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# Aquaporin-4 deletion in mice reduces encephalopathy and brain edema in experimental acute liver failure

Kakulavarapu V. Rama Rao [a,*], A.S. Verkman [c,d], Kevin M. Curtis [b,e], Michael D. Norenberg [a,b,e,*]

[a] Department of Pathology, University of Miami Miller School of Medicine, Miami, FL 33101, USA
[b] Department of Biochemistry & Molecular Biology, University of Miami Miller School of Medicine, Miami, FL 33101, USA
[c] Department of Medicine, University of California, San Francisco, CA 94143, USA
[d] Department of Physiology, University of California, San Francisco, CA 94143, USA
[e] Veterans Affairs Medical Center, Miami, FL 33125, USA

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ABSTRACT

Brain edema and associated astrocyte swelling leading to increased intracranial pressure are hallmarks of acute liver failure (ALF). Elevated blood and brain levels of ammonia have been implicated in the development of brain edema in ALF. Cultured astrocytes treated with ammonia have been shown to undergo cell swelling and such swelling was associated with an increase in the plasma membrane expression of aquaporin-4 (AQP4) protein. Further, silencing the AQP4 gene in cultured astrocytes was shown to prevent the ammonia-induced cell swelling. Here, we examined the evolution of brain edema in AQP4-null mice and their wild type counterparts (WT-mice) in different models of ALF induced by thioacetamide (TAA) or acetaminophen (APAP). Induction of ALF with TAA or APAP significantly increased brain water content in WT mice (by 1.6% ± 0.3 and 2.3 ± 0.4%, respectively). AQP4 protein was significantly increased in brain plasma membranes of WT mice with ALF induced by either TAA or APAP. In contrast to WT-mice, brain water content did not increase in AQP4-null mice. Additionally, AQP4-null mice treated with either TAA or APAP showed a remarkably lesser degree of neurological deficits as compared to WT mice; the latter displayed an inability to maintain proper gait, and demonstrated a markedly reduced exploratory behavior, with the mice remaining in one corner of the cage with its head tilted downwards. These results support a central role of AQP4 in the brain edema associated with ALF.

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## Introduction

Brain edema is a potentially lethal complication of acute liver failure (ALF), with an approximately 55–60% mortality (Escorsell et al., 2007; Lee et al., 2008). There is currently no effective treatment for the brain edema other than emergency liver transplantation (Farges et al., 1996; Hoofnagle et al., 1995; Vaquero et al., 2003). Cytotoxic brain edema, principally due to astrocyte swelling, is the major neuropathological finding in ALF (Jover et al., 2006; Kato et al., 1989; Martinez, 1968; Norenberg, 1977; Traber et al., 1989). Employing neuroimaging techniques, several reports have documented a significant intracellular accumulation of water in brain parenchyma in human and experimental ALF of various etiologies, indicating the presence of cytotoxic brain edema. For review, see (Chavarria et al., 2011).

Several lines of evidence indicate that elevated blood and brain ammonia levels play major roles in the development of brain edema in ALF. For reviews, see (Blei, 1997). Blood and brain ammonia levels have been shown to correlate with the degree of encephalopathy and brain edema (Clemmesen et al., 1999; Kato et al., 1989; Ong et al., 2003; Traber et al., 1987), and ammonia is known to induce cell swelling in cultured astrocytes (Norenberg et al., 1991; Olson et al., 1992; Zwingmann et al., 2000) and in brain slices (Ganz et al., 1989).

The mechanisms by which ALF causes astrocyte swelling are not completely clear. It has recently been shown that altered ionic homeostasis, largely mediated by activation of the $Na^+$,$K^+$,$2Cl^-$ cotransporter (NKCC1) and associated cytoplasmic osmolar imbalance are involved (Jayakumar et al., 2008, 2011). Such osmotic imbalance must be accompanied by the entry of water into cells so as to achieve osmotic equilibrium.

