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

Gry Fluge Vindedal^{a,b}, Anna E. Thoren^b, Vidar Jensen^b, Arne Klungland^{c,d}, Yong Zhang^e, Michael J. Holtzman^e, Ole Petter Ottersen^f, and Erlend A. Nagelhus^{a,b,g}

^aCentre for Molecular Medicine Norway, Nordic EMBL Partnership, University of Oslo, 0318 Oslo, Norway.

^bGliaLab and Letten Centre, Division of Physiology, Department of Molecular Medicine, Institute of Basic Medical Sciences, University of Oslo, 0317 Oslo, Norway.

^cInstitute of Medical Microbiology, Oslo University Hospital, Rikshospitalet, 0027 Oslo, Norway.

^dDepartment of Molecular Medicine, Institute of Basic Medical Sciences, University of Oslo, 0317 Oslo, Norway.

^ePulmonary and Critical Care Medicine, Department of Medicine, Washington University School of Medicine, Saint Louis, Missouri 63110, USA.

^fLaboratory of Molecular Neuroscience, Division of Anatomy, Department of Molecular Medicine, Institute of Basic Medical Sciences, University of Oslo, 0317 Oslo, Norway.

^gDepartment of Neurology, Oslo University Hospital, Rikshospitalet, 0027 Oslo, Norway.

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*^{fllox/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.

Keywords

alpha-syntrophin; aquaporin; AQP4; astrocytes; brain edema; cerebrospinal fluid; CSF; endfeet; extracellular space; ependyma; Foxj1; glia; glymphatic; interstitial fluid; neuron-glia; paravascular; water homeostasis

Corresponding author: Erlend Nagelhus, Department of Molecular Medicine, Institute of Basic Medical Sciences, University of Oslo, 0317 Oslo, Norway. e.a.nagelhus@medisin.uio.no.

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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 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 subarachnoid 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 subarachnoid space.

Materials and methods

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.

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 sec 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 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; 1mg/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) (1:1,000; 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.

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 (Fistream 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).

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 μ l 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 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 stable 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 μ l/min for 5 min by a pump (kdScientific, model 100 series), as previously described (Iloff et al., 2012).

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.

Results

AQP4 immunofluorescence differed between genotypes. In wild-type mice (Fig. 1A,F), AQP4 immunolabeling was most intense subjacent to pia and around blood vessels, with lower intensities in parenchymal membranes and basolaterally in ependymal cells. Global *Aqp4*^{-/-} mice were devoid of AQP4 in all these locations (Fig. 1B,G), while *α -syntrophin*^{-/-} mice showed a selective loss of perivascular AQP4 (Fig. 1C,H). *Aqp4* floxed mice (Fig. 1D,I) showed similar AQP4 labeling as wild-types. The *Foxj1-Cre:Aqp4*^{f/f} mice, the novel ependymal-conditional *Aqp4* knockouts (Fig. 1E,J), lacked AQP4 in basolateral ependymal membranes but retained AQP4 both perivascularly and subpially. None of the genotypes showed AQP4 immunofluorescence labeling over the choroid plexus epithelium (Fig. 1K-O), in line with previous reports (Nielsen et al., 1997; Yang et al., 2011). Hematoxylin-eosin staining of coronal brain sections from ependymal-conditional *Aqp4* knockouts and controls (n=3 for each genotype) did not reveal apparent abnormalities in the cellular architecture in the vicinity of the third and lateral ventricles (Fig. 2). Specifically, the ependymal cells displayed similar morphology and organization in mutant (Fig. 2B, D, G and H) and control

mice (Fig. 2A, C, E and F). These findings contrast those of Li et al., (2009), who reported disorganized ependymal layer in global *Aqp4*^{-/-} mice.

Basal brain water content was significantly higher in global *Aqp4*^{-/-} mice than in wild-types (Fig. 3A). Whereas brain dry weight did not significantly differ between the genotypes (0.1057 ± 0.0009 g vs. 0.1030 ± 0.0009 g in *Aqp4*^{-/-} mice and wild-types, respectively, n=10 in each group, p=0.0572, unpaired *t* test), brain wet weight was 6% higher in *Aqp4*^{-/-} compared to wild-type mice (0.4882 ± 0.004 g vs. 0.4601 ± 0.004 g, respectively, p<0.001, unpaired *t* test; Fig.3B). Neither *α-syntrophin*^{-/-} nor *Foxj1-Cre:Aqp4*^{fl/fl} mice differed from their controls in basal brain water content (Fig. 3 C,D) or brain wet weight (not shown).

