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Research Article

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# Cerebral Expression of NKCC1 in Rats with Acute and Chronic Hyperammonemia

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## Abstract

Acute Liver Failure (ALF) is a severe medical condition, primarily defined by high plasma-ammonia levels, multiorgan failure and development of Hepatic Encephalopathy (HE), while chronic liver failure is defined by chronic low level hyperammonemia and development of HE. Newly research showed that increased cerebral expression of the ion-channel $Na^+$, $K^+$, $Cl^-$ co-transporter I (NKCC1) is associated with brain edema development in several medical clinical conditions. We aimed to determine if brain cortical expression of NKCC1 and the level of its phosphorylated activated state, phos-NKCC1, were associated to increased brain water content, increased Intracranial Pressure (ICP), and development of HE in acute and chronic hyperammonemia. As a model of acute hyperammonemia, we used porta-cava anastomosed rats infused with ammonia (PCA). As a model of chronic hyperammonemia and chronic liver failure, we used rats receiving bile duct ligation over 28 days (BDL28). The PCA group developed brain edema and high ICP, though brain level of NKCC1 and phos-NKCC1 were stabile. In chronic liver failure, we found low level HE and stabile brain water content and ICP, while the level of phos-NKCC1 was elevated 2.6 times the PCA-CONTROL ($p<0.001$), and stabile level of NKCC1. From these findings, we conclude that phos-NKCC1 could be involved in development of HE in chronic hyperammonemia. Regarding acute hyperammonemia, our data indicates that NKCC1 and phos-NKCC1 are without pathophysiological impact on the observed brain edema and high ICP.

## Keywords

Phos-NKCC1; Intracranial pressure; Bile duct ligation; Porta cava anastomosis; Aquaporin-4

## Abbreviations

ALF: Acute Liver Failure; HE: Hepatic Encephalopathy; NKCC1: $NA^+$; $K^+$, $Cl^-$ Co-transporter I; Phos-NKCC1: Phosphoryleret NKCCI; PCA: Porta Cava Anastomosis; BDL28: Bile Duct Ligated rats 28 days; ICP: Intracranial Pressure; Aqp4: Aquaporin-4; BBB: Blood Brain-Barrier; Wb: Western blotting

## Introduction

Acute Liver Failure (ALF) is associated with a high risk of Hepatic Encephalopathy (HE) and brain edema, which may proceed into brain herniation and death. The pathophysiology behind this condition has been explored in decades. High arterial ammonia concentration [1-3], high cerebral glutamine and alanine levels [4,5], and elevated levels of pro-inflammatory cytokines [6], have been suggested to be key components in the formation of brain edema. Yet, it remains unknown how water circulates in between the brain compartments in HE and brain edema in the event of ALF [7], and why patients with chronic liver failure are at high risk of developing HE and a low degree brain edema [8]. In the clinical setting, increased attention in decreasing the plasma ammonia level in ALF has emerged, in an attempt to decrease the risk of brain edema and death [9], which understates the importance of ammonia in this condition. *In vitro* studies with astrocytic cells exposed to high ammonia concentration showed up regulation of NKCC1 in the cellular plasma-membrane [10], and recent molecular research showed that the ion-channel NKCC1 influences on development of brain edema observed in animal studies of both traumatic, ischemic, and cytotoxic brain edema [11-14]. NKCC1 is an electroneutral co-transporter of the ions $Na^+$, $K^+$ and $Cl^-$ into the cells, mediating secondary osmotically driven influx of water molecules by channels with water transporting properties, such as Aquaporin-4 (aqp4) [14]. NKCC1 is widely expressed in the body and exerts crucial cellular ionic homeostatic functions [14]. In the brain, NKCC1 is expressed in several different cell types, such as endothelial cells, glial cells, epithelial cells of the plexus choroideus, and the neurons. The main function of NKCC1 in the brain is believed to be regulation of cellular volume, while in endothelial cells, they also participate in the regulation of water flow across the Blood Brain-barrier (BBB) [14].

