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Role of Ammonia Species in Mammalian Nervous Tissue

Bachelor Thesis

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Content

Abbreviations: BBB – blood-brain barrier; CBF – cerebral blood flow; CNS – central nervous system; Gln – glutamine; GS – gutamine synthetase; Glu – glutamate; HE – hepatic encephalopathy; MAS – malate-aspartate shuttle; MK801 - (5R,10S)-5-methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine hydrogen maleate; MSO – methionine sulfoximine; NHE – Na^+/H^+-exchanger; NMDAr – *N*-methyl-D-aspartate receptor; NMR – nuclear magnetic resonance; NOS – nitric oxide synthase; PET – positron emission tomography; PS – permeability-surface area product; TCA cycle – tricarboxylic acid cycle

Keywords: ammonia, ammonium, nervous tissue, brain, mammals, metabolism

Klíčová slova: amoniak, nervová tkáň, mozek, savci, metabolismus

Abstract

In this thesis, role of ammonia/um in mammalian nervous tissue is discussed. Several points of view are taken into account and some perspectives are offered. Ammonia species is a vital substance in healthy human brain with both regulatory and metabolic functions. However, there are a few research areas being paid surprisingly little attention, *e.g.* molecular mechanisms of ammonium transport across the brain cell membrane (hitherto knowledge is summarized in section *2*). Another field of interest still awaiting solution is the transport of ammonia/um from blood to brain (section *4*). There has been a dispute whether the permeability of the blood-brain barrier correlates with blood ammonia/um concentration or not.

In the brain itself, there is an orderly exchange of ammonia/um between astrocytes and neurons. Astrocytes provide neurons with substantial biochemical help and protection. Ammonia/um participates in some of the key reactions of such assistance (section *5*). However, in pathological levels, ammonia/um causes severe neuropsychiatric diseases and can be deadly. Therefore, a vast portion of research has focused on hyperammonemic conditions, their causes and consequences. This issue far exceeds the extent of this thesis. Nevertheless, the most important facts and findings are summarized in section *6*.

(Tato bakalářská práce se zabývá rolí amoniaku v savčí nervové tkáni, a to hned z několika hledisek. Amoniak má totiž zásadní význam pro normální fungování lidského mozku, kde je jedním z klíčových metabolitů a kde plní i některé regulační funkce. Přesto v této souvislosti existují oblasti výzkumu, které zůstávají stranou vědeckého zájmu, například molekulární mechanismy transportu amoniaku přes membránu mozkové buňky (dosavadní poznatky jsou shrnuty v sekci 2). Problémem, který zůstává nevyřešen, je také transport amoniaku z krve do mozku (sekce 4). Diskutuje se především o tom, jestli propustnost hematoencefalické bariéry koreluje s hladinou amoniaku v krvi.

V samotném mozku amoniak podléhá systematické výměně mezi astrocyty a neurony. Astrocyty zajišťují neuronům důležitou biochemickou pomoc a ochranu, jejíž součástí jsou i některé klíčové reakce, kterých se amoniak účastní (sekce 5). Při zvýšených koncentracích ale amoniak vyvolává závažná neuropsychiatrická onemocnění a může způsobit i smrt. Proto se na takzvanou hyperammonemii a na její příčiny a následky soustřeďuje značná pozornost. Tato problematika dalece přesahuje rozsah této práce. Přesto jsou nejdůležitější fakta shrnuta v sekci 6.)

1. Introduction

Ammonia/um* plays a vital role in cell metabolism. It was very likely to be present in early forms of life and it has participated in essential biochemical reactions ever since. It is a characteristic part of amino acids and, therefore, is present in all proteins, of both structural and enzymatic function.

In nervous tissue, ammonia/um is of great regulatory and signal importance. There is a significant flux from neurons to supportive glial cells (astrocytes) and *vice versa.* Ammonia/um undergoes several key reactions including formation of alanine from pyruvate, glutamine from glutamate and glutamate from α-ketoglutarate. However, in pathological concentrations ammonia/um causes severe psychiatric disturbances, brain edema and death. In chronic hyperammonemia, hepatic encephalopathy unfolds and many mental functions are impaired.

2. Ammonium transport across cell membranes

Unlike other small gases (*e.g.* CO_2, O_2, NO), NH_3 is only moderately soluble in lipids [25]. Therefore, its motility across most of the cell membranes is much lower. Moreover, there are several membrane types further reducing their permeability to ammonia by replacing lipids with proteins (*e.g.* internal membrane of rat mitochondria, apical membranes of bladder cells) [9 *in* 25].

Since the excretion of NH_4^+ is crucial for mammalian acid-base homeostasis accounting for as much as two thirds of daily net acid excretion [24 *in* 7] and this process takes place in the kidney, most of the research of the ammonium transport so far have been focused on renal cells. However, the molecular mechanisms of the transport in brain may be similar and in some cases the similarity has already been shown.

2.1. Methods of measuring NH_4^+ fluxes

The flux of ammonia/um into the cells is most often infered from changing in intracellular pH (pH_i). The supply of extracellular space with ammonia/um usually leads to increase in pH_i, since NH_3 diffuses across the cell membrane and combines with H^+ [1]. However, when

* There is a slight confusion over the terminology. In this thesis, "ammonia" refers to the neutral NH_3 form and "ammonium" to the protonated NH_4^+ form. When both forms are considered, "ammonia/um" is given.

a system transporting NH_4^+ is present on the membrane, the pH_i will decrease due to an influx of protonated ammonium ions and subsequent partial dissociation [13].

Another method applied to investigation of ammonia/um influx is nuclear magnetic resonance (NMR). Both ^{14}N and ^{15}N isotopes can be used in this case. Intracellular and extracellular ammonia/um are distinguished by addition of shift reagent, which changes the resonance frequency of available (extracellular) ammonia/um [6].

2.2. Ways of NH_4^+ influx

Since NH_4^+ is a fair substitution for the K^+ ion (minimum pore diameter 3.00 Å and 2.66 Å, respectively [33]), many types of transporters have affinity to both.

2.2.1. K^+ channels

Potassium channels are to a certain extent permeable to NH_4^+. Unfortunately little has been done to clarify mechanisms of their selectivity and conductance properties over the recent history. Choe *et al.* [17] studied two inwardly rectifying potassium channels (ROMK2, IRK1) using recombinant oocytes of *X. laevis* and determined the permeability ratios (P_{NH4+}/P_{K+}) to be 0.09 and 0.10, respectively (tab. 1).

