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ORIGINAL ARTICLE

Profiling of astrocyte properties in the hyperammonaemic brain: Shedding new light on the pathophysiology of the brain damage in hyperammonaemia

U. Lichter-Konecki

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Summary Acute hyperammonaemia (HA) causes cerebral oedema and severe brain damage in patients with urea cycle disorders (UCDs) or acute liver failure (ALF). Chronic HA is associated with developmental delay and intellectual disability in patients with UCDs and with neuropsychiatric symptoms in patients with chronic liver failure. Treatment often cannot prevent severe brain injury and neurological sequelae. The causes of the brain oedema in hyperammonaemic encephalopathy (HAE) have been subject of intense controversy among physicians and scientists working in this field. Currently favoured hypotheses are astrocyte swelling due to increased intracellular glutamine content and neuronal cell death due to excitotoxicity caused by elevated extracellular glutamate levels. While many researchers focus on these mechanisms of cytotoxicity, others emphasize vascular causes of brain oedema. New data gleaned from expression profiling of astrocytes acutely isolated from hyperammonaemic mouse brains point to disturbed water and potassium homeostasis as regulated by astrocytes at the brain microvasculature and in the perisynaptic space as a potential mechanism of brain oedema development in hyperammonaemia.

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U. Lichter-Konecki
Center for Neuroscience Research,
Washington DC, USA

U. Lichter-Konecki (✉)
Division of Genetics & Metabolism,
Children's National Medical Center,
111 Michigan Ave., NW,
Washington, DC 20010-2970, USA
e-mail: ulichter@cnmc.org

U. Lichter-Konecki
Department of Pediatrics,
George Washington University Medical Center,
Washington, DC, USA

Abbreviations

ALF	acute liver failure
Aqp4	aquaporin 4
Cx43	connexin 43
FACS	fluorescence activated cell sorting
GFAP	glial fibrillary acidic protein
(E)GFP	(enhanced) green fluorescent protein
HA	hyperammonaemia
HAE	hyperammonaemic encephalopathy
HE	hepatic encephalopathy
Kir	inward-rectifying potassium channel
OTC(D)	ornithine transcarbamylase (deficiency)
qRTPCR	quantitative reverse transcription polymerase chain reaction
spf	sparse fur
UCD	urea cycle disorder

Introduction

Urea cycle disorders (UCDs) are inborn errors of metabolism which are caused by a deficiency of one of the enzymes or transporters that constitute the urea cycle. The main function of the hepatic urea cycle is

the excretion of nitrogen as urea. When its function is impaired, nitrogen accumulates, for example as glutamine and as ammonia, resulting in hyperammonaemia (HA). Elevated blood ammonia levels cause hyperammonaemic encephalopathy (HAE), although the ammonia level at which a patient becomes encephalopathic varies. In liver failure the urea cycle is not the only pathway that is compromised, but elevated ammonia levels are still considered to be one of the main causes of hepatic encephalopathy (HE; Desjardins et al 2001). Depending on the amount of residual urea cycle function, environmental factors and management, patients either develop acute HAE causing cerebral oedema and severe brain damage or chronic HAE. Patients with UCDs and chronic HAE show developmental delay, intellectual disability and attention deficit/hyperactivity disorder, and may have seizures (Batshaw et al 1980, 1986; Msall et al 1984, 1988; Maestri et al 1999; Gyato et al 2004; Gropman et al 2007). In a similar way, acute HE and cerebral oedema occur in acute liver failure, while chronic HE is seen in chronic liver failure and manifests with neuropsychiatric disturbances. What causes acute or chronic HAE or HE has been subject of many studies and is still under investigation. After Norenberg (1977) described astrocyte swelling in animals with hepatic encephalopathy, astrocytes became a main focus of HAE and HE research.

Astrocytes and brain function

The notion that astrocytes are the primary site of ammonia toxicity in brain is also suggested by the physiological function of astrocytes as they are the main site of ammonia, glutamate, and glutamine metabolism in brain (Marcaggi and Coles 2001). In addition to the role astrocytes play in brain metabolism, regulation of glutamate signalling and maintenance of potassium homeostasis are two very important astrocytic functions (Kofuji and Newman 2004). Extracellular glutamate and potassium levels which increase during neuronal activity must be immediately lowered to avoid prolonged glutamate receptor activation and widespread depolarization (Felipo and Butterworth 2002; Kofuji and Newman 2004). Astrocytes regulate extracellular glutamate and potassium levels through uptake and dispersion. Following uptake into the cell, glutamate is either allocated through gap junctions or metabolized with ammonia in the glutamine synthesis reaction and potassium ions are dispersed through gap junctions (Giaume et al 1997; Hansson et al 2000). The glutamine synthesized from glutamate and ammonia is transferred back into neurons, where it is deamidated by glutaminase for glutamate re-utilization (Marcaggi and Coles 2001). Thus ammonium may have an important physiological function in the astrocyte–neuron glutamate/glutamine shuttle (Marcaggi and Coles 2001). Astrocytes which are coupled by gap junctions form an astrocyte syncytium that allows the bi-directional transfer of ions and small molecules, such as K^+ (Sontheimer et al 1990; Wallraff et al 2006) and glutamate, between neighbouring cells. In this way the syncytium enables spatial buffering of K^+ and spatial allocation of neurotransmitters and metabolites (Giaume et al 1997; Hansson et al 2000; Haydon 2001). The astrocyte syncytium can respond to different concentrations of molecules in the extracellular space by regulating the permeability of its gap junctions (Hansson et al 2000).

