



Neurobiology of ammonia

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Abstract

Hyperammonemia resulting from inherited urea cycle enzyme deficiencies or liver failure results in severe central nervous system dysfunction including brain edema, convulsions and coma. Neuropathologic evaluation in these disorders reveals characteristic alterations of astrocyte morphology ranging from cell swelling (acute hyperammonemia) to Alzheimer Type II astrocytosis (chronic hyperammonemia). Having no effective urea cycle, brain relies on glutamine synthesis for the removal of excess ammonia and the enzyme responsible, glutamine synthetase, has a predominantly astrocytic localization. Accumulation of ammonia in brain results in a redistribution of cerebral blood flow and metabolism from cortical to sub-cortical structures. In addition to changes in astrocyte morphology, increased brain ammonia concentrations result in alterations in expression of key astrocyte proteins including glial fibrillary acidic protein, glutamate and glycine transporters and “peripheral-type” (mitochondrial) benzodiazepine receptors. Such changes result in alterations of astrocytic volume and increased extracellular concentrations of excitatory and inhibitory substances. In addition, the ammonium ion has direct effects on excitatory-inhibitory transmission via distinct mechanisms involving cellular chloride extrusion and postsynaptic receptor function. Acute ammonia exposure leads to activation of NMDA receptors and their signal transduction pathways. Chronic hyperammonemia also results in increased concentrations of neuroactive L-tryptophan metabolites including serotonin and quinolinic acid. Therapy in hyperammonemic syndromes continues to rely on ammonia-lowering strategies via peripheral mechanisms (reduction of ammonia production in the gastrointestinal tract, increased ammonia removal by muscle).

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1. Introduction

Increased ammonia concentrations have deleterious effects on central nervous system (CNS) function. Depending upon the age of the species and the magnitude and duration of exposure, ammonia toxicity may result in severe neurological symptoms including seizures, mental retardation and coma and in neuronal cell damage and loss. Brain edema sufficient to cause increased intracranial pressure and death by brain herniation is a frequent complication of severe acute hyperammonemia.

This review article will briefly summarise the nature of the hyperammonemic syndromes, their associated neuropathologic characteristics and will systematically address the major mechanisms proposed to explain the molecular basis of ammonia's neurotoxicity.

Ammonia is a molecule with many properties. Depending upon pH, it is an ion (NH_4^+) with an ionic radius and properties similar to that of K^+ or a gas (NH_3) with free access across cellular and subcellular membranes. Ammonia is an important substrate for several enzymic reactions in the brain and is a product of others. Ammonia is an acid, a base and, at elevated concentrations, is toxic to both neuronal and astrocytic elements in the CNS.

In aqueous solution, ammonia (NH_3) is in equilibrium with the ammonium ion (NH_4^+). The ratio of $\text{NH}_3/\text{NH}_4^+$ is a function of pH as defined by the Henderson–Hasselbach equation:

$$\log_{10} \left[\frac{\text{NH}_3}{[\text{NH}_4^+]} \right] = \text{pH} - \text{p}K_a$$

At 37 °C, the $\text{p}K_a$ of ammonia is 9.15 (Bromberg et al., 1960). Consequently, under normal physiological conditions, more than 98% of ammonia is present as NH_4^+ .

Large quantities of ammonia normally enter the portal vein circulation from protein digestion in the gastrointestinal system. However, arterial ammonia levels are maintained at low concentrations (in the 50–100 μM range), a finding which illustrates the efficiency with which the liver normally removes gut-derived ammonia. Reduced hepatic capacity for ammonia removal associated with inherited urea cycle disorders or liver injury results in hyperammonemia and a spectrum of neuropsychiatric symptoms.

2. The hyperammonemic syndromes

A number of human disorders are associated with hyperammonemia. In some of these disorders, hyperammonemia is the primary defect; in others, it is secondary. Acute (ALF) or chronic liver failure and other metabolic conditions such as hypoglycemia and seizures may also result in increases of blood and/or brain ammonia.

2.1. Inherited hyperammonemias

In infants with inborn errors of urea cycle enzymes, blood ammonia concentrations may be as high as 1 mM and, if the hyperammonemia is not treated promptly, severe neurological symptoms including seizures and coma occur. Surviving children have a high incidence of mental retardation and cerebral palsy.

A complete urea cycle is only expressed in the liver although other tissues, including brain, may express some of the constituent enzymes. Inborn errors of each of the individual enzymatic steps of the urea cycle have been described. However, the most frequently encountered primary inherited deficit of a urea cycle enzyme involves ornithine transcarbamylase (OTC; or ornithine carbamoyl transferase, EC 2.1.3.3). OTC is a mitochondrial enzyme which catalyses the conversion of ornithine to citrulline (Fig. 1).

OTC deficiency in humans is inherited as an X-linked trait. More than 100 OTC gene mutations have been described, the majority of which are single base substitutions (Tuchman et al., 1996). In a study of 26 children with inherited urea cycle disorders who survived neonatal hyperammonemic coma and were maintained on a regimen of nitrogen restriction and stimulation of alternative nitrogen-excreting pathways, a significant negative correlation was observed between duration of hyperammonemic coma and IQ scores at 1 year of age (Msall et al., 1984).

Organic acidemias due to inborn errors of β -ketothiolase, propionyl CoA carboxylase or methylmalonyl CoA mutase deficiencies are also accompanied by marked hyperammonemia.

2.2. Acquired hyperammonemias

Liver failure resulting from the ingestion of toxins (including ethanol), viral infections or autoimmune disease results

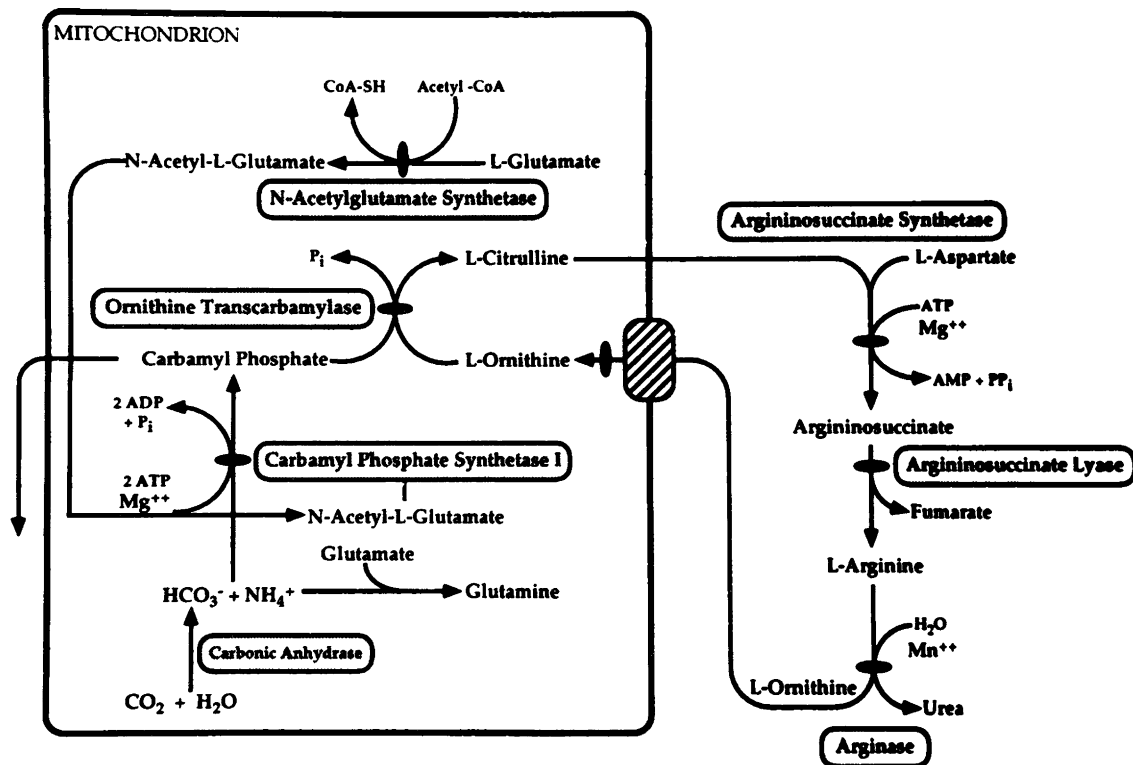


Fig. 1. The urea cycle. Inborn errors of each of the individual enzymatic steps of the cycle have been described. Inherited urea cycle enzymopathies result in hyperammonemia and severe neurological impairment, which may include seizures and mental retardation.

in increased blood and brain ammonia concentrations. As liver function deteriorates, a severe neuropsychiatric disorder known as hepatic encephalopathy (HE) develops. Infusion of ammonia to produce blood ammonia levels comparable to those observed in patients in hepatic coma leads to a clinical syndrome and neuropathological changes that are indistinguishable from HE. Hepatic encephalopathy occurs in one of two common forms. In acute liver failure, HE is characterised by a rapid progression of symptoms starting with altered mental status progressing to stupor and coma within hours or days. Arterial ammonia concentrations in patients with ALF and severe encephalopathy are in the 0.3–0.5 mM range (Clemmensen et al., 1999). Experimental ALF in laboratory animals results in blood ammonia concentrations as high as 1 mM at coma stages of encephalopathy. Brain ammonia concentrations in these animals are in the 1–5 mM range (Swain et al., 1992a,b).

In contrast to ALF, chronic liver failure (cirrhosis) of either alcoholic or non-alcoholic etiology results in significant spontaneous portal-systemic shunting of portal blood and more modest increases in arterial ammonia concentrations, which are generally found to be in the 0.1–0.2 mM range (Lockwood et al., 1991). Hepatic encephalopathy in chronic liver failure (sometimes referred to as portal-systemic encephalopathy) develops slowly and is often precipitated by ammoniagenic conditions such as ingestion of a protein load, constipation or a gastrointestinal bleed. In the cirrhotic liver, both urea cycle enzyme activity and the activity

of glutamine synthetase (GS) are significantly decreased (Haussinger, 1983; Haussinger and Gerok, 1984).

