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# Early upregulation in nasal epithelium and strong expression in olfactory bulb glomeruli suggest a role for Aquaporin-4 in olfaction

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**Abstract Aquaporin-4 (AQP4) has been reported to be upregulated post-partum in pregnancy and in early lung development. Several technical challenges exist in measuring AQP4 protein levels, among them sensitivity to detergent solubilization, sample heating and gel composition. Here we have optimized quantification of AQP4 using immuno-blots. Using improved methodology we find no evidence for AQP4 upregulation post-partum or in the early lung development. However, in the nasal epithelium AQP4 is upregulated as early as in the brain. Furthermore, AQP4 is strongly expressed in the glomerulus, the synaptic unit of the olfactory bulb, suggesting a role for AQP4 in olfactory function.**


*Keywords:* Aquaporins; AQP4; Membrane protein; Olfaction

## 1. Introduction

Aquaporins are a sequence-similarity family of 12 mammalian water channels that facilitate water and glycerol transport over cell membranes [1]. Whereas the role of some aquaporins yet remains to be elucidated, aquaporin-facilitated water transport is particularly well studied in the kidneys, where several aquaporins are expressed: AQP1 in the proximal tubuli and descending thin-limb, and AQP2, AQP3 and AQP4 in the collecting ducts. The action of the anti-diuretic hormone arginine vasopressin increases water reabsorption in the collecting duct by recruiting AQP2 to the apical membrane, in response to increased plasma osmolality and decreased effective circulating volume, while AQP3 and AQP4 provide water transport over the basolateral membrane [1–3].

AQP4 is expressed most strongly in the brain in rats [4,5], humans [5,6] and mice [7]. A series of studies over the last 12 years have established that AQP4 is found at the interfaces between the brain and blood, and brain and cerebrospinal fluid, respectively. AQP4 is enriched in astroglial endfeet facing blood vessels, and in the ependymal cells lining the brain ventricles [5,8,9]. These studies have led to the hypothesis that AQP4 is involved in water homeostasis in the mammalian brain [8]. Important clues about the role of AQP4 in the development of brain water imbalance have been revealed by knockout studies [10]. Strikingly, AQP4 knockout mice were more resistant than wild type to the formation of cellular or cytotoxic edema after water intoxication [11].

Eclampsia is a common cause of maternal and foetal mortality during and after pregnancy, involving hypertension and edema. A previous study reported immunoblot data indicating strongly elevated AQP4 protein levels in pregnant and post-partum rats, consistent with a pregnancy-induced upregulation of AQP4 [12]. If confirmed, this would provide important insight into the regulation of the AQP4 gene in particular, and in water regulation in eclampsia in general.

Highly hydrophobic integral membrane proteins such as AQP4 can behave atypically upon SDS–PAGE [13,14], the electrophoresis technique of choice for quantitative measurements of individual membrane proteins in tissue lysates. In this study, we optimized procedures for obtaining reliable AQP4 SDS–PAGE immunoblot signals, with respect to detergent choice, sample heating and gel composition, finding that these factors all affect the quantity of AQP4 monomers.

By improved technique we have reinvestigated the important claim of AQP4 upregulation in the brain of post-partum rats [12]. Our results indicate no upregulation of neither AQP4 protein, nor AQP4 mRNA in the rat brain post-partum. We have also investigated the reported upregulation of AQP4 in early lung development [15], finding no significant upregulation of AQP4. However, comparing AQP4 expression in the nose, which has been reported in two previous studies [16,17], with the reported upregulation of AQP4 in the cerebellum after P7 [18], we find that AQP4 is upregulated as early in the nose as in the brain. As this suggested a role for AQP4 in olfaction, we investigated the expression pattern of AQP4 in the olfactory bulb by immunohistochemistry, discovering that AQP4 is strongly expressed in the glomerulus, the multicellular synaptic compartment of the olfactory bulb.

## 2. Materials and methods

### 2.1. Membrane fractionation

Male PVG rats were killed by elevated $CO_2$ and decapitated. The brain was dissected and the cerebrum separated from the cerebellum and brain stem and put into chilled 50 mM HEPES–NaOH pH 7.4, 2 mM EDTA, 0.32 M sucrose and protease inhibitor cocktail (Roche). The tissue was homogenized in a glass-homogenizer (Kontes), then centrifuged $1000 \times g$ 10 min, yielding the P1 (nuclear pellet) and S1 (post-nuclear supernatant) fractions. S1 was subjected to another round of centrifugation at $164000 \times g$ for 30 min using a Beckman Ti70 rotor. The resulting supernatant S2 (cytosolic fraction) was decanted and the pellet P2 (crude membrane fraction) was resuspended in 10 ml of the same buffer and centrifuged once more under identical conditions, yielding the P2′ (washed membrane fraction). The P2 was

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resuspended in 5 ml of 50 mM HEPES pH 7.4, 2 mM EDTA and protease inhibitor cocktail and stored at −20 °C.