CNS reactions to acute hyperammonaemia

Since the first observation of astrocyte swelling in portacaval-shunted rats by Norenberg (1977), swollen astrocytes have been regarded by many as the cellular correlate of the brain oedema in acute HA. The synthesis of glutamine from glutamate and ammonia in astrocytes is not only a physiological reaction but also the only pathway by which the brain can detoxify ammonia. There is increased astrocytic glutamine synthesis in hyperammonaemia, leading to increased intracerebral glutamine concentrations. Glutamine has been considered to act as an osmolyte, causing cellular swelling. This has led to the proposal of inhibition of glutamine synthesis as a therapy for HAE (Tanigami et al 2005). Besides glutamine synthesis, many other pathways and mechanisms have been under consideration as the cause of HAE over the past 30 years.

Among the main hypotheses being tested are: (i) elevated extracellular glutamate causing excitotoxicity; (ii) elevated glutamine having an osmotic effect and causing cell swelling; (iii) increased water transport through Aqp4 water channels effecting astrocyte swelling; (iv) altered glucose metabolism leading to disturbed energy metabolism; (v) ammonia interfering with the normal flux of potassium ions; and (vi) toxicity caused by oxidative and nitrosative stress due to increased free radical production and increased nitric oxide synthesis.

Glutamate-induced excitotoxicity

The excitotoxicity hypothesis stems from the fact that astrocytic glutamate transporters were found to be

downregulated during hyperammonaemia. This may be caused by direct or indirect effects of ammonia on glutamate transporters and receptors (Felipo and Butterworth 2002). When astrocytic glutamate transporters are downregulated, extracellular glutamate levels may rise (Chan and Butterworth 1999; Zhou and Norenberg 1999; Chan et al 2000; Desjardins et al 2001; Felipo and Butterworth 2002). Decreased astrocytic glutamate transport could thus lead to overstimulation of neurons by increased extracellular glutamate levels and result in neuronal cell death by excitotoxicity. Elevated concentrations of extracellular glutamate during HA have been reported in many studies as reviewed, for example, by Felipo and Butterworth (2002) and Butterworth (2007); however there is little evidence for excitotoxicity and only limited neuronal cell death in the models studied (Butterworth 2007).

Disturbed energy metabolism

Ammonia stimulates phosphofructokinase, the key enzyme of glycolysis. Ammonium may play a significant role in regulating glycolysis in astrocytes under physiological conditions *in vivo* (Tsacopoulos and Magistretti 1996). Increased ammonia levels, on the other hand, inhibit α-ketoglutarate dehydrogenase in brain (Lai and Cooper 1986). Increased α-ketoglutarate levels were measured in the brain of hyperammonaemic mice (Ratnakumari et al 1994). Stimulation of phosphofructokinase as well as inhibition of α-ketoglutarate dehydrogenase, the key enzyme of the TCA cycle, will cause increased formation of lactate in brain. A block in the TCA cycle will lead to compromised brain energy metabolism.

Altered potassium homeostasis

The altered potassium homeostasis hypothesis stems from the fact that NH_4^+ has properties similar to those of the potassium ion. An important astrocytic function is the maintenance of potassium homeostasis. NH_4^+ can cross cell membranes through ion channels or membrane transporters, and can replace K^+ or H^+ on different transporters. In some cell types, NH_4^+ is also a substrate for NA^+/K^+-ATPases. In cultured mouse astrocytes ammonium was shown to enter the cell through Ba^{2+} sensitive channels which are inward rectifying K^+ channels (Marcaggi and Coles 2001). Ammonia may thus interfere with potassium channel or potassium transporter function. An elevated extracellular K^+ concentration was measured in the parietal cortex of healthy rats infused with ammonium acetate to cause HA (Sugimoto et al 1997).