It is important to bear in mind that, in addition to ammonia, chronic liver failure results in the accumulation of other toxins including manganese (Pomier Layrargues et al., 1995), mercaptans and short-chain fatty acids (Zieve et al., 1974), all of which may have deleterious effects on brain function.

3. Neuropathology of hyperammonemic syndromes

3.1. Mature brain

Acute and chronic liver failure in adults give rise to distinct neuropathological changes. In the case of ALF, cytotoxic brain edema characterised by swelling of astrocytes and their processes occurs (Fig. 2A). Cell swelling may be so severe as to cause raised intracranial pressure and, as a consequence, brain herniation. Brain herniation is the major cause of mortality in ALF. The occurrence of brain herniation in patients with ALF is predicted by increased arterial ammonia concentrations (Clemmensen et al., 1999).

Chronic liver failure, on the other hand, results in characteristic morphological changes to astrocytes in addition to a mild form of cell swelling known collectively as Alzheimer type II astrocytosis (Fig. 2B). Alzheimer type II astrocytes have a large pale nucleus, prominent nucleolus and show a margination of their chromatin pattern. Alzheimer type II

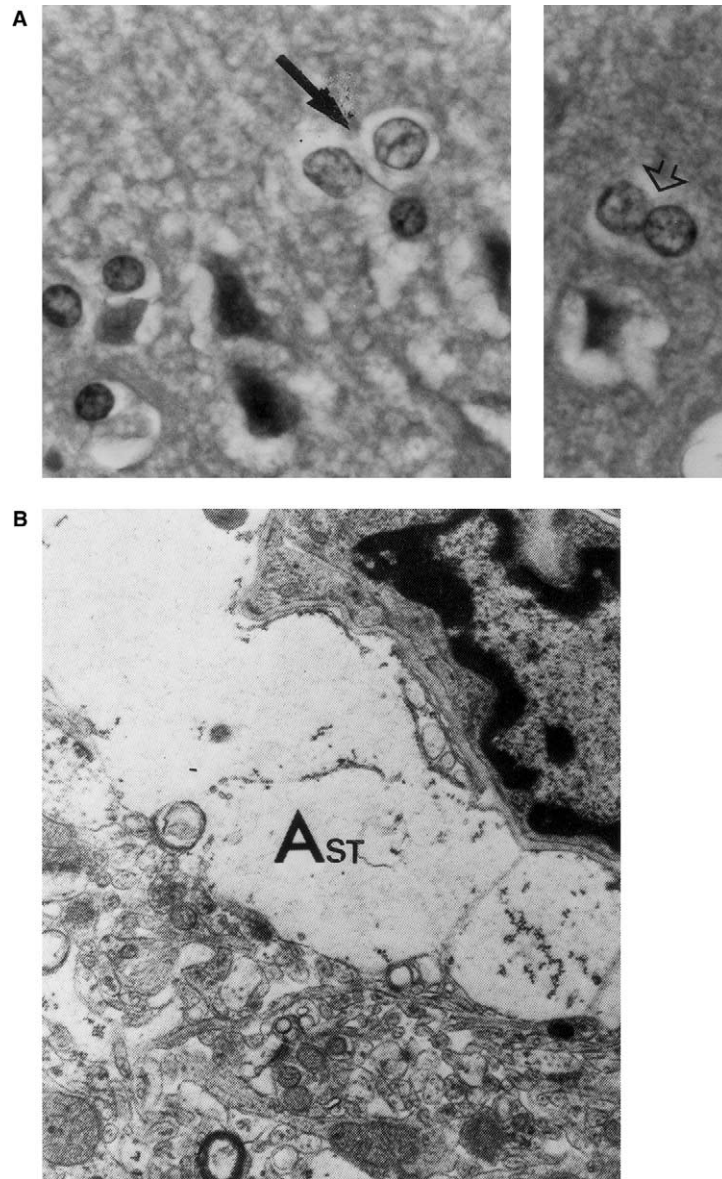


Fig. 2. Neuropathology of hyperammonemic syndromes. Chronic hyperammonemia resulting from urea cycle enzymopathies or liver cirrhosis results in Alzheimer type II astrocytes (A). Alzheimer type II cells (arrow, left-hand panel) show enlarged nuclei and margination of chromatin in this H+E stained section of frontal cortex from a 51-year-old cirrhotic patient who died in hepatic coma. Doublets suggestive of a proliferative response are frequently seen (right-hand panel, arrow head). Acute hyperammonemia resulting from acute liver failure or Reye syndrome results in astrocytic swelling. Plate (B) represents an electron micrograph showing swelling of a perivascular astrocyte (Ast) from a patient with acute liver failure who died of brain herniation (modified from Kato et al., 1992).

cells are encountered in many types of hyperammonemic syndromes including HE, congenital urea cycle disorders, urease-treated mice and in the brains of rats following surgical portacaval anastomosis. They are most prominent at coma stages of HE. Exposure of primary rat astrocyte cultures to millimolar concentrations of ammonia also results in Alzheimer type II changes (Norenberg, 1981).

Neuronal cell loss is rarely observed in mature brain exposed to hyperammonemia. However, an uncommon condition known as acquired hepatocerebral degeneration (non-Wilsonian) has been described (Victor et al., 1965) in

cirrhotic patients who had undergone multiple episodes of HE. This disorder is characterised by neuronal cell loss in basal ganglia and cerebellar structures.

3.2. Developing brain

Developing brain is more susceptible to acute or chronic hyperammonemia compared to the adult brain. In addition to Alzheimer type II astrocytosis, neuropathological evaluation of infants and young children who died with congenital OTC deficiency reveals cerebral atrophy, cystic degeneration,

ventricular enlargement and delayed myelination (Harding et al., 1984; Dolman et al., 1988; Harper and Butterworth, 1997). Cerebral edema sufficiently severe to result in intracranial hypertension and uncal herniation (similar to that observed in ALF) has also been described in patients with congenital OTC deficiency (Michalak and Butterworth, 1997a,b). Computed tomographic (CT) abnormalities have been reported in a case of OTC deficiency with 7% residual enzyme activity (Takayanagi et al., 1984). Such abnormalities included diffuse hypointensities in frontal and parietal lobes, and it was suggested that a substantial portion of neuropathologic damage in congenital OTC deficiency may have occurred in utero (Harding et al., 1984; Filloux et al., 1986).

In a mouse model of congenital OTC deficiency, a selective loss of medium spiny neurons together with increased numbers of microglia and oligodendroglia was reported in striatum (Robinson et al., 1985), a pattern which is characteristic of an excitotoxic mechanism of neuronal cell death.

4. Brain ammonia uptake and synthesis

Ammonia enters the brain from blood by diffusion rather than via a saturable transport system, and it has been demonstrated that the brain uptake index for ammonia is independent of arterial ammonia levels over a wide range of concentrations (Cooper et al., 1979). Ammonia free base (NH_3) permeates cell membranes more freely than does NH_4^+ . However, it has been estimated that up to 25% of ammonia may enter the brain as NH_4^+ at physiological pH values. The rate constant for protonation of NH_3 has been calculated to be $4.3 \times 10^{10} \text{ M}^{-1} \text{ s}^{-1}$ yielding a relaxation time of, approximately 12 s. Since the blood transit time through brain is of the order of seconds, the NH_4^+ to NH_3 conversion rate is too rapid to limit the rate at which ammonia enters the brain (Cooper and Plum, 1987). The lower permeability of NH_4^+ implies that blood–brain barrier transfer of ammonia is dependent upon arterial blood pH and systemic alkalosis exacerbates ammonia toxicity (Warren, 1958) consistent with a higher rate of diffusion of NH_3 into brain at higher blood pH values. Ammonia extraction by brain varies inversely with cerebral blood flow (CBF) since, at low rates of flow, the contact time for a given volume of blood with the blood–brain barrier increases, and the probability of entering the brain increases (Phelps et al., 1977; Raichle and Larson, 1981).

Since diffusion of ammonia into brain is pH dependent, the pH gradient between blood and brain may affect brain ammonia concentrations. Assuming a blood pH of 7.4 and a brain intracellular pH of 7.1, the Henderson–Hasselbalch equation predicts a ratio of brain to blood ammonia concentrations under normal physiological conditions of 2 (Cooper and Plum, 1987). Experimental values range from 1.5 to 3.0. However, in hyperammonemia, values as high as 8 have been reported (Warren and Schenker, 1964). For example,

experimental ALF in the absence of encephalopathy results in blood ammonia concentrations in the 0.1–0.3 mM range (Mans et al., 1994; Rose et al., 1998). At coma stages of encephalopathy in these animals, blood ammonia concentrations rise to 0.5–1.0 mM (Mans et al., 1994). Concentrations of blood ammonia of a comparable magnitude have been reported in patients with ALF (Clemmensen et al., 1999). However, brain ammonia concentrations as high as 1–5 mM have been described at coma stages of encephalopathy in animals with acute liver failure (Swain et al., 1992a,b), some 5–10-fold higher than corresponding blood ammonia concentrations in these animals. Brain ammonia concentrations at symptomatic stages following the administration of toxic doses of ammonium salts are likewise in the 3 mM range (Kosenko et al., 1994).