### 2.2. Total fraction homogenates

Rat brains from non-pregnant or post-partum (day 0–5) adult female Wistar rats were dissected and cerebellum/brain stem separated from cerebrum. The tissue was homogenized in 1% SDS, 10 mM sodium phosphate pH 7.4, 150 mM NaCl, 5 mM EDTA, protease inhibitor cocktail and then sonicated. Homogenates were centrifuged $1000 \times g$ 10 min to pellet any insoluble material and yielding the total fraction in the supernatant. One percent SDS solubilizes brain tissue completely as the homogenate goes into a clear solution after sonication when used at appropriate tissue weight: buffer volume ratios. All fractions were assayed for total protein using the detergent-compatible DC-Kit (Bio-Rad) with BSA diluted in $H_2O$ as a reference (the buffer alone gave only negligible readings).

### 2.3. AQP4 detergent solubilization assay

Fifty μg of mouse brain membranes were diluted to 1 g/L in 50 mM HEPES pH 8.0, 1 mM DTT and one of a series of detergents: 60 mM CHAPS (Sigma), 5% v/v Triton X-100 (Electron microscopy sciences), 5% DOC (Fluka), 5% v/v NP-40 substitute (Fluka), 5% v/v Decon90 (Decon laboratories limited) or 1% w/v SDS (Fluka). Triton-X-100 samples were sometimes supplemented with 150 mM NaCl or 8 M urea. In addition, 48 detergents from the Detergent Screening Kit for Crystallization (Sigma–Aldrich) were tested (data not shown). All samples were incubated at room temperature with agitation for 1 h, then centrifuged at $23\,000 \times g$ for 15 min. The resulting 50 μl supernatant was mixed with 30 μl 6× loading buffer (SDS in excess was used to prevent a detergent competition effect), 45 μl loaded on gel. Pellet was resuspended in 45 μl of 1× loading buffer, whole fraction was loaded. Gels were 10% SDS–PAGE for this assay.

### 2.4. SDS–PAGE and immunoblotting

1× sample loading buffer was 1.7% SDS, 60 mM Tris pH 6.8, 5% glycerol, and was supplemented with 100 mM DTT and 0.01% v/v β-mercaptoethanol where indicated. Molecular weight markers were SeeBluePlus2 and MagicMarkXP (both Invitrogen). Two setups were used for electrophoresis and blotting: First, pre-cast 10% or 12% bis–tris gels and the mini-cell (Invitrogen) were used. Bis-tris gels were run with MOPS-SDS buffer (Invitrogen) at 200 V for ~60 min or until the dye reached the bottom. Blotting was carried out according to the manufacturer's (Invitrogen) instructions using bis–tris blotting buffer with 20% methanol and 0.2 μm PVDF (Bio-Rad). Immunodetection was performed as described below.

Second, the Mini-Protean III cell from Bio-Rad was used for self-cast gels according to Laemmli (16). Gels measured 1.5 mm × 83 mm × 73 mm and the stacking gel was 15 mm. For conventional SDS–PAGE, gels consisted of 10% or 12% total acrylamide monomer ($C = 2.6\%$), 375 mM Tris pH 8.8, 0.1% SDS (resolving gel) and 4% or 5% total acrylamide monomer ($C = 2.6\%$), 125 mM Tris pH 6.8, 0.1% SDS. For Laemmli-Urea, gels were identical to the conventional gels with the exception of 3 M urea was included in both the resolving and the stacking gel. Urea concentrations from 2.8 M to 6 M were tested and no difference was found in terms of AQP4 monomer resolution. All self-cast gels were polymerized with the TEMED/persulfate system.

Self-cast gels were blotted with the Criterion cell (Bio-Rad). Briefly, after electrophoresis gels were equilibrated in Towbin buffer (25 mm Tris, 192 mM glycine) supplemented with 20% methanol and blotted onto 0.2 μm PVDF (Bio-Rad) at 100 V for 30 min (increasing blotting time to 90 min did not show improved transfer) with the same buffer. Successful transfer was approved by PonceauS staining (Sigma). Blots were destained with $H_2O$ followed by 0.1 M NaOH, then blocked for 15 min or more in 5% non-fat dried milk powder (Applichem) in TBST (20 mM Tris pH 7.6, 137 mM NaCl, 0.05% Tween20 (Sigma)) supplemented with 0.05% $NaN_3$.