Oxidative/nitrosative (O/N) stress due to increased free radical production and increased nitric oxide synthesis

As reviewed by Norenberg and colleagues (2007), ammonia causes free radical production and a decrease in antioxidant enzyme activity, and antioxidants prevented astrocyte swelling after ammonia exposure. In addition to oxidative stress there is also nitrosative stress in HAE as an upregulation of nitric oxide synthase (NOS) activity and expression was detected in animal models for HE (Rao et al 1997). Increased amounts of NO were found in the brains of such animals and NOS inhibitors prevented astrocyte swelling *in vivo* and *in vitro*. How oxidative/nitrosative stress would cause astrocyte swelling is not known, but a mechanism under consideration is induction of the mitochondrial permeability transition (MPT). In this scenario, oxidative stress would cause opening of the permeability transition pore (PTP) of the inner mitochondrial membrane and ions would flow, changing the membrane potential. This would lead to mitochondrial dysfunction and more free radical production, causing cell damage. The PTP is thought to consist of an adenine nucleotide translocator, a voltage-dependent anion channel, the peripheral benzodiazepine receptor, Bcl-2, hexokinase, and creatine kinase (Norenberg et al 2007).

Mitochondrial dysfunction may also be the underlying mechanism in glutamine-induced astrocyte swelling (Norenberg et al 2007). Glutamine was shown also to induce the MPT (Rama Rao et al 2003a) and free radical production (Jayakumar et al 2004). Ultimately this could be an ammonia effect inside the mitochondria as glutamine is hydrolysed to ammonia and glutamate inside the organelle. With this consideration, Albrecht and Norenberg (2006) formulated the 'Trojan horse hypothesis' whereby glutamine is synthesized from glutamate and ammonia in the cytosol of astrocytes, and then hydrolysed inside the astrocytic mitochondria, which will lead to high ammonia levels inside the mitochondria, MPT, free radical production, and cell swelling. In this model, glutamine would be the 'Trojan horse' carrying the ammonia into the mitochondria.

The mechanisms described above could explain a cytotoxic effect of ammonia and imply that the brain oedema associated with HA is a cytotoxic oedema. However, other work supported and emphasized a vasogenic cause of the oedema (Felipo and Butterworth 2002). The number of hypotheses regarding the nature of ammonia toxicity in the brain indicates that a major cause of HAE has not been identified and that several mechanisms may contribute.

Previously used research tools

Animal models for acute and chronic HAE and HE

Animal models employed previously to investigate the brain under hyperammonaemic conditions were portacaval-shunted rats with chronic liver failure; portacaval-shunted rats with hepatic artery ligation causing acute liver failure (ALF); urease-treated or ammonium acetate-infused healthy rats (Felipo and Butterworth 2002); and the sparse fur (*spf*) mouse that is partially deficient in ornithine transcarbamylase (OTC; EC 2.1.3.3). OTC deficiency (OTCD OMIM 311250; DeMars et al 1976) is an X-linked urea cycle disorder; *spf*/Y mice with OTCD are small, have sparse fur, and may become symptomatic with HA in a 30-day time window that extends from weaning to the end of their shortened lifespan (Gushiken et al 1985; Inoue et al 1987; Robinson et al 1995). Gene knockout mouse models for other UCDs have been created, namely for citrullinaemia (Patejunas et al 1994) and argininosuccinic aciduria (Sutton et al 2003), but these mice typically die in the perinatal period, making them difficult to study. A mouse model for argininaemia also exists (Iyer et al 2002), but in this urea cycle disorder there is neurological involvement implicating additional mechanisms of neurological damage. As outlined above, the *spf* mouse is the best currently available animal model for urea cycle disorders and only has a partial enzyme deficiency. The mouse has to be stressed to ensure that it is hyperammonaemic at the time of the experiment. A proven method of stressing the urea synthesis capacity of the *spf* mouse is feeding it an arginine-free diet (D'Hooge et al 2000). *spf* mice will be hyperammonaemic after a defined time on the diet because the arginine deficiency further compromises their urea cycle function by decreasing the amount of arginine available as ornithine precursor, with ornithine necessary for normal urea cycle function. A limitation of the *spf* mouse in experimental settings is that animals have variable phenotypes and are not all affected to the same degree.

A shortcoming of animal models in which acute hyperammonaemia is achieved by injection of ammonium chloride or ammonium acetate is that close to lethal doses of ammonia have to be injected to overcome the ureagenesis capacity of the healthy rodent liver and achieve hyperammonaemia. These very high doses of ammonia acutely delivered to the animal are unlikely to mimic the pathophysiology of HAE typically seen in patients as developing over several days.

