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The effects of ammonia and portal-systemic shunting on brain metabolism, neurotransmission and intracranial hypertension in hyperammonaemia-induced encephalopathy

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Background/Aims: The pathogenetic factors contributing to encephalopathy in portacaval shunted rats with hyperammonaemia were studied.

Methods: Hyperammonaemia was induced by ammonium-acetate infusions in portacaval shunted rats (2.8 mmol·kg bw⁻¹·h⁻¹; AI-portacaval shunted rats) and in sham-portacaval shunted rats (6.5 mmol·kg bw⁻¹·h⁻¹; AI-NORM rats). Severity of encephalopathy was quantified by clinical grading and EEG spectral analysis. Changes in brain metabolites were assessed by amino acid analysis of brain cortex homogenates, whereas changes in amino acids with neurotransmitter activity were assessed in cerebrospinal fluid; brain water content was measured by subtracting dry from wet brain weights and intracranial pressure was measured by a pressure transducer connected to a cisterna magna cannula.

Results: Although similar increased blood and brain ammonia concentrations were obtained in both experimental groups, only AI-portacaval shunted rats developed encephalopathy, associated with a significant increase in intracranial pressure.

Conclusions: These results indicate that hyperammonaemia alone does not induce encephalopathy, whereas portal-systemic shunting adds an essential contribution to the pathogenesis of encephalopathy. It is hypothesised that the larger increase in brain glutamine in AI-portacaval shunted rats than in AI-NORM rats is responsible for increased brain concentrations of aromatic amino acids, for cell swelling and for extracellular release of glutamate and aspartate. This might promote encephalopathy. If cell swelling is not restricted, intracranial hypertension will develop.

Other significant differences were: higher concentrations of brain glutamine and aromatic amino acids, higher concentrations of cerebrospinal fluid glutamine, aromatic amino acids, glutamate and aspartate in AI-portacaval shunted rats than in AI-NORM rats.

Key words: Aromatic amino acids; Cell swelling; Cerebrospinal fluid; Glutamate; Glutamine; Hepatic encephalopathy; Hyperammonaemia; Intracranial pressure; Portacaval shunting; Rat.
which can arise separately or in combination (for review see Ferenci et al. (7)).

1. The vasogenic type, which is due to an increased permeability of the blood brain barrier (BBB), resulting in an increase in extracellular fluid (8,9). The permeability of the BBB may be increased by toxins like ammonia (10,11).

2. The cytotoxic type, which results in an accumulation of intracellular fluid, as a consequence of insufficient cellular osmo-regulation (9,12-14). Increased intracellular water content may be caused by an increased cellular concentration of glutamine, a well-known intracellular osmolyte.

Takahashi et al. (5) and Blei et al. (4) have demonstrated that inhibition of brain glutamine synthase (GS) by L-methionine sulfoximine (MSO) in rats with hyperammonaemia results in a decrease of brain oedema and intracranial pressure (ICP), but normalisation of brain water content and ICP was not achieved. This suggests that other, unknown factors may play a role. Furthermore, the effect of decreasing brain glutamine concentration on the severity of encephalopathy could not be determined, because of anaesthesia of the animals in these studies.

For these reasons, this study was designed to determine other factors contributing to the pathogenesis of hyperammonaemia-induced encephalopathy associated with intracranial hypertension. This was achieved by comparing two experimental rat models with equal hyperammonaemia: encephalopathic PCS rats infused with ammonium-acetate (referred to as AI-PCS rats), and non-encephalopathic sham-PCS rats with similar increased blood and brain ammonia concentrations achieved by a higher amount of ammonium-acetate infusion (referred to as AI-NORM rats).

In contrast to other studies (4,5), we quantified the severity of encephalopathy by clinical grading and EEG spectral analysis. Possible contributing factors could be detected by determination of amino acid and ammonia concentrations in blood, cerebral cortex (mainly reflecting brain metabolism) and in cerebrospinal fluid (CSF, representative for neurotransmission). Intracranial hypertension was assessed by a pressure transducer connected to a cisterna magna cannula, and brain oedema was assessed by subtracting dry from wet brain weights.