In chronic liver failure, prior to the onset of encephalopathy, blood ammonia concentrations are increased three-fold in both experimental animals and humans (Lockwood et al., 1991), and corresponding brain concentrations are in the 0.3–0.5 mM range (Girard et al., 1993). However, following precipitation of coma by administration of ammonium salts to animals with chronic liver failure, brain ammonia concentrations increase to 3–5 mM (Hindfelt et al., 1977; Girard et al., 1993). These increases in the blood–brain ammonia ratio in liver failure may result from increased permeability of the blood–brain barrier to ammonia. Consistent with this latter possibility, positron emission tomography (PET) studies using $^{13}\text{NH}_3$ in cirrhotic patients with mild HE reveal increases in the brain permeability/surface area product (Fig. 3) consistent with increased blood–brain barrier permeability of ammonia in chronic hyperammonemia (Lockwood et al., 1991).

At least 16 enzymatic pathways in brain result in the formation of ammonia. One of the most important is glutamate dehydrogenase, which catalyses the reversible oxidative deamination of glutamate. It has been proposed that in both normal and hyperammonemic conditions, glutamate dehydrogenase is ammonia producing, particularly in astrocytes and, in this way, may provide a mechanism for the removal of excess nitrogen from certain catabolised amino acids (Cooper and Plum, 1987). A catabolic role for glutamate dehydrogenase is also consistent with the finding of decreased brain glutamate concentrations in a wide range of hyperammonemic syndromes (Lavoie et al., 1987a; Swain et al., 1992a,b; Ratnakumari et al., 1994).

L-Glutaminase is widespread in brain and is particularly abundant in nerve endings of glutamatergic neurons where it forms an integral part of the glutamate–glutamine cycle in which a molecule of ammonia is transferred from the astrocyte to the neighbouring neuron (see Section 6.3.1). There is evidence to suggest that at least part of the increased glutamine encountered in brain in hyperammonemia results from inhibition of glutaminase (Tyce et al., 1981). Enzymes of the purine nucleotide cycle may also be responsible for generating a significant fraction of brain ammonia (Schultz and Lowenstein, 1978).

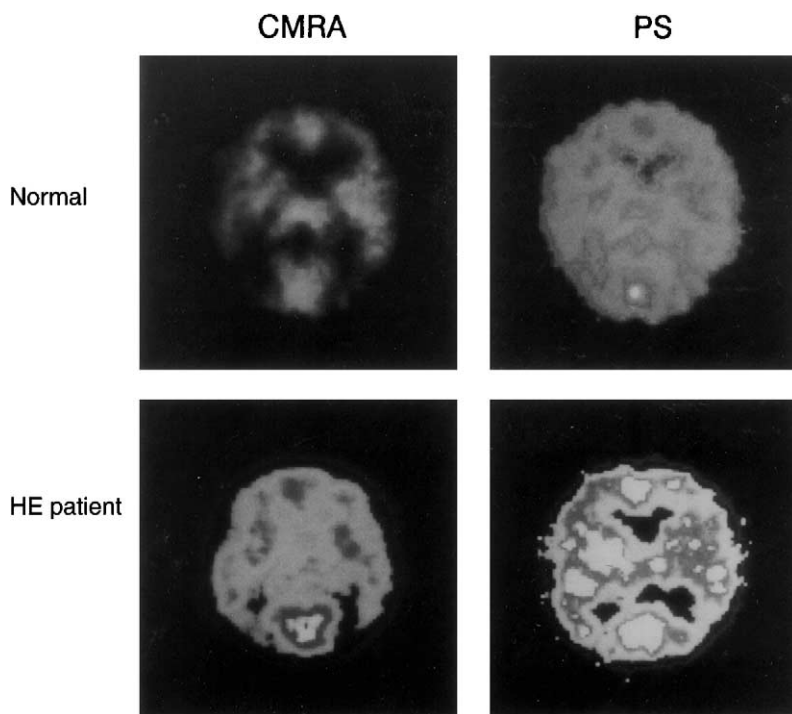


Fig. 3. Positron emission tomographic images from a normal subject compared to a cirrhotic patient with mild hepatic encephalopathy (HE) and increased arterial ammonia concentrations. Cerebral metabolic rate for ammonia (CMRA) is increased three-fold in the patient and blood–brain barrier permeability (permeability/surface area product, PS) is increased two-fold (modified from Lockwood et al., 1991).

5. Brain ammonia metabolism

Metabolic trapping of ammonia is the principal mechanism responsible for maintaining brain ammonia levels at around 0.05 mM under normal physiological conditions. Since brain lacks carbamoyl-phosphate synthase I and ornithine transcarbamylase, it is unable to remove ammonia in the form of urea. Consequently, brain ammonia is metabolised almost exclusively to glutamine via the GS reaction. The GS reaction has a very rapid turnover time with a half life of, approximately 3 s. Glutamine synthesis remains the predominant route for ammonia removal in brain under both normal and hyperammonemic conditions (Cooper and Plum, 1987).

Immunohistochemical studies reveal that GS expression is limited almost exclusively to astrocytes (Norenberg and Martinez-Hernandez, 1979). Thus, it is the astrocyte rather than the neuron that is uniquely responsible for ammonia detoxification in brain. Surprisingly, in contrast to peripheral tissue such as skeletal muscle, there is no significant induction of GS expression in brain in hyperammonemic states (Cooper et al., 1985; Lavoie et al., 1987b). Moreover, since the enzyme functions at near maximal capacities under normal physiological conditions (Cooper and Plum, 1987), hyperammonemia rapidly exceeds the brain's capacity to synthesise glutamine, and ammonia concentrations rise significantly. It has been proposed that ammonia-induced activation of the nitric oxide–cGMP signal transduction

pathway in brain could lead to a further limitation in GS capacity (see Section 6.4).

6. Effects of ammonia on brain function

6.1. Cerebral blood flow

Hyperammonemia is associated with profound effects on CBF. These effects are dependent upon the severity and duration of hyperammonemia and show region selectivity. For example, in chronic mild hyperammonemia associated with liver cirrhosis, CBF is decreased in proportion to the deterioration of neuropsychiatric status (Posner and Plum, 1960; James and Garassini, 1971). However, recent studies using non-invasive and imaging techniques reveal that, rather than a generalised reduction of CBF in these patients, there is a redistribution of flow characterised by a reduction in cortical structures and a concomitant increase in some sub-cortical areas (O'Carroll et al., 1991).

The cerebral metabolic rate for glucose (CMR_{glucose}), a parameter that remains tightly coupled to CBF both under normal physiological conditions and in chronic mild hyperammonemia, shows a similar redistribution pattern (Lockwood et al., 1993), a pattern which no doubt reflects the regional ammonia-induced effects on brain metabolism and on excitation/inhibition mechanisms (see Sections 6.2–6.7).

Interestingly, CMR_{glucose} rises acutely immediately following portacaval anastomosis surgery (James et al., 1972),

and infusions of ammonia solutions to anaesthetised dogs were found to result in a lightening of anaesthesia and increased glucose metabolic rates (James et al., 1974). In a separate series of studies, intracarotid infusions of ammonia sufficient to cause EEG slowing were found to result in increased $\text{CMR}_{\text{glucose}}$, which was confined to deep grey matter structures (Lockwood et al., 1982). These findings again underline the regional selectivity of the cerebral metabolic changes in hyperammonemia.

Acute liver failure results in increased CBF at late stages of encephalopathy that are associated with brain edema (Aggarwal et al., 1994; Wendon et al., 1994). Increased CBF in ALF has been attributed to a consequence of ammonia toxicity and, in particular, to ammonia's metabolism to glutamine in the astrocyte (Takahashi et al., 1992). Furthermore, in ALF, CBF may become uncoupled from cerebral metabolism and loss of cerebrovascular autoregulation may also result (Larsen, 1996).

6.2. Mitochondrial function and brain energy metabolism

Ammonia causes significant alterations of mitochondrial function and, consequently, changes in cerebral energy metabolism. As with CSF changes, these effects depend upon the concentration and duration of ammonia exposure. Ammonia stimulates glycolysis in brain extracts by activation of phosphofruktokinase (Sugden and Newsholme, 1975) and acute ammonia toxicity in normal rats leading to brain ammonia concentrations in the 1.4–1.5 mM range results in increased brain glucose utilisation (Hawkins et al., 1973). Increased brain glucose concentration in acute hyperammonemia may be the consequence of an increased in expression of the endothelial cell/astrocytic glucose transporter GLUT-1 as was recently reported in experimental ALF (Desjardins et al., 2001). Increased brain glucose uptake was accompanied by increased brain lactate concentrations, which occurred without any loss of high energy phosphates. The increased brain glucose uptake and lactate accumulation due to acute ammonia exposure appears to be predominantly an astrocytic phenomenon since expression of the neuronal glucose transporter GLUT-3 is not affected by ammonia (Desjardins et al., 2001).

In contrast to the situation in acute moderate hyperammonemia, exposure of experimental animals to lethal doses of ammonium salts and to brain ammonia concentrations in excess of 3 mM, results in reductions of brain ATP concentrations (McCandless and Schenker, 1981; Kosenko et al., 1994). Reduced brain ATP levels have also been described in mice with congenital urea cycle disorders (Ratnakumari et al., 1992). Two distinct mechanisms have been proposed to explain ammonia-induced reductions in brain ATP:

- (1) inhibition of the tricarboxylic acid cycle (TCA);
- (2) a mechanism involving *N*-methyl-D-aspartate (NMDA) receptors (see Section 6.2).