Blots were incubated with rabbit-anti-AQP4 antibody (Chemicon AB3068) at a final concentration of 1 μg/ml in the blocking solution overnight at 4 °C. Blots were washed in TBST, then incubated in alkaline phosphatase conjugated goat-anti-rabbit 1:10 000 in TBST (Amersham, ECF Kit) 1 h at room temperature, washed for several hours in TBST and finally incubated 5 min with the ECF substrate before scanning on a Typhoon 9410 scanner (Amersham). Images were processed using ImageQuantTL-software (Amersham). Blots were stripped and reprobed according to the manufacturer's instructions (ECF Kit, Amersham). Antibodies for this purpose were mouse-anti-β-actin (Abcam ab8226) diluted 1:2000 and mouse-anti-GFAP (Chemicon mAB360) diluted 1:20 000.

### 2.5. RNA isolation, cDNA synthesis and TaqMan real-time PCR

Fifty milligram pieces of tissues were dissected from animals killed by carbon dioxide gassing and decapitation, and stored briefly in RNAlater (Ambion), before being homogenized and RNA extracted using RNeasy (Qiagen). The RNA concentration was quantified using NanoDrop (NanoDrop Technologies, Wilmington, USA) UV spectrometry, and 2 μg RNA was converted into cDNA using High Capacity cDNA Archive Kit (Applied Biosystems). AQP4 expression was measured using TaqMan assay Rn00563196_m1 (Applied Biosystems) on a 7900 HT Fast Real-Time PCR System (Applied Biosystems).

### 2.6. Immunohistochemistry

Male adult PVG rats were transcardially perfused with 25 ml 2% dextran in 0.1 M Naphosphate (NaPi) buffer (pH 7.4), then perfused with 500 ml 4% paraformaldehyde in 0.1 M NaPi at 50 ml/min. The teeth were excised and the nose region decalcified 24 h in 10% formic acid, then cryoprotected 24 h in 30% sucrose in 0.1 M NaPi. 16 μm cryosections were cut on a Leica CM3050 S cryostat. Sections were incubated 20 min in blocking buffer (3% BSA, 0.5% TritonX-100, 0.05% azide in TBS buffer), then incubated overnight at 4 °C with primary antibody (goat-anti-AQP4, SantaCruz sc9888, 2 μg/ml) or non-immune goat IgG (Sigma I9140, 2 μg/ml) in blocking buffer. After 3× 5 min washes in TBS, sections were incubated for 1 h in secondary antibody (donkey-antigoat, Jackson #705-165-147, 1:1000 dilution) in blocking buffer. After 3× 5 min washes in TBS, the sections were mounted with Prolong Antifade Gold with DAPI (Invitrogen), and images taken on a Zeiss LSM PASCAL Axioplan 2 Imaging confocal microscope. DAPI pictures were captures with the same microscope in epifluorescence mode.

## 3. Results and discussion

### 3.1. Detergent influence on AQP4 monomer signal

Agre and coworkers have proposed that AQP4 exists *in vivo* as a heterotetramer [19], a hypothesis supported by recent structural studies [20]. For reliable AQP4 monomer signal on SDS–PAGE of AQP4, complete breakdown of tetramers is essential. The choice of protein extraction parameters, in particular detergents, can play a key role in obtaining appropriate AQP4 signals upon SDS–PAGE/immunoblotting. We developed a simple solubilization assay to measure the detergent effect on AQP4 protein extraction.

Membrane fractions, obtained by ultracentrifugation, were incubated in various detergents, then centrifuged at $23\,000 \times g$, and the supernatant and pellet tested for presence of AQP4. With no detergent present in the buffer, all AQP4 signal remained in the membrane pellet (P) after centrifugation, and no signal was solubilized to the supernatant (S) (Fig. 1A, lanes 2 and 3).

Using CHAPS or Triton X-100, parts of the AQP4 signal is observed in the supernatant (Fig. 1A, lanes 5 and 7). Interestingly, while most of the AQP4 signal in the pellet is visible as monomer, dimer and tetramer (Fig. 1A, lane 4 and 6, "1×", "2×", "4×"), in the detergent solubilized fraction in the supernatant, only the ~120 kDa tetramer band is visible (Fig. 1A, lanes 5 and 7, "4×").

The AQP4 tetramer can be seen even more clearly if the Triton solubilization mixture is supplemented with 150 mM NaCl (Fig. 1A, lanes 8 and 9), or 8 M urea (lanes 10 and 11). Now AQP4 is almost depleted in the pellet (lanes 8 and 10), and