**Materials and Methods**

**Animals and surgical procedures**

Male Wistar rats (200–300 g, HSD, Zeist, The Netherlands; 12-h light cycle: 8 a.m. – 8 p.m.) were used, and animal welfare was in accordance with the guidelines of the University of Amsterdam.

One week before the ammonium-acetate infusion, a portacaval shunt (PCS) was performed in rats under ether anaesthesia according to the technique of Lee et al. (15). In “control” rats a sham-PCS operation was performed, in which the abdomen was opened and the portal vein clamped for 7.5 min, after which the abdomen was sutured. PCS and sham-PCS rats were kept in separate cages. PCS rats were fed standard laboratory chow (RMH 1410 Hope Farms, The Netherlands) and water *ad libitum*, and sham-PCS rats were pair-fed with the PCS rats.

On the day of the infusion the carotid artery and the jugular vein of PCS or sham-PCS rats was cannulated under ether anaesthesia in order to take blood samples from the artery and to give an intravenous ammonium-acetate infusion. At the time when the rats had recovered from anaesthesia, PCS rats received a bolus injection of ammonium-acetate (0.5 mmol/kg body weight), after which an ammonium-acetate infusion of 2.8 mmol·kg·bw⁻¹·h⁻¹ was given for 6 h. Sham-PCS rats received a higher dose of ammonium-acetate infusion (6.5 mmol·kg·bw⁻¹·h⁻¹) in order to obtain the same steady-state blood ammonia concentrations as in the PCS rats (1000–1300 μM). The pH of the ammonium-acetate solutions was 7.3–7.4. During the infusion, body temperature was monitored rectally and was maintained between 36°C and 37°C with the aid of a heating lamp.

Thus, two different rat models were compared with each other: PCS rats and Sham-PCS rats with *similar* increased steady state blood concentrations of ammonia. These two models are referred as AI-PCS rats vs. AI-NORM rats.

**Experimental procedures**

Three different series of experiments were performed in both groups:

In the *first series of experiments* (*n=6/7 per group*) the development of encephalopathy was determined by clinical grading of encephalopathy, together with
EEG spectral analysis. Encephalopathy was graded clinically according to the level of consciousness (Table 1).

Five days before a (sham)-PCS was performed four golden skull electrodes were implanted as published previously (16). EEG spectral analysis was carried out for four EEG power band regions within the range of 1-26.5 Hz. After Fourier transformation power density spectra were obtained. Parameters calculated for the range 1-26.5 Hz were the EEG left index and mean dominant frequency (MDF). EEG left index was defined as the logarithm of the ratio between the power of the low frequency (1-7.4 Hz) and the high frequency (13.5-26.5 Hz). Normal rats show an EEG left index of ~ 0.60 and rats in coma ~ 0.80-0.90. These logarithmic values are comparable with the linear values of 5-10 for normal rats and 15-25 for rats in coma, published by our group previously (17). During the development of hepatic encephalopathy MDF decreases from 16 to 18 Hz to 11-13 Hz (own observations).

In the second and third series of experiments, a cisterna magna cannula was implanted according to the technique of Boer et al. (18), 3 days before the ammonium-acetate infusion was given. After the rat was anaesthetised (AI-PCS rats 30 mg/kg bw and AI-NORM rats 60 mg/kg bw nembutal i.p.), a stainless-steel cannula (o.d. 0.8 mm) was positioned by hand and fixed to the skull by a round plate soldered at an angle of 45° using dental cement.

In the second series of experiments (n=5 per group) aliquots of 50 μl cerebrospinal fluid (CSF) from a cisterna magna cannula were taken at the start and at the end of ammonium-acetate infusion for measurement of ammonia and amino acid concentrations. CSF samples for amino acid determinations were frozen in liquid nitrogen immediately after sampling, and stored at -70°C until required for HPLC analysis.

In the third series of experiments (n=10/13 per group) the cisterna magna cannula was connected to a pressure transducer (Viggo-Spectramed DTX/Plus™ disposable transducer kit) and ICP was monitored at the start and at the end of the infusion.