Cell culture models

Cell culture models have also been widely used to study the effect of high ammonia levels on cell physiology. The most commonly used cell culture model involves monocultures of primary cortical astrocytes isolated from newborn rats and treated with dibutyryl cyclic AMP (dBcAMP) for 7–14 days to induce differentiation after the initial 2 weeks of culture. These astrocytes were typically exposed to an ammonium chloride concentration of 5 mM or 10 mM in the cell culture medium for 72 h, while controls were exposed to equivalent concentrations of sodium chloride (Rama Rao et al 2003b). Besides problems with the ion load in the control culture medium, a major shortcoming of this monoculture model is that it does not allow one to study the complex interactions between astrocytes and neurons on one hand and astrocytes and the brain vasculature on the other, while important pathophysiological mechanisms cumulating in HAE may result from these interactions. Braissant and colleagues (2002) therefore studied aggregate cell cultures containing neurons and glial cells. Although the brain aggregate model has great advantages over studying monocultures, it still does not allow for the analysis of the astrocyte brain vasculature interaction.

Gene expression analysis

Changes in the expression of a number of astrocyte-specific genes have been observed in hyperammonaemic rodents and in cultured rodent astrocytes exposed to ammonia (see below). Except for one study (Song et al 2002), only the expression of single genes or of a few genes was investigated.

Excitatory amino acid transporter (EAAT)-1 and -2 and glycine transporter-1 (GLYT-1)

Several authors have reported loss of expression of EAAT-1, an astrocytic glutamate transporter, in cultured cortical astrocytes exposed to ammonia (Chan and Butterworth 1999; Zhou and Norenberg 1999; Chan et al 2000), and in the brain of animal models for HA (Desjardins et al 2001). In the latter, a significant decrease in GLYT-1 (astrocytic glycine transporter-1) expression associated with elevated extracellular glycine concentration and a decrease in astrocytic EAAT-2 expression were also reported. Loss of EAAT2 expression was associated with increased extracellular glutamate concentration (Felipo and Butterworth 2002). This led to the hypothesis of excitotoxic neuronal damage in HAE (see above).

Aquaporin 4 (Aqp4)

Aqp4 is a channel protein that allows water transport at blood–brain and brain–CSF interfaces. It is expressed in astrocytic endfeet at the brain vasculature. Aqp4 localization and function suggests an important role for water homeostasis in the brain. Rama Rao and colleagues (2003b) reported a time-dependent upregulation of Aqp4 expression in astrocyte cultures exposed to ammonia. Aqp4 expression was increased after 10 h of ammonia exposure and continued to increase until cell swelling occurred. In other work, the regulation of Aqp4 expression was shown to depend on the type of cell injury and oedema. Aqp4 expression was significantly decreased during the early phase of focal ischaemia, hypoxia or diffuse traumatic brain injury when primarily cytotoxic brain oedema is present. This decrease was considered to be protective for the cells at that time. Aqp4 expression increased with oedema development during the later phase of focal ischaemia (Kiening et al 2002).

Facilitative glucose transporter (GLUT1)

Increased mRNA and protein levels of the facilitative glucose transporter (GLUT1) were reported in the cortex of portacaval-shunted rats and in cultured astrocytes exposed to ammonia, indicating that ammonia causes increased cerebral glucose uptake (Belanger et al 2006).

New research tools

Animal models for *in vivo* analyses

Because of the complex function of astrocytes in their interactions with neurons, synapses and the brain vasculature, astrocytes should be investigated in their brain environment rather than in isolation to accurately assess alterations in their cellular function. A tool that allows the study of specific cell types in the context of the brain is the expression of green fluorescent protein (GFP) in a specific cell type using a GFP transgene and a cell type-specific promoter. Cells expressing GFP can then be isolated from a suspension of dissociated brain tissue by fluorescence activated cell sorting (FACS) and their properties studied. Nolte and colleagues (2001) made a transgenic mouse with enhanced GFP (EGFP) expressed under the control of the glial fibrillary acidic protein (GFAP) promoter. GFAP is a protein that is only expressed in astrocytes in the developed brain. Use of the GFAP promoter thus allows for the tissue-specific expression of EGFP in astrocytes in the postnatal brain. The transgenic mouse was characterized and shown to express EGFP in about 50% of its cortical astrocytes, 80% of its striatal astrocytes, and 100% of its cerebellar astrocytes at 8–12 weeks of age (Wehner et al 2003).

