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ORIGINAL PAPER

Brain expression of the water channels Aquaporin-1 and -4 in mice with acute liver injury, hyperammonemia and brain edema

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Abstract Cerebral edema is a feared complication to acute liver failure (ALF), but the pathogenesis is still poorly understood. The water channels Aquaporin-1 (Aqp1) and -4 (Aqp4) has been associated with brain edema formation in several neuropathological conditions, indicating a possible role of Aqp1 and/or Aqp4 in ALF mediated brain edema. We induced acute liver injury and hyperammonemia in mice, to evaluate brain edema formation and the parallel expression of Aqp1 and Aqp4 in ALF. Liver injury and hyperammonemia were induced by +D-galactosamine (GLN) plus lipopolysaccharide (LPS) intraperitoneally and intravenous ammonia-acetate (NH_4^+), the GLN+LPS+NH_4^+ group. The vehicle control group (CONTROL) was treated with NaCl and phosphate-buffered saline. The GLN+LPS+NH_4^+ group showed significantly elevated p-alanine aminotransferase, p-INR and p-ammonium vs. CONTROL ($p<0.001$). Cortical brain water content was significantly elevated in the GLN+LPS+NH_4^+ group vs. CONTROL, mean (SEM) 80.8 (0.3) vs 80.0(0.1) % ($p<0.05$). Western blot of membrane enriched cortical brain tissue showed significantly upregulation of Aqp4 in the GLN+LPS+NH_4^+ group vs. CONTROL, mean AU (SEM) 100775(14820) vs. 58857(6266) ($p<0.05$), and stationary levels for Aqp1. *Aqp1* and *Aqp*4 mRNA were stationary. This study indicates that Aqp4, but not Aqp1, may be of importance in the pathogenesis of cortical brain edema in mice with ALF.

Keywords Brain water content · Acute liver failure · Experimental mouse model · +D-galactosamine · Lipopolysaccharide

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Introduction

Regulation of brain water content is of critical importance for normal function of the central nervous system, and major changes may result in cellular swelling and brain edema. Indeed, many neuropathological conditions including: traumatic injury, ischemia, neoplasms, and hyponatremia are associated with cerebral edema (Badaut et al. 2002). In acute liver failure (ALF) cerebral edema is a feared complication, which pathophysiologically has been associated with hyperammonemia and the release of cytokines (Vaquero et al. 2003) from the failing liver (Jalan et al. 2004; Chastre et al. 2010; Bemeur et al. 2010).

That ammonia is of importance for development of cytotoxic brain edema in ALF, was visualized in in vitro studies of astrocytic cell cultures, showing cellular swelling in conditions with high ammonium concentrations (Norenberg et al. 1991; Swain et al. 1992; Rama Rao et al. 2003). In addition, animal in vivo studies of the pathophysiologic relation between brain astrocytic swelling and ALF showed connection to hyperammonemia (Ganz et al. 1989; Swain et al. 1992). Clinically, hyperammonemia

and high intracranial pressure has been associated (Tofteng et al. 2006) and secondly, in vivo MRI studies of patients suffering from ALF, demonstrated concomitant brain edema (Ranjan et al. 2005). However, the mechanisms involved in the ammonia and inflammatory mediated influx of water into the astrocytic cells, causing intracellular swelling and brain edema, remains poorly understood.

Identification of the water channel class of aquaporins and knowledge of their physiologic functions, has given new pathophysiological insight regarding evolvement of brain edema. Especially Aquaporin-4 (Aqp4) has been of interest, due to its physiological profile and its abundant expression in brain glial tissue. This water channel works as a bi-directional non-gated transporter of water, activated by osmotic gradients. Aqp4 is primarily expressed in the astrocytic endfeet, mediating close contact with the endothelial basal area, but also expressed in ependymal cells (Badaut et al. 2002; Amiry-Moghaddam et al. 2004; Wolburg-Buchholz et al. 2009). Aqp4 is found in two major iso-variants, i.e the M1 and the more abundant M23 (Hirt et al. 2009; Chastre et al. 2010), as well as several other splice variants, though the physiologic impact and exact distribution of these variants are not well understood (Moe et al. 2008). The role of Aqp4 in development of brain edema, under various pathophysiologic conditions, has been explored in experiments using *Aqp4* (-/-) and *α-syntrophin* (-/-) knock out mice, the latter a protein involved in the cellular localization of Aqp4. These experiments showed reduced brain swelling and improved neurological outcome in the knock out animals (Manley et al. 2000; Vajda et al. 2002; Amiry-Moghaddam et al. 2004; Papadopoulos and Verkman 2005). Regarding the ALF related biochemical parameters such as ammonia and the cytokine IL-1 beta, ammonia upregulates astrocytic expression of Aqp4 protein (Rama Rao et al. 2003), while IL-1 beta upregulates the astrocytic mRNA expression of Aqp4, shown in vitro (Chastre et al. 2010). These studies indicate, that Aqp4 could play a role in the formation of brain edema, in conditions of liver injury and hyperammonemia, such as ALF.

