consuming Na^+,K^+-ATPase-mediated Na^+ extrusion. This is in agreement with the observation by Robinson and Jackson [102] that astrocytic glutamate transporters, mitochondrial enzymes and Na^+,K^+-ATPase are co-localized and co-precipitate. These authors also suggest that glutamate transport (and thus presumably its subsequent intense metabolism [63]) plays an essential role in regulation of brain energetics. The inhibition of glutamate metabolism shown in Fig. 3 might therefore have considerable adverse effects.

Ammonia Effects on Lactate and Pyruvate

Brain glucose consumption is increased in brains of acutely hyperammonemic animals [103–105]. An ammonia-induced stimulation of glycolysis has been demonstrated in brain slices [106, 107], rats with ALF [108] and bile duct-ligated rats [109]. Production of lactate is increased in cultured astrocytes (Fig. 4), but not in cultured neurons [110]. In contrast the rate of pyruvate production is decreased, leading to a reduced pyruvate/lactate ratio, consistent with the decreased cytosolic [NAD^+/NADH] ratio observed by Hindfelt and Siesjo[104] and Gjedde et al. [111]. The effect of ammonia on pyruvate/lactate ratio is more potent in cells that had not been treated with dBcAMP, where the pyruvate/lactate ratio progressively decreases within the entire concentration range 0.1–3.0 mM [110]. Addition of glutamate to the incubation medium diminished the reduced pyruvate/lactate ratio, consistent with the previously discussed inhibition of MAS and of glutathione synthesis due to enhanced glutamate utilization for glutamine production during hyperammonemic conditions. However, addition of glutamate had no effect on the increased lactate production (Fig. 4), suggesting that the main reason for this is not reduced oxidative metabolism. Ammonia activates brain phosphofructokinase, a rate-limiting and highly regulated enzyme in glycolysis [112–116]. This may be due to the similarity between NH_4^+ and K^+ (see below), since highly elevated K^+ concentrations have a similar, although less marked effect and prevent an additional effect of ammonia [115].

The pathophysiological importance of the ammonia-induced increase in lactate production may be minor since lactate can be exported from brain during hyperammonemic conditions [105], but it occurs early during the disease [117], and its magnitude is proportional to the severity of the condition [108].

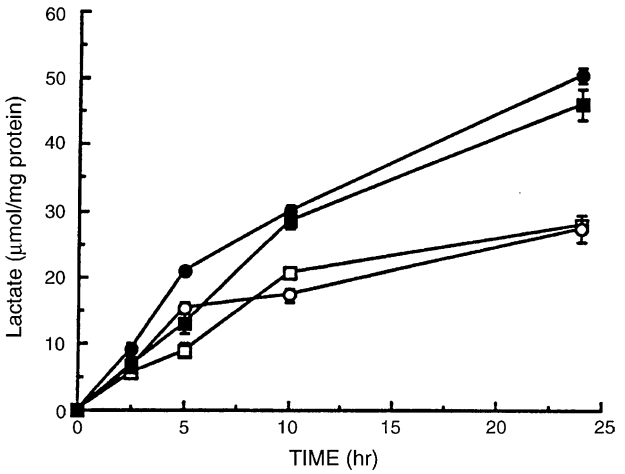

Fig. 4 Accumulation of lactate in the incubation medium of 3–4-week-old primary cultures of mouse astrocytes as a function of the length of the incubation period under control conditions (*open symbols*), i.e., incubation in normal serum-free tissue culture medium with addition of 3 mM sodium chloride, and after incubation in normal serum-free tissue culture medium with addition of 3 mM ammonium chloride (*filled-in symbols*). The cells had either been grown in tissue culture medium supplemented with 0.25 % dBcAMP from the age of 2 weeks and were accordingly morphologically differentiated (*squares*), or they had been grown for 3–4 weeks without dBcAMP supplementation and were accordingly morphologically undifferentiated (*circles*). Results are means ± SEM values (if extending beyond the symbols) of 4–20 individual experiments using cultures obtained from at least two different batches. All values obtained in the presence of ammonia are significantly different ($P < 0.05$ or better) from corresponding control values. From Kala and Hertz [110]