At the end of the third series of experiments, rats were sacrificed by exsanguination under complete ether anaesthesia. The cerebrum was promptly removed and the wet weight was measured on an analytical balance. It was then placed in an oven of 160°C for 72 h. Brain water content was calculated by subtracting dry from wet brain weights.

In addition, brain water content of six normal rats was measured in order to quantify possible brain oedema of AI-NORM and AI-PCS rats. Finally, ICP and brain water content were also measured in another four PCS rats, to whom sodium-acetate infusion was given with the same osmolarity as ammonium-acetate (referred as NaI-PCS).

Biochemical and blood pressure determinations
In all series of experiments blood samples were taken from the carotid artery in order to measure blood ammonia concentration (0.05 ml every hour) or plasma amino acid concentrations (0.2 ml, at the start and at the end of the experiment). Mean arterial blood pressure was also measured via the carotid artery every hour, using a pressure transducer (Viggo-Spectramed DTX/Plus™ disposable transducer kit). In the first and second series cerebral cortex was separated from white matter and frozen in liquid nitrogen. Frozen cortex and white matter were stored at -70°C until determination of ammonia and amino acids in cerebral cortex.

Ammonia concentration was measured with the Blood Ammonia Checker II (Kyoto Daiichi Kagaku Co. Ltd.) (19). Amino acid concentrations were measured by HPLC analysis as described by van Eijk et al. (20).

Statistical analysis
Results are presented as means ±SEM. Statistical analysis was performed by means of repeated measurement analysis of variance (ANOVA) for blood ammonia concentrations, clinical grading and EEG activity, and Student’s t-test for blood ammonia concentration at the start of the infusion, brain and CSF ammonia concentrations, amino acid concentrations, ICP and brain water content. P-values<0.05 were considered to be significant.

Results
General aspects
Both AI-PCS and AI-NORM rats showed a decrease in body weight of ~ 15% at 1 week after (sham)PCS operation. However, the liver/body weight ratio was lower in AI-PCS rats: 2.00±0.09% vs. 3.12±0.12% in AI-NORM rats, indicating liver atrophy induced by portacaval shunting.

Blood ammonia concentration in AI-PCS rats was significantly higher at the start of the ammonium-acetate infusion than in AI-NORM rats (408±20 μM vs. 47±9 μM, p<0.0001), but after 1 h infusion similar steady-state concentrations of 1000 to 1300 μM were obtained in both groups (Fig. 1). Brain ammonia concentrations at the end of the
experiment were similar in both groups (Table 3). CSF ammonia concentration also showed only a significant difference at the start of the ammonium-acetate infusion (Table 4). The mean arterial pressure was similar in both groups, and varied between 90 and 100 mmHg during the whole experiment.

Severity of encephalopathy
Clinical grading of encephalopathy and EEG spectral analysis revealed that only the AI-PCS rats developed encephalopathy. AI-PCS rats reached clinical grade 3 after 6 h ammonium-acetate infusion compared to clinical grade 1 in AI-NORM rats (p<0.0001, Fig. 2). EEG left index in AI-PCS rats increased from 0.590±0.023 to 0.855±0.058, whereas EEG left index in AI-NORM rats did not exceed 0.70 (p<0.0001, Fig. 3). The profile of MDF also differed significantly between the groups: MDF in AI-PCS rats showed a decrease from 17.9±0.5 to 12.9±0.8, but MDF in AI-NORM rats remained more or less constant with a value of 15.0 to 16.0 (p<0.0001, Fig. 4.)
Amino acid concentrations in CSF
Analysis of amino acids in CSF revealed that Asp, Glu, Gln, Tyr, Phe and Trp were all significantly increased 1 week after PCS compared to 1 week after sham-PCS (Table 4). They were all further increased in AI-PCS rats after 6 h ammonium-acetate infusion and were at that time all significantly higher than in AI-NORM rats, which also showed a significant increase in these amino acids after 6 h infusion. Other amino acids did not show significant differences between the two groups (data not shown).