The water channel Aquaporin-1 (AQP1) is also expressed in brain, though with a markedly different expression pattern and physiologic impact than Aqp4. Its expression in brain is restricted to the ventricular-facing surface of the choroid plexus and it participate primarily in the production of cerebrospinal fluid, but Aqp1 has also been localized in rat astrocytic cells following brain injury (McCoy and Sontheimer 2010). Aqp1 has been associated with development of brain edema under various brain pathologic conditions such as glial neoplasia (Oshio et al. 2005) and sub-arachnoid haemorrhage (Badaut et al. 2003), indicating that Aqp1 could play a role in ALF-mediated brain edema.

In the present study, we aimed to evaluate the cortical brain water content and the parallele cortical brain expression of the water channels Aqp1 and Aqp4, in conditions resembling acute liver injury and hyperammonemia in mice. To accomplish this, we used the well characterized, liver specific, mouse model of acute toxic liver injury, i.e. the +D-galactosamine (GLN) plus lipopolysaccharide (LPS) model (Zhou et al. 2003; Eipel et al. 2007), combined with infusion of ammonia-acetate (NH_4^+), to visualize the effect of hyperammonemia.

Materials and methods

Experimental animal model

Male C57 BL/6 mice (microbiologically approved) (Taconic, Ry, Denmark) weighing 25–26 g were used. The animals were acclimatized for 5 days before the experiments, free access to food and water and 12/12 h day/night cycle. Animal protocols were approved by the Danish Council for Supervision with Experimental Animals (protocol number 2007/561-1271).

Experimental acute liver injury was induced with a combination of GLN (Sigma-Aldrich, Brøndby, Denmark) 800 mg/kg and LPS Escherichia coli 0111:B4 (Sigma-Aldrich) 10 μg/kg, diluted in a total volume of 100 μL 0.9% NaCl saline, and injected intraperitoneally (i.p.). GLN and LPS works synergistically inducing apoptosis and inflammation in liver (Zhou et al. 2003; Eipel et al. 2007). After 3 h of experimental time we induced hyperammonemia. To perform this, in a reproducible way, we first induced anaesthesia in the animals with Ketamine (Intervet, Skovlunde, Denmark) 100 mg/kgi.p. and Xylazine (Intervet) 10 mg/kg i.p. (Eipel et al. 2007), followed by treatment with oxygen enriched air at a percentage of 40%. An intravenous tail catheter Terumo SURFLO 25G (Terumo, Leuven, Belgium) was inserted and infusion of NH_4^+ (Sigma-Aldrich) (diluted in phosphate buffered saline) at a rate of 120 μmol/kg/min (100 μl/hour) was conducted, until a total experimental time of approximately 6.5 h was reached. The control groups, which didnt receive infusion of ammonia, received same volume of phosphate buffered saline i.v.. The temperature was kept constant at 37°C with a rectal thermistor coupled to a heating pad (AgnTho, Lidingo, Sweden). Blood glucose was measured twice during the experimental procedure with a glucometer, Precision Xceed (Abbot, Copenhagen, Denmark), to detect hypoglycemia. At end experiment, a hearth blood sample was drawn and the animals were quickly decapitated. Brain cortical tissue was frozen in liquid nitrogen and kept at −80°C. In pre-experimental surveys, mice treated with GLN plus LPS and NH_4^+ expressed signs of acute liver injury, indicated by

elevated p-alanine aminotransferase (ALT), p-INR, hyperammonemia and elevated brain water content, compared to the vehicle control group (CONTROL), treated with NaCl i.p. and phosphate buffered saline i.v.. This resulted in our animal model set up with the GLN+LPS+NH_4^+ group, as the group mimicking ALF, six intermediate control groups: GLN+LPS, GLN+NH_4^+, LPS+NH_4^+, LPS, NH_4^+, GLN, and the CONTROL. We also found, that the average survival time was shortest for the GLN+LPS+NH_4^+ group, approximately 6.5 h, which we choose as the limiting experimental time. The total treatment time for all the groups was an average of 391.2 min (SD 22.2), no significant regulation was observed, compared to the CONTROL.