The activity of NKCC1 is regulated by phosphorylation (phos-NKCC1) [13,15], which mediates translocation of NKCC1 to the cellular plasma membrane, where it performs its action. The state of hyperammonemia observed in both acute and chronic liver failure, has in *in vivo* animal experiments shown increased oxidative/nitrosative stress and increased activity of several different protein-kinases, such as oxidative stress response kinase and Mitogen-activated Protein Kinases (MAPK's) [6,13]. These proteins and their respective pathways are believed to be partly responsible for the phosphorylation of NKCC1, and thereby, regulation of its activity.

In this study, we focused on the impact of hyperammonemia on the brain function, exploring the function of specific ion channels. We aimed to determine if the expression of NKCC1 and the level of phos-NKCC1 were associated to alterations in brain water content, and/or intracranial pressure (ICP), and development of HE in rat models of acute and chronic hyperammonemia, resembling acute and chronic liver failure. We hypothesise that both acute and chronic hyperammonemia may induce increased expression, and/or activation of NKCC1 in the brain, and that these alterations could be important pathophysiological factors in the development of brain edema, high ICP, and HE in these conditions.

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## Methods

### Animal models

We performed a rat model of hyperammonemia, the Porta Cava Anastomosis plus ammonia model (PCA), and a model of chronic liver failure with chronic hyperammonemia, the 28 days Bile Duct Ligation group (BDL28). We used three months old male Wistar rats (biologically approved) (Charles River, Sulzfeld, Germany), with initial weight of 239-350 gms. The animals were housed in cages with free access to food and water, and kept at 12:12-hour's light/dark cycle, with constant temperature and humidity. The animals rested for three days before the experimental procedures were performed. A total of 29 Wistar rats were divided into four groups. Power analysis estimating "N" were performed: N=16 for each group. All animals survived the surgical procedure, and all animal experiments were performed in accordance with the European Communities Council Resolves of 24 November 1986 (86/609/EEC), and approved by the Danish State Research Inspectorate (J. No. 2006/561-1244 and 2009/561-1632).

### Treatment groups

1) Porta-cava anastomosis plus ammonia infusion, (PCA), (N=7)
2) Control group corresponding to the PCA model, (PCA-CONTROL), (N=7)
3) Bile duct ligation, 28 days, (BDL28), (N=8)
4) Control group corresponding to the BDL28 model, (BDL28-CONTROL), (N=7)

The reason for discrepancy in N, in between the groups 3 and 4, were due to errors in the plan of the experiment. No deaths of animals were recorded, and no selection where performed.

### Experimental procedures

In Isoflurane anaesthesia (Abbott, Scandinavia, Solna, Sweden), the PCA group underwent abdominal surgery with performance of end-to-side V. porta to V. cava anastomosis [16]. Surgery was completed within 15 minutes, and the abdomen was sutured in two layers. In the post-operative period, animals received buprenorphine as analgesic.

In Isoflurane anaesthesia, the BDL28 group underwent abdominal surgery with performance of two double ligations of the hepatic bile duct at ductus hepaticus communis, with a subsequent cut of the structure. Surgery was completed within 15 minutes, and the abdomen was sutured in two layers. In the post-operative period, the animals received buprenorphine as analgesic.

In Isoflurane anaesthesia, the control groups PCA-CONTROL and BDL28-CONTROL, both received sham operations, with opening of the abdomen and exposure of liver tissue to free air for 15 minutes, with subsequent suture of the abdomen in two layers, followed by buprenorphine as analgesic.

24 hours after surgery for the PCA and the PCA-CONTROL groups and 28 days after surgery for the BDL28 and BDL28-CONTROL groups, collection of data were performed. Animals were induced with anaesthesia, initially Isoflurane, in order to perform insertion of a femoral arterial and a venous line, whereafter anaesthesia was continued with Pentobarbital (Mebumal) i.v. The animals were coupled onto a respirator (Hallowel EMC, E-vet, Haderslev, Denmark), placed in a stereotactic frame (Kopf Instruments, Dusseldorf, Germany), followed by insertion of a fluid filled catheter in cisterna magna for measurement of ICP. The PCA group received i.v. infusion of ammonia-acetate ($NH_4^+$) diluted in Phosphate Buffered Saline (PBS), which were conducted at an infusion rate of 55 µmol/kg/min (20 ml/hour). This infusion was continued until the ICP made a steep increase and the blood pressure became un-stable, indicating that the animal was about to incarcerate the brain. The PCA-CONTROL group was treated with infusion of PBS, receiving same volume as the PCA group. Continuous measurement of mean arterial blood-pressure (MAP), ICP and two arterial blood samples were performed under the infusion of ammonia. The PCA and the PCA-CONTROL group were treated with ammonia infusion, for respectively: mean 293 min (SD 12.3) *vs.* 292 min (11.3) ($p>0.05$). In the BDL28 and the BDL28-CONTROL group, measurement of ICP and MAP were performed over a period of approximately 15 minutes.