In favour of mechanism (1), McKhann and Tower (1961) reported ammonia-induced inhibition of the TCA cycle in brain with accumulation of α -ketoglutarate and pyruvate. Subsequently, Lai and Cooper (1986) described a significant inhibition of the rate-limiting TCA cycle enzyme α -ketoglutarate dehydrogenase (α KGDH) in brain mitochondrial preparations exposed to ammonia with an EC_{50} of 2 mM. Consistent with α KGDH inhibition and a consequent reduction in entry of pyruvate into the tricarboxylic acid cycle are the findings of increased brain lactate concentrations in various hyperammonemic disorders (Hawkins et al., 1973; Hindfelt et al., 1977; McCandless and Schenker, 1981; Therrien et al., 1991; Mans et al., 1994; Chatauret et al., 2001). Furthermore, hypoxia significantly exacerbates the effects of lethal injections of ammonium salts in mice (Warren and Schenker, 1960). Cerebrospinal fluid lactate concentrations are increased in both acute (Chatauret et al., 2001) and chronic (Therrien et al., 1991) liver failure and are positively correlated with the severity of HE in these disorders. Increased CSF lactate has also been reported in human HE (Yao et al., 1987). Moreover, prevention of the brain lactate increase in experimental ALF by mild hypothermia results in prevention of encephalopathy and brain edema (Chatauret et al., 2001). Also in favour of a mechanism involving inhibition of the TCA cycle by ammonia is the report that increased ammonia levels in animals injected with U^{14}C glucose resulted in a reduction in the amount of label incorporated into the amino acids glutamate and GABA (Prior and Visek, 1972).

In support of mechanism (2), it has been shown that ammonia-induced depletion of brain ATP *in vivo* is prevented by administration of a wide range of glutamate (NMDA) receptor antagonists (Kosenko et al., 1994). Based upon these observations, it was suggested that ammonia-induced activation of NMDA receptors results in ATP depletion via the activation of Na^+ , K^+ , ATPase as well as by decreased synthesis of ATP due to impairment of Ca^{2+} homeostasis (Kosenko et al., 2000). These mechanisms are further discussed in Section 6.2.

In contrast to the situation involving the administration of lethal doses of ammonia, there is little convincing evidence to suggest that hyperammonemia resulting from acute or chronic liver failure results in a loss of ATP in brain at least until stages of encephalopathy characterised by prolonged coma (Mans et al., 1994; Hindfelt et al., 1977). Likewise, studies in cirrhotic patients with end-stage liver failure using spectroscopic techniques have so far not provided convincing evidence for a primary cerebral energy deficit (Taylor-Robinson et al., 1994; Lockwood et al., 1997). There are several possible explanations for the absence of cerebral energy deficit in chronic liver failure. Proliferation of astrocytic mitochondria has been reported in conditions of chronic hyperammonemia (Gregorios et al., 1985; Norenberg and Lapham, 1974), a phenomenon that has been attributed to increased energy requirements. In addition, it has been reported that chronic hyperam-

monemia similar in magnitude to that observed in end-stage chronic liver failure leads to down-regulation of functional NMDA receptors (Peterson et al., 1990; Marcaida et al., 1995). Another possible mechanism responsible for the lack of energy depletion in chronic hyperammonemia is the finding of impairment of signal transduction pathways associated to NMDA receptors (Hermenegildo et al., 1998). Both mechanisms could effectively minimise the impact of mechanism (2) and prevent loss of ATP due to NMDA receptor activation. Consistent with these possibilities, it has been shown that chronic moderate hyperammonemia in rats completely prevents the depletion of ATP induced by subsequent acute lethal injections of ammonia (Kosenko et al., 1993).

6.3. Astrocyte function

As discussed above, it is the astrocyte which bears the brunt of ammonia removal by the brain and it is the astrocyte (rather than the neuron) which manifests characteristic neuropathologic changes in hyperammonemia. Recent studies demonstrate that exposure to ammonia either in vitro or in vivo results in alterations in astrocyte morphology (as described in Section 3.1) and in changes in expression of key astrocytic proteins.

6.3.1. Glial fibrillary acidic protein (GFAP)

GFAP is the major protein of intermediate filaments in differentiated astrocytes (Eng, 1985). Results of a recent study reveal that GFAP mRNA and protein were significantly reduced in frontal cortex of rats with acute hyperammonemia resulting from hepatic devascularisation (Bélanger et al., 2002). These findings were selective for GFAP; expression of a second glial filament protein S-100 β was unchanged in brain in this model of ALF. It was suggested that the loss of GFAP and the resulting impairment of visco-elastic properties of the astrocyte could favorize the cell swelling and subsequent brain edema, which is characteristic of acute hyperammonemic syndromes. Exposure of cultured astrocytes to millimolar concentrations of ammonia has consistently been shown to result in a loss of GFAP expression (Neary et al., 1994; Bélanger et al., 2002), and it was suggested that ammonia exposure under these conditions led to a destabilisation of GFAP mRNA (Neary et al., 1994).

GFAP expression in brain has been studied in moderate, chronic hyperammonemia resulting from either experimental or human liver failure and has been reported to be decreased or unchanged depending upon the brain region. GFAP-immunolabelling of cerebral cortical astrocytes was reportedly decreased following end-to-side portacaval anastomosis in the rat (Norenberg, 1977) and in cerebrum of patients with chronic liver failure (Sobel et al., 1981). However, a subsequent study revealed that GFAP-immunolabelling of cerebellar Bergmann glia in human chronic liver failure was unaltered (Krill et al., 1997).

6.3.2. Glutamate (and other astrocytic amino acid) transporters

The removal of neuronally-released glutamate from the synaptic cleft is achieved by high affinity, energy-dependent glutamate transporters. There is a convincing body of evidence both from in vitro and in vivo studies to suggest that ammonia exposure results in alterations in expression and activity of these transporters (Butterworth, 2002).

Rat hippocampal slices perfused with low millimolar concentrations of ammonia show decreased capacity for uptake of the non-metabolisable glutamate analogue D-aspartate (Schmidt et al., 1990). Furthermore, exposure of these preparations to serum extracts from patients with end-stage liver failure and encephalopathy led to similar reductions in high affinity D-aspartate uptake and a significant inverse correlation was observed between the uptake inhibition and ammonia content of the serum extracts from these patients. Previous studies had revealed a significant inhibition by ammonia of high affinity uptake of glutamate by synaptosomal preparations from rats (Mena and Cotman, 1985) and studies of glutamate uptake by synaptosomes from experimental animals with acute hyperammonemia associated with ALF also showed a significant decrease in uptake capacity (Oppong et al., 1995).

Astrocytes play a key role in the synaptic clearance of neuronally-released glutamate, and to this end, these cells express the high affinity glutamate transporters EAAT-1 and EAAT-2 in forebrain. A neuronally localised glutamate transporter EAAT-3 does not appear to be localised on nerve terminals and is therefore not considered to play a major role in the removal of synaptically-released glutamate. EAAT-4 is a neuronal transporter limited to cerebellar Purkinje cells, and expression of EAAT-5 is confined to the retina. Thus, most of brain, particularly cerebral cortex, hippocampus and midbrain structures, appears to rely primarily on astrocytic transporters for the effective removal of glutamate from the synapse (Fig. 4). Exposure of cultured rat cortical astrocytes to ammonia results in a significant loss in expression of EAAT-1 mRNA and protein (astrocytes cultured under the conditions used express only the EAAT-1 transporter). This ammonia-induced loss of EAAT-1 expression was accompanied by a parallel reduction in capacity of these cells to transport D-aspartate (Chan and Butterworth, 1999; Chan et al., 2000).

Furthermore, expression of the other astrocytic glutamate transporter EAAT-2 mRNA and protein is decreased in frontal cortical extracts from rats with acute hyperammonemia resulting from hepatic devascularisation (Knecht et al., 1997) and in the brains of mice with ALF due to thioacetamide hepatotoxicity (Norenberg et al., 1997). Again, loss of EAAT-2 expression in the brains of ALF rats was accompanied by a significant loss of capacity for high affinity uptake of D-aspartate by brain preparations from these animals (Butterworth, 2002). Moreover, the loss of EAAT-2 expression in frontal cortex of rats with ALF due to hepatic devascularisation is accompanied by increased extracellular

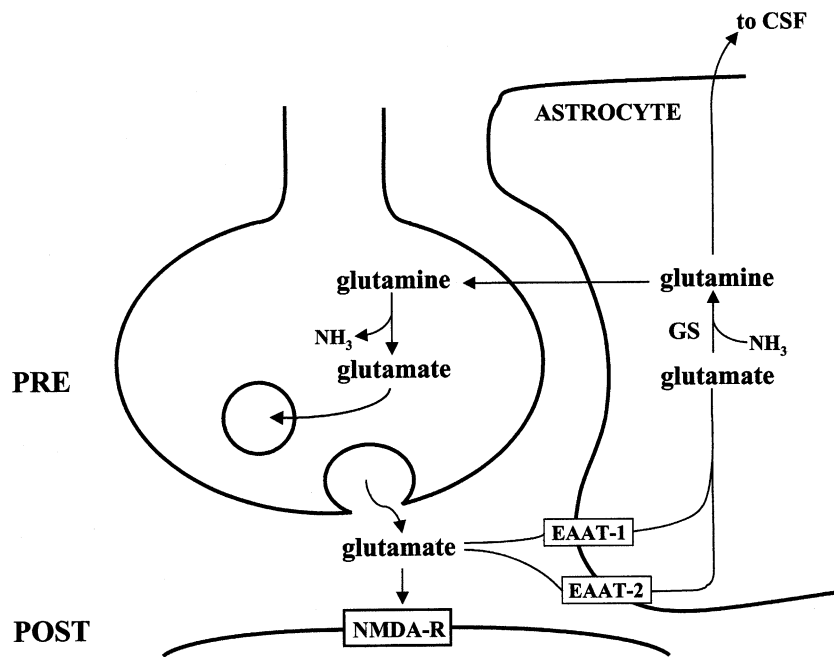


Fig. 4. The glutamate synapse and the so-called “glutamate–glutamine cycle” whereby glutamate is released into the synaptic cleft from the presynaptic neuron (PRE) where it acts on postsynaptic (POST) receptors (for simplification, only NMDA receptor (NMDA-R) is shown here). Excess glutamate is then uptaken into astrocytes via the glutamate transporters EAAT-1 and/or EAAT-2. Exposure of brain to increased ammonia results in reduced expression of EAAT-1 and EAAT-2, and increased extracellular glutamate concentrations.

brain concentrations of glutamate (Michalak et al., 1998), a phenomenon which has been confirmed in many models of acute hyperammonemia and which could be implicated in the pathogenesis of the HE and brain edema, which are characteristic of ALF in both humans and experimental animals (see Table 1).