Analysis of the mouse transcriptome

Microarray expression analysis is a molecular genetic tool that enables the investigator to analyse changes that occur in the mouse or human transcriptome under a specific pathophysiological condition. In other words, the investigator is able to look at changes in RNA levels of over 34 000 mouse genes at once in the condition under study; 34 000 is a number of genes considered sufficient to cover the mouse genome. The results of a microarray hybridization are analysed using new data analysis tools. These tools allow one to determine in an instant which of the genes showing expression changes are involved in the same molecular mechanism or belong to the same canonical pathway. The data analysis tools also allow the identification of networks of genes with regulatory interactions. One such analysis tool is the publicly available *DAVID* (Database for Annotation, Visualization and Integrated Discovery of the *DAVID* and *EASE* bioinformatics resources of the National Institute of Allergy and Infectious Diseases (NIAID)); others are commercially available pathway analysis systems such as Ingenuity Pathway Analysis (Ingenuity Systems, www.ingenuity.com). A list of those genes that were found to be significantly differentially expressed can be loaded into the database of the respective analysis program. *DAVID* allows identification of affected gene ontology groups in which genes are grouped according to criteria such as biological process, molecular function, or cellular components while (Ingenuity) Pathway Analysis allows identification of affected pathways or networks. The significance of the association between the data set and a canonical pathway is measured (i) by determining a ratio of the number of genes from the dataset that map to the pathway divided by the total number of genes that map to the canonical pathway, and (ii) by using Fisher's exact test to calculate a *p*-value determining the probability that the association between the genes in the dataset and the canonical pathway is explained by chance alone. All microarray expression analysis results are confirmed by a second independent method, usually a quantitative reverse transcription polymerase chain reaction (qRTPCR). This method also measures expression changes at the RNA level but by different means.

Using this approach, a project was initiated to test the hypothesis that microarray expression analysis of ammonia-exposed brain astrocytes would permit the identification of astrocyte-specific genes that show expression changes after ammonia exposure. A mouse model needed to be generated that would allow the study of the expression profiles of astrocytes from hyperammonaemic brains without interference from the expression profiles of other cell types. If specific groups of genes emerged from microarray analysis, identification of major pathways affected by hyperammonaemia in astrocytes would allow one to focus research on the pathogenesis of HAE. OTC-deficient *spf* mice (DeMars et al 1976) were crossed with the GFAP-EGFP transgenic mouse (Nolte et al 2001; Wehner et al 2003) to generate an animal model for HAE with green fluorescent astrocytes. The animal model enabled the purification of astrocytes from dissociated hyperammonaemic mouse brains by FACS and the study of their expression profiles by microarray analysis. Affected *spf* males and unaffected male littermates were fed an arginine-free diet (mentioned under animal models) to render affected animals hyperammonaemic at the time of the experiment. The need for the diet introduces a potentially confounding factor into the experiment because affected animals may eat relatively less food than controls although they are fed the same arginine-free diet. Furthermore, the diet could generate arginine deficiency in the affected animals but not in the controls. Weighing the animals at the beginning and at the end of the dietary intervention would allow one to assess differences in food intake. This was not done in our preliminary study. However full plasma and full hindbrain tissue amino acid profiles were measured in each animal and the arginine levels were not significantly lower in the plasma and in the brains of the affected animals used for microarray analysis than in controls (Tables 1 (plasma) and 2 (brain)). The amino acid profiles also showed significantly higher brain glutamine concentrations in affected animals versus controls (Table 2) indicating high brain ammonia levels in affected animals.

Affected animals had significant hyperammonaemia at the time of brain harvesting (Lichter-Konecki et al 2008). When compared with healthy littermates, affected mice showed changes in pathways previously described as altered in HA including glycolysis/gluconeogenesis pathways, oxidative phosphorylation, glutathione metabolism, and the citric acid cycle. There were also changes in pathways not previously investigated in HA such as calcium signalling pathways and xenobiotic metabolism (for details see Lichter-Konecki et al 2008) as well as chemokine signalling.

Table 1 Comparison of amino acid concentrations: plasma levels (nmol/ml)