After the experiment, the animals were sacrificed in anaesthesia and the brain was rapidly removed and split into two halves. Cortex was removed from one hemisphere and placed in fluid nitrogen, while the cortical brain water content was measured from the contralateral hemisphere. Small samples from the liver, approximately 5% of the total mass, were removed and fixated in 1.5% formaldehyde and embedded in paraffin.

### Brain water measurement

Animals were sacrificed in anaesthesia, and brain cortex was dissected and transferred onto pre-weighed glass scales. Wet weight of the cortical brain tissue was measured on a high precision scale (Sartorius AG, Goettingen, Germany), and the sample was dried for 48 hours at 120°C. Dried brain material acclimatized in an exicator, and was subsequently weighed. The percentage of water content of the brain cortex was calculated according to the following formula: ((wet weight−dry weight)/wet weight)×100 [16].

### Histopathologic evaluation of liver damage

Histopathologic evaluation of liver damage was performed by a trained pathologist, using standard light microscopy of Haematoxylin and Eosin stained liver sections. The samples were evaluated concerning following parameters: Apoptosis, parenchymal inflammation/necrosis, portal inflammation and fibrosis, all parameters were graded 0-3. The grading was based on common principals of pathology; no specific criterias were used, except regarding scoring of fibrosis/cirrhosis, which were graded by the Metavir criterias.

Apoptosis was recognized as hepatocytes with condensed, homogenic cytoplasma, and a pycnotic and dark nucleus, often presenting as a rounded cell deadherent from the neighbouring hepatocytes in the trabecules. Inflammation was notified as focal, dispersed areas of aggregates of inflammatory cells (lymphocytes and Kupffer cells), in the sinusoidal spaces and in the trabecules, accompanied by destruction of hepatocytes.

### Evaluation of neurobehavioral changes (hepatic encephalopathy)

HE was evaluated in the BDL28 group, just prior to anaesthesia. The PCA group and PCA-CONTROL groups were not evaluated regarding HE because of the long experimental setup in anaesthesia. The stage of HE was determined by a neuro-behavioral evaluation, much similar to the assessment in humans: stage 1: lethargy; stage 2: mild ataxia; stage 3: lack of spontaneous movement and loss of righting reflex, but still responsive; stage 4: coma and lack of response to pain [17].

### Biochemical analysis of blood samples

Blood samples were centrifuged at 3500 rpm for 10 minutes at 4°C. Plasma concentrations of Alanine aminotransferase (ALT), Ammonium, Creatinine, Bilirubin, Alkaline Phosphatases, Sodium, and coagulation status with International Normalized Ratio (INR) were measured by routine techniques. Glucose was measured on peripheral blood samples. Arterial blood samples were analyzed on a radiometer ABL 500 (Radiometer, Copenhagen, Denmark) regarding pH, oxygen saturation ($sO_2$), $pCO_2$, and $pO_2$.