Analysis of protein expression by Western blotting

Frozen cortical brain tissue from mice were homogenized in a Potter Elverhjem (B. BRAUN, Melsungen, Germany) at high speed for 4 min 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, Copenhagen, Denmark). The homogenate was centrifuged in an Eppendorf 5415c centrifuge (Eppendorf, Hamburg, Germany) at 4000 × *g* for 15 min at four °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 min to produce a membrane-enriched pellet (Vajda et al. 2000). The resultant pellet was resuspended in 50 mM HEPES buffer, 2% Sodium Dodecyl Sulphate (SDS) and one dissolved tablet of protease-inhibitor cocktail, for 2.5 h at 20°C. Protein concentration was measured using BIORAD DC Kit (Bio-Rad Laboratories, Copenhagen, Denmark) and a photometer Pharmacia LKB-Ultrospec. III (GE-Healthcare, Hillerød, Denmark). The membrane enriched protein samples were loaded onto 4–12% Invitrogen mini-cell-system (Invitrogen) (150 V, 50 min) with three μg protein per lane for Aqp4 and 10 μg protein per lane for Aqp1, the standard marker SeeBlue PLUS 2 was also applied (Invitrogen). No heating prior to sample loading was performed for detection of Aqp4, while samples for analysis of Aqp1 were heated to 70°C for 10 min. All procedures were performed under denaturing conditions. Protein was transferred to PVDF-membranes (Invitrogen) by electroelution (30 V, 60 min). 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 1 h, the PVDF membrane was incubated overnight at four °C with the primary antibodies diluted in 5% low fat milk and TBST buffer (Aqp1 polyclonal antibody, ab15080, 1:2000, Abcam, Cambridge, UK; Aqp4 polyclonal antibody, SC9888, 1:5000, Santa Cruz Biotechnology, Heidelberg, Germany). The membranes were subsequently washed in TBST for 30 min and then incubated at room temperature for 2 h with horseradish peroxidase conjugated secondary antibody diluted in the same buffer (Aqp1, P0448, 1:2000, Dako, Glostrup, Denmark; Aqp4, SC2020, 1:5000, Santa Cruz Biotechnology). After incubation the membrane was washed with TBST for 30 min. Finally, the antibody detection was performed using the Enhanced Chemiluminescence system (PerkinElmer®, Massachusetts, USA) and camera detecting system LAS 9000® with software ImageGauge 2006® (FujiFilm, Stockholm, Sweden). An internal control was made, to ensure equal protein amount in the samples (Beta-actin polyclonal antibody, #4967, 1:1000, Cell Signaling, Medinova, Birkerød, Denmark).

Analysis of mRNA expression by dot blotting

Total RNA was isolated with RNeasy® mini lipid kit (Qiagen Sciences, Maryland, USA). The total amount of RNA in the samples was measured using a NANO-DROP photometer (Thermo Fisher Scientific, Göteborg, Sweden). *Aqp1* and *Aqp4* mRNA quantification were performed by dot blot analysis with cDNA-probes against mouse *Aqp1* and *Aqp4*: (Primers:

Aqp1 forward: 5′GCCCTAGGCTTCAATTACC CACTGG 3′,
Aqp1 reverse: 5′GGGCACCCCCAATGAACGGCCC CACCC 3′
Aqp4 forward: 5′GCTGCGGCAAGGCGGTGG GGTAAGTG 3′,
Aqp4 reverse: 5′GGATGCCGGCTCCAAT GATGGCCCCCAGGC 3′).

32PdCTP-labeling of the *Aqp1* and the *Aqp4* cDNA probes were performed by in vitro transcription using Maxiscript In Vitro Transcription Kit (Amersham Biosciences, Hillerød, Denmark), followed by purification on NICK Spin Columns (Stratagene, La Jolla, USA). Five μg of total RNA was immobilized onto a PVDF nylon membrane using a Schleicher & Schuell Minifold SRC 072/0 (Schleicher & Schuell, Dassel, Germany). Pre-hybridization was performed at 68°C for 30 min, followed by hybridization with the probe at 68°C for 1 h in Quick Hybridisation Solution (Boehringer Mannheim, Ingelheim, Germany). Subsequently, the membrane was washed at 25°C in two × 3.0 M NaCl and 0.3 M Sodium Citrate (SSC) and 0.1% SDS for 15 min and secondly at 68°C in 0.1 × SSC and 0.1% SDS for 30 min. Signals were detected using Imaging Plate BASIII (FujiFilm) and the hybridization signal was analyzed by FUJI FLA 9000 STARION® (FujiFilm). The signals were densitometrically evaluated using ImageGauge 2006 software (FujiFilm). Specificity of the used probes was ascertained by Northern blotting technique, using mouse cortical brain tissue, showing single bands at expected sizes.