Intracranial pressure and brain water content
Figure 5 shows a different profile of ICP in AI-PCS rats compared to AI-NORM rats. ICP in AI-PCS rats at the end of the experiment was significantly higher than in AI-NORM rats (p<0.01). After ammonium-acetate infusion, ICP increased in 9 of the 13 AI-PCS rats in which values of 15 to 20 mmHg were obtained, whereas ICP in most AI-NORM rats remained constant with a maximal value of 6 mmHg.

Table 4
CSF amino acid and ammonia (NH₃) concentrations in AI-PCS rats and AI-NORM rats at the start of the ammonium-acetate infusion (t=0 h) and at the end of the infusion (t=6 h). Values are expressed in μM as mean±sem.

<table>
<thead>
<tr>
<th></th>
<th>t=0 h</th>
<th>t=6 h</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>AI-NORM</td>
<td>AI-PCS</td>
</tr>
<tr>
<td></td>
<td>n=5</td>
<td>n=5</td>
</tr>
<tr>
<td>Asp</td>
<td>2.85±0.53</td>
<td>4.82±0.65°</td>
</tr>
<tr>
<td>Glu</td>
<td>7.05±1.47</td>
<td>28.98±0.65°</td>
</tr>
<tr>
<td>Gln</td>
<td>469.2±30.1</td>
<td>2543±494.0°</td>
</tr>
<tr>
<td>Tyr</td>
<td>5.57±0.41</td>
<td>25.90±4.46°</td>
</tr>
<tr>
<td>Phe</td>
<td>4.24±0.46</td>
<td>18.32±2.70°</td>
</tr>
<tr>
<td>Trp</td>
<td>1.02±0.21</td>
<td>4.40±0.41°</td>
</tr>
<tr>
<td>NH₃</td>
<td>24.6±3.28</td>
<td>172±13.7°</td>
</tr>
</tbody>
</table>

° p<0.05 AI-PCS at t=0 or 6 h vs. AI-NORM at t=0 h.
°° p<0.01 AI-PCS at t=0 or 6 h and AI-NORM at t=6 h vs. AI-NORM at t=0 h.
°°° p<0.05 AI-PCS at t=6 h vs. AI-NORM at t=0 h.
°°°° p<0.01 AI-PCS at t=6 h vs. AI-NORM at t=0 h.
°°°°° p<0.05 AI-PCS at t=6 h vs. AI-NORM at t=6 h.
°°°°°° p<0.01 AI-PCS at t=6 h vs. AI-NORM at t=6 h.

Table 5
Brain water content in normal rats (NORM), PCS rats that had received Na-acetate infusion (NaI-PCS), AI-NORM rats and AI-PCS rats. Values are expressed as mean±sem.

<table>
<thead>
<tr>
<th></th>
<th>NORM</th>
<th>NaI-PCS</th>
<th>AI-NORM</th>
<th>AI-PCS</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n=6</td>
<td>n=4</td>
<td>n=10</td>
<td>n=13</td>
</tr>
<tr>
<td>Brain water%</td>
<td>80.9±0.07</td>
<td>81.16±0.09°</td>
<td>81.39±0.15°</td>
<td>81.99±0.15°</td>
</tr>
</tbody>
</table>

° p<0.05 NaI-PCS or AI-NORM vs. NORM.
°° p<0.01 AI-PCS vs. NORM.
°°° p<0.001 AI-PCS vs. NaI-PCS or AI-NORM.
Table 5 shows that the brain water content of all AI-PCS rats was significantly higher compared to normal rats and compared to AI-NORM rats. The brain water content of AI-NORM rats was also significantly higher compared to normal rats. In addition, brain water content of PCS rats infused with Na-acetate (NaI-PCS rats, which did not develop encephalopathy, data not shown) was significantly higher than that of normal rats, significantly lower than that of AI-PCS rats, but not significantly different from that of AI-NORM rats.

Discussion

In agreement with other studies (21,22), portacaval shunting in male Wistar rats resulted in a decrease in liver/body weight ratio to ~0.02, indicating liver atrophy. Since the sham-operated rats (AI-NORM group) were pair-fed with the PCS rats (AI-PCS group), possible influences of nutrient deficiencies on the different parameters measured, were equalised.