### Analysis of NKCC1/phos-NKCC1 protein expression by Western blotting

Frozen cortical brain tissue were homogenized in an UltraThurax (IKA, Staufen, Germany) at high speed for 30 seconds on ice, in dissection buffer containing 0.32 M sucrose, 50 mM HEPES buffer (pH-7.4) (Invitrogen™, Tåstrup, Denmark), two mM EDTA (Invitrogen™), one dissolved tablet of protease-inhibitor cocktail completeMINI®, and one tablet of phosphatase inhibitor (Roche Diagnostics, Copenhagen, Denmark). The homogenate was centrifuged in an Eppendorf 5415c centrifuge (Eppendorf, Hamburg, Germany) at 4000×g for 15 minutes at 4°C to remove whole cells, nuclei and mitochondria, and the protein concentration was measured using BIORAD DC Kit (Bio-Rad Laboratories, Copenhagen, Denmark) and a photometer Beckman Coulter DU®730 (Ramcon, Birkerød, Denmark). Protein was loaded onto 4-12% Invitrogen mini-cell-system (Invitrogen™) (150V, 50 minutes) with 20 µg protein per lane. SeeBlue PLUS2 and Magic Mark (Invitrogen™) standard markers were also loaded. Samples were heated prior to loading, and all procedures were performed under denaturing conditions. Protein was transferred to a PVDF-membrane NuPAGE® 4-12% Bis-Tris gel (Invitrogen™) by electroelution (30V, 180 minutes, room temperature with measurement of temperature every 30 min to prevent overheating). After blocking with 5% low fat milk in TBST (10 mM Tris-HCl, 150 mM NaCl, 0.1% Tween 20, pH-7.4) for one hour, the PVDF membrane was incubated overnight at 4°C with the primary antibody diluted in 5% low fat milk and TBST-buffer (Polyclonal antibody R5, a gift from Professor B. Forbush, Yale University, USA; Polyclonal antibody NKCC1, 1:5000, Nordic Biosite, Copenhagen, Denmark). The membrane were subsequently washed in TBST buffer for 30 minutes, and incubated at room temperature for two hours with horseradish peroxidase-conjugated secondary antibody diluted in the same buffer. After incubation, the membrane was washed with TBST for 30 minutes. Finally, detection of bound antibody was performed using the Enhanced Chemiluminescence system (PerkinElmer®, Massachusetts, USA), and the camera detecting system LAS 9000® with software Image Gauge® 2006 (FujiFilm, Stockholm, Sweden). All Western blots were visually controlled with respect to protein loading (Beta-actin polyclonal antibody, #4967, 1:1000, Cell Signaling, Medinova, Birkerød, Denmark).

### Analysis of aqp4 protein expression by Western blotting

Frozen cortical brain tissue was homogenized in a Potter Elverhjem (B. BRAUN, Melsungen, Germany) at high speed for four minutes on ice, in dissection buffer containing 0.32 M sucrose, 50 mM HEPES buffer (pH 7.4) (Invitrogen, Tåstrup, Denmark), two mM EDTA (Invitrogen), and one dissolved tablet of protease-inhibitor cocktail completeMINI® (Roche Diagnostics). The homogenate was centrifuged in an Eppendorf 5415c centrifuge (Eppendorf) at 4000×g for 15 minutes at 4°C to remove whole cells, nuclei and mitochondria. Subsequently, the supernatant was centrifuged at 200000×g in a Beckmann 50.3 Ti Centrifuge (Beckman Coulter, Fullerton, USA) for 30 minutes to produce a membrane-enriched pellet. The resultant pellet was resuspended in 50 mM HEPES buffer, 2% Sodium Dodecyl Sulfate (SDS), and one dissolved tablet of protease-inhibitor cocktail, for 2.5 hours at 20°C. Protein concentration was measured using BIORAD DC Kit (Bio-Rad) and a photometer Pharmacia LKB-Ultrospec.III (GE-Healthcare, Hillerod, Denmark). The membrane enriched protein samples were loaded onto 4-12% gels (Invitrogen). The standard marker SeeBlue PLUS 2 was also applied (Invitrogen). No heating prior to sample loading was performed. All procedures were performed under denaturing conditions. Protein was transferred to PVDF-membranes NuPAGE® 4-12% Bis-Tris gel (Invitrogen™) by electroelution (30V, 60 minutes). After blocking with 5% low fat milk in 10 mM Tris-HCl, 150 mM NaCl and 0.1% Tween 20, pH 7.4 (TBST) for one hour, the PVDF membrane was incubated overnight at 4°C, with the primary antibodies diluted in 5% low fat milk and TBST-buffer (aqp4 polyclonal antibody, SC9888, 1:5000, Santa Cruz Biotechnology). The membranes were subsequently washed in TBST-buffer for 30 minutes, and then incubated at room temperature for two hours, with horseradish peroxidase conjugated secondary antibody diluted in the same buffer (Aqp4, SC2020, 1:5000, Santa Cruz Biotechnology). Detection where performed as stated in the NKCC1 Wb protocol. An internal control was made to ensure equal protein amount in the samples (Beta-actin polyclonal antibody, #4967, 1:1000, Cell Signaling).