Ammonia Effects on Cyclic GMP

Cyclic GMP and Nitric Oxide

Lymphocytes from patients with liver cirrhosis show reduction of intracellular cyclic guanosine monophosphate (cGMP) and less than normal activation of soluble guanylate cyclase (sGC) by NO, which correlates with the degree of encephalopathy [118]. cGMP modulates some forms of learning and memory by activating cGMP-dependent protein kinase and phosphorylation of the glutamate receptor GluR1, which results in insertion of AMPA receptors in the synaptic membrane and increased magnitude of long-term potentiation (LTP) [119]. At least two pathways modulate cGMP levels in brain. One of these is the glutamate-NO-cGMP pathway which is impaired by ammonia due to reduced activation of sGC by NO [120–123]. Impairment of this pathway in brains and neurons of rats with hyperammonemia or hepatic encephalopathy may be partly responsible for their reduced ability to learn [124, 125]. Moreover, restoration of cGMP levels in brain by administering phosphodiesterase inhibitors, cGMP or anti-inflammatory drugs improves learning ability in patients with hepatic encephalopathy [118].

Cyclic GMP and Natriuretic Peptides

cGMP synthesis is also elicited by stimulation of the natriuretic peptide receptor 2 (NPR-2) with its natural ligand, C-type natriuretic peptide [126, 127]. NPR-2 is expressed both in neurons and astrocytes [128, 129]. Atrial natriuretic peptide seems to be enriched in astrocytes [130], and although it is also expressed in neurons it is possible that natriuretic peptides modulate neuronal activity via their effect on glial cells [131]. It is consistent with this possibility that Zielinska et al. [125] showed that hyperammonemia interferes with this pathway in rat cerebral cortical slices and that it does so in astrocytes. In slices from control animals CNP stimulated cGMP synthesis to a similar extent as the NO donor, *S*-nitroso-N-acetylpenicillamine (SNAP) used at optimal concentrations. Inhibition of specifically astrocytic oxidative metabolism with fluoroacetate reduced cGMP synthesis by ~50 %, and in slices from animals with ammonium acetate-induced hepatic encephalopathy it was decreased by 68 %. This inhibition was absent after treatment with fluoroacetate indicating that the CNP-dependent cGMP synthesis occurred in the fluoroacetate-inhibitable astrocytic compartment, not in the fluoroacetate-resistant neurons.

Effects of Hyperammonemia Due to Similarity with Catalytic and Signaling Effects of K^+ on the Na^+,K^+-ATPase

Brain Edema, Na^+,K^+-ATPase, NKCC1 and Inflammation

Brain edema is a serious and often fatal complication in ALF [109, 132, 133] and in inborn errors of metabolism [134]. A major reason for the development of brain edema is the similarity between NH_4^+ and K^+. NH_4^+ can potently replace K^+ in stimulation of the Na^+,K^+-ATPase and active Na^+ transport [135, 136] as well as the associated increase in oxygen consumption [137, 138]. Expression and phosphorylation of NKCC1, a cotransporter of Na^+, K^+, 2 Cl^- and water [139–141] are increased in ammonia-treated cultured astrocytes and in brain slices from thioacetamide-rats with a delay of 3–6 h (Fig. 5) [142, 143]. Similarly, Dai et al. [144] found an increase in cell volume of cultured astrocytes after 12 h' exposure to 3 mM ammonia. Kelly and Rose [145] showed in hippocampal slices that intracellular Na^+ in astrocytes but not in neurons rapidly increased by 25–30 mM after exposure to a high concentration of NH_4Cl (5 mM). This increase was prevented by bumetanide, a specific NKCC1 inhibitor. As discussed by Hertz et al. [146] these findings all indicate that the ammonia induced swelling is secondary to stimulation of NKCC1 and that ammonia acts more potently than K^+, which needs to be increased by 10 mM to activate the cotransporter.