	NH_4^+	Rb^+	Tl^+
ROMK2	0.09	0.42	2.58
IRK1	0.10	0.49	1.49

Tab. 1. Relative permeability of K^+ channels to monovalent cations (P_{X+}/P_{K+}).

Compared to other monovalent cations included in this study (Rb^+, Tl^+), the permeability to ammonium was minute (almost 30 times smaller in case of ROMK2 and Tl^+).* Nevertheless, K^+ channels (especially subtypes blocked by Ba_2^+) remain to be considered essential for NH_4^+ influx into mammalian astrocytes, since brain areas with intensive ammonia/um metabolism reduce concentration of extracellular potassium allowing NH_4^+ to enter the cells [34]. Nagaraja and Brookes [13] studied acidification of mouse cerebral astrocytes after treatment with NH_4Cl and they identified flux through Ba_2^+-sensitive K^+ channels as the main way of ammonium entry, since Ba_2^+ reduced the acidification by up to 80%. Permeability of these channels to NH_4^+ has recently been affirmed on cultured rabbit medullary thick ascending limb cells [15].

2.2.2. Na^+/K^+-ATPase

Na^+/K^+-ATPase is able to transport ammonium instead of potassium, as well. It has been known for over fifty years [30 *in* 25], although not all members of this transporter family are

* It has been proposed that in evolution, K^+ channels became less permeable to ammonium as animals left water environment and turned to the terrestrial way of life. It is supposed to have occured due to the change in major substance of excretion from urea to uric acid [25, 31].

NH_4^+-sensitive. Crayfish neurons, for example, have been described to possess ammonium-activated Na^+/K^+-ATPase [21]. It has been proposed that this mechanism contributes to the toxic effect of ammonia species, since increased NH_4^+ in the extracellular space stimulates export of Na^+ decreasing its intracellular concentration to fatal levels [36].

2.2.3. Cotransport

Bergeron *et al.* [4] examined the capability of the cation-chloride cotransporter (CCC) family to translocate ammonium at the K^+ site. They took into account both potassium-chloride and sodium-potassium-chloride cotransporters either present in wide range of tissues (NKCC1, KCC1, KCC3 and KCC4) or tissue-specific (NKCC2 in kidney, KCC2 in brain). All these transporters were revealed to be able to translocate NH_4^+ and it is presumed that chloride cotransport is an important way of NH_4^+ entry into glial cells [32]. Moreover, cation-chloride cotransporter of bee retinal glial cells seems to be highly selective for NH_4^+ over K^+ [1]. As for the Na^+-K^+-Cl^- cotransport the stoichiometry is believed to be 1:1:2. However, some recent studies proposed other rates: 2:1:3 as well as 1:1:1 [11].

Beside K^+, NH_4^+ can replace Na^+ at some exchangers' cation sites. It has been shown on the Na^+-H^+ exchanger (NHE) of thick ascending limb of rat kidney [26]. However, only some NHE isoforms can transport NH_4^+, while others can not [19]. Investigation of NHE in nervous tissue remains to be done, although this transporter plays an important role in pH regulation in brain cells [8].

2.3. Ammonium-specific transport

Until recently, no mammalian ammonium-specific transporter had been known. All transporting systems mentioned above are able to translocate ammonium but, presumably, it is not their primary function. There were two mammalian transmembrane proteins, however, described as ammonium-specific transporters in recent works by Jahn *et al.* [2] and Nakhoul *et al.* [7].

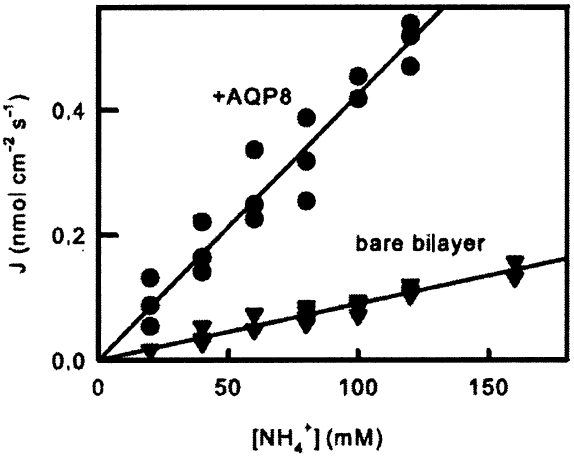

Fig. 1. Transmembrane flux *J* of ammonium across plain lipid bilayer and with incorporated aquaaminoporin after application of NH_4Cl. The "inner" concentration of NH_4^+ was 1 mM [3].

Aquaporins are often involved in conducting not only water, but also other compounds. In 2004, an aquaporin with ammonia/um specificity was discovered. It was the Aqp8 protein present in both plants and mammals and it was entitled "aquaamminoporin" (fig. 1) [2]. Although investigation of Aqp8 is still under way,

several important features have already been discovered. Aquaaminoporin leaks both NH_3 and NH_4^+ contributing significantly to the acid-base equilibrium. Its permeability to ammonia/um is double of that to water, and last but not least, the porin was able to complement deffect in ammonia/um uptake in yeast [3]. However, an experiment with mice having AQP8 gene knocked-out revealed no significant differences from control suggesting that the physiological importance of the porin is only minute, if any [5].

Two types of Rh-family proteins were identified as putative ammonium transporters. Rh glycoproteins are well known for their function in erythrocytes, where they form Rh complexes with major antigenic properties [7]. RhBG and RhCG are nonerythroid Rh glycoproteins abundant in human liver, skin, kidney as well as central nervous system [20], *i.e.* important sites of ammonia species metabolism. Moreover, the gene sequences are homological to ammonium transporters of yeast (Mep) and bacteria (Amt) and all these proteins share similar structure [7].

3. Normal and pathological ammonia/um levels

Normal ammonia/um concentration is about 40-50 μM in human brain [22, 96, 111]. Marcaggi and Coles [25] give a table of ammonia/um levels in brain of different mammal species (not including human) per wet weight of the tissue. The values range from 0.18 to 0.30 mmol (kg wet wt.)$^{-1}$. In blood, normal ammonia/um concentration in healthy fasting human is approximately 15-20 μM [90, 91]. However, it was shown more than a hundred years ago, in the laboratory of Nobel laureate I.P. Pavlov, that ammonia/um levels in blood differ severalfold according to previous diet [53].