Both groups showed severe hyperammonaemia with blood ammonia concentrations of 1000–1300 µM. This was reflected in an ~3-fold higher cerebral cortex concentration of ammonia, which is in agreement with others (see review, Mousseau et al. (23)). However, only the AI-PCS rats developed encephalopathy, as indicated by the increase in clinical grading and in EEG left index, and by the decrease in MDF. From this observation it can be concluded that ammonia itself is not the sole factor that causes encephalopathy, whereas portal-systemic shunting adds an important contribution to the development of hyperammonaemia-induced encephalopathy. We addressed the interesting question of which differences at the cerebral level between both groups could be responsible for the development of hyperammonaemia-induced encephalopathy.

Our data show the following significant cerebral differences in AI-PCS rats compared to AI-NORM rats after 6 h severe hyperammonaemia:

1. Higher concentrations of brain glutamine and aromatic amino acids (AAA), 2. higher concentrations of CSF glutamine, glutamate, aspartate and AAA, and 3. increased intracranial pressure and higher brain water content.

Ad 1. Higher concentrations of brain glutamine and aromatic amino acids

It is well known that an increase in brain glutamine and AAA concentrations is already present after 1 day of PCS in rats (21,24), and that the increase is even more pronounced in (sub)acute liver failure with brain glutamine concentrations of 20 to 25 mM (4,17,25,26). The following explanations are available for the rise in brain glutamine concentration after PCS: a) Increased brain glutamine synthesis by glutamine synthase (GS); b) Inhibition of brain glutaminase activity; and c) Decreased glutamine efflux from brain to blood. The last explanation seems unlikely, since it has been suggested that glutamine efflux is increased after portal-systemic shunting (27). Furthermore, de Jong et al. (28) have shown that glutamine efflux in PCS rats is increased. The other two explanations are both possible, because increased brain ammonia concentrations can inhibit glutaminase activity (Kᵢ value of ammonia is 180 µM, 30). But it has been suggested that GS activity is already maximal at normal brain ammonia levels (0.2–0.3 mmol/kg ww), and several studies have found no change or even less GS activity in brain tissues of PCS rats (31,32).

However, GS activity is usually measured in vitro under optimal conditions with enzyme-saturating concentrations (4 mM NH₄Cl, 20 mM glutamate, 10 mM ATP, 10 mM Mg²⁺, 31, 32), whereas brain concentrations of glutamate, ATP, Mg²⁺ and Mn²⁺ in vivo are significantly lower: 11–14 mM, 2.5 mM, 0.7 mM and <4 µM, respectively (33). This is in agreement with a ¹⁵N-MRS study of Kanamori et al. (34,35), which showed that the in vivo activity of brain GS in rats (3.5 µmol·h⁻¹·g⁻¹) is only 1–2% of the in vitro activity (275–350 µmol·h⁻¹·g⁻¹), which suggests that substrates and/or co-factors other than ammonia kinetically limit GS activity in vivo. A recent clinical study of Krieger et al. (36) has shown that blood and brain concentrations of manganese, one of the co-factors of GS, are increased in chronic HE. Therefore, it can be concluded that portal-systemic shunting can induce increased cerebral glutamine concentrations by inhibition of glutaminase through increased ammonia concentrations and/or by stimulation of glutamine synthesis through increased ammonia and GS co-factor concentrations.

Increased brain concentrations of AAA can be partially explained by increased plasma concentrations of AAA, which compete with other neutral amino acids for the BBB transport system of neutral amino acids. Furthermore, Jeppsson et al. (37) showed a positive correlation between brain Gln and brain AAA concentrations in PCS rats.

Ad 2. Higher concentrations of CSF glutamine, glutamate, aspartate and AAA

The significantly higher concentrations of CSF glutamate, aspartate, glutamine and AAA concentrations...
in AI-PCS rats compared to AI-NORM rats may also be explained by the larger increase in cerebral cortex glutamine concentration in these rats. The large increase in intracellular osmolyte glutamine induces a small release of glutamine, glutamate and aspartate to the extracellular compartment. Although the extracellular concentrations of the last two amino acids were in the range of μM, release of Glu and Asp has also been demonstrated in other studies in which cell swelling (see ad 3.) was induced (38,39). Increased CSF concentrations of AAA are most likely the consequence of increased brain concentrations of AAA, as discussed above (see ad 1).