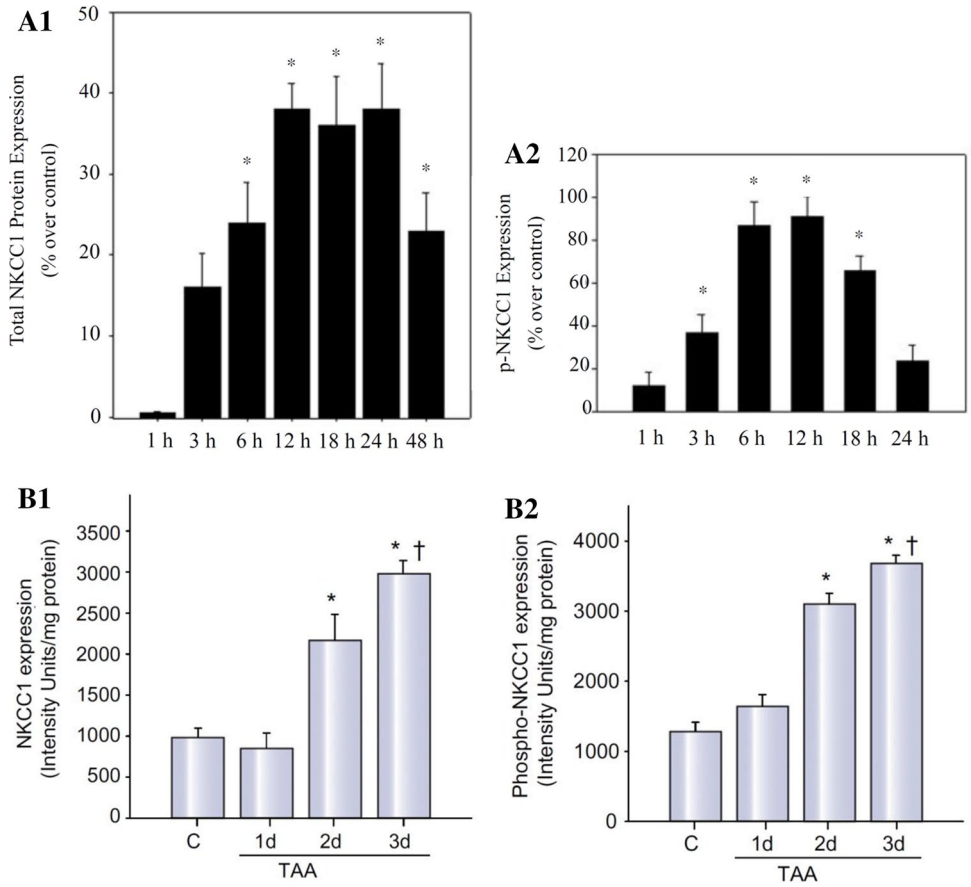

Fig. 5 Similarity between effects of ammonia (5 mM) on protein expression of both NKCC1 and phosphorylated NKCC1 (p-NKCC1) in cultured rat astrocytes (**a1, a2**) and the effect of thioacetamide-induced acute liver failure in rats in vivo, leading to hyperammonemia (**b1, b2**). The cultures had been grown in almost the same manner as the cultures used by us and were 98 % GFAP- and glutamine synthetase-positive with microglia constituting the rest of the cells. After solubilization in lysis buffer, cellular protein levels were measured and equal amounts of protein were subjected to gel electrophoresis and transferred to nitrocellulose membranes, which after blocking with non-fat dry milk were incubated with respective antibodies. Primary antibodies were used at 1:1000 to detect total NKCC1 (**a1**) and R5-phosphorylated NKCC1 (**a2**). Antibody to α-tubulin was used as housekeeping gene and results expressed as the ratios between the NKCC1 genes and tubulin after optical density of the bands had been determined with Chemi-Imager digital imaging system. In the cultures both total NKCC1 and p-NKCC1 show a significant ($P<0.05$) increase after 3 h of ammonia exposure (*asterisk*) and maximum response is perhaps reached slightly earlier for p-NKCC1 (6 h) than for total NKCC1 (12 h). The reason that the response is not immediate is that it is dependent upon oxidative and nitrosative damage of NKCC1. In the rat experiments thioacetamide (300 mg/kg body wt) was given daily for between 1 and 3 days and protein expression of NKCC1 (**b1**) and p-NKCC1 (**b2**) determined as in **a1, a2**. Note significant ($P<0.05$) increases after 2 days (*asterisk*) and a further significant increase (*dagger symbol*) after 3 days. The responses are identical in **a, b** (a maximum~threefold increase), but it occurs faster in (**a**) than in (**b**). **a** From Jayakumar et al. [142] and **b** from Jayakumar et al. [143]