Hyperammonemia (section *6.*) is a state of increased levels of blood ammonia/um. Usually it is a consequence of urea cycle deficiency or liver failure, either acute or chronic. Since ammonia/um concentration in brain reflects the one in blood, hyperammonemia leads to elevated brain ammonia/um and may cause encephalopathy, *i.e.* disturbance of mental functions, which is, in this case, called hepatic encephalopathy (HE). HE affects some neuropsychiatric functions and features, such as neuro-muscular coordination, personality, consciousness (due to modified neurotransmission), and leads to somnolence and sometimes coma or even death [59, 67, 83]. Basal ganglia, thalamus and cerebellum are the primary targets of hyperammonemia in brain [61]. *In vitro*, hyperammonemia is studied usually by exposing the brain-cell culture to ammonia/um concentrations from tenths to ones mM. *In*

vivo, up to 5 mM ammonia/um concentration in brain during acute liver failure has been described [105].

4. Blood-brain barrier

Although as much as 98% of total ammonia species is at physiological pH in NH_4^+ form, most of it enters the brain as gaseous NH_3. Therefore, higher ammonia/um uptake is in coincidence with blood alkalosis [111]. Since the blood-brain barrier (BBB) does not readily leak ions [75 *in* 47], NH_3 had been believed for a long time to be the only form being able to cross the BBB. A few works, however, give evidence that NH_4^+ transport does occur [78].*

Recently, a fierce discussion was iniciated on the BBB permeability to ammonia/um under hyperammonemic conditions. In the late 70s pioneering experiments outlined simple relation: the higher ammonia/um concentration in blood, the higher permeability of BBB [51, 79]. This was supposed to be the direct way the encephalopathy is induced by hyperammonemia. However, recent experiments with dynamic PET (positron emission tomography) challenge this view suggesting the deleterious mechanism of elevated blood ammonia/um is slightly different.

To describe permeability of BBB to ammonia/um several parameters must be introduced [78]: 1/ extraction fraction (*E*) describes clearence of ammonia/um by one passage, *i.e.* the fraction that is removed from the blood flow:

$$E = \frac{a - v}{a}$$

where a is arterial and v is venous concentration of ammonia/um; 2/ cerebral blood flow (*CBF*) is the blood supply to the brain per minute; 3/ permeability-surface area product (*PS*) describes ammonia/um uptake across BBB per volume/weight of the brain tissue:

$$PS = -CBF \cdot \ln(1 - E).$$

PS is the direct indicator of permeability of BBB to ammonia/um. In healthy humans, E and PS were determined by Lockwood *et al.* [81 *in* 61] 0.20 ± 0.04 and 0.13 ± 0.03 $ml.g^{-1}.min^{-1}$, respectively.

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* In addition, ammonia/um was suggested to cross the BBB bound to a branched-chain amino acid, especially leucine [66]. After entering the brain leucine undergoes transamination reaction

leucine + α-ketoglutarate ↔ α-ketoisocaproate + glutamate

thus contributing to the regulation of glutamate levels in brain.

Rate of ammonia/um transfer from blood to brain, E and/or PS, can be obtained using different methods. Strauss *et al.* [52] measured ammonia/um concentrations in both arteries entering nervous tissue and veins leaving it (fig. 2). Their results indicate that elevated levels of blood ammonia/um lead to increase in extraction fraction and, therefore, support the early proposal that the BBB permeability to ammonia/um increases with rising blood levels [52, 80]. However, these findings do not take into account the possibility that ammonia/um leaves the brain also in a different form, *e.g.* bound to an amino acid, which is quite likely.

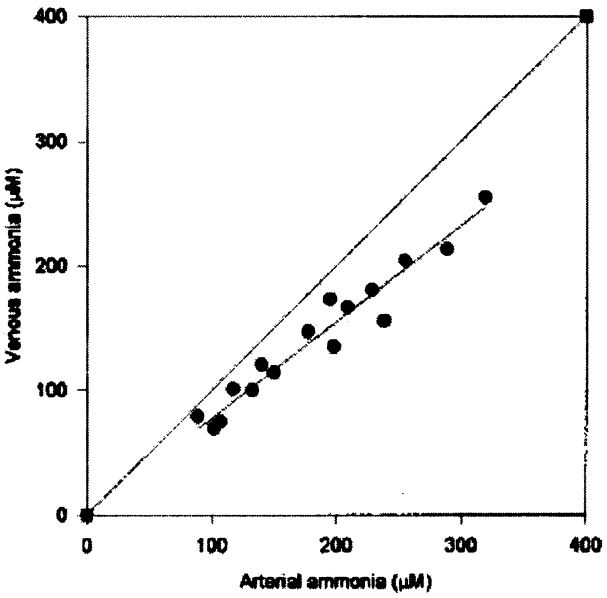

Fig. 2. Extraction fraction of ammonia/um is increased in hyperammonemic conditions. The slope of the regression line is 0.75 ± 0.05 [52].

Important data concerning this efflux were obtained from ^{13}N-ammonia/um dynamic PET. Keiding *et al.* [77, comments in 82] determined several characteristics of ammonia/um uptake in a study including patients with cirrhosis, with cirrhosis and HE and healthy controls that were injected intravenously with ^{13}N-ammonia/um. They measured not only general permeability-surface area product (PS_{BBB}) but also net conversion of blood ammonia/um into astrocytic glutamine (PS_{met}), *i.e.* PS_{BBB} plus subsequent ammonia/um conversion to glutamine (tab. 2; see *5.2.1.*). With such a correction BBB permeability to ammonia/um in patients with HE did not differ from controls. Moreover, general flux of ammonia/um (PS_{BBB}) decreased