Gravimetric analysis

A bromobenzene (Sigma-Aldrich) / kerosene (Sigma-Aldrich) gradient column was used to measure changes in brain specific gravity (Marmarou et al. 1978). After decapitation, the brain was quickly removed and kept in a sealed container at four °C. The cortical tissue was dissected into 2×2×2 mm samples and swiftly placed on a premade bromobenzene / kerosene column. After 2 min, the equilibration point was read and the specific weight was calculated from a standard curve, the latter made using K_2SO_4 samples (Sigma-Aldrich) with known specific density. The room temperature was kept constant. Between five and 10 samples per animal was applied to the column and an average of the equilibration point was made. Significantly increased brain water content, compared to the CONTROL, was interpreted as the presence of brain edema.

Histopathologic evaluation of liver damage

Evaluation of histopathologic liver damage was performed by a trained pathologist, using standard light microscopy of haematoxylin and eosin stained preparations of liver samples. The samples were evaluated concerning following parameters: Apoptosis, parenchymal inflammation / necrosis, portal inflammation and fibrosis, all parameters were graded 0–3. 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 hepatic encephalopathy (HE)

HE was evaluated for the GLN+LPS+NH_4^+ group and the control groups. To enable clinical HE observations, ammonia was applied i.p.. The stage of hepatic encephalopathy was determined by a neurobehavioral scale, and the end experiment data was read at time = 6.5 h or when the animals reached HE level four. The stages were as follows: 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 (Larsen et al. 1994).

Biochemical analysis of blood samples

The blood samples were centrifuged at 3500 RPM for 10 min at four °C. p-ALT, p-ammonium, p-INR, p-creatinine, total p-bilirubin and p-sodium were measured by routine techniques. Glucose was measured on peripheral blood samples.

Statistical analysis

Data were evaluated using Students T-test, ANOVA One-Way Analysis with Bonferroni correction against control and Kruskall Wallis with Dunnett's correction against control. All data were analysed regarding normality by the Kolmogorov-Smirnov test. If the data passed the test, we proceeded with parametric statistic analysis. A p-value lower than 0.05 was considered statistically significant. Statistical analysis were performed with SigmaStat® software (Systat Software, Washington, USA) and Graphic presentations was set with SigmaPlot® software (Systat Software).

Results

Evaluation of brain water content for the GLN+LPS+NH_4^+ and the control groups were performed, showing significantly increased brain water content in the GLN+LPS+NH_4^+ group compared to the CONTROL ($p<0.05$) (Table 1).

To determine the clinical impact of liver failure and hyperammonemia in mice, we evaluated the level of HE in all groups. The level of HE was elevated to level four in the GLN+LPS+NH_4^+- and the GLN+LPS-groups, none of the other groups scored above level two and the CONTROL scored HE level 0 (Table 1). The groups GLN+LPS+NH_4^+ and GLN+LPS were both close to expire, when reaching HE level four, close to the 6.5 h treatment limit, though the GLN+LPS+NH_4^+ group tended to reache HE level four before the GLN+LPS group.

Signs of cellular liver damage with significantly elevated p-ALT, p-INR and total p-bilirubin was found in the GLN+LPS+NH_4^+ group compared to the CONTROL ($p<0.001$), (Table 1). p-ALT was siginificantly elevated in the GLN+LPS+NH_4^+- and the GLN+LPS-groups compared to the CONTROL ($p<0.001$), while we found significantly increased levels of p-ammonium in all groups receiving NH_4^+ compared to the CONTROL ($p<0.001$). INR was elevated in the GLN+LPS+NH_4^+-, GLN+LPS-, LPS+NH_4^+- and the GLN-groups compared to the CONTROL ($p<0.001$). p-creatinine was significantly increased in the GLN+LPS+NH_4^+-, LPS+NH_4^+, and the NH_4^+-groups, compared to the CONTROL ($p<0.001$). p-total bilirubin was found significantly increased in the GLN+LPS+NH_4^+-, GLN+NH_4^+-, and the LPS+NH_4^+-groups compared to the CONTROL, ($p<0.001$). Sodium was stationary in all groups compared to the CONTROL, ($p>0.05$), while glucose was