The increase in NKCC1 activity is secondary to ion-induced astrocytic depolarization [147, 148] and opening of L-channels for Ca^{2+} [149]. An ammonia-induced increase in $[Ca^{2+}]_i$ and in expression of the gene for an L-type Ca^{2+} channel $Ca_v1.2$ in cultured astrocytes treated with 3 mM ammonia [150–152] is consistent with the operation of this pathway. Increased $[Ca^{2+}]_i$ also occurs in brain slices exposed to as little as 1–2 mM ammonia, and inhibition of GS results in a significantly larger $[Ca^{2+}]$ increases [153], perhaps due to impaired ammonia detoxification. The reason for the delayed response at relative low ammonia concentrations is a gradually developing, marked nitrosative and oxidative damage after ammonia exposure [154–157] which lowers the threshold for NKCC1 activation. The presence of inflammation is in agreement with a good correlation between arterial pro-inflammatory cytokines and intracranial pressure in patients with ALF [158].

Re-distribution of water between extracellular and intracellular spaces alone cannot cause brain edema, which in addition requires uptake from the systemic circulation. Effects on endothelial cells in the blood–brain barrier are necessary for this component of the swelling. This is similar to the situation during brain ischemia/reperfusion, where NKCC1 plays a major role in the pathway importing ions and water into brain parenchyma [159]. In rats subjected to permanent middle cerebral artery occlusion immune-electron microscopy

has demonstrated a predominant expression of NKCC1 at the luminal membrane of the blood-brain barrier [160]. It is therefore important that cultured cerebral endothelial cells treated with ammonia also react with oxidative/nitrosative stress [161] and that the transcription factor NF-κB is activated in cortical endothelial cells from thioacetamide-treated animals [162]. A multitude of inflammatory mediators are increased in hepatic encephalopathy and microglia is activated by ammonia [157]. In liver cirrhosis patients with and without hepatic encephalopathy expression levels are altered for more than 1000 genes related to oxidative stress, microglia activation, receptor signaling, inflammatory and anti-inflammatory pathways, cell proliferation, and apoptosis [163]. As a consequence of the role of inflammation in hepatic encephalopathy anti-inflammatory therapy is becoming of major importance in treatment of liver failure [164].

NKCC1 is also expressed in GABAergic neurons. Using very high plasma ammonia concentrations in non-anesthetized mice Rangroo Thrane et al. [20] concluded that over-activation of NKCC1 in these neurons compromised inhibitory neurotransmission, which could be prevented by bumetanide. The plasma ammonia concentrations (4 mM) were one order of magnitude larger than those seen in hepatic encephalopathy, all animals died within 1 h, and death was only postponed by ~10 min by bumetanide treatment. This study is accordingly relevant for the acute and deadly toxicity caused by ingestion of very high concentrations of ammonia. This does not exclude that neuronal NKCC1 stimulation may contribute to the pathophysiology of hepatic encephalopathy, although the decreased inhibitory transmission is in disagreement with the increased GABA formation demonstrated by Leke et al. [75, 76]. However, the concern expressed by Hadjihambi et al. [165] that the expression of NKCC1 also on astrocytes and endothelial cells may produce off-target actions of bumetanide is unjustified and in complete disagreement with the beneficial effects of NKCC1 inhibition described above.

Endogenous Ouabains

The role of endogenous ouabains in Na^+,K^+-ATPase-mediated K^+ uptake in astrocytes will be briefly discussed, since (a) Na^+,K^+-ATPase is stimulated by both NH_4^+ and K^+; (b) Na^+,K^+-ATPase activity is required to create the ion gradients driving NKCC1 [166]; and (c) stimulation of the Na^+,K^+-ATPase by an increased extracellular K^+ concentration opens a pathway mediated by endogenous ouabains [167]. Because simultaneous stimulation of the intracellular Na^+-stimulated site of the Na^+,K^+-ATPase is required for its stimulation by elevation of the extracellular K^+ concentration the K^+-induced stimulation requires a concomitant increase in intracellular Na^+ [146, 168–170]. Astrocytes are non-excitable cells and their intracellular Na^+ concentration is therefore not increased when neuronal excitation raises extracellular K^+, except as a consequence of glutamate uptake [171]. Experiments in cultured astrocytes known to mimic other signaling events and gene expression in astrocytes in situ [172] have shown that signaling mediated by nanomolar concentrations of ouabain opens an astrocytic Na^+ channel and thereby enables K^+-stimulated K^+ uptake in astrocytes [146, 169, 173]. This effect is probably essential for an initial Na^+,K^+-ATPase-mediated K^+ uptake in astrocytes during clearance of elevated extracellular K^+ following neuronal activity, at least in astrocytes that have not accumulated Na^+ during glutamate uptake. That elevated extracellular K^+ initially stimulates astrocytic K^+ uptake in brain tissue and optic nerve is now well established [174–179]. Subsequently the accumulated K^+ is released [180], probably after re-distribution over a wider area [181] which prevents a second rise in extracellular K^+ and allows neuronal uptake.