		CBF (min^{-1})	PS_{BBB} (min^{-1})	PS_{met} (min^{-1})
cirrhosis with HE (n = 8)	cortex	0.41 ± 0.04	0.21 ± 0.02	0.20 ± 0.01
	basal ganglia	0.60 ± 0.05	0.28 ± 0.03	0.27 ± 0.01
	cerebellum	0.56 ± 0.06	0.20 ± 0.02	0.24 ± 0.01
cirrhosis without HE (n = 7)	cortex	0.44 ± 0.03	0.31 ± 0.03	0.23 ± 0.04
	basal ganglia	0.59 ± 0.04	0.37 ± 0.07	0.32 ± 0.04
	cerebellum	0.58 ± 0.04	0.27 ± 0.04	0.27 ± 0.03
healthy controls (n = 5)	cortex	0.47 ± 0.03	0.34 ± 0.03	0.20 ± 0.01
	basal ganglia	0.62 ± 0.05	0.44 ± 0.03	0.26 ± 0.02
	cerebellum	0.64 ± 0.04	0.34 ± 0.02	0.23 ± 0.01

Tab. 2. CBF, PS_{BBB} and PS_{met} in patients with cirrhosis, with cirrhosis and HE and in healthy controls. The values of patients with HE do not statistically significantly differ from controls in case of PS_{met} and are lower in case of PS_{BBB} ($p < 0.05$) [77]. Note that PS determined by Lockwood *et al.* [81 *in* 61] is close to (PS_{BBB} - PS_{met}) in this study.

significantly in patients with HE. This effect might be an adaptation of the brain to chronic exposure to pathological ammonia/um levels which occurs in HE [77].

Whether BBB permeability changes or not, the delivery of ammonia/um to different brain areas varies, since the CBF shows regional alterations in hyperammonemia. The flow is reduced in cortex, whereas subcortical structures (thalamus, lenticular nucleus, cerebellum) show increased CBF [61, 111].

5. Ammonia/um and metabolite trafficking in CNS

In central nervous system (CNS), significant flux of ammonia/um between two types of cells – neurons and astrocytes – is apparent. It is a consequence of one of the major functions of astroglia: absorption of released neurotransmitter and recovery of neuron's signalling potential at the same time. Early works concerning the role of ammonia species in CNS soon put to the limelight glutamate [10 *in* 16] as a substance tightly linked to ammonia/um.

5.1. Glutamate

The amino acid glutamate is a major excitatory neurotransmitter accounting for 80-90% of all synapses [14]. It plays, therefore, a key role in most of the brain functions including cognition, memory and learning [35]. Turnover of glutamate is rapid, it keeps shuttling between cells along with being oxidized and synthesized *de novo* (for extensive review see [12]). Neurons (with help from astrocytes) sustain their glutamate pool high (~10 mM compared to extracellular <10 μM [66]) in order to be able to generate signal quickly.

5.2. Glutamate-glutamine cycle

After releasing glutamate to the synaptic cleft neurons replenish their glutamate pool via glutamate-glutamine cycle (fig. 3). Glutamate is trapped by astrocytes (although a little is reabsorbed by neurons) where it combines with ammonium and is converted by glutamine synthetase to glutamine. Glutamine is then released to the extracellular matrix and is, in turn, captured by neurons. Neuronal phosphate-

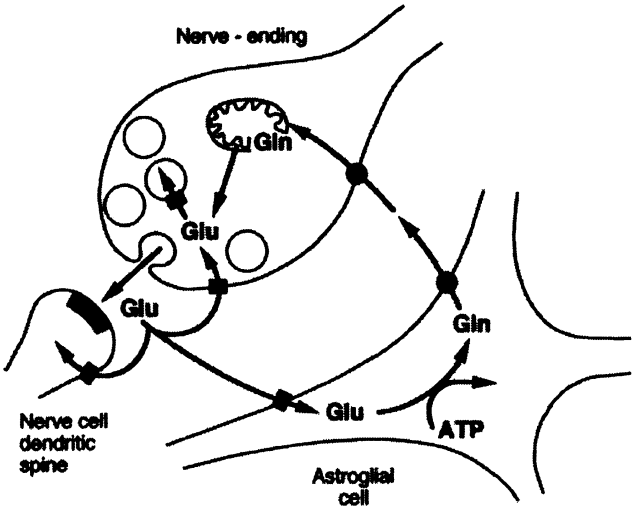

Fig. 3. Glutamate-glutamine cycle [12].

activated glutaminase hydrolyses glutamine releasing glutamate and ammonium and the cycle restarts.

5.2.1. Glutamine synthesis

The reaction

$$Glu + NH_4^+ + ATP \leftrightarrow Gln + H_2O + ADP + P_i$$

is catalyzed by glutamine synthetase (GS; EC 6.3.1.2*), which is present almost exclusively in astrocytes [43, 55]. Its affinity to ammonium in rat brain is relatively high with K_m of 0.18 mM [63 *in* 25] or even 0.042 mM [62 *in* 25], accordingly. A lot of helpful information on the glutamate-glutamine cycle was obtained after treatment with methionine sulfoximine (MSO), specific inhibitor of glutamine synthetase. It led to 50% decrease in glutamate release by neurons and to increase in glutamate content in astrocytes [16]. But GS is not only important because of the glutamate-glutamine cycle. It also ensures detoxification during hyperammonemia (see *6.1.*).

5.2.2. Glutamine hydrolysis

The reaction

$$Gln + H_2O \leftrightarrow Glu + NH_4^+$$

is catalyzed by phosphate-activated glutaminase (EC 3.5.1.2). Unlike GS, glutaminase is present not only in neurons (as one may expect due to its role in glutamate-glutamine cycle) but also in astrocytes [64], although its activity is lower there. Glutaminase, in this case, triggers oxidative degradation of glutamine [22]. The enzyme is inhibited by NH_4^+ with K_i = 0.5 mM [65 *in* 25].

5.3. Lactate-alanine shuttle

In order to maintain the glutamate-glutamine cycle function, there has to be a continual flux not only of glutamine from astrocytes to neurons but also of ammonia/um backwards. The means of ammonia/um return into astrocytes has not yet been fully clarified. Beside transport and diffusion of plain ammonia/um [42], the need of a carrier has been taken into consideration.

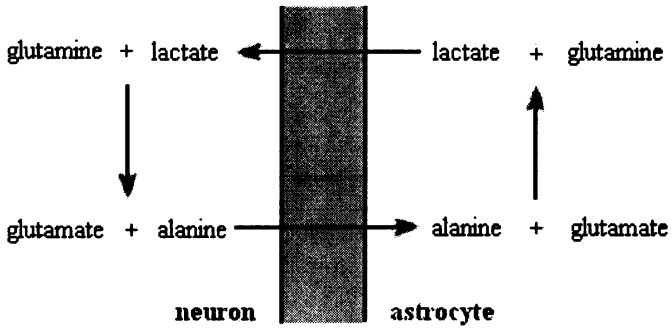

Fig. 4. Lactate-alanine shuttle. The reactions are bidirectional which is not indicated.