Intracisternal infusion of aCSF at 2 μl/min for 5 min was associated with a temporary elevation of ICP in both *Aqp4*^{-/-} and wild-type mice (Fig. 4). Neither peak value (3.40 ± 0.21 and 3.25 ± 0.32 mm Hg in *Aqp4*^{-/-} and wild-type mice, respectively, n=8 in both groups, p=0.62, unpaired *t* test) nor decay (160.5 ± 14 and 152.7 ± 22, p=0.09, unpaired *t* test) differed between the genotypes. This is in line with the idea that the gene deletion interferes with water drainage upstream of, rather than downstream of, the subarachnoidal space.

Discussion

To identify the exit routes for brain water is a matter of utmost importance, given the clinical implications of impaired water drainage. A failing balance between brain water production and brain water efflux will cause the intracranial pressure to increase to levels that might ultimately be detrimental to brain function.

Several lines of evidence suggest that the water channel AQP4 serves as an exit route for excess brain water. Notably, intraparenchymal infusion of isotonic solution leads to a more pronounced ICP elevation in mice lacking AQP4 (Papadopoulos et al., 2004). Moreover, deletion of *Aqp4* exacerbates vasogenic brain edema in multiple disease models (Papadopoulos and Verkman, 2013). Direct evidence for the role of glial AQP4 water channels in draining brain water came from our study using glial-conditional *Aqp4*^{-/-} mice (Haj-Yasein et al., 2011). Specifically, we showed that glial-conditional *Aqp4*^{-/-} mice displayed a delayed clearance of brain water in the postnatal phase and increased basal brain water content in the adult stage. The latter work indicates that brain water contents can be seen as a proxy for the capacity of water efflux routes. The rationale is that in the presence of relative impediments to water efflux, the equilibrium between cerebral and extracerebral water pools will be skewed towards more brain water. Hence we assessed brain water contents in three different mouse lines, with differential deletions of AQP4, in order to identify the AQP4 pools that are critical for water efflux.

Obviously, much would be gained by having access to more direct ways of differentiating between putative efflux routes for water. Pending relevant tracers and considering the limitations of the use of tritiated water (Hladky and Barrand, 2014), we have to make do with the current proxy. ICP changes following intracerebral water injections are indirect measures of water flux and will be confounded by changes in volume reserve capacities (due

to increased brain weight and hence an increased brain volume). Thus, such measurements do not provide an advance over the present experimental approach.

The most salient observation was that global *Aqp4*^{-/-} animals showed an increase in basal brain water contents and brain wet weight while knockout of the α -syntrophin dependent pool or ependymal pool of AQP4 failed to show such changes. If one looks at the interfaces between brain and extracerebral liquid spaces – i.e., candidate sites for brain water drainage – the difference between *Aqp4*^{-/-} mice and *α -syntrophin*^{-/-} mice is that the latter line contains a sizeable residual pool of AQP4 subjacent to the pial membrane (Amiry-Moghaddam et al., 2003). By inference, this result suggests that water clearance is critically dependent on the subpial pool of AQP4 – i.e., that this pool must be fully or partially intact for efficient drainage to occur. Conversely, the absence of water accumulation in brains with selective loss of perivascular AQP4 (the *α -syntrophin*^{-/-} mice) suggests that the latter pool of AQP4 is not critical for water efflux. Unfortunately, no animal line exists that has a selective depletion of the subpial pool of AQP4. Such a line could help provide quantitative data on the relative contribution of the perivascular vs. subpial pool of AQP4. An alternative explanation of our findings is that the brain water accumulation in *Aqp4*^{-/-} animals reflects loss of water channels or developmental abnormalities downstream of the subarachnoidal space. This possibility is not consistent with our ICP measurements following intracisternal aCSF injections. These injections produced the same pressure increase in *Aqp4*^{-/-} mice as in wild-types.

An ependymal-conditional *Aqp4*^{-/-} mouse line was generated specifically for this study in order to resolve whether elimination of ependymal AQP4 would cause water retention. No difference was found between ependymal knockouts and control mice, neither for basal brain water content nor for brain wet weight. This suggests that AQP4-dependent water movement through the ependymal layer is not critical for water clearance from brain.

Conclusion

The present study suggests that depletion of astrocytic AQP4 causes water to accumulate in brain. An accumulation of brain water does not occur when deletion is restricted to the perivascular pool of AQP4, indicating that the other major pool of astrocytic AQP4 (the subpial pool) is the one that is critical for water efflux. That the subpial pool of AQP4 may become limiting for water efflux adds a new aspect to current concept of brain water circulation. The current concept has largely neglected the role of transpial water transport. Our finding that depletion of perivascular AQP4 failed to affect brain water contents must not be taken to suggest that this pool is not involved in water transport between blood and brain. Indeed, a series of studies provide convincing evidence that this AQP4 pool serves as an influx route of water in cerebral edema. Since AQP4 allows bidirectional transport of water, the inference is that perivascular AQP4 must double as an efflux route. What the present study indicates is that the deletion of this route can be compensated for by the sizeable pool of subpial AQP4. This conclusion stands to be corroborated once knockouts are available that offer selective removal of the latter pool.