Amino acid	Microarray – Arginine-free diet					QTPCR – Arginine-free diet					QTPCR – 1% Arginine diet				
	Unaffected		Affected			Unaffected		Affected			Unaffected		Affected		
	N	Mean±SD	*N*	Mean±SD	*p*-value[a]	*N*	Mean±SD	*N*	Mean±SD	*p*-value[a]	*N*	Mean±SD	*N*	Mean±SD	*p*-value[a]
Gln[b]	6	526.9±122.3	5	686.0±267.6	0.2733	5	433.0±95.8	4	569.0±120.1	0.0864	5	502.8±125.3	3	685.7±232.7	0.2967
Arg	5	111.9±29.1	3	78.1±93.3	0.8815	5	22.2±9.3	4	35.5±24.8	0.4624	3	54.7±46.0	3	334.9±483.2	0.2752
Orn	6	126.4±29.1	3	181.6±128.8	0.7963	5	61.4±28.9	4	119.1±107.2	0.6242	4	95.9±49.4	3	424.4±257.2	0.0771
Citr	6	74.4±17.4	5	33.0±17.1	**0.0062**	5	66.4±10.1	4	35.6±19.1	**0.0275**	4	76.6±29.5	3	60.6±25.8	0.7237
Ala	6	533.9±142.7	5	282.3±187.2	0.0679	5	341.7±66.6	4	259.7±134.0	0.4624	5	439.3±267.5	3	704.3±167.2	0.1797
Glu	6	177.5±127.7	5	85.5±43.8	0.0446	5	84.0±29.6	4	71.6±32.4	0.4624	3	80.5±28.9	3	453.6±386.9	0.1266
Asp	4	28.9±9.6	3	40.8±20.4	0.4795	4	16.1±7.5	3	15.1±4.2	0.7237	3	14.8±10.9	3	148.4±129.1	0.1266
Lys	6	625.9±248.3	3	625.5±401.5	0.7963	5	444.4±153.5	4	462.0±210.4	0.8065	4	529.0±58.4	3	1735.1±1352.5	**0.0339**
Val	6	207.6±38.8	5	300.4±190.2	0.7150	5	137.6±37.9	4	173.8±87.2	0.6242	4	136.3±69.2	3	467.4±244.7	0.0771

[a] *p*-value from Wilcoxon rank sum test comparing mean amino acid levels between Unaffected and Affected.
[b] Pooled glutamine levels of the three groups together were previously published in Lichter-Konecki et al (2008); splitting the animals into three groups changed the difference between the plasma glutamine levels of affected and unaffected animals to 'not significant'.

Table 2 Comparison of amino acid concentration: brain levels (nmol/per 100 mg wet weight of hindbrain tissue[a])

Amino acid	Microarray – Arginine-free diet					QTPCR – Arginine-free diet					QTPCR – 1% Arginine diet				
	Unaffected		Affected			Unaffected		Affected			Unaffected		Affected		
	N	Mean±SD	*N*	Mean±SD	*p*-value[b]	*N*	Mean±SD	*N*	Mean±SD	*p*-value[b]	*N*	Mean±SD	*N*	Mean±SD	*p*-value[b]
Gln[c]	6	280.1±117.6	6	901.3±264.2	**0.0039**	5	532.2±84.9	3	1131.1±321.5	**0.0253**	5	321.5±68.5	3	1132.7±556.0	**0.0253**
Arg	6	59.1±14.2	6	79.1±33.0	0.4233	5	179.8±51.6	4	101.4±60.1	**0.0500**	5	51.6±12.3	3	49.0±12.8	0.8815
Orn	6	2.7±1.2	6	4.7±2.2	0.0651	5	9.2±2.5	4	8.7±8.1	0.4624	5	2.3±0.6	3	2.5±0.9	0.9999
Citr	6	10.9±3.4	6	16.4±11.4	0.6310	5	75.2±24.7	4	24.3±21.0	**0.0268**	5	9.4±3.5	3	6.0±0.8	0.1797
Ala	6	113.8±24.7	6	150.2±57.0	0.3367	5	300.5±75.0	4	162.9±105.2	0.1416	5	101.6±14.1	3	104.6±17.5	0.6547
Glu	6	136.4±43.2	6	146.3±61.5	0.6310	5	201.1±24.3	4	172.4±101.6	0.6242	5	188.9±56.0	3	225.6±40.9	0.2302
Asp	6	448.4±73.9	6	425.8±77.6	0.7488	5	677.3±177.2	4	528.4±304.1	0.2372	5	530.3±149.6	3	12.9±3.3	0.0253
Lys	6	64.8±15.1	6	98.7±36.9	0.0547	5	215.8±66.3	4	116.7±79.3	0.1416	5	56.2±9.6	3	78.5±15.7	0.1011
Val	6	39.1±8.0	6	57.6±22.8	0.1495	5	149.0±56.5	4	75.7±54.8	0.1416	5	33.4±5.3	3	35.8±4.5	0.4561

[a] Amino acids were extracted from hindbrain tissue by sulfosalicylic acid precipitation.
[b] *p*-value from Wilcoxon rank sum test comparing mean amino acid levels between Unaffected and Affected.
[c] Pooled glutamine levels of the three groups together were previously published in Lichter-Konecki et al (2008).

Similarities and differences were detected when comparing the results of the microarray analysis with previous work. For instance, RNA levels of antioxidant enzymes of glutathione metabolism such as γ-glutamyltranspeptidase, glutathione synthetase, glutathione *S*-transferase, isocitrate dehydrogenase, and selenium-glutathione peroxidase were downregulated (Lichter-Konecki et al 2008). Murthy and colleagues (2000) reported an initial decrease with subsequent increase of glutathione levels in cultured astrocytes exposed to NH_4Cl for 3 days. This, as well as the Aqp4 expression data obtained (see below) may indicate that the stressed *spf* mouse as a model reflects mostly the initial phase of HAE.