Table 1 Brain water, Hepatic encephalopathy (*HE*), biomarkers

	Brain water % mean (SEM) (n=4–11)	HE median (n=5)	ALT (U/L) mean (SEM) (n=4–6)	Ammonium (μmol/L) mean (SEM) (n=4–6)	INR mean (SEM) (n=5–6)	Creatinine (μmol/L) mean (SEM) (n=4–6)	Total Bilirubin (μmol/L) mean (SEM) (n=4–6)	Sodium (mmol/L) mean (SEM) (n=5–6)	Glucose (mmol/L) mean (SEM) (n=5–6)
GLN+LPS+NH_4+	80.8 (0.29)*	4	5443 (1278.4)*	2472 (232.9)*	5.4 (0.90)*	60 (13.3)*	34.5 (8.9)*	161 (4.7)	14.4 (3.7)
CONTROL	80.0 (0.13)	0	66 (17.7)	75 (12.8)	1.0 (0.00)	7 (0.8)	1.8 (0.3)	148 (2.5)	21.9 (3.0)
GLN+LPS	80.6 (0.19)	4	1591 (1035.5)*	165 (54.8)	2.4 (0.24)*	18 (6.1)	4.8 (1.3)	147 (6.9)	10.9 (0.7)*
GLN+NH_4+	80.1 (0.12)	1	145 (63.6)	1703 (307.6)*	1.4 (0.05)	16 (3.0)	9.0 (1.8)*	158 (2.6)	22.2 (1.6)
LPS+NH_4+	80.1 (0.19)	2	278 (76.8)	4301 (294.9)*	1.7 (0.55)*	26 (3.3)*	31.0 (14.0)*	169 (5.7)	17.8 (1.0)
LPS	80.5 (0.09)	1	48.5 (2.9)	114 (9.4)	1.5 (0.27)	13 (1.7)	1.5 (0.6)	150 (10.0)	16.8 (1.6)
NH_4+	79.9 (0.11)	1	51 (9.8)	1404 (227.2)*	1.0 (0.02)	25 (3.2)*	7.0 (0.6)	151 (2.6)	19.1 (1.7)
GLN	79.8 (0.20)	1	120 (21.6)	159 (41.4)	1.5 (0.05)*	24 (8.4)	2.5 (0.3)	154 (3.0)	12.4 (0.5)*

Data end experiment for all groups, presented as mean (median) ± SEM

GLN galactosamine; *LPS* lipopolysaccharide; *NH_4^+* ammonium; *CONTROL* vehicle control group treated with NaCl i.p. and phosphate buffered saline i.v.; *HE* hepatic encephalopathy; *ALT* alanine aminotransferase; *INR* international normalized ratio

Significant differences were found regarding: Brain water content: (GLN+LPS+NH_4^+ vs CONTROL), p-ALT (GLN+LPS+NH_4^+ and GLN+LPS vs CONTROL), p-ammonium (GLN+LPS+NH_4^+, GLN+NH_4^+, LPS+NH_4^+ and NH_4^+ vs CONTROL), p-INR: (GLN+LPS+NH_4^+, GLN+LPS, LPS+NH_4^+ and GLN vs CONTROL) p-creatinine: (GLN+LPS+NH_4^+, LPS+NH_4^+, and NH_4^+ vs CONTROL), total p-bilirubin: (GLN+LPS+NH_4^+, GLN+NH_4^+ and LPS+NH_4^+ vs CONTROL), Glucose (GLN+LPS and GLN vs CONTROL)

Significant result, group vs CONTROL (*)

significantly lowered in the GLN+LPS- and GLN-groups, compared to the CONTROL ($p<0.01$).

Liver parenchyma for the GLN+LPS+NH_4^+ group and the CONTROL were evaluated histopathologically (median; GLN+LPS+NH_4^+ ($n=6$)/CONTROL ($n=5$)): Apoptosis: 3/0, inflammation/necrosis: 1/0, portal inflammation: 1/0 and fibrosis: 0/0.

Finally, we investigated the protein and mRNA expression of Aqp1 and Aqp4. Aqp1 protein expression in brain cortical tissue was low and evenly distributed in all the groups, both visually and densitometrically evaluated by Western blotting with pooled samples (Fig. 1A). Due to the evenly and low expression of Aqp1, no single sample Western blot are presented. Results with densitometric data AU ($n=6$): GLN+LPS+NH_4^+: 8178, CONTROL: 6949, GLN+LPS: 8201, GLN+NH_4^+: 6543, LPS+NH_4^+: 5993, LPS: 6466, NH_4^+: 6771 and GLN: 6261. The GLN+LPS+NH_4^+- and the GLN+LPS-groups showed increased levels of Aqp1, both to a level of 1.18 times the CONTROL, (no error bars available due to pooled data). Beta-actin internal control, showed low variation in the amount of protein in the samples. Analysis of *Aqp1* mRNA, showed low absolute levels and the ratios were all regulated with less than one fold, compared to the CONTROL (Fig. 1B). Results with densitometric data, mean Photo Stimulated Luminiscence (PSL) (SEM) ($n=6$): GLN+LPS+NH_4^+: 1095 (84), CONTROL: 1309 (67), GLN+LPS: 1341 (127), GLN+NH_4^+: 1942 (188), LPS+NH_4^+: 1524 (73), LPS: 1461 (120), NH_4^+: 1860 (137) and GLN: 1918 (126). The GLN+NH_4^+-, NH_4^+- and GLN-groups showed significantly upregulated *Aqp1* mRNA compared to the CONTROL ($p<0.05$) (*). Regarding Aqp4, the GLN+LPS+NH_4^+ group showed increased amount to 1.64 times the CONTROL, evaluated by Western blotting with pooled samples (Fig. 2A). Results, densitometric data AU, ($n=6$): GLN+LPS+NH_4^+: 74473, CONTROL: 45549, GLN+LPS: 33569, GLN+NH_4^+: 42908, LPS+NH_4^+: 53289, LPS: 49720,