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Highlights

- Aquaporin-4 (AQP4) water channels in astrocytes regulate basal brain water content
- AQP4 is not involved in draining CSF through arachnoid villi or lymphatic vessels
- Ependymal AQP4 does not have any major effect on basal brain water content

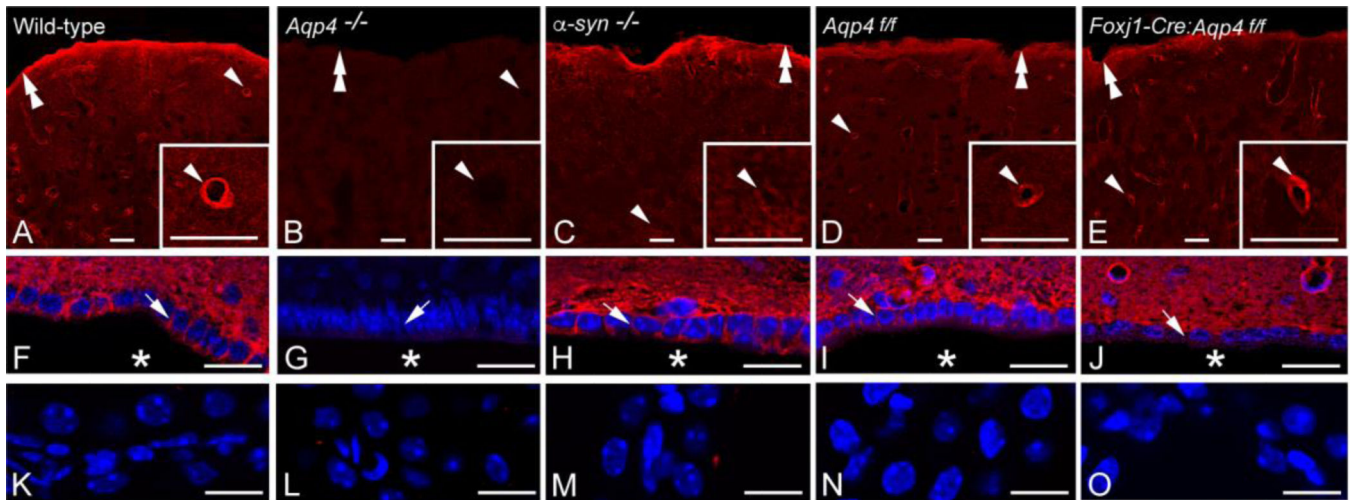


Figure 1.

Effect of gene knockout on AQP4 distribution. AQP4 immunofluorescence labeling (red) of the cortex (A-E), periventricular area (F-J; nuclear labeling in blue) and choroid plexus (K-O; nuclear labeling in blue) of wild-type (A, F, K), global *Aqp4*^{-/-} mice (B, G, L), α -syn trophin^{-/-} mice (C, H, M), homozygous *Aqp4* floxed mice (“*Aqp4*^{fl/fl}”) (D, I, N) and ependymal-conditional *Aqp4*^{-/-} mice (“*Foxj1-Cre:Aqp4*^{fl/fl} mice”) (E, J, O). **A, F, K:** In wild-type mice AQP4 labeling is most pronounced around cortical blood vessels (arrowhead; same vessel shown at higher magnification in inset) and along the pial surface (double arrowhead). Distinct AQP4 immunolabeling is evident in the basolateral membrane (arrow) of the ependyma covering the third ventricle (asterisk). The choroid plexus epithelium is devoid of AQP4 immunolabeling. **B, G, L:** Global *Aqp4* deletion abolishes AQP4 immunolabeling. Labels as in A, F. **C, H, M:** In α -syn trophin^{-/-} mice AQP4 labeling is lost around cortical blood vessels but still prominent along the pial surface and in the basolateral ependymal membrane. Labels as in A, F. **D, I, N:** AQP4 labeling in *Aqp4*^{fl/fl} mice is indiscriminately from that of wild-types. Labels as in A, F. **E, J, O:** In *Foxj1-Cre:Aqp4*^{fl/fl} mice the AQP4 immunofluorescence signal is lost from ependymal membranes but otherwise similar to that of litter controls (*Aqp4*^{fl/fl} mice) and wild-types. Labels as in A, F. Scale bars: 20 μ m.