With regard to O/N stress, microarray analysis detected decreased RNA levels for the antioxidant enzymes glutathione peroxidase and superoxide dismutase 1 and increased RNA levels of the oxidative stress marker haem oxygenase 1. This was consistent with previous findings (Norenberg et al 2007). No increase in nitric oxide synthase expression, previously reported by Rao and colleagues (1997), was detected. This could have been due to the low-arginine diet, with arginine being a necessary precursor for nitric oxide synthesis; however no brain arginine deficiency was documented in the experimental animals. With regard to induction of MPT and the constituents of the PTP as reviewed by Norenberg and colleagues (2007) (see above) the voltage-dependent anion channel number 1 of the outer mitochondrial membrane and hexokinase 1 were downregulated in microarray analysis, indicating a possible effect of ammonia on these components.

Microarray expression analysis results mentioned thus far were not confirmed by an independent second method and not tested for a diet effect. This was, however, done for the expression analysis results discussed in the following, which became the focus of the study.

Apart from the results for the canonical pathways discussed above, microarray analysis also revealed downregulation of three channels that are very important for water and potassium homeostasis in brain. These were the astrocytic gap-junction channel connexin 43 (Cx43), the water channel Aqp4, and the astrocytic inward-rectifying potassium channel (Kir) subunit Kir5.1 (Lichter-Konecki et al 2008). It was plausible that their function could be directly influenced by extracellular ammonium concentrations given the effect ammonium has on potassium channels (see below). Because of this and because of their important function for potassium and water homeostasis in brain, as well as for inter-astrocyte signalling, the expression changes of these three channels were

analysed further. They were investigated by qRTPCR as an independent second method using new samples. qRTPCR confirmed significant downregulation of all three channels as well as of the Kir4.1 subunit of the astrocytic inward-rectifying potassium channel. Furthermore, expression of these channels at the RNA level was investigated in animals with different amounts of arginine in their diet and a diet effect could be ruled out as the cause of the expression changes observed for these channels in the hyperammonaemic *spf* mice (Lichter-Konecki et al 2008).

The astrocytic gap junction channel connexin 43

As described above, dispersion of molecules such as glutamate and potassium, through the gap junctions of the astrocyte syncytium, serves a protective function by maintaining potassium homeostasis in brain and by attenuating peak extracellular glutamate levels. The degree of this dispersion can be regulated by changing gap junction permeability or gap junction gene expression.

The primary gap junction channels of astrocytes in the brain are formed by Cx43 protein subunits (Dermietzel et al 1991; Iacobas et al 2004). Astrocytic gap junctional intercellular communication (GJIC) was not previously analysed in hyperammonaemic states. Alterations in Cx43 expression have, however, been detected in other neurological disorders (Huntington and Alzheimer diseases) by microarray analysis (Iacobas et al 2004), and decreased expression was associated with increased neuronal apoptosis in cerebral ischaemia (Nakase et al 2003).

Rouach and colleagues (2000) showed that neuronal activity increases astrocytic Cx43 expression. Increased immunoreactivity for Cx43 was also seen 2 days after mild ischemic injury in rat hippocampus and striatum (Hansson et al 2000). Experimental evidence that astrocytic GJIC may not always serve a protective function for the brain but can also lead to spread of injury was published by Lin and colleagues (1998), who showed *in vitro* that astrocytic gap junctions can propagate cell injury.

Besides the role that astrocytes may play in brain damage caused by acute HA, these cells may also be involved in brain injury caused by chronic HA. Processes of astrocytes envelop synapses and astrocytes play an important part in the regulation of neurotransmitter levels and levels of other signalling molecules in the perisynaptic space (Haydon 2001). Their gap junctions are not only permeable by the neurotransmitter glutamate and by potassium ions but also by such important signalling molecules as calcium ions as well as other molecules. Because of these properties, astrocytes are considered to be intimately involved in synaptic transmission and synaptic plasticity and one can conclude that their functional state may affect learning and memory (Robitaille 1998; Frisch et al 2003; Allen and Barres 2005). It is thus conceivable that decreased gap junctional connectivity when present in the chronic disease state could contribute to the development of intellectual disability, one of the long-term effects of chronic HA in children.