In contrast to the situation in acute hyperammonemia, chronic exposure of brain to ammonia, resulting from, for example, portacaval anastomosis in the rat, does not appear to consistently result in a loss of glutamate transport capacity. Although some studies provide evidence demonstrating that extracellular concentrations of glutamate are increased

Table 1
Effects of hyperammonemia or liver failure on CSF or extracellular brain glutamate concentrations: a review of the literature

| Species | Experimental model | CSF/Brain region | Effect | Reference |
|-----------------------------------|---|---------------------------|----------------------------|------------------------------|
| (a) Acute hyperammonemia | | | | |
| Rat | Ammonium acetate administration | Cerebral cortex | Increased | Moroni et al. (1983) |
| Rabbit | Galactosamine-induced liver failure | Hippocampus | Increased | Hamberger and Nyström (1984) |
| Rat | Liver ischemia | Cerebral cortex | Increased | Bosman et al. (1992) |
| Rat | Liver ischemia | CSF | Increased | Swain et al. (1992a,b) |
| Rat | Thioacetamide-induced acute liver failure | CSF | Slight increase | Swain et al. (1992a,b) |
| Rabbit | Liver ischemia | Cerebral cortex | Increased | De Knecht et al. (1994) |
| Rabbit | Ammonia infusion | Cerebral cortex | Increased | De Knecht et al. (1994) |
| Rat | Liver ischemia | Frontal cortex | Increased | Michalak et al. (1996) |
| Rat | Thioacetamide-induced liver failure | Hippocampus | Increased | McArdle et al. (1996) |
| Rat | Thioacetamide-induced liver failure | Striatum | Increased | Hilgier et al. (1999) |
| Rat | Liver ischemia | Frontal cortex | Increased | Rose et al. (2000) |
| Rat | Acute ammonia administration | Cerebellum | Increased | Hermenegildo et al. (2000) |
| (b) Chronic hyperammonemia | | | | |
| Rat | Portacaval shunt | Striatum | No effect | Tossman et al. (1983) |
| Human | Liver cirrhosis with hepatic encephalopathy | Cerebrospinal fluid (CSF) | Increased | Watanabe et al. (1984) |
| Rat | Portacaval shunt | Cortex, striatum | Slight increase, no effect | Tossman et al. (1987) |
| Rat | Portacaval shunt | CSF | Increased | Therrien et al. (1991) |
| Rat | Continuous ammonia infusion | Striatum, cortex | Increased, no effect | Suzuki et al. (1992) |
| Rat | Portacaval shunt + ammonium acetate | Frontal cortex | No effect | Rao et al. (1995) |

Table 2
Effects of hyperammonemia or liver failure on binding of ligands to the NMDA receptor

| Species | Experimental model | Brain region | Effect | Reference |
|---------|---|---|-------------------------|--|
| Rat | Portacaval shunt | Cortex, hippocampus, striatum, thalamus | Decreased | Peterson et al. (1990) |
| Rat | Repeated i.p. injection of ammonia | Cerebellum | Decreased | Rao et al. (1991) |
| Mouse | OTC-deficient (spf) mouse | Most regions | Decreased | Ratnakumari et al. (1995), Rao and Qureshi (1999) |
| Mouse | OTC-deficient (spf) mouse | Frontoparietal cortex, striatum | Decreased, no change | Hopkins and Oster-Granite (1998) |
| Rat | Chronic moderate hyperammonemia | Hippocampus | Decreased | Marcaida et al. (1995) |
| Rat | Chronic moderate hyperammonemia | Cultured cerebellar neurons | Decreased | Marcaida et al. (1995) |
| Rat | Prenatal exposure | Cultured cerebellar neurons | Decreased | Miñana et al. (1997) |
| Rat | Thioacetamide-induced acute liver failure | Hippocampus striatum, cortex | Decreased, no effect | Saransaari et al. (1997) |
| Human | Cirrhotic patients | Cortex | No change | Dodd et al. (1992) |

in brain in chronic liver failure and/or chronic hyperammonemia (Moroni et al., 1983; Tossman et al., 1987), other studies could not confirm these findings (Tossman et al., 1983; Rao et al., 1995; Tables 1 and 2).

More recent studies show that other astrocytic amino acid transporters are also modified by exposure to ammonia. Acute hyperammonemia resulting from ALF due to liver ischemia leads to a significant loss of expression of the astrocytic glycine transporter GLYT-1 in cerebral cortex (Zwingmann et al., 2001) and a concomitant increase in concentration of glycine in brain extracellular fluid of comatose animals (Michalak et al., 1996). Exposure of cultured astrocytes likewise leads to a significant reduction in the cortical expression of GLYT-1. A major function of glycine in cerebral cortex is modulation of the glutamate (NMDA) receptor on which there is a specific glycine neuromodulatory site. Stimulation of this site by increased extracellular glycine could result in increased glutamatergic transmission.

6.3.3. “Peripheral-type” benzodiazepine (ω 3) receptors

The “peripheral-type” benzodiazepine receptor (PTBR) is a multimeric complex comprising three sub-units namely an 18 kDa isoquinoline carboxamide binding protein (IBP), a 34 kDa voltage-dependent anion channel (Porin) and a 30 kDa adenine nucleotide carrier (McEnery et al., 1992).

Treatment of rat cortical astrocytes with ammonia for up to 24 h results in increased binding of ^3H -PK11195, a highly selective antagonist ligand for the PTBR (Itzhak and Norenberg, 1994). Administration of ammonium salts to mice for 3 days leads to increases in density of ^3H -PK1195 binding sites (Itzhak et al., 1995), and increases in densities of these sites were also reported in the brains of mice with hyperammonemia due to deficits in the urea cycle enzyme ornithine transcarbamylase (Rao et al., 1993a). In this latter case, increased binding sites were apparent in both brain and peripheral tissue suggesting the presence of a circulating factor (probably ammonia).

Using RT-PCR, it was subsequently shown that end-to-side portacaval anastomosis in the rat, which results

in chronic moderate hyperammonemia, led to a significant increase in expression of PTBR isoquinoline binding protein mRNA in frontal cortical extracts (Desjardins et al., 1997, 2001). Concomitant with these changes was a significant increase in ^3H -PK11195 binding sites (Giguère et al., 1992; Desjardins et al., 2001; Desjardins and Butterworth, 2002). Increased densities of binding sites for ^3H -PK11195 have also been reported in autopsied brain tissue (frontal cortex, caudate nuclei) of cirrhotic patients who died in hepatic coma (Lavoie et al., 1990). Following this report, increased densities of binding of the PET ligand ^{11}C -PK11195 were described in basal ganglia and frontal cortical regions of the brains of cirrhotic patients (Cagnin et al., 2001), and these increased ^{11}C -PK11195 sites were found to be positively correlated with the severity of cognitive impairment in this patient population.

Since the PTBR is localised predominantly on astrocytic mitochondria in mammalian brain (Bender and Hertz, 1987), it is not surprising that alterations of PTBR expression are associated with altered mitochondrial function following ammonia exposure. For example, chronic hyperammonemia following portacaval anastomosis in the rat results in astrocytic proliferation (Zamora et al., 1973), and proliferation of mitochondria has been described in both cultured astrocytes (Norenberg and Lapham, 1974) and C6 glioma cells (Shiraishi et al., 1991) exposed to ammonia.

There is also evidence to suggest that changes in expression of the PTBR may be an integral part of the Alzheimer type II changes that are characteristic of astrocytes in chronic hyperammonemia (see Section 3.1). The Alzheimer type II change in the astrocyte in chronic hyperammonemia comprises both a swelling and proliferative component (Norenberg, 1987). It was recently reported that increased expression of the PTBR IBP was correlated with the presence of Alzheimer type II changes in autopsied brain tissue from cirrhotic patients who died in hepatic coma (Bélanger et al., 2001).

In addition to a likely role in astrocytic energy metabolism, increased expression of PTBRs may stimulate the synthesis of so-called “neurosteroids” in brain

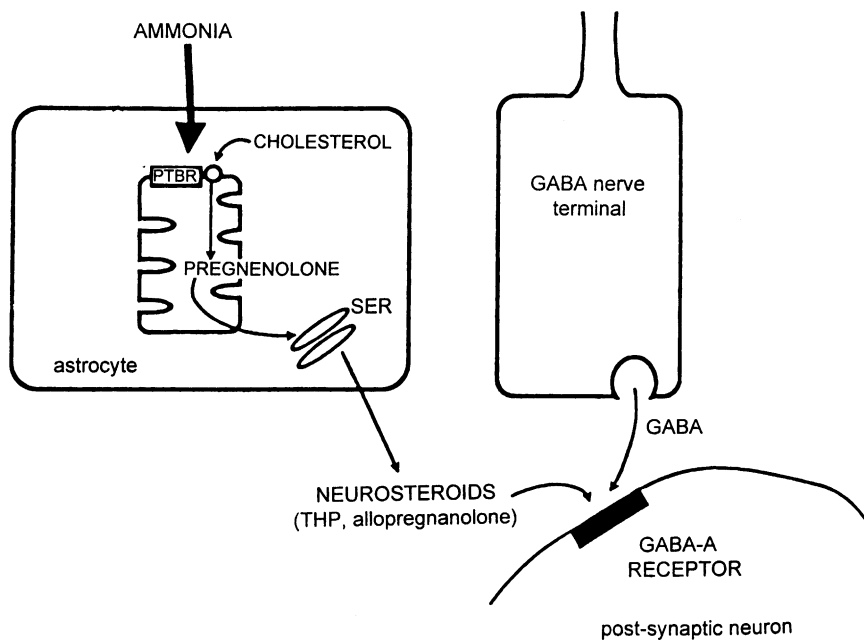


Fig. 5. Hypothesis whereby ammonia stimulates the “peripheral-type” benzodiazepine receptor (PTBR) localised on the astrocytic outer mitochondrial membrane resulting in increased transport of cholesterol and pregnenolone. Pregnenolone is the precursor of “neurosteroids” some of which have positive allosteric modulatory properties on the neuronal GABA-A receptor leading to increased inhibitory neurotransmission.

via the stimulation of cholesterol transport across the mitochondrial membrane. Studies have shown that, following uptake, action of the P450 enzyme system converts cholesterol to pregnenolone, the parent compound of the neurosteroids. In particular, two members of the neurosteroid family namely 3α -hydroxy- 5α -pregnan-20-one and $3,21$ -dihydroxy- α -20-one, are potent positive allosteric modulators of the GABA-A receptor in brain (Krueger and Papadopoulos, 1992). Increased synthesis of the precursor steroid pregnenolone has been reported in the brains of hyperammonemic mice (Itzhak et al., 1995), and it was proposed that increased synthesis of the neurosteroids with GABA-A receptor agonist properties could contribute to the neuroinhibition that is characteristic of hepatic encephalopathy (Hazell and Butterworth, 1999; Desjardins and Butterworth, 2002).

