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Removal of aquaporin-4 from glial and ependymal membranes causes brain water accumulation

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ABSTRACT

There is a constitutive production of water in brain. The efflux routes of this excess water remain to be identified. We used basal brain water content as a proxy for the capacity of water exit routes. Basal brain water content was increased in mice with a complete loss of aquaporin-4 (AQP4) water channels (global *Aqp4*$^{-/-}$ mice), but not in mice with a selective removal of perivascular AQP4 or in a novel mouse line with a selective deletion of ependymal AQP4 (*Foxj1*-Cre:*Aqp4*$^{flox/flox}$ mice). Unique for the global *Aqp4*$^{-/-}$ mice is the loss of the AQP4 pool subjacent to the pial membrane. Our data suggest that water accumulates in brain when subpial AQP4 is missing, pointing to a critical role of this pool of water channels in brain water exit.

1. Introduction

There are several unresolved questions regarding the handling of water in the central nervous system (Hladky and Barrand, 2014). One important question that still needs to be resolved is how water is drained from the brain. There is a constitutive production of cerebrospinal fluid through the choroid plexus and of interstitial fluid from the brain microvasculature, and this production has to be balanced by an efflux of fluid to blood or subarachnoid space. There is also a net production of water from the brain's glucose metabolism that must be offset by water efflux (MacAulay and Zeuthen, 2010).

The predominant brain aquaporin – aquaporin-4 (AQP4) – is a bidirectional water channel that plays a critical role as an influx route of water in brain edema (Manley et al., 2000; Amiry-Moghaddam et al., 2003). An increasing body of evidence suggests that AQP4 doubles as an exit route for excess water (Papadopoulos and Verkman, 2013; Nagelhus and Ottersen, 2013). We have addressed this issue in a glial conditional *Aqp4* knockout mouse and found that this line showed a delayed clearance of brain water in the postnatal phase and increased basal brain water content in the adult phase (Haj-Yasein et al., 2011). The latter study left an important question unresolved: which of the several AQP4 pools in brain is responsible for water exit? Glial-conditional *Aqp4* knockout animals are deficient in AQP4 in three

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different locations: 1, in astrocytic membrane domains facing the basal lamina of brain capillaries; 2, in astrocytic membrane domains facing pia; and 3, in the basolateral membrane domains of ependymal cells. A priori, each of these three pools of AQP4 could have been responsible for the effects observed in our glial conditional *Aqp4*$^{-/-}$ animals. Further, it was not ruled out that glial conditional *Aqp4*$^{-/-}$ mice could have had developmental anomalies that impeded drainage of water downstream of the subarachnoidal space. To resolve these questions we compared brain water contents in three different mouse lines: 1, a global *Aqp4* knockout line, lacking each of the three AQP4 pools described above (Thrane et al., 2011); 2, α-syntrophin$^{-/-}$ mice, lacking the AQP4 pool normally present in pericapillary astrocytic membranes (Neely et al., 2001); 3, *Foxj1*-Cre:*Aqp4*$^{f/f}$ mice, a mouse line that was specifically generated for the present study and that harbored a selective deletion of the ependymal pool of AQP4. In addition, we measured the intracranial pressure (ICP) response after intracisternal liquid infusions to determine whether a genetically induced increase in brain water content could be due to inadvertent obstructions of outflow routes from the subarachnoidal space.

2. Materials and methods

2.1. Animals

The experiments were conducted on male adult (12–26 weeks, weighing 21–35 g) constitutive *Aqp4*$^{-/-}$ (Thrane et al., 2011) and *α-syntrophin*$^{-/-}$ mice (Adams et al., 2000) with C57BL/6J mice as controls, and novel ependymal-conditional *Aqp4*$^{-/-}$ mice. The latter mice were generated by crossing *Aqp4* floxed mice (Haj-Yasein et al., 2011) with *Foxj1*-Cre mice (Zhang et al., 2007) and subsequently breeding offspring with each other. Breeders were homozygous for the *Aqp4* floxed allele (*Aqp4*$^{f/f}$) and either non-carrier or heterozygous carrier of *Foxj1*-Cre. We obtained similar amounts of ependymal-conditional *Aqp4*$^{-/-}$ mice ("*Foxj1*-Cre:*Aqp4*$^{f/f}$ mice") as *Foxj1*-Cre negative litter controls ("*Aqp4*$^{f/f}$ mice"). The animals were allowed ad libitum access to food and drinking water. All animal experiments were approved by the Animal Care and Use Committee of the Institute for Basic Medical Sciences, University of Oslo.