Na^+,K^+-ATPase activity is increased in primary cultures of rat astrocytes exposed acutely or chronically to ammonia [182, 183] and in astrocytes from animals treated chronically with thioacetamide [184]. Kala et al. [183] investigated if this could represent an up-regulation of the Na^+,K^+-ATPase, compensating for exposure to an increased concentration of endogenous ouabains. They found an increase of ouabain(s) in the incubation media of cultured astrocytes exposed to 3 mM ammonia for 4 days. This is accompanied by increased expression of the astrocytic α2 subunit of the Na^+,K^+-ATPase [185], which is abolished by AG1478, an inhibitor of the epidermal growth factor receptor (EGFR). This receptor is a key component of a pathway mediated by endogenous ouabain(s) in cultured astrocytes [144, 152, 169]. This astrocytic pathway [169] leads like many other signaling pathways initiated by G-protein-coupled receptors [186, 187] to metalloproteinase-mediated release of a growth factor and 'transactivation' and phosphorylation of the EGFR [188]. Stimulation of the EGFR activates two pathways, one leading to Ca^{2+} release and glycogenolysis which is needed for uptake of K^+, and the other to phosphorylation of extracellular-regulated kinases one and two ($ERK_{1/2}$) via Ras, Raf and MEK. Phosphorylation of ERK, increased ROS production and swelling in ammonia-exposed cultured astrocytes is abolished by the ouabain antagonist canrenone (Fig. 6) [144]. This is consistent with observations in cardiac myocytes that ouabain increases ROS, an effect that was antagonized by a dominant negative Ras, suggesting Ras involvement in ROS generation [189]. Ouabain-induced nitrosative damage is also likely in cardiac myocytes, since iNOS, the form expressed in astrocytes is increased by ouabain [190].

Two endogenous steroids with ouabain-like activity, the cardenolide, endogenous ouabain and the bufadienolide, marinobufagenin are found in small but equal amounts in brain cortex [191]. Synthesis of marinobufagenin has