Water entry in some cell types is known to be mediated largely by aquaporin (AQP) water channels (King and Agre, 1996). AQP4 is the principal water channel in astrocytes (Nielsen et al., 1997), where it is involved in the development of brain edema in various neurological conditions, including ischemic stroke, traumatic brain injury, brain tumors and hyponatremia (Bloch et al., 2005; Manley et al., 2004; Papadopoulos and Verkman, 2007; Verkman et al., 2006). Knock-out mice lacking AQP4 are resistant to the development of the cytotoxic brain edema in hyponatremia and ischemic stroke (Manley et al., 2000).

Previous studies have documented that treatment of cultured astrocytes with ammonia resulted in increased AQP4 levels, which correlated

* Corresponding authors at: Department of Pathology, University of Miami Miller School of Medicine, PO Box 016960, Miami, FL 33101, USA. Fax: +1 305 585 5311.
*E-mail addresses:* mnorenbe@med.miami.edu (M.D. Norenberg), vkakulavarapu@med.miami.edu (K.V. Rama Rao).
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well with the degree of cell swelling (Rama Rao et al., 2003). Additionally, silencing AQP4 in cultured astrocytes prevented the cell swelling following exposure to ammonia (Rama Rao et al., 2010). An increase in brain plasma membrane AQP4 was also documented in experimental ALF (Eefsen et al., 2010; Rama Rao et al., 2010). Together, these studies invoke a major role for AQP4 in the astrocyte swelling/brain edema associated with ALF. Despite these findings, one report found unaltered levels of brain AQP4 in experimental ALF (Wright et al., 2010).

To more comprehensively assess the role of AQP4, the present study employed AQP4-null mice to examine the role of AQP4 in the evolution of brain edema and associated neurological deficits in ALF using two models produced by the hepatotoxins thioacetamide (TAA) and acetaminophen (APAP). We found a robust development of brain edema in wild-type (WT) mice, which was associated with an increase in AQP4 content in the plasma membrane, although total cortical levels remained unchanged. Brain edema was remarkably reduced in AQP4-null mice following ALF, and these mice showed a delayed onset of coma. These findings suggest that AQP4 is an important determinant in the brain edema associated with ALF.

## Materials and methods

### *Transgenic AQP4-null mice*

AQP4-null mice with a CD1 background originally developed by Ma et al. (1997) were used in the present study. WT mice were generated by crossing CD1 males (Charles River Laboratories) with B6D2F1 females (Jackson Laboratories), and littermates of 10–14 weeks old (35–45 g) containing 50% of CD-1 and B6D2F1 background were used.

### *Thioacetamide (TAA) model of ALF*

TAA (100 mg/kg, i.p.) was used to induce ALF as described previously by Jayakumar et al. (2011). These mice have been previously characterized with regard to blood levels of glucose, lactate, creatinine, and hemoglobin (Avraham et al., 2011; Caballero et al., 2001; Chiang et al., 2011; Lim et al., 2011; Sarhan et al., 1993). In brief, TAA was injected i.p. daily for 3 days. To prevent hypoglycemia and dehydration, mice were given 0.5 ml/kg of fluid therapy every 12 h, s.c. (5% dextrose and 0.45% saline containing 20 mEq/l of potassium chloride). Control groups for both WT-type and AQP4-null mice received normal saline (vehicle used for TAA). Mice were euthanized by decapitation at the onset of coma (Grade V HE) (Gammal and Jones, 1989).

### *Acetaminophen (APAP) model of ALF*

The APAP model of ALF was induced in mice following the same protocol described for TAA, except that mice were fasted for 12 h before the injection of APAP (Kon et al., 2004; Shah et al., 2013). APAP (500 mg/kg) was freshly dissolved in pre-heated saline and a single dose was injected i.p. to WT and AQP4-null mice. Similar to the TAA model of ALF, these mice have been previously characterized with regard to blood levels of glucose, lactate, creatinine, and hemoglobin (Lim et al., 2010; Das et al., 2010; Grace-Lynn et al., 2012; Shah et al., 2013). To prevent hypoglycemia and dehydration, 3–4 h following APAP injection, mice were given 0.5 ml/kg of fluid therapy, s.c. (5% dextrose and 0.45% saline containing 20 mEq/l of potassium chloride). Control groups for both WT-type and AQP-4-null mice received normal saline (vehicle used for APAP). Following injection, mice had free access to food and water. All APAP-treated animals developed coma at $8 \pm 1.5$ h, at which time they were euthanized by decapitation. Cerebral cortices were rapidly removed and then frozen at −80 °C for subsequent Western blot studies. Both AQP4-null mice and WT-mice treated with hepatotoxins were euthanized at identical time points, i.e., at the time when WT-mice developed coma, so as to make a comparative analysis on brain water content in these mice.