Inward rectifying potassium channels Kir4.1/Kir4.1 and Kir4.1/Kir5.1

Following neuronal activity, astrocytic Kir channels are thought to siphon increased extracellular potassium towards sinks, such as capillaries, and to set the resting membrane potential of the cell (Neusch et al 2001). The heteromeric potassium channel Kir4.1/Kir5.1 and the homomeric channel Kir4.1/Kir4.1 are considered to be the main astrocytic Kir channels facilitating potassium uptake (Hibino et al 2004; Kofuji et al 2000). In the mouse neocortex, the heteromeric Kir4.1/Kir5.1 channel was detected perivascularly and both the homomeric Kir4.1/Kir4.1 and the heteromeric Kir4.1/Kir5.1 channels were shown to be present in perisynaptic astrocytic processes (Hibino et al 2004). Neither of these channels was found in neurons (Hibino et al 2004). Kir4.1 and Kir5.1 also co-localize with Cx43 and Aqp4 channels in astrocytic endfeet. There is experimental evidence that Kir4.1 and Aqp4 form clusters at the plasma membrane through binding of these channels to the dystrophin glycoprotein complex (DGC; Hibino et al 2004; Kofuji and Newman 2004). The co-localization of Kir4.1, Kir5.1, Cx43 and Aqp4 in astrocytic endfeet at capillaries and at the pia implies that regulation of extracellular potassium levels may be coupled with water transport (Hibino et al 2004; Nagelhus et al 2004). Furthermore, the fact that Cx43, Aqp4 and Kir4.1/Kir5.1 channel expression showed a similar response to HA also supports co-regulation of these three channels.

As reviewed by Marcaggi and Coles (2001) and more recently observed by Westhoff and Wylie (2006), the ammonia ion can mimic and replace the potassium ion at channels, transporters and receptors and has been shown to traverse aquaporins (Holm et al 2005). The changes in the expression of three channels that facilitate potassium and water homeostasis in brain may therefore be a direct result of increased amounts of ammonium ions in brain. Given their potential permeability for ammonium ions, astrocytes may downregulate Cx43, Aqp4 and Kir4.1/Kir5.1 channel

gene expression to reduce ammonium uptake at the brain vasculature and thereby protect the brain. Both Kir4.1/Kir5.1 and Cx43 are important for spatial buffering of K^+ ions (Sontheimer et al 1990) and for their siphoning from a site of neuronal activity to the vasculature where K^+ ions are expelled into the bloodstream through the Kir4.1/Kir5.1 channel and water through the Aqp4 channel. A downregulation of these channels that would be protective for the brain with regard to ammonia uptake would in turn interfere with K^+ and water homeostasis and could lead to the development of brain oedema due to water retention and to widespread depolarization of neurons caused by elevated extracellular potassium levels. An acute consequence of neuronal depolarization could be increased neuronal activity and seizures (Basile 2002).

The observation of decreased expression of Aqp4 during HA in mouse brain astrocytes in the study described differed from a previous report about Aqp4 expression in cultured astrocytes. Rama Rao and colleagues (2003b) demonstrated an increase of Aqp4 expression in astrocyte cultures exposed to ammonia. This discrepancy may be due to the difference between the *in situ* and the *in vitro* systems, as the complex interplay between the perisynaptic space, the astrocyte syncytium, and the brain vasculature will only be revealed when studying cells from intact tissue. It is also possible that the cell culture system models a different phase of HAE as Aqp4 expression changes during the course of specific brain injuries as described for focal ischemia (Kiening et al 2002). In oedematous brain *Aqp4* was found to be upregulated, while *Kir4.1* was upregulated in damaged brain (Saadoun et al 2003). Reactive astrocytes surrounding a glial scar were reported to show enhanced expression of Kir channels, suggesting that they may buffer potassium away from the lesion (Bordey et al 2000).

The study described here analysed expression changes at the RNA level. To what degree the differences in RNA levels influence the functionality of the channels examined has to be investigated by follow-up studies. Furthermore, the analysis focused on astrocytes as they are assumed to be the primary site of ammonia toxicity in brain. If ammonia also traverses other potassium channels and interacts with cation transporters and receptors, then the function of other cell types in the brain should also be significantly altered during HAE.

In summary, gene expression profiling of astrocytes from hyperammonaemic mice indicated that the brain damage of acute hyperammonaemic encephalopathy may be caused by a protective regulatory response of the astrocyte syncytium to high blood ammonia levels, downregulating channels that could be used by ammonia to enter the brain. This downregulation could in turn have the negative consequence of water retention and extracellular potassium elevation and result in brain oedema and neuronal dysfunction. Chronic downregulation of these channels could cause perturbation of neuronal function because of chronic elevation of potassium ions in the extracellular space (Fickbohm and Willard 1999). Neuronal function could further show long-term alteration because of reduced inter-astrocyte signalling through lesser astrocytic gap junctions (Allen and Barres 2005; Panatier et al 2006; Robitaille 1998). If this is correct Cx43, Aqp4, Kir4.1 and Kir5.1 would be important targets for pharmacological strategies aimed at arresting or preventing acute and chronic HAE.

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