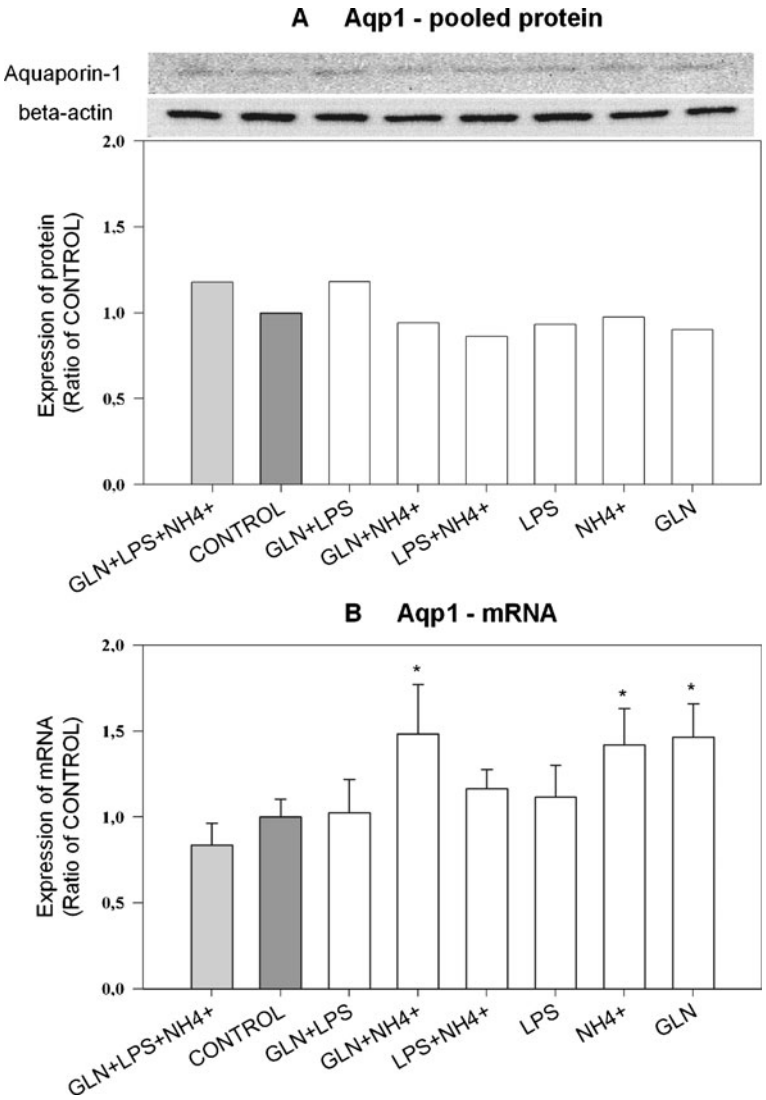
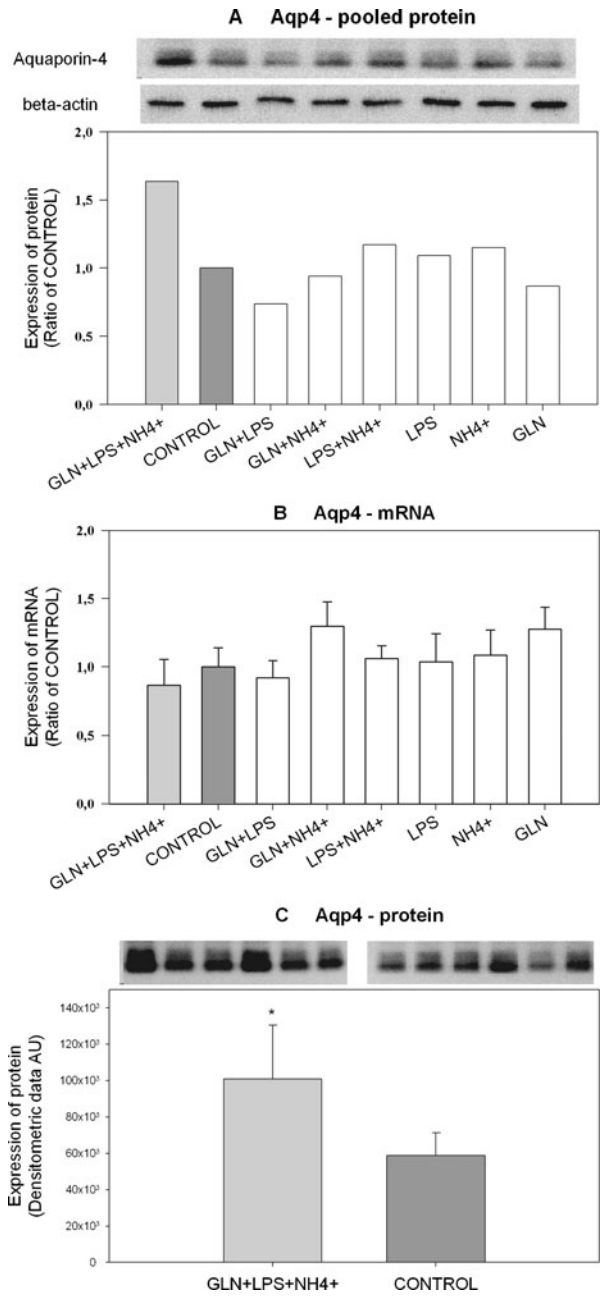