The increase in extracellular concentrations of the above-mentioned amino acids in hyperammonaemia-induced encephalopathy is in agreement with other experimental studies who studied (sub)acute encephalopathy (40-43). However, Rao et al. (44) did not find similar increases. A possible explanation for this discrepancy is that this group studied rats 4 weeks after PCS, which makes it very likely that formation of numerous collaterals to the liver had given rise to re-vascularisation of the liver and diminished portal-systemic shunting. Moreover, they induced hyperammonaemia by an intraperitoneal injection of 3.85 mmol/kg bw ammonium acetate. This was followed by deep coma after 40 min and was reversible after 100 min.

Ad 3. Increased intracranial pressure and higher brain water content
A small increase in brain water content was already present in PCS rats and in sham-PCS rats with hyperammonaemia, the latter in agreement with other studies (4,5,10,11,45). Blei et al. did not show an increase in brain water content in rats, 1 day after a PCS. Nevertheless, our results indicate that hyperammonaemia itself can induce some degree of brain oedema. The increase in brain water content was highest in rats with hyperammonaemia-induced encephalopathy (i.e. an increase of ±1.00% compared to normal rats, comparable with the data of Blei et al. (4)) and was of such a magnitude that intracranial hypertension could develop.

Both the increase in brain glutamine and the increase in CSF glutamate and aspartate may be responsible for the increase in brain water content and ICP in AI-PCS rats, probably resulting in cytotoxic brain oedema. Since glutamine is a well-known intracellular osmolyte, an increase of this amino acid can result in cell swelling, as has been shown by Norenberg et al. (46). Thus, the higher increase in brain glutamine concentrations in AI-PCS rats may explain the more pronounced development of brain oedema, resulting in intracranial hypertension. Another possibility might be that in PCS rats the compensatory mechanisms for maintenance of cell volume are more exhausted than in control rats because of the longer standing hyperammonaemia. An extra ammonia challenge in such PCS rats would produce HE more quickly. The possible role of glutamine in the development of brain oedema in rats with ammonia intoxication has been studied by other groups, who showed that brain oedema could be partially diminished by decreasing brain glutamine concentration by use of the GS inhibitor L-methionine sulfoximine (4,5). The increase in CSF glutamate and aspartate may also cause cell swelling. Increased extracellular concentrations of glutamate and aspartate, both excitatory neurotransmitters which can bind the N-Methyl-D-Aspartate receptor (NMDA), may result in NMDA-mediated glutamate neurotoxicity including cell swelling (47,48). Since NMDA receptors have been demonstrated on neurons (49), astrocytes (50) and on brain capillaries (51), this phenomenon could contribute to the increase in ICP in AI-PCS rats.

Based on these results, we would like to propose the following hypothesis concerning encephalopathy in PCS rats with severe hyperammonaemia: shunting around the liver will result in an increase in the systemic circulation of several factors that are normally metabolised in the liver, and other substances that are usually excreted into the bile. As a direct consequence, brain GS might be stimulated for instance by ammonia and manganese (see Krieger et al. (36)).

The extra large increase in brain glutamine concentration in AI-PCS rats will promote encephalopathy by induction of cell swelling, by extracellular release of glutamate and aspartate and by increased brain concentrations of AAA, or by exhaustion of compensatory mechanisms of cell volume regulation. Since AAA are precursors of the monoamines, elevated brain AAA concentrations may result in altered activity of these neurotransmitters, although the literature on this aspect is conflicting (review by Bergeron et al (52)). A possible consequence of increased extracellular concentrations of glutamate and aspartate is NMDA-receptor overstimulation, promoting cell swelling and finally even neuron degeneration. If cell swelling is not restricted, life-threatening intracranial hypertension will develop.

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