### Statistical analysis

All data were analyzed regarding normality by the Kolmogorov-Smirnov test, and were following analyzed by parametric statistic analysis (Student's t-test). $p<0.05$ were considered statistically significant. Statistical power analysis to estimate "N" were perfomed using the formula: $N=(Z_\alpha+Z_\beta)^2\times s^2/(\text{Minimal relevant diff.})^2$, (unpaired testing). Statistical analysis was performed with Sigma Stat software (Systat Software, Washington, USA), and Graphic presentation was set with SigmaPlot software (Systat Software).

## Results

In acute hyperammonemia, the PCA model we showed significantly increased levels of p-ammonia, brain water content, and ICP. Liver pathology showed non-inflammatory liver injury with centrilobular ischemia with various involvement of the lobules, and modest neutrophili (N=2). The PCA-CONTROL group showed normal liver tissue (N=7). Biochemically, we found significantly

increased levels of: p-ALT (p<0.05), p-ammonia (p<.0001), p-alkaline phosphatases (p<0.05), p-creatinine (p<0.05), and p-sodium (p<0.01) (Table 1). Clinically, the PCA group showed significantly increased ICP (p<0.0001) and elevated brain water content (p<0.05), when compared to the PCA-CONTROL (Table 1). MAP was significantly lowered in the PCA group (p<0.01), when compared to the PCA-CONTROL, while all other physiological parameters were stabile and in normal range. In the PCA group, we found no difference in the expression of NKCC1 (p>0.05) or level of phos-NKCC1 (p>0.05), when compared to the PCA-CONTROL (Figure 1). The level of phos-NKCC1 tended against down-regulation (p=0.06). The protein expression of aqp4 was stabile, PCA *vs.* CONTROL: arbitrary units (SD); 3994972 (336706) *vs.* 4057584 (312781) (p>0.05) (Figure 2).

**Table 1:** End data for the PCA *vs.* PCA-CONTROL, presented as mean (SD).

| Biomarkers and Physiological Data for the PCA Group | | |
|---|---|---|
| **Mean (SD)** | **PCA** | **PCA-CONTROL** |
| Ammonium (µmol/L) | 1209.3 (283.1) * | 57.8 (36.9) |
| INR (U/L) | 1.8 (0.3) | 1.4 (0.1) |
| ALT (U/L) | 950.0 (1001.9) * | 57.4 (23.7) |
| Bilirubin (µmol/L) | 3.9 (15.8) | 2.0 (0.5) |
| Creatinine (µmol/L) | 21.6 (8.1) * | 15.3 (0.5) |
| Alkaline phosphatase IU/L | 148.1 (46.4) * | 107.9 (19.1) |
| Sodium (mmol/L) | 149.0 (3.5) * | 144.0 (2.6) |
| Glucose (mmol/L) | 8.2 (2.6) | 7.6 (1.3) |
| Brain water % | 80.88 (0.6) * | 80.20 (0.3) |
| ICP (mmHg) | 11.4 (2.6) * | 2.3 (0.9) |
| HE | - | - |
| MAP (mmHg) | 74.3 (12.6) | 93.9 (7.7) * |
| pH | 7.43 (0.05) | 7.46 (0.02) |
| $pCO_2$ (mmHg) | 40.9 (2.4) | 39.5 (2.0) |
| $SO_2$% | 98.4 (1.1) | 98.6 (0.03) |

**Abbreviations:** INR: International Normalized Ratio; ALT: Alanine Aminotransferase; ICP: Intracranial Pressure; HE: Hepatic Encephalopathy; MAP: Mean Arterial Pressure. (*=significant result).

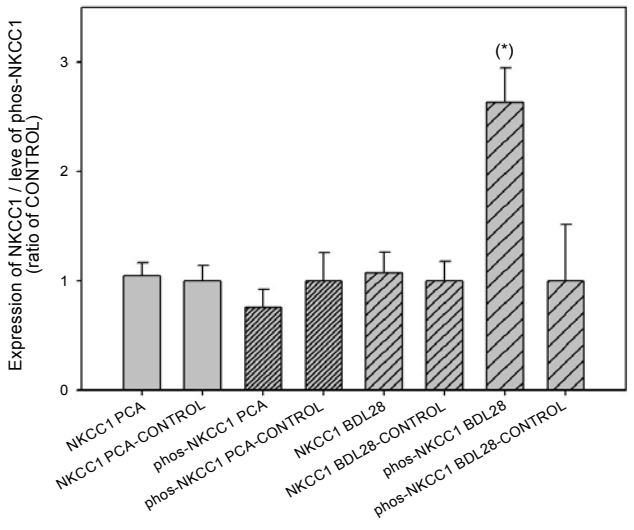


**Figure 1:** Densitometric results for brain cortical tissue evaluated for NKCC1 and phos-NKCC1 in the PCA and the BDL28 groups.