6.3.4. Other astrocytic proteins

GS is the enzyme primarily responsible for ammonia removal by brain and is almost exclusively localised in astrocytes (Figs. 4 and 5). Not surprisingly, in conditions of hyperammonemia resulting either from inherited urea cycle enzymopathies, the administration of ammonium salts or acute or chronic liver failure, brain glutamine concentrations have consistently been found to be increased (Ratnakumari et al., 1994; Lavoie et al., 1987b; Laubenberger et al., 1997). However, there is little convincing evidence to suggest that GS gene or protein expression or GS activities are significantly induced in brain in conditions of hyperammonemia. For example, chronic hyperammonemia resulting from portacaval

anastomosis in the rat results in unaltered or decreased activities of GS in brain (Cremer et al., 1975; Girard et al., 1989, 1993; Colombo et al., 1977; Cooper et al., 1985). The extent to which decreased glutamine degradation (in addition to increased glutamine synthesis) in brain contributes to the increase of brain glutamine in hyperammonemia has not been assessed.

Astrocyte swelling is characteristic of acute hyperammonemic syndromes. It is not surprising, therefore, that the recent literature contains preliminary reports of alterations in expression of proteins that are implicated in cell volume regulation. Such reports include a loss in expression of the astrocytic water channel protein aquaporin IV (Margulies et al., 1999) and the astrocytic glucose transporter GLUT-1 (Desjardins et al., 2001) in brain in experimental ALF.

6.4. Synaptic transmission (electrophysiology)

Ammonium ions affect both excitatory and inhibitory synaptic transmission in the mammalian brain by a variety of mechanisms (see Szerb and Butterworth, 1992 for review). Depression of excitatory synaptic transmission by NH_4^+ has been described both in vivo (Théoret and Bossu, 1985; Raabe, 1990) and in vitro using either extracellular (Théoret et al., 1985; Fan et al., 1990) or intracellular (Alger and Nicoll, 1983) recordings.

In vivo perfusion of the lateral ventricle with ammonium leads to inhibition of excitatory neurotransmission (Théoret and Bossu, 1985), and intravenous infusions of ammonium salts have been reported to suppress the monosynaptic excitatory reflex in spinal cord (Raabe, 1990). Three mechanisms

(two presynaptic, one postsynaptic) have been proposed to explain the depression of excitatory transmission by ammonium ions. Presynaptically, ammonium reduces the release of transmitter (glutamate) by inhibition of its synthesis from glutamine in the nerve terminal (Hamberger et al., 1982) or by prevention of the action potential from invading the presynaptic terminal (Raabe, 1990). Postsynaptically, ammonium ions may reduce the effectiveness of release glutamate by a direct effect on glutamate receptors (Fan et al., 1990). To these proposed effects must now be added the inhibitory effect of ammonia on glutamate uptake into the perineuronal astrocyte (an effect which should potentially enhance rather than inhibit excitatory transmission, see Section 6.1). A more comprehensive coverage of the effects of ammonia on glutamate receptors and their signal transduction pathways is provided in Section 6.4.

A depression of postsynaptic hyperpolarising IPSP's by ammonia is well established (Lux, 1971; Raabe and Lin, 1983) in several neural preparations including spinal motor neurons, trochlear motor neurons and neocortical pyramidal tract neurons. In these *in vivo* experiments, ammonium ions were found to reduce or abolish the membrane hyperpolarisation via an effect on chloride ion extrusion (Raabe, 1987).

6.5. The glutamate system

Acute hyperammonemia associated with experimental ALF leads to significant reductions of brain glutamate (Tyce et al., 1981; Mans et al., 1994). Reductions in brain glutamate have also been reported in chronic hyperammonemia resulting from portacaval shunting in the rat (Hindfelt et al., 1977; Giguère and Butterworth, 1984) as well as in autopsied brain tissue from cirrhotic patients who died in hepatic coma (Lavoie et al., 1987b).

Brain glutamate is contained in at least four distinct compartments namely glutamatergic nerve terminals, GABA terminals (where glutamate serves as precursor), an astrocytic pool associated with ammonia metabolism (glutamine synthesis) and a multi-cellular metabolic pool associated with energy metabolism. A reduction of glutamate from the nerve terminal pools could result in impaired excitation/inhibition whereas a loss from the astrocytic pool could lead to impaired capacity for ammonia detoxification (lack of substrate for GS). There is evidence in support of both of these possibilities. For example, evidence from studies with radiolabelled substrates suggests that exposure to ammonia leads to a limitation in the capacity of brain to remove additional blood-borne ammonia (Cremer et al., 1975; Ukida et al., 1988) and to decreased incorporation of glucose into glutamate and GABA (Prior and Visek, 1972).

There is now an overwhelming body of evidence to suggest that exposure of brain to increased ammonia concentrations has serious adverse effects on the glutamate neurotransmitter system. As already mentioned, ammonia causes alterations in expression and activities of astrocytic glutamate transporters (Section 6.1). In addition, ammonia

has potent effects on glutamate receptors and on glutamate receptor-mediated signal transduction pathways.

There are two major classes of glutamate receptors in the mammalian CNS. Ionotropic glutamate receptors gate ion channels and their activation results in entry of Na^+ , K^+ and Ca^{2+} ions into the cell. There are two subtypes of ionotropic glutamate receptors classified according to their neuropharmacologic characteristics, namely the NMDA subtype and the AMPA-Kainate (or non-NMDA) subtype. These receptor subtypes have distinct molecular (sub-unit) compositions as well as cellular and regional distributions in the CNS. A second major class of glutamate receptors, the metabotropic receptors, do not directly gate ion channels; rather they are coupled to G proteins leading to the modulation of specific enzymes and ion channels such as phospholipase C and D and adenylate cyclase. Both acute and chronic hyperammonemia have deleterious effects on glutamate receptor expression and function. However, the nature of these effects is quite distinct depending upon the magnitude and duration of ammonia exposure.

6.5.1. Acute ammonia exposure

The mortality due to acute ammonia toxicity *in vivo* is prevented by the administration of a wide range of NMDA receptor antagonists (Marcaida et al., 1992; Hermenegildo et al., 1996), and studies using *in vivo* cerebral microdialysis show that acute ammonia exposure results in activation of the NMDA receptor-coupled nitric oxide–cyclic GMP signal transduction pathway in brain (Fig. 6, Hermenegildo et al., 2000). Moreover, a significant positive correlation has been reported between the increase in cGMP produced as a result of acute ammonia exposure and the severity of neurological symptoms. Importantly, studies of the time-course of these changes reveal that NMDA receptor activation and cGMP increases due to ammonia exposure precede any increases in extracellular brain glutamate in these animals. Furthermore, both the increased brain cGMP concentrations and the subsequent increase in extracellular glutamate were prevented by administration of the NMDA receptor antagonist MK801 (Hermenegildo et al., 2000).

A role for activation of NMDA receptors in the neurological symptoms associated with an experimental animal model of ALF was also reported by Vogels et al. (1997) who showed that administration of the non-competitive NMDA receptor antagonist memantine led to a significant improvement in neurological status in rats with acute hyperammonemia resulting from hepatic devascularisation.

The most likely explanation for activation of NMDA receptors by ammonia involves depolarisation leading to removal of the Mg^{2+} block on the NMDA receptor and consequently increased NMDA-induced currents as had previously been shown to occur in electrophysiological studies (Fan and Szerb, 1993, discussed in Section 6.2).