* Nomenclature Committee of the International Union of Biochemistry and Molecular Biology; http://www.chem.qmul.ac.uk/iubmb/enzyme/

The first nitrogen carrier proposed to be shuttling between astrocytes and neurons was leucine [49] but it has not been widely accepted as a donor of ammonia/um in glutamine synthesis in astrocytes. The most recent hypothesis by Waagepetersen *et al.* [23] seems more likely. They studied fate of labeled ^{15}N-alanine in neuronal-astrocytic cultures and came to a conclusion that it is the pivotal carrier of NH_4^+. In both neurons and astrocytes, the central metabolite pyruvate is in equilibrium with lactate as well as alanine. Lactate is released by astrocytes and then taken up by neurons where it is transaminated to alanine, which undergoes the way back to astrocytes. The release of lactate by astrocytes is in direct response to glutamate uptake [50] indicating that the lactate-alanine shuttle (fig. 4) is the counterpart of glutamate-glutamine cycle. However, this hypothesis remains to be tested [58].

5.4. Glutamate metabolism in neurons and astrocytes

Since glutamate is not able to cross the blood-brain barrier efficiently [35], its concentration in brain must be maintained by brain cells themselves. In addition, glutamate serves as an energy fuel and is oxidized in the TCA cycle [27], therefore *de novo* synthesis must occur. Nevertheless, neurons are not capable of such synthesis, since they lack the key enzyme pyruvate carboxylase [16] (fig. 5).

Hence, there is a wide-spread belief that neurons are not able to refresh their signalling potential independently and rely completely on support from astrocytes. However, this view

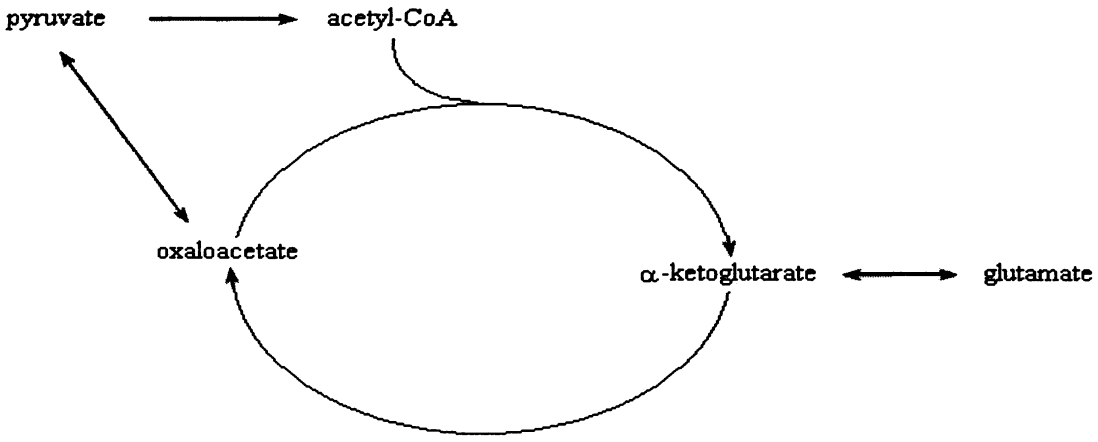

Fig. 5. In the tricarboxylic acid (TCA) cycle, one acetyl group generated in glycolysis combines with one molecule of oxaloacetate forming citrate. It is importatnt that one citrate starting the cycle regenerates one oxaloacetate at the very end. Therefore, when any of the TCA cycle intermediates is consumpted (*e.g.* α-ketoglutarate to form glutamate), the cycle stops, since there is no reborn oxaloacetate to condense with acetyl group. Net production of amino acids or other molecules synthesized from TCA cycle intermediates requires external source of oxaloacetate. Mostly it is ensured by pyruvate carboxylase, which forms oxaloacetate from pyruvate generated in glycolysis. Note that the whole molecule of glucose is necessary to produce one molecule of glutamate: one pyruvate to enter the TCA cycle and form α-ketoglutarate, another one to replenish the oxaloacetate pool. Up to 20% of glucose content in brain was estimated to be used to maintain glutamate steady-state level [22, 45].

is in contradiction to early findings by Hertz [37 *in* 18] that the release of glutamate can exceed the glutamine uptake by neurons, and has been challenged by a few recent works. Kam and Nicoll [28] observed persistent neurotransmitter release after removal of glutamine from pure neuronal cultures and Hassel and Brâthe [18] suggested that neurons might be able to supplement glutamate pool via pyruvate carboxylation by malic enzyme.

5.5. Energy metabolism in CNS

Both astrocytes and neurons utilize the major brain energy substrate glucose via usual catabolic pathway: glycolysis and subsequent pyruvate oxidation in TCA cycle. In this case, however, neuronal dependence on astrocytes seems doubtless. Neurons do not synthesize glycogen, the main reservoir of glucose and energy, and lack its degrading enzyme glycogen phosphorylase [22]. These are the privilege of astrocytes, which reveals vastly important during hypoglycemia, when astrocytes provide neurons with substantial protection [40]. There are two fundamental metabolites that may be transferred from astrocytes to neurons as energy supplies. First of all glucose itself; Swanson *et al.* [38 *in* 22] outlined the correlation between brain activation and glycogen breakdown suggesting that astrocytes produce glucose so that neurons, after receiving it, could release transmitters. And secondly lactate (see *5.3.*); the pivotal role of glucose in neuronal metabolism (especially during activation) was challenged by Pellerin and Magistretti [50]. They proposed that glycolysis and oxidative degradation in the brain are compartmented: astrocytes utilize glucose only to lactate, which is then handed over to neurons where it enters the TCA cycle. This can probably be applied to bee retina, since its astrocytes lack mitochondria, whereas neurons contain lots of them [29], but might be too simplifying in case of mammalian brain. No consensus has been reached so far whether neuronal energy metabolism demands just lactate, glucose or both [39, 41, 46].