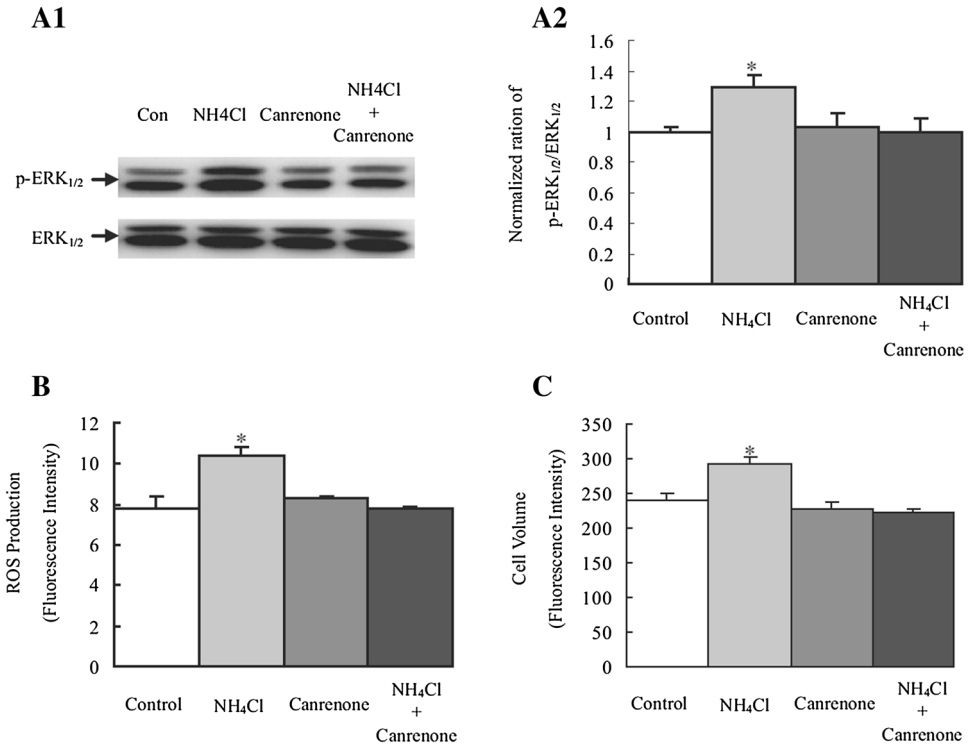

Fig. 6 Ammonia-induced ERK phosphorylation, ROS production and cell swelling is inhibited by canrenone, an inhibitor of ouabain. **a** Astrocyte cultures grown as described in legend of in Fig. 3 were incubated with 0 or 3 mM NH_4Cl in the absence (control: no NH_4Cl, no canrenone) or presence of 100 μM canrenone for 20 min. **a1** Immunoblots from a representative experiment. Similar results were obtained from three independent experiments using cultures from three different batches. Bands of 44 and 42 kDa represent p-ERK_1 (phosphorylated ERK_1) and p-ERK_2 (phosphorylated ERK_2), respectively (*upper rows*), or total ERK_1 and ERK_2 (*lower rows*). Average ERK phosphorylation was quantitated as ratios between p-$ERK_{1/2}$ and $ERK_{1/2}$ (**a2**). The ratio between p-$ERK_{1/2}$ and $ERK_{1/2}$ in control group was designated a value of one. S.E.M. values are indicated by *vertical bars*. **b** Cells were incubated as in (**a**) for 2 h. ROS was determined as fluorescence intensity of oxidized carboxy-H_2DCFDA. Fifteen and twenty cells were selected in each coverslip, and three coverslips were used in each experimental group. **c** Cells were incubated as in (**a**) for 12 h Cell volume was determined as fluorescence intensity of calcein. Fifteen and twenty cells were selected in each coverslip, and three coverslips were used in each experimental group. *Statistically significant ($P<0.05$) difference from control group. From Dai et al. [144]

recently been studied by Fedorova et al. [192] in adrenocortical and placental cells and found to occur from cholesterol via the acidic bile acid pathway, which is controlled by enzyme mitochondrial sterol 27-hydroxylase (CYP27A1). In macrophages the needed cholesterol trafficking from the outer to the inner mitochondrial membrane is mediated by a highly regulated multimeric protein complex. This complex comprises mitochondrial TSPO (translocator protein 18 KDa or mitochondrial peripheral benzodiazepine receptor) and VDAC (voltage-dependent anion channel) together with additional proteins [193, 194]. TSPO is expressed in both astrocytes and microglia [195]. In cultured retinal microglia its antagonists Ro5-4864 and PK11195 both decrease ROS production, whereas only Ro5-4864 decreases TNFα [196]. TSPO is activated in hyperammonemic animals and in brain tissue from patients with hepatic encephalopathy, resulting in increased synthesis of allopregnanolone and tetrahydrodeoxycorticosterone, neurosteroids which have positive modulatory effect on the $GABA_A$ receptor complex [197]. The induced sedation may be one reason for a therapeutic effect of partial inverse agonists of the benzodiazepine receptor in hepatic encephalopathy [198], but a potential reduction of endogenous ouabains might be even more important. This might not only apply to such a serious effect as brain swelling but also to relatively early manifestations of hepatic encephalopathy like the memory impairment found by Zielinska et al. [125] to be caused by deficient cGMP simulation by NPR-2. Since marinobufagonin stimulates collagen synthesis [199] it is possible that endogenous ouabains even play a role in the development of liver cirrhosis.

Concluding Remarks

The symptoms in hyperammonemic diseases depend on the concentration of ammonia in the systemic circulation and as a consequence in the brain. Ammonia detoxification

increases brain glutamine and decreases glutamate, which has metabolic consequences. Another major reason for ammonia toxicity is the resemblance between NH_4^+ and K^+, which both stimulate NKCC1 and the Na^+,K^+-ATPase in astrocytes and endothelial cells. In ALF these effects lead to brain swelling, which is generally delayed because it depends upon additional oxidative and nitrosative damage of NKCC1, induced via signaling by nanomolar concentrations of endogenous ouabains. These effects can be prevented by an ouabain antagonist which might become useful in the therapy of hyperammonemic disorders.

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