Activation of NMDA receptors leads to induction of nitric oxide synthase and increased production of nitric oxide (Fig. 6). This nitric oxide-mediated pathway may also be

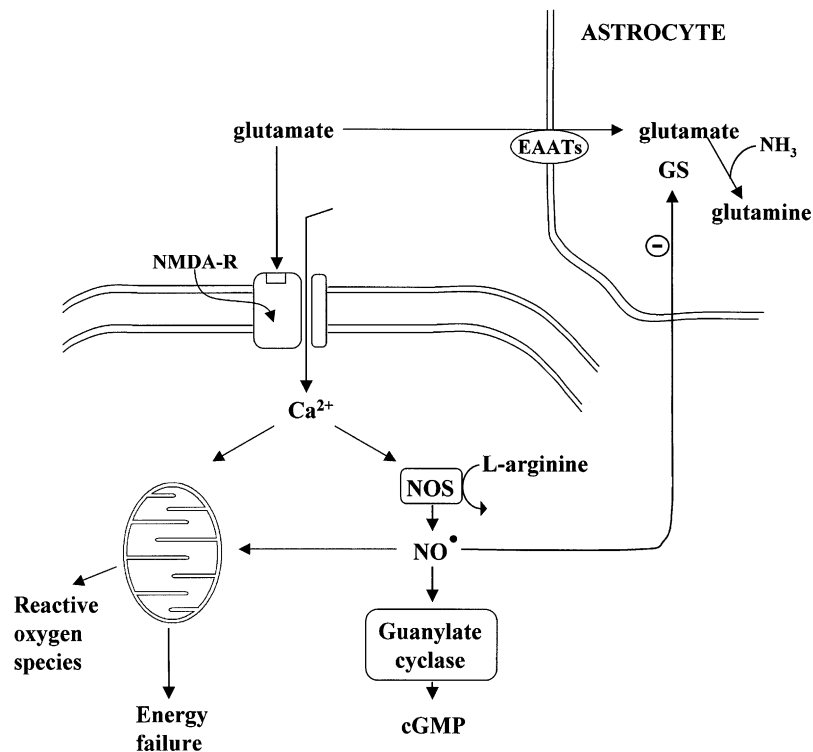


Fig. 6. The NMDA receptor (NMDA-R)-mediated nitric oxide (NO)-cyclic GMP signal transduction pathway. Acute exposure to ammonia results in stimulation of this pathway leading to increased entry of Ca^{2+} , stimulation of nitric oxide synthase (NOS), and production of NO, which stimulates guanylate cyclase and cGMP synthesis. Increased NO production may cause damage to mitochondria resulting in production of reactive oxygen species and energy failure. NO may also diffuse out to astrocytes where it inhibits glutamine synthetase (GS) the predominant ammonia-removing pathway in the brain.

stimulated by ammonia's activation of uptake of L-arginine, the obligate precursor for nitric oxide production (Rao et al., 1997). Evidence in support of this activation of the nitric oxide signal transduction pathway by ammonia has also been provided by studies demonstrating that the selective nNOS inhibitor, nitroarginine, inhibits the toxic and metabolic effects of acute hyperammonemia (Kosenko et al., 1995).

It has been shown that blocking of either NMDA receptors or NOS leads to a significant increase of both brain glutamine concentration and of GS activity in rats injected with large doses of ammonia (Kosenko et al., 1994, 1995). These results support the notion that NO production inhibits GS in brain. In order to directly assess this possibility, primary astrocyte cultures were treated with the NO-generating agent SNAP, which resulted in a 50% inhibition of GS activity (Miñana et al., 1997). These findings directly demonstrate that NO production maintains a tonic control over GS activity in brain (Fig. 6), a conclusion that is strengthened by the observation that blocking NMDA receptors or NOS reduces the tonic inhibition of GS by NO and increases GS activity. This clearly implies that GS is modulated by glutamatergic activity and also that acute hyperammonemia, by activation of NMDA receptors and NO production, may result in a deficit in GS and a further compromise in the brain's ability to remove additional blood-borne ammonia creating a

vicious cycle whereby ammonia concentrations in brain are further increased.

As discussed in Section 6.3, acute ammonia toxicity leads to increased brain lactate, altered NAD⁺/NADH ratios and, if sufficiently severe, a loss of ATP content in brain (Schenker et al., 1967; Hindfelt and Siesjo, 1971; Hawkins et al., 1973; Hindfelt et al., 1977; Kosenko et al., 1993, 1994). Two possible mechanisms have been proposed to explain this effect:

- (1) Activation of NMDA receptors by ammonia leads to increased entry of Ca^{2+} and Na^{+} . In order to maintain Na^{+} homeostasis, Na^{+} is extruded from the neuron by $\text{Na}^{+}/\text{K}^{+}$ ATPases causing a net consumption of ATP. This ammonia-induced increase of $\text{Na}^{+}/\text{K}^{+}$ ATPases is completely prevented by prior administration of the NMDA antagonist MK801 (Kosenko et al., 1994). $\text{Na}^{+}/\text{K}^{+}$ ATPase is modulated by phosphorylation by protein kinase C, and when PMA, an activator of protein kinase C, is added to brain extracts from rats with acute hyperammonemia, $\text{Na}^{+}/\text{K}^{+}$ ATPase activities are normalised suggesting that ammonia acts by decreasing PKC-dependent phosphorylation (and consequent activation) of the enzyme (Kosenko et al., 1994). This activation of $\text{Na}^{+}/\text{K}^{+}$ ATPase following activation

of NMDA receptors has been reproduced in cultured neurons (Marcaida et al., 1995). In this latter system, inhibitors of the Ca^{2+} -dependent protein phosphatase calcineurin prevent activation of Na^+/K^+ ATPase by glutamate (Marcaida et al., 1995) indicating that activation of calcineurin is implicated in the effects of acute ammonia toxicity on the phosphorylation state of Na^+/K^+ ATPase.

- (2) Acute ammonia toxicity also results in increased mitochondrial Ca^{2+} content, an effect which may result in dysfunction of key mitochondrial (energy-related) enzymes and the generation of superoxide radicals (Kosenko et al., 1997, 2000). Consistent with this mechanism, studies have consistently shown an inhibitory effect on the tricarboxylic acid cycle enzyme α -ketoglutarate dehydrogenase following exposure to ammonia (Lai and Cooper, 1986). Loss of activities of succinate dehydrogenase (Kosenko et al., 1997) and of state 3 respiration have also been reported in brain extracts from acute hyperammonemic animals. Acute ammonia toxicity leads to increased production of superoxide radical by brain mitochondria and to decreased activities of the free radical scavenging enzymes glutathione peroxidase, superoxide dismutase and catalase (Kosenko et al., 1999). Increased superoxide radical production and accumulation of intramitochondrial Ca^{2+} induced by acute ammonia toxicity are inhibited by treatment with either MK801 (Kosenko et al., 1999) or nitroarginine (Kosenko et al., 1998) again suggesting a role of the NMDA receptor–nitric oxide pathway in the pathogenesis of these changes.

Acute exposure to ammonia also leads to degradation of MAP-2 in brain, an effect which is prevented by MK801 pretreatment implicating an involvement of NMDA receptor activation (Saez et al., 1999). Evidence suggests that this results from increased entry of Ca^{2+} leading to activation of the Ca^{2+} -dependent protease calpain, which then degrades MAP-2 with potential degradation of the neuronal microtubular network and ultimately cell dysfunction and death.

In contrast to the NMDA receptor system which is clearly activated in conditions of acute hyperammonemia, non-NMDA (AMPA-Kainate) sites have been less thoroughly investigated. Acute exposure of rat cortical wedges to millimolar concentrations of ammonia was found to result in a reduction in the degree of depolarisation caused by AMPA (Lombardi et al., 1994). A subsequent study revealed that densities of AMPA-Kainate binding sites were significantly reduced in the brains of rats with acute hyperammonemia resulting from hepatic devascularisation (Michalak and Butterworth, 1997a,b).

Two reports suggest that metabotropic glutamate receptors and their signal transduction pathways may also be susceptible to acute ammonia exposure (Lombardi et al., 1994; Saez et al., 1999).

6.5.2. Chronic ammonia exposure

In contrast to acute hyperammonemia, chronic hyperammonemia leads to a *depression* of excitatory neurotransmission by impairment of NMDA receptor function and by inhibition of NMDA receptor-mediated signal transduction pathways.

Chronic hyperammonemia results in decreased binding of ^3H -MK801 to membranes from rat hippocampus (Marcaida et al., 1995). These findings are consistent with earlier reports of decreased binding of ^3H -glutamate to NMDA-sensitive sites in synaptic membrane preparations from rats made chronically hyperammonemic by administration of ammonium salts (Rao et al., 1991) as well as in the brains of rats following end-to-side portacaval anastomosis (Peterson et al., 1990), a procedure which results in chronic moderate hyperammonemia. ^3H -MK801 binding was also found to be reduced in the brains of mice with chronic hyperammonemia due to a congenital defect of ornithine transcarbamylase (Ratnakumari et al., 1995). Immunoblotting studies failed to reveal any significant loss of expression of the NMDA R1 sub-unit of the receptor due to chronic exposure to ammonia suggesting a post-translational modification of receptor function. The decrease in binding of ligands to NMDA receptors in animal models of chronic hyperammonemia has been reproduced in cultured cerebellar neurons exposed chronically to 1 mM ammonia (Marcaida et al., 1995). This treatment also led to impaired activation of NMDA receptors as assessed by measuring the increase in intracellular Ca^{2+} in these cells. Subsequent addition of the phorbol ester PMA (a potent activator of protein kinase C) to the neurons in culture led to restoration of the NMDA receptor deficit caused by chronic ammonia exposure suggesting that the functional deficit was primarily due to decreased protein kinase C-mediated phosphorylation (Marcaida et al., 1995; Grau et al., 1996).

Chronic exposure of cultured rat cerebellar granule cells to submillimolar concentrations of ammonia did not impair the activation of NMDA receptors by glutamate. However, this treatment led to a significant impairment in NMDA receptor-mediated signal transduction at the level of the glutamate–nitric oxide–cyclic GMP (cGMP) pathway (Hermenegildo et al., 1998). Chronic exposure to 0.1 mM ammonia did not impair glutamate (or NMDA)-mediated increases in intracellular Ca^{2+} but did result in significant inhibition of the glutamate (NMDA) receptor-mediated formation of cGMP in a dose and time-dependent manner. Exposure of these cells to ammonia did not affect activation of nitric oxide synthase but did cause a reduction in the synthesis of cGMP from the nitric oxide-generated agent SNAP. Taken together, these findings suggest that the site of inhibitory action of chronic ammonia treatment in the NMDA receptor signal transduction pathway is at the level of guanylate cyclase activation by nitric oxide. Similar findings of an inhibition of this pathway by chronic hyperammonemia were demonstrated in vivo in chronically hyperammonemic rats in the absence (Montfort et al.,

in press) or presence (Hermenegildo et al., 1998) of liver failure. On the other hand, chronic hyperammonemia associated with portacaval anastomosis was shown to result in a significant increase of cerebellar nNOS gene and protein expression (Rao et al., 1995), a finding that would be expected to result in induction of the NMDA receptor signal transduction pathway in the brains of these animals.