Fig. 1 Brain expression of Aquaporin-1 (Aqp1) protein and mRNA. Abbreviations: *GLN* galactosamine; *LPS* lipopolysaccharide; *NH_4^+* ammonium; *CONTROL* vehicle control group treated with NaCl i.p. and phosphate buffered saline i.v. **A** Western blot of Aqp1 with pooled protein samples showing a band at 28 kDa, ($n=6$). Each bar represents a ratio of the CONTROL, no error bars due to pooled protein samples. Beta-actin internal control, show low variation in the amount of protein in the samples. Aqp1 was found elevated 1.18 times the CONTROL for both the GLN+LPS+NH_4^+- and the GLN+LPS-groups. **B** *Aqp1* mRNA evaluated by dot blotting ($n=6$). Each bar shows mean PSL values as a ratio of CONTROL, error bars represents SEM. Very low absolute figures and very low regulation of the ratios, less than one fold changes were observed. The groups GLN+NH_4^+, NH_4^+ and GLN were significantly upregulated compared to the CONTROL ($p<0.001$) (*)

NH_4^+: 52349 and GLN: 39564. The LPS+NH_4^+-, LPS- and the NH_4^+-groups all showed increased levels of Aqp4 to respectively 1.17, 1.10 and 1.15 times the CONTROL (no error bars available due to pooled data). Beta-actin internal control showed low variation in the amount of protein in the samples. Regarding *Aqp4* mRNA, no significantly differences were observed compared to the CONTROL ($p>0.05$) (Fig. 2B). Results, densitometric data, mean PSL (SEM) ($n=6$): GLN+LPS+NH_4^+: 9597 (1021), CONTROL: 11044 (768), GLN+LPS: 10201 (667), GLN+NH_4^+: 14332 (992), LPS+NH_4^+: 11704 (527), LPS: 11449 (1146), NH_4^+: 11983 (1011) and GLN: 14097 (884). We showed significantly higher expression of Aqp4 protein in the GLN+LPS+NH_4^+ group compared to the CONTROL, by single sample Western blot for the respective groups (Fig. 2C). Results with densitometric data, mean AU (SEM) ($n=6$): GLN+LPS+NH_4^+: 100775 (14820) ; CONTROL: 58857 (6266). The GLN+LPS+NH_4^+ group showed significantly increased Aqp4 to 1.71 times the CONTROL ($p<0.05$).



Discussion

In order to assess if the brain expression of the water channels Aqp1 and/or Aqp4 is regulated in conditions resembling ALF, with possible impact on the brain water content, we evaluated the expression of the respective proteins in conditions with acute liver injury and hyperammonemia in mice. We demonstrated significantly increased level of Aqp4 protein, but not Aqp1, protein in a membrane-enriched fraction of cortical brain tissue, obtained from mice induced with acute liver injury, hyperammonemia and brain edema, the GLN+LPS+NH_4^+ group. In this experiment, neither isolated liver injury with GLN, stimulation with low level LPS nor isolated hyperammonemia were capable of inducing brain edema, and these compounds showed no effect on the expression of Aqp1 nor Aqp4. Though it was only the synergistic combination of hyperammonemia and acute liver injury, reflected by significantly elevated ALT, INR and billirubin, that induced significantly increased brain water content, paralelled by significantly increased cortical brain expression of Aqp4. This imply, that Aqp4 could be of pathophysiologic