6. Hyperammonemic conditions: effects on cell metabolism and brain functions

6.1. Astrocytes

After entering astrocytes abundant ammonia/um disturbs equilibrium of the glutamine synthesis reaction (see *5.2.1.*), since it is an almost exclusive way of metabolism of excessive ammonia/um in brain [56 *in* 96]. It forces the cell to overproduce glutamine at the expense of reducing or even depleting glutamate pool. Since glutamine is an active osmolyte, the first apparent effect of hyperammonemia is astrocyte swelling which is further augmented by

upregulated expression of aquaporin-4 [54] and can be counteracted by inhibition of GS [84]. Involvement of both oxidative- and nitrosative-stress factors has been determined, as well, since antioxidants and NOS-inhibitors prevent astrocytes from ammonia/um-induced swelling [57, 95]. However, these effects remain to be fully elucidated, since NO inhibits GS [108] and swelling could be, therefore, assumed to be boosted after NOS inhibition. In acute/fulminant liver failure, cell swelling usually leads to brain edema, which is followed by increase in intracranial pressure, brain herniation and death [44, 68]. In addition to glutamine synthesis, downregulated expression of glial fibrillary acid protein (the major component of astrocytic intermediate filaments) has been suggested to enhance swelling and deteriorate brain edema in hyperammonemia [74].

In order to cope with abundant ammonia/um astrocytes must supply the GS reaction with glutamate. It was predicted to be achieved via the anaplerotic pathway in which one mol of glucose is fissured into one mol of acetyl-CoA and one mol of oxaloacetate, both of which combine to form one mol of glutamate without producing energy (fig. 5). If this were the case, the fraction of cerebral glucose "wasted" in this pathway should rise in hyperammonemia. Indeed, this has been observed [112, 113]. Conversion into glutamine, however, cannot deal with all excessive ammonia/um in liver failure, since the GS operates

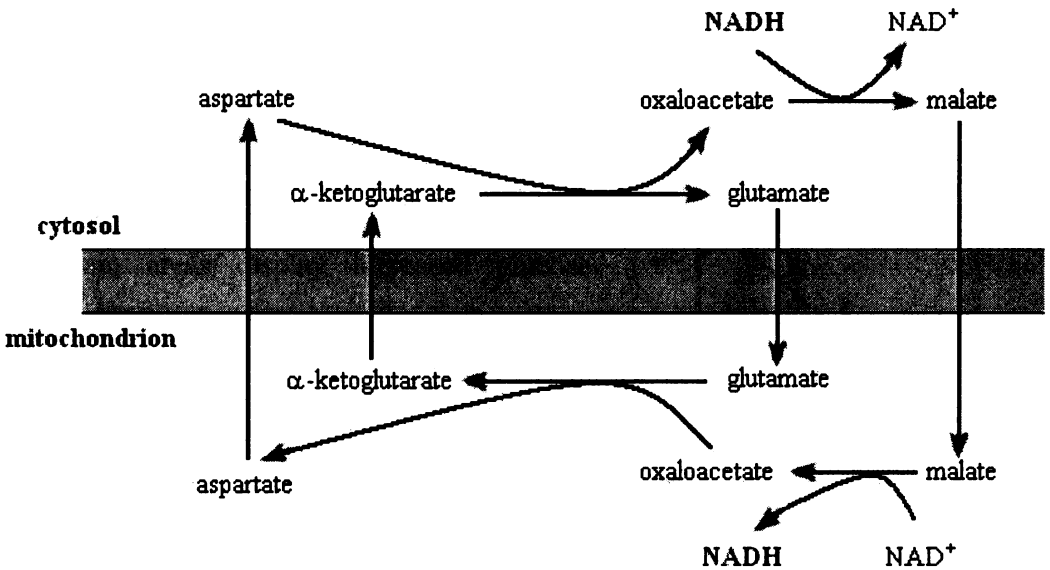

Fig. 6. Malate-aspartate shuttle. Since NAD^+/NADH cannot cross the mitochondrial membrane *per se*, electrons are delivered to the electron transport chain via malate-aspartate shuttle: oxaloacetate is reduced to malate by cytosolic malate dehydrogenase; malate is then transported into mitochondrion where it is reoxidated to oxaloacetate by mitochondrial malate dehydrogenase; mitochondrial aspartate aminotransferase transaminates oxaloacetate to aspartate which is transported to the cytosol; the cycle is finished when oxaloacetate is restored by cytosolic aspartate aminotransferase. The backflux of amino group from cytosol to mitochondrion is ensured by shuttling of glutamate and α-ketoglutarate. Aspartate and malate are transported by cotransporters in exchange with glutamate and α-ketoglutarate, respectively (not indicated). Shortage of glutamate prevents mitochondrial oxaloacetate from being transaminated and, therefore, blocks the shuttle.

close to its limits even in normammonemic conditions [76 *in* 111]. Thus hyperammonemia does not allow the astrocytic glutamate pool to be restored. Conversely, the pool is further reduced by two more factors. 1/ Glutamate uptake is weakened by downregulating both major astrocytic transporters Glt-1 (EAAT2) and Glast (EAAT1) in hyperammonemia [72, 88]. 2/ Synthesis of glutathione, for which glutamate is a precursor, is stimulated by ammonia/um [100].

6.1.1. Redox state

The ammonia/um-induced glutamate depletion has a few severe consequences in astrocytes. One of them is impairment of malate-aspartate shuttle (MAS) by which reducing equivalents generated in glycolysis and other redox reactions are transported from cytosol into mitochondrial matrix for use in electron transport chain and oxidative phosphorylation (fig. 6). In consequence, NAD^+/NADH ratio is decreased in cytosol and increased in mitochondria.

Nevertheless, cytosolic NAD^+ needs to be replenished, anyway, in order to serve as an electron acceptor in glycolysis. Dysfunction of MAS forces the cell to "ferment", *i.e.* to reduce pyruvate to lactate in reaction

$$\text{pyruvate} + \text{NADH} \rightarrow \text{lactate} + NAD^+$$

which leads to increased production of lactate and decreased pyruvate/lactate ratio, the indicator of redox potential of the cell [48] (fig. 7). Furthermore, lactate accumulation is boosted by glycolysis stimulation which occurs in hyperammonemia (though some evidence suggest not all brain areas display increased glucose utilization [86]). It is due to both direct ammonia/um-mediated activation of glycolytic enzyme phosphofructokinase [106] and upregulated expression of glucose transporter Glut-1 [102]. Much of the abundant lactate is released into blood circulation causing convulsion in experimental hyperammonemic rats [70].