6.6. The GABA system

The GABA system has been extensively studied in hyperammonemic syndromes and results of these studies are conflicting. It has been demonstrated in *in vitro* experiments that concentrations of ammonia in the 0.5–2.5 mM range may directly result in alterations of the binding of agonist ligands such as ³H-muscimol to the GABA-A receptor (Ha and Basile, 1996). Ammonia exposure of rat brain preparations also leads to modification of the capacity of benzodiazepines to enhance binding of ³H-muscimol to its receptor suggesting an effect of ammonia on the coupling between these sites on the GABA-A receptor complex (Basile, 2002). It was suggested that these effects of ammonia resemble those produced by barbiturates. It remains to be established whether such mechanisms occur *in vivo* in hyperammonemic syndromes.

There is currently little convincing evidence to support the “GABA theory of HE” as originally formulated, namely that gut-derived GABA gains access to brain resulting in increased neuroinhibition (Schafer and Jones, 1982). This theory was based upon findings from studies in experimental animals with galactosamine-induced ALF where increases of brain GABA and alterations of GABA-related enzymes and receptor sites were reported (Schafer et al., 1983; Baraldi et al., 1984). However, subsequent studies failed to confirm these changes in other experimental animal models of liver failure (Butterworth and Giguère, 1986; Roy et al., 1988; Maddison et al., 1987a,b) or in autopsied brain tissue from cirrhotic patients who died in hepatic coma (Lavoie et al., 1987a,b; Butterworth et al., 1988).

On the other hand, there is evidence to support the notion that substances such as benzodiazepines and neurosteroids with positive allosteric modulatory properties at the GABA-A receptor may be increased in brain and thus contribute to the inhibition characteristic of HE in liver failure (Basile, 2002). As discussed in Section 6.1 of this review, both acute and chronic liver failure result in increased expression and densities of PTBR sites with the consequent potential to stimulate the synthesis of neurosteroids such as allopregnanolone with potent GABA-A receptor stimulatory properties.

Previous studies had revealed evidence of increased brain concentrations of “benzodiazepine-like” substances in brain liver failure in both experimental animals and humans (Olasmaa et al., 1990; Mullen and Jones, 1996). However, some of these compounds in human brain could have been attributable to previously prescribed benzodiazepine medi-

cation in these patients (Perney et al., 1998). Administration of flumazenil, a highly selective antagonist of the benzodiazepine modulatory site on the GABA-A receptor may be effective in some cases of HE (Pomier Layrargues et al., 1994).

6.7. The serotonin system

Administration of ammonium salts to rats results in increased transport of L-tryptophan across the blood–brain barrier of these animals (Grippon et al., 1986). Similarly, rats made chronically hyperammonemic by injection of urease manifest increased L-tryptophan uptake into brain and increased production of the 5HT metabolite 5-hydroxyindoleacetic acid (5HIAA, Batshaw et al., 1986), a finding which is indicative of increased 5HT turnover or metabolism in the brain in hyperammonemia. Increased 5HIAA concentrations have also been reported in CSF of children with hyperammonemia caused by congenital urea cycle disorders, and plasma ammonia levels are found to correlate with CSF 5HIAA in these patients (Batshaw et al., 1990).

There is abundant evidence of increased 5HT metabolism/turnover and increased 5HIAA production in brain in liver failure. Patients with ALF manifest increased CSF concentrations of 5HIAA (Knell et al., 1974), and CSF 5HIAA levels are increased in cirrhotic patients in hepatic coma (Young et al., 1975). Studies in autopsied brain tissue from cirrhotic patients who died in hepatic coma reveal increased 5HIAA concentrations (Jellinger et al., 1978; Bergeron et al., 1989) as well as increased mRNA and activity of the 5HT-degrading enzyme monoamine oxidase (A isoform, Mousseau et al., 1997). Results of a recent study in rats with graded portacaval shunts suggest that the degree of portal-systemic shunting is directly correlated with brain concentrations of 5HIAA (Lozeva et al., 2001).

Several mechanisms have been proposed to explain the increase in brain 5HT turnover in conditions of hyperammonemia. These mechanisms include: (a) stimulation of the uptake of the precursor amino acid L-tryptophan, (b) increased MAO-A expression in brain and (c) a reserpine-like effect of ammonium on vesicular monoamine stores resulting in decreased storage and increased intracellular oxidation of 5HT leading to decreased availability for release from the presynaptic nerve terminals (Erecinska et al., 1987). Mechanisms (b) and (c) would be expected to result in a central 5HT deficit in hyperammonemia. In favour of a 5HT synaptic deficit in hyperammonemic syndromes are the findings of an upregulation of postsynaptic 5HT₂ binding sites in autopsied brain tissue from patients with chronic liver disease who died in hepatic coma (Rao et al., 1993b). However, studies in congenital hyperammonemic animals reveal a loss of 5HT₂ sites (Robinson et al., 1992) suggesting that ammonia toxicity in developing brain may involve distinct mechanisms involving the serotonergic system compared to the brain of the adult.

Decreased brain uptake of L-tryptophan in hyperammonemia could also result in increased synthesis of other metabolites some of which are neuroactive or neurotoxic. Brain concentrations of quinolinic acid, an excitotoxic metabolite of L-tryptophan, are increased in both inherited and acquired hyperammonemias (Robinson et al., 1995; Moroni et al., 1986), and the L-tryptophan metabolite tryptamine is increased in brain in chronic liver failure (Young and Lal, 1980). However, the precise roles of the changes in these metabolites and their relationship to ammonia neurotoxicity or HE in liver failure have yet to be established.

7. Therapy in hyperammonemic syndromes

7.1. Ammonia-lowering strategies

Dietary protein reduction remains a corner-stone in the treatment of hyperammonemic syndromes. However, since skeletal muscle is an important organ involved in ammonia removal, in order to minimise the adverse effects on muscle function, protein reduction is carefully controlled and is generally maintained at around 40–50 g per day. Reduction of intestinal ammonia production also forms an important part of the treatment regimen, which may include non-absorbable disaccharides, such as lactulose or antibiotics to reduce gut ammonia production (Cordoba and Blei, 1997).

Sodium benzoate has been found to be effective in the treatment of hyperammonemic episodes in children (Batshaw and Brusilow, 1980) with congenital urea cycle enzymopathies and may also be effective in cirrhotic patients (Sushma et al., 1992). L-Ornithine-L-aspartate is effective as an ammonia-lowering agent in patients with chronic liver disease (Kircheis et al., 1997) by a mechanism that appears to involve both stimulation of residual hepatic urea synthesis and by increased glutamine synthesis by the muscle (Rose et al., 1998). Studies in experimental animals suggest that this drug may also be useful in the lowering of blood ammonia in ALF (Rose et al., 1999).

7.2. Neuropharmacology

As the central nervous system effects of hyperammonemia become better characterised, future treatments will target cerebral mechanisms, many of which have been identified in experimental animal models of acute and chronic hyperammonemias. For example, it has been suggested that NMDA receptor antagonists may be beneficial in the prevention of ammonia's toxic effects on the brain both in the absence (Marcaida et al., 1992) and presence (Vogels et al., 1997) of liver failure. Drugs to enhance monoaminergic and cholinergic function have also been proposed as alternative approaches to therapy in a variety of hyperammonemic syndromes (Morgan et al., 1980; Michalak and Butterworth, 1997a,b).

8. Summary

Ammonia has potentially deleterious effects on the central nervous system. Depending upon the severity and duration of exposure, these effects may include seizures, cognitive deficits leading to coma and, in development, mental retardation and cerebral palsy. Neuropathological studies in hyperammonemic syndromes reveal predominantly astrocytic changes consisting of cell swelling (acute hyperammonemia) and Alzheimer type II astrocytosis (chronic hyperammonemia). Exposure of brain to increased ammonia concentrations results in altered expression of key astrocytic proteins including glial fibrillary acidic protein, the glutamate transporter EAAT-2, mitochondrial "peripheral-type" benzodiazepine receptors as well as glutamine synthetase and the water channel protein aquaporin IV.

Ammonia has profound effects on brain metabolism and brain perfusion. Chronic mild hyperammonemia resulting from chronic liver failure leads to region-selective alterations in the cerebral metabolic rate for glucose. On the other hand, acute hyperammonemia leads to increased cerebral blood flow and accumulation of lactate in brain. In acute liver failure, cerebral blood flow may become uncoupled from cerebral metabolism leading to a loss of cerebrovascular autoregulation. Chronic liver failure leads to brain ammonia concentrations in excess of 0.5 mM, which inhibit α -ketoglutarate dehydrogenase in brain. This leads to accumulation of lactate but no cerebral energy deficit until deep coma (isoelectric) stages of encephalopathy. On the other hand, lethal doses of ammonium salts in the absence of liver failure result in loss of ATP, a finding which has been attributed to inhibition of the tricarboxylic acid cycle or to stimulation of NMDA receptors and activation of the nitric oxide-cGMP signal transduction pathway.

Ammonia also has electrophysiological actions and NH_4^+ may prevent action potentials from invading presynaptic nerve terminals or may directly stimulate some glutamate receptors. Hyperammonemic syndromes are also associated with increased turnover of the monoamines serotonin and dopamine in brain, a finding which may result from increased availability of amino acid precursors and/or increased expression of the catabolic enzyme monoamine oxidase.

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