All experimental procedures followed guidelines established by National Institute of Health Guide for the Care and Use of Laboratory animals and were approved by our Institutional Animal Care and Use Committee (IACUC).

### *Preparation of plasma membranes from cerebral cortex*

Plasma membrane enriched fractions were isolated following the method of Marples et al. (1995). In brief, cortical tissue was homogenized in 0.32 M sucrose–EDTA buffer containing a protease inhibitor cocktail (PIC, Roche Diagnostics), and centrifuged at 3000 *g* for 5 min. The pellet was frozen at −80 °C for 1 h to fracture the cells; then thawed and homogenized in 50 mM Tris–HCl (pH 8) containing PIC. The homogenates were centrifuged at 35,000 *g* for 30 min and the pellets were rehomogenized 2 times in 50 mM Tris–HCl buffer. The final pellet containing the plasma membrane enriched fraction was dissolved in 0.25 ml of lysis buffer containing 50 mM Tris–HCl (pH 7.4), 150 mM NaCl, 10% SDS, 1% NP-40, 5% sodium-deoxycholate and PIC.

### *Immunoblotting*

The protein concentration in plasma membranes was determined by the bicinchoninic acid method (BioRad). Equal quantities of plasma membrane and tissue lysates were subjected to SDS-PAGE using 12% gels (Tris–HCl, pH 7.4) and then electrophoretically transferred to PVDF membranes. Blots were blocked with 5% nonfat dry milk in tris-buffered saline (TBS) containing Tween 20 (20 mM Tris–HCl, 150 mM NaCl, pH 7.4, and 0.05% Tween 20; TBS-T) for 2 h at room temperature and then incubated with rabbit anti-AQP4 (1:3000, Millipore), overnight at 4 °C. PVDF membranes were then washed with TBS-T and incubated with HRP-conjugated secondary antibodies for 2 h at RT. After washing, membranes were visualized using enhanced chemiluminescence (ECL-plus; Amersham Biosciences, Piscataway, NJ). Optical densities of the bands were measured with the Chemi-Imager digital imaging system (Alpha Innotech, San Leandro, CA), and the results were quantified with the Sigma Scan Pro program (St. Louis, MO) as a proportion of the signal of a plasma membrane marker protein ($Na^+$–$K^+$-ATPase).

### *Immunohistochemistry*

Mice were anesthetized and transcardially perfused with heparinized saline for 1 min, followed by fixation in 4% paraformaldehyde for 15 min. The heads were left in the same fixative for an additional 24 h at 5 °C and cryoprotected with 30% sucrose in PBS. Coronal sections of brain were obtained and 20 μm thick sections were prepared with a cryostat. Frozen sections were blocked with 10% goat serum and incubated with specific antibodies to AQP4 (1:100) (Chemicon, CA) and GLUT 1 (1:100) overnight at 4 °C. Sections were washed with tris-buffered saline (TBS) containing 0.1% Triton X 100 (TBS-T); incubated with fluorescent AlexaFlour-FITC and AlexaFlour-Rhodamine conjugated secondary antibodies (1:500) for 2 h; covered with commercial mounting media (Vector Laboratories), and examined with a laser scanning confocal microscope (Olympus, Japan). Fluorescent images were captured by randomly moving the microscope stage 5 $mm^2$ in all 4 directions.

### *Measurement of brain edema*

Brain water content was determined by the wet/dry weight method. Approximately 10 mg tissue (3–4 pieces from each animal) of cerebral cortex was dissected; wet weights of tissue determined; tissue dried overnight in an oven at 120 °C; and dry weights determined. The differences in wet/dry weights were converted to percent water content [(tissue wet weight − tissue dry weight) / wet weight × 100].