Fig. 2 Brain expression of Aquaporin-4 (Aqp4) protein and mRNA. Abbreviations: *GLN* galactosamine; *LPS* lipopolysaccharide; *NH_4^+* ammonium; *CONTROL* vehicle control group treated with NaCl i.p. and phosphate buffered saline i.v. **A** Western blot of Aqp4 with pooled protein samples, showing bands at 32–34 kDa, ($n=6$). Each bar represents a ratio of the CONTROL, no error bars due to pooled protein samples. Beta-actin internal control, showed low variation in the amount of protein in the samples. For the GLN+LPS+NH_4^+ group, Aqp4 was elevated 1.64 times the CONTROL, which was the highest difference found, corresponding well to the visual impression of the Aqp4 Western blot. The LPS+NH_4^+-, LPS-, and the NH_4^+–groups all showed increased levels of Aqp4 to respectively 1.17, 1.10 and 1.15 times the CONTROL. **B** *Aqp4* mRNA evaluated by dot blotting ($n=6$). Each bar shows mean PSL values as a ratio of the CONTROL, error bars represents SEM. Very low regulation of the ratios, less than one fold changes were observed. No significant results were obtained ($p>0.05$). **C** Statistical evaluation of the expression of Aqp4 protein in the GLN+LPS+NH_4^+ group ($n=6$) versus the CONTROL ($n=6$). Western blot with single samples per lane, (the blot has been separated for better overview). The GLN+LPS+NH_4^+ group showed significantly upregulation of Aqp4 compared to the CONTROL, mean (SEM), ($p<0.05$) (*). The visual impression and the densitometric results for the Western blot with single samples per lane, showed similar proportions as for the Western blot performed with pooled protein samples (Fig. 2A). Significant results, group vs CONTROL (*)

importance in the formation of increased brain water content and that pro-inflammatory factors, escaping the failing liver, could act synergistically with ammonia in upregulating Aqp4, mediating the subsequent brain edema.

The importance of Aqp4 in the pathophysiology of brain edema, was demonstrated in mice over-expressing Aqp4 in astrocytic cells (Yang et al. 2008). These mice were induced with cytotoxic brain edema, and the experiment showed correlation between high brain water content and high expression levels of Aqp4. Accordingly, Aqp4 was assumed to be the rate-limiting factor for development of cytotoxic brain edema. This is in agreement with the present work, indicating that acute upregulation of Aqp4 protein in brain cortical tissue is unfavourable, with the potential to induce brain edema. Our experimental results are in line with other studies of cytotoxic brain edema, including studies of stroke (Manley et al. 2000), cardiac arrest with brain ischemia (Xiao et al. 2004) and bacterial meningitis (Papadopoulos and Verkman 2005), which all showed involvement of Aqp4 in the formation of the increased brain water content. An animal model of ischemic cytotoxic brain edema, demonstrated regulation of Aqp4 over hours to days, indicating that Aqp4 may play a role at various stages in development and resolvement of brain edema (Ribeiro et al. 2006). The exact physiologic impact of this regulation, however, remains unknown.

The expression of Aqp4 protein in this experiment, was shown to be independent of the mRNA expression, which is in agreement with a previous investigation (Vajda et al. 2000). This indicate, that post-translatory mechanisms for regulation of Aqp4 protein expression and mechanisms for regulation of Aqp4 cellular trafficking, could be present and activated (Madrid et al. 2001; Zelenina et al. 2002; Carmosino et al. 2007). Further studies are needed to establish the exact pathways, regulating the brain expression of Aqp4 protein in ALF.

In our study, *Aqp*1 mRNA and protein expression in cortical brain tissue showed very low absolute levels, regardless of the overall brain water content. The visual impression and densitometric results for Aqp1, evaluated by Western blotting, corresponded well to the overall impression of the absolute *Aqp1* mRNA level (Fig. 1A and B). Though it did not correspond to the significant upregulation of *Aqp1* mRNA, found in the intermediate control groups GLN+NH_4^+, NH_4^+ and GLN. That the Aqp1 protein level doesn't strictly follow the mRNA-level, we explain by the very discrete regulations, less than one fold, which may not be visualized on the protein level. That the overall absolute Aqp1 protein level was very low and none regulated in the group with brain edema, and stabile throughout all the treatment groups, compared to the CONTROL, indicates that Aqp1 has no influence on the brain water content in mice with acute liver injury and hyperammonemia. This notion, is consistent with a study of stroke induced cytotoxic brain edema (Ribeiro et al. 2006).

In summary, we found significantly increased Aqp4 protein in membrane enriched cortical brain tissue and unchanged expression of Aqp1 protein, under pathophysiologic conditions with acute liver injury, hyperammonemia and brain edema in mice. We speculate, that this upregulation of Aqp4 in brain cortical tissue, is of pathophysiological importance for the evolvement of brain edema in mice with acute liver injury and hyperammonemia, and therefore could be a future target for control of brain edema in ALF.

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