Data are presented as ratio of the CONTROL, whiskers show (SD). (* =significant result p<0.001)

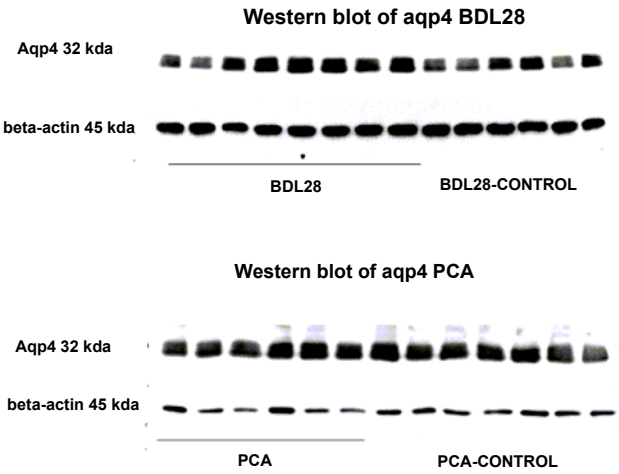


**Figure 2:** Western-blot of aqp4 for the PCA *vs.* PCA-CONTROL and BDL28 *vs.* BDL28-CONTROL.

No significant results present.

In our model of chronic liver failure with chronic hyperammonemia, the BDL28 model, we found pathological, biochemical, and physiological signs of chronic liver failure. Liver pathology for the BDL28 group showed proliferation of the bile ducts observed in periportal areas with varying amounts of fibrosis, and a few sections showed signs of nodule formation (N=8). The BDL28-CONTROL group showed normal pathologic features, although a mild lymphocytic portal infiltration without involvement of the interface was found (N=7). Biochemically, the BDL28 group showed significantly increased p-ammonia (p<0.01), p-bilirubin (p<0.0001), p-alkaline phosphatases (p<0.001), p-ALT (p<0.01), and no signs of impaired kidney function, hypoglycemia or anoxia (Table 2). We observed stabile ICP (p>0.05) and stabile brain water content (p>0.05), and all other observed physiological parameters were stabile and in normal physiologic range, when compared to the BDL28-CONTROL. The BDL28 group showed signs of fatigue with HE level 0-1 at end experiment, whereas the BDL28-CONTROL group showed no signs of HE. Regarding the BDL28 model, we found stabile level of NKCC1 (p>0.05), while the level of phos-NKCC1 was significantly elevated 2.6 times the BDL28-CONTROL (p<0.001) (Figures 1 and 3). The protein expression of aqp4 was stabile: BDL28 *vs.* CONTROL: arbitrary units (SD); 830633 (211056) *vs.* 644842 (95936) (p>0.05) (Figure 2).

## Discussion

Ammonia is believed to be the corner-stone in ALF mediated brain edema and development of HE. Newly research results revealed an association between development of brain edema and the ion-channel NKCC1 in several conditions such as ALF, stroke and traumatic brain damage; though a study exploring the direct effect of ammonia in this particular setting seemed well indicated. In this study, we aimed at

millimolar concentrations of p-ammonia, in order to explore the pathophysiologic effect of ammonia in mediating brain edema, high ICP and HE, and the possible association to the ion-channel NKCC1. Our study showed that development of brain edema and high ICP in acutely induced hyperammonemia could not be associated to elevated cerebral cortical expression of NKCC1 or increased level of phos-NKCC1 (Figure 1). The experimental model used is designed to induce high cerebral levels of ammonia and accommodate high level of physiological control. Physiologically, the animals did not suffer from dehydration, anoxia, hypothermia, diminished kidney function, and only expressed modest hypotension. Regarding ammonia and its experimental distribution in brain and physiologic safety, earlier research results in the PCA-model has shown that approximately 2/3 of the maximum plasma ammonia level is reached within the first 60 minutes from start of the ammonia infusion, and a close correlation between plasma ammonia levels and brain ammonia levels has been described [6,18]. Regarding acute heart toxicity due to divalent ions, ammonia has shown to be without impact on the heart rate, but no electrocardiogram has been evaluated [19].