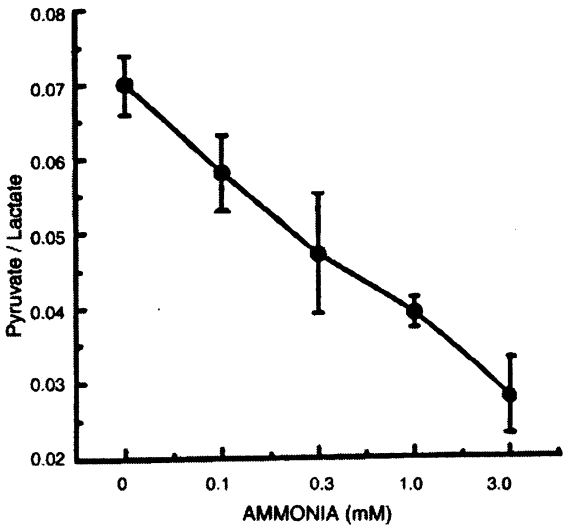

Fig. 7. Pyruvate/lactate ratio in cultures of mouse astrocytes incubated with NH_4Cl for 2.5 h [48].

6.1.2. Energy failure

Accelerated glycolysis does not imply intensified TCA cycle and larger energy yields. In hyperammonemia, the opposite is true. TCA cycle is slowed down, production of its metabolites (hence also amino acids) is disturbed and ATP levels steeply decrease [69]. Several factors have been proposed to contribute to this effect. $^{14}CO_2$ production from ^{14}C-pyruvate was diminished after ammonia/um treatment in cultured astrocytes [89] indicating a direct impact of ammonia/um on pyruvate dehydrogenase complex. This implication is supported by the fact that α-ketoglutarate dehydrogenase complex, TCA cycle enzyme of similar structure, is impaired by ammonia/um, as well [110 *in* 96] (fig. 8). In addition, MAS inhibition was proposed by Murthy and Hertz [60] to worsen the TCA cycle operation, since addition of aspartate (as a precursor of intramitochondrial oxaloacetate, fig. 6) accelerated pyruvate carboxylation in normal astrocytic cultures, whereas addition of glutamate had no

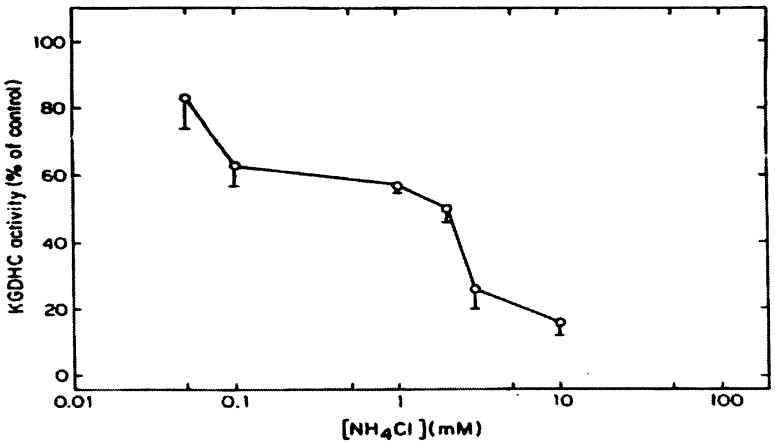

Fig. 8. α-ketoglutarate dehydrogenase complex (KGDH) is blocked by ammonia/um. Accumulated α-ketoglutarate enters the transamination reaction to form glutamate which combines with free ammonia/um to form glutamine (see above); thus the impairment of KGDH is an important way of ammonia/um detoxification, though it is at the expense of rapid decline in energy yields [99].

effect. In hyperammonemic conditions (in which MAS function is affected) glutamate supply and/or inhibition of GS with MSO increased the rate of pyruvate decarboxylation due to reigniting MAS.

All the deleterious effects of ammonia/um mentioned so far are astrocyte-specific and do not take place in neurons. Thus astrocytes are the primary targets of hyperammonemia which may be a reflection of their major function: to protect neurons from being damaged.

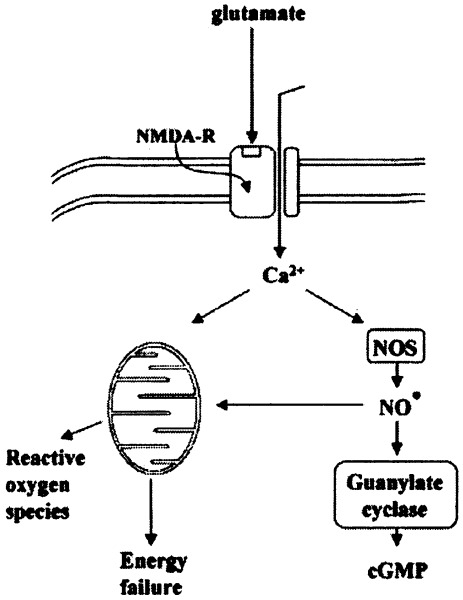

Fig. 9. Activation of NMDAr elicits increase in cGMP via nitric oxide-cyclic GMP signal transduction pathway. Stimulated receptor opens Ca^{2+} channel. Calcium ions leak in the cell, bind calmodulin and subsequently activate nitric oxide synthase (NOS). Generated NO activates guanylate cyclase, which results in increased cGMP concentration [111]. However, the NO-cGMP pathway is inhibited in chronic hyperammonemia as an adaptation to long-term NMDAr activation. This may disturb some cerebral functions such as circadian rhythms and learning [94]. In addition, Ca^{2+} enters mitochondria and affects energy metabolism and stimulates production of free radicals [114].

Nevertheless, neurons are affected by pathological levels of brain ammonia/um, as well, and this impact might be complex and deadly.

6.2. Neurons

Psychiatric significance of hyperammonemia (section *3.*) is predominantly due to altered glutamatergic neuronal activity. Elevated ammonia/um affects different types of glutamate receptors, signal transduction as well as intracellular effectors of the concerned signal pathway and their substrates.