2.2. Light microscopic immunocytochemistry

For all fixation protocols, the animals were deeply anesthetized by an i.p. injection of a Zoletil-Xylazine-Fentanyl cocktail (zolazepam 188 mg/kg, tiletamine 188 mg/kg, xylazine 4.5 mg/kg, fentanyl 26 μg/kg) before transcardiac perfusion with 0.1 M phosphate buffer (PB; pH 7.4) with 2% dextran for 15 s and fixative for 20 min (flow rate 8 ml/min). The perfused animals were stored at 4 °C overnight in situ. The brain was removed and cryoprotected by undergoing sucrose step (10, 20, and 30% in PB), and coronal sections were cut at 16 μm thickness on a cryostat. The sections were either used for hematoxylin-eosin staining (n = 3 for each genotype) or immunocytochemistry. Light microscopic immunocytochemistry was carried out using a method of indirect fluorescence, as described previously (Nagelhus et al., 1998). We used rabbit affinity-purified isolated antibody against AQP4 (Sigma A5971; 1 mg/ml; 1:500), diluted in 0.01 M PB with 3% normal goat serum, 1% bovine serum albumin, 0.5% Triton X-100, and 0.05% sodium azide, pH 7.4, and revealed by donkey secondary antibodies with indocarbocyanine (Cy3) (1:1000: Jackson ImmunoResearch Laboratories, West Grove, PA). The secondary antibody was diluted in the same solution as the primary antibody with the omission of sodium azide. The cortical sections were mounted with ProLong Gold antifade reagent with DAPI (Life Technologies), and viewed and photographed with a model "LSM 510 META" microscope (Zeiss) equipped with a model "Imager.M1 AX10" epifluorescence unit (Zeiss), using 561 nm for Cy3 visualization and 408 nm for DAPI visualization. For cortical sections, a 20×/0.8 Plan-Apochromat objective was used, and for the ventricles we used a 40×/1.3 Oil Plan-Neofluar objective.

2.3. Measurement of brain water content and weight

Brain water content and brain weight was measured with the wet/dry mass method (Haj-Yasein et al., 2011). Animals were sacrificed by cervical dislocation and the brains were immediately dissected out intact and in a standardized fashion to ensure a non-biased brain mass sample collection. All mice were prepared at the same circadian time. The pia mater was kept in place and a transverse cut was made by a rectangular razor blade through the brainstem just posterior to the cerebellum and perpendicular to the axis of the brainstem. Each brain sample was massed in a pre-weighed 10 ml glass vial, before being manually homogenized with a spatula against the inside of same vial. The vial was then massed with the brain sample once again, and wet brain sample mass was calculated from the difference. The samples were dried in a vacuum oven (Fistreem International) for 24 h at 80 °C and − 1000 mbar. After drying, each vial with dried brain was again massed. Percentage brain water content was calculated as (wet mass − dry mass) × 100 / (wet mass).

2.4. Measurements of intracranial pressure

Mice were anesthetized with an i.p. injection of a Zoletil-Xylazine-Fentanyl cocktail (zolazepam 188 mg/kg, tiletamine 188 mg/kg, xylazine 4.5 mg/kg, fentanyl 26 μg/kg). Body temperature was monitored with a rectal probe and kept at 37 °C by a temperature-controlled heating pad (Harvard Apparatus). Tracheostomy was performed and mice mechanically ventilated (model "SAR-1000" ventilator, CWE Inc.) with room air at 100 breaths per minute (bpm), volume 0.25–0.35 ml/min (depending on size of the mouse). Blood gases, blood pressure and oxygen saturation were monitored using MouseOx (StarrLife Sciences) with thigh sensor for mice. Oxygen saturation was at all times above 90%. An incision in the neck region was made and muscle was bluntly dissected to localize the cisterna magna. A 30 GA needle connected to tubing and a 50 μl Hamilton syringe was inserted into the cisterna magna and fixed with tissue adhesive (LiquiVet Adhesive, Oasis). A small craniotomy (1 mm in diameter, 3 mm posterior to bregma, 2 mm lateral from midline) was made in the skull and the ICP catheter (SPR-1000, Millar) connected to a pressure transducer (PCU 2000) inserted 2 mm under the dura towards bregma. When a stabile pressure was measured, baseline values were recorded for 1 min, before artificial cerebrospinal fluid (aCSF, containing (in mM): 124 NaCl, 2 KCl, 1.25 KH_2PO_4, 2 $MgSO_4$, 2 $CaCl_2$, 26 $NaHCO_3$, and 12 glucose, pH 7.3) was infused at 2 μl/min for 5 min by a pump (kdScientific, model 100 series), as previously described (Iliff et al., 2012).

2.5. Data analyses

The genotypes of the mice were for practical reasons known to the investigator when collecting samples and performing measurements. Genotypes were confirmed by re-genotyping biopsies after completion of data analysis. Data on basal brain water content and weight was analyzed using Prism (Version 6 for PC, GraphPad Software). Unpaired *t*-test was used for comparison between mutant mice and the respective controls.

Data on ICP was recorded in Clampex 10.4, imported into Matlab and maximum values calculated. Further processing and analysis of data were made in Excel. Unpaired *t*-test was used for comparison between mutants and the respective controls. For all comparisons $p < 0.05$ was considered statistically significant.