Our experimental findings are in contrast to earlier research results which showed up regulation of NKCC1 and higher level of phos-NKCC1 in cortical brain tissue in thioacetamide-induced ALF with brain edema development and three times elevated brain ammonia level, induced over days [13]. From this it was stated that ammonia, *via* increased kinase activity, could induce increased expression of NKCC1 and higher level of phos-NKCC1 with subsequently development of brain edema. Our research showed that five hours of exposure to high plasma ammonia levels with induction of brain edema and high levels of ICP, did not induce increased expression of NKCC1 or increased level of phos-NKCC1, though phos-NKCC1

**Table 2:** End data for the BDL28 *vs.* BDL28-CONTROL, presented as mean (SD).

| Biomarkers and Physiological Data | | |
|---|---|---|
| **Mean (SD)** | **BDL28** | **BDL28-CONTROL** |
| Ammonium (µmol/L) | 100.7 (41.6) * | 33.4 (8.6) |
| INR (U/L) | 1.8 (0.4) | 1.6 (0.1) |
| ALT (U/L) | 168.3 (57.5) * | 62.0 (17.3) |
| Bilirubin (µmol/L) | 131.4 (30.5) * | 3.0 (0.6) |
| Creatinine (µmol/L) | 17.4 (1.7) | 17.3 (2.1) |
| Alkaline phosphatase IU/L | 274.1 (88.3) * | 122.7 (18.4) |
| Sodium (mmol/L) | 142.9 (2.1) | 146.9 (5.4) |
| Glucose (mmol/L) | 5.2 (0.8) | 5.7 (0.7) |
| Brain water % | 79.35 (0.3) | 79.48 (0.2) |
| ICP (mmHg) | 1.1 (0.3) | 1.1 (0.6) |
| HE | 0-1 | 0 |
| MAP (mmHg) | 113.4 (11.4) | 113.9 (9.6) |
| pH | 7.47 (0.02) | 7.46 (0.03) |
| $pCO_2$ (mmHg) | 40.2 (2.6) | 39.0 (4.2) |
| $SO_2$% | 98.3 (1.6) | 98.8 (0.2) |

**Abbreviations:** INR: International Normalized Ratio; ALT: Alanine Aminotransferase; ICP: Intracranial Pressure; HE: Hepatic Encephalopathy; MAP: Mean Arterial Pressure. (*=significant result).

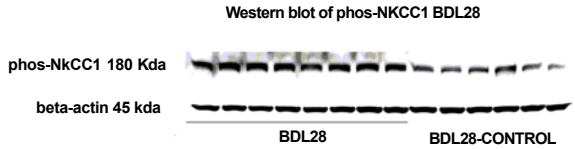


**Figure 3:** Western-blot of phos-NKCC1 evaluated for the BDL28 *vs.* the BDL28-CONTROL, (p<0.001).

tended against down regulation. This last statement could be due to a type-II statistical error, when taken into consideration the relatively small "N" used, and the power-calculation showing an estimated "N" to 16 animals in each group. According to *in vitro* studies of cellular cultures exposed to millimolar concentrations of ammonia, the expression of NKCC1 and the level of phos-NKCC1 is significantly elevated even after 5 hours [10], which is comparable to the treatment time for our PCA rats. Increasing the infusion time of ammonia is not an option, due to highly increased ICP and risk of incarceration by end experiment. From this, other mechanisms than regulation of expression and activity of NKCC1 seems to be of importance in development of brain edema in acute hyperammonemia, as observed in our *in vivo* animal study.

Also, our results for the PCA group are in contrast to studies of traumatically and ischemically induced brain edema [14,20-22], in which NKCC1 has been shown to serve impact. The reason for this discrepancy could be sought in the different pathophysiologic mechanisms underlying the different types of edema development. Brain edema in ALF and hyperammonemia primarily relies on cytotoxic factors, though vasogenic factors are also believed to be of importance [23,24]. Traumatic and ischemic induced brain edema is believed to be induced by a combination of several pathogenic mechanisms, though primarily vasogenic, but also ionic and cytotoxic factors are of importance [14]. The BBB is also impaired in both traumatic and ischemic brain edema, which is not the case in primary cytotoxic brain edema [14].