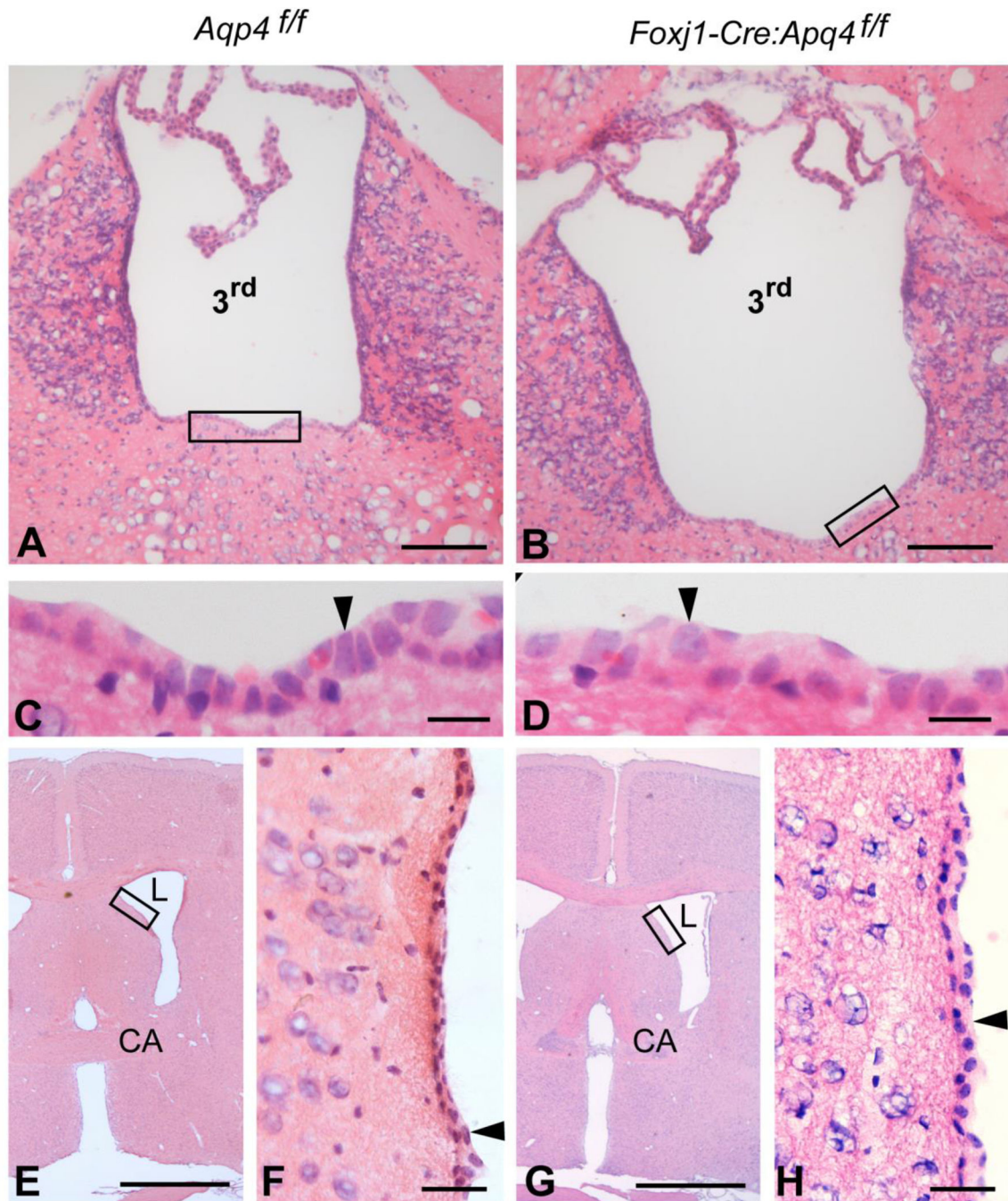


Figure 2.

Ependymal-conditional $Aqp4^{-/-}$ mice do not reveal apparent abnormalities in the ependymal architecture. **A–H:** Hematoxylin-eosin staining of coronal brain sections revealed comparable morphology of the ependyma (arrowheads) lining the third (3rd) and lateral (L) ventricles in control (“ $Aqp4^{f/f}$ ”; **A, C, E and F**) and ependymal-conditional $Aqp4^{-/-}$ (“ $Foxj1-Cre:Aqp4^{f/f}$ ”; **B, D, G and H**) mice. Boxed areas in **A, B, E and G** are enlarged in **C, D, F and H**, respectively. CA, commissura anterior. Scale bars: **A, B:** 100 μm ; **C, D:** 10 μm ; **E, G:** 1 mm; **F, H:** 30 μm .