6.2.1. NMDA receptor activation

In mammalian brain, there are many types of glutamate receptors divided into two main groups: 1/ metabotropic receptors associated with G proteins and 2/ ionotropic receptors operating Na^+, K^+ and Ca^{2+} channels. *N*-methyl-D-aspartate ionotropic glutamate receptor (NMDAr) is the most explored subtype in respect of the vulnerability to hyperammonemia [115]. Most of the research has been conducted using specific infibitor of NMDAr MK801.

Since glutamate uptake by astrocytes is impaired (see *6.1.*) and its reuptake by neurons themselves is minor, extracellular glutamate level (unlike the intracellular one) is increased in hyperammonemic conditions. Moreover, ammonia/um induces glutamate release from neurons [101]. Therefore, NMDAr can be presumed to be overstimulated. This hypothesis was tested by Hermenegildo *et al.* [93]. They measured extracellular levels of cGMP as an indicator of NMDAr activity (fig. 9).

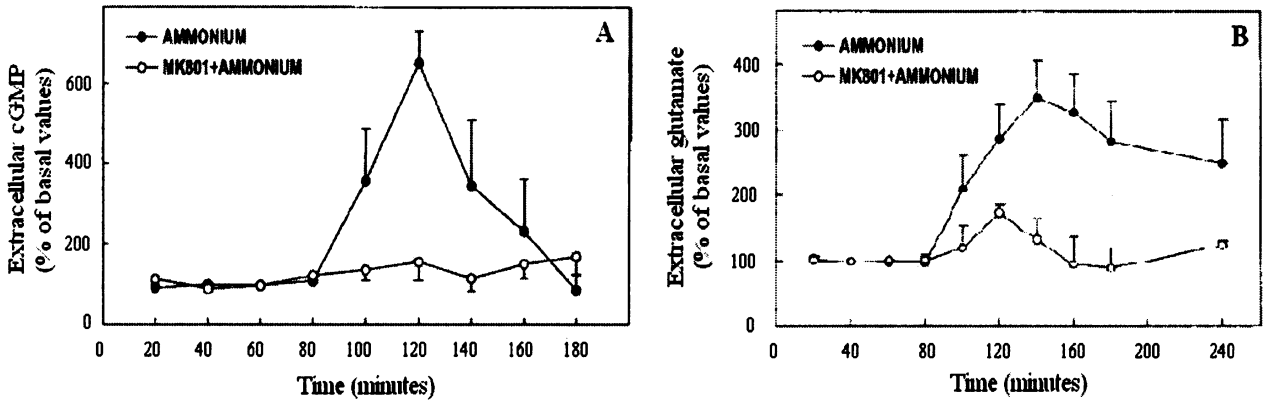

Fig. 10. Time course of cGMP and glutamate levels in rat brain *in vivo* after ammonia/um treatment. **A** – cGMP concentration reflects activation of NMDAr by ammonia/um. Application of the specific antagonist of NMDAr (5R,10S)-5-methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine hydrogen maleate (MK801) abolished the effect. **B** – Glutamate concentration uprise is not a cause, but rather a consequence of NMDAr activation, since it is inhibited by MK801 [93].

Hyperammonemia, indeed, stimulated NMDAr (fig. 10-A). However, it could not be considered a consequence of elevated extracellular glutamate, since the latter occurred afterwards. Conversely, the glutamate level increased in response to NMDAr activation (fig. 10-B).

But what is the true cause of ammonia/um-induced NMDAr activation then? And how does NMDAr activation propulse the glutamate release by neurons? The receptor is normally blocked by Mg^{2+} in a voltage-dependent manner [71]. The ion is released after membrane depolarization leaving the receptor prone to activation. NH_4^+ causes such depolarization which might be the trigger of NMDAr activity [93]. In addition, acute liver failure leads to reduced expression of astrocytic glycine transporter Glyt-1 and concomitant increase in extracellular glycine, which serves as an agonist of NMDAr [107].

The release of glutamate by neurons in response to NMDAr activation could be explained by another effect of ammonia/um – ATP depletion (see *6.1.2.*). NMDAr contributes to ATP depletion by inhibiting protein kinase C as well as by activating calcineurin (Ca^{2+}/calmodulin-dependent protein phosphatase), both of which result in higher activity of less-phosphorylated Na^+/K^+-ATPase and increased consuption of ATP [87, 97]. This can reverse the sodium-dependent glutamate uptake and lead to extrusion of glutamate to the extracellular space [116].

6.2.2. Neuronal damage and cell death

Most of the calcium ions having entered the neuron after NMDAr activation are taken up by mitochondria (fig. 9) [73]. Abundant intramitochondrial Ca^{2+} causes failure of enzymes of the respiratory chain and production of free radicals [114]. Furthermore, enhanced formation of nitric oxide was determined to contribute to the mitochondrial damage [103]. Oxidative stress is augmented by changes in antioxidant enzymes catalase, glutathione peroxidase and superoxide dismutase and by depletion of glutathione [109]. In consequence, increased lipid peroxidation has been observed [85]. All these NMDAr-mediated effects of hyperammonemia are responsible for the neuronal necrosis [92]. In addition to necrosis, ammonia/um triggers apoptosis in neurons [98].

To complete the list of deleterious effects ammonia species has in neurons via NMDAr, degradation of microtubule-associated protein MAP-2 should be mentioned. MAP-2 binds tubulin and enables its polymerization. Its Ca^{2+}-dependent proteolysis causes collapse of neuronal microtubular network [104].

7. Summary

Ammonia species is essential for nervous tissue. It is present both free and bound to molecules such as amino acids. The barter of free ammonia/um among brain cells is enabled by nonspecific transporters. However, a couple of putative ammonium-specific transporters have been identified recently. Their role is still to be determined as the research is under way.

There is a dispute over the blood-brain barrier permeability to ammonia/um (especially under hyperammonemic conditions). The results are contradictory and there is no consensus on whether the permeability increases, remains the same or even decreases in hyperammonemia.

Ammonia/um takes part in several key reactions of cell metabolism in brain: formation of glutamate from α-ketoglutarate, alanine from pyruvate and glutamine from glutamate. The latter is a part of glutamate-glutamine cycle by which neurons sustain their neurotransmitter pool. The way ammonia/um is returned to astrocytes within the cycle is uncertain.

Hyperammonemia is a state of increased levels of ammonia/um in the human body. It leads to severe damage of both astrocytes and neurons, disturbs brain functions and may cause even death due to either brain edema or neurotransmission hyperactivation.

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