In chronic liver failure, hyperammonemia is thought to be one of the major pathophysiological factors in development of HE, partly due to impact on cerebral ion-channels mediating changes in the distribution of water in the brain [25,26]. In the BDL28 model, the animals showed signs of fatigue and were evaluated to HE grade 0-I. The liver sections showed signs of fibrosis and bile duct proliferation, but no signs of inflammation. These findings, combined with the observed elevated level of phos-NKCC1 and stabile expression of NKCC1, leads us to conclude that phos-NKCC1 is increased due to exposure to long term hyperammonemia, or exposure to liver derived cytokines escaping the injured liver, with possible nitrosative/oxidative action, and subsequently, development of HE [13].

Co-expression of NKCC1/phos-NKCC1 and aqp4 is a potential edema inducing combination [14]. We aimed to establish both the expression of NKCC1/level of phos-NKCC1, and the expression of aqp4, in order to evaluate on this subject. No co-expression were found, aqp4 were found with stabile expression in both acute hyperammonemia and chronic hyperammonemia, and are thereby, without any influence in both conditions.

This study explored, amongst others, the time frame as a

parameter in induction of brain edema and HE, in the event of acute and chronic hyperammonemia, and the impact on the level of expression or activity of NKCC1. The PCA model induces high grade hyperammonemia and low grade brain edema over a short period of time, without impact on expression or activity of NKCC1. Long term exposure to hyperammonemia induces increased activity of NKCC1 and low level HE in the BDL28 model. This leaves us to discuss if the exposure to ammonia in the PCA model is too short to serve pathophysiological impact on the brain. Though as stated earlier, *in vitro* studies has shown significantly increased activity of NKCC1 after hours of exposure to ammonia, and the PCA model express an adequate physiological response. The study by Jayakumar et al. [13] showed significantly increased NKCC1 and phos-NKCC1 in the period after the first and second day, after induction of ALF, showing three times increase in brain ammonia level. In conjunction with the above, NKCC1 could be of importance in inducing brain edema in ALF, expressing low degree brain ammonia levels developed over days, though in acute hyperammonemia developed within 24 hours, the mechanism of brain edema seems to be independent of ion channels and water channels. Also, it seems that chronic exposure to moderate hyperammonemia over weeks has impact on the activity of NKCC1, and thereby, on development of HE, while unknown compensatory pathophysiological mechanisms is probably activated preventing brain edema development.

Blocking agents of NKCC1, such as Bumetanide and Furosemide, could be of value to further study the function of NKCC1 in brain. They may also be of importance in future therapy of brain pathological changes related to chronic liver disease and hyperammonemia, developed over a prolonged period of time. The effect of Bumetanide has been shown to prevent brain edema in animal experiments of ALF, developed over days [14], brain ischemia, and traumatic brain injury [21]. The exact mechanism behind this effect is not clearly established, but could theoretically be due to an inhibitory effect on the brain endothelial luminal pool of NKCC1, or a direct diuretic effect by inhibition of NKCC1 in the kidneys. The background for these statements is the fact that these two agents do not penetrate the intact BBB very well. Though one *in vivo* animal study indicates that Bumetanide has the ability to penetrate the BBB to some extend [13].

In conclusion, we showed that acute hyperammonemia developed over hours, induces brain edema and elevated ICP, independently of the expression of NKCC1 or level of phos-NKCC1. We, therefore, conclude that other pathophysiological mechanisms must be of importance in mediating brain edema in this clinical condition. In chronic hyperammonemia, we found that the level of phos-NKCC1 was increased significantly, and was associated with low level increase in HE, but was not associated with changes in brain water content or changes in ICP. No co-expression of NKCC1/increased level of phos-NKCC1 and aqp4 where found. From this, we conclude that NKCC1 is activated by chronic hyperammonemia, and that phos-NKCC1 could be of importance in mediating HE in chronic liver failure, but further studies are needed.

## Conflict of Interest

The authors declare that they have no conflicts of interest.

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