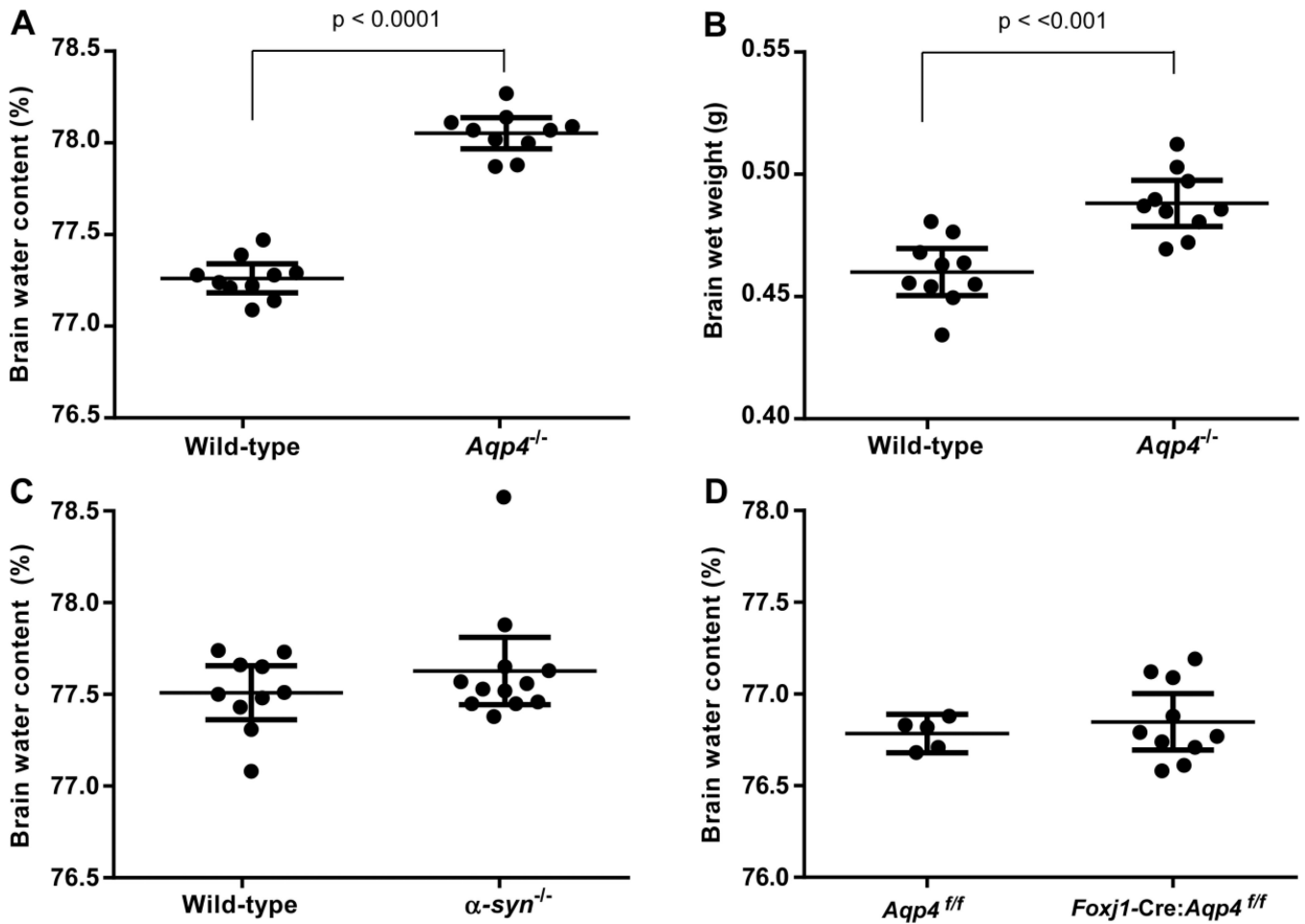


Figure 3.

Impact of gene deletion on basal brain water content and brain wet weight. **A, B:** In $Aqp4^{-/-}$ mice ($n=10$) basal brain water content and brain wet weight are significantly elevated compared to wild-types ($n=10$; p values are indicated, unpaired t test). **C, D:** Neither $\alpha\text{-syn}^{-/-}$ mice ($n=12$) nor $Foxj1\text{-Cre:Aqp4}^{fl/fl}$ mice ($n=10$) differed significantly from their controls ($n=10$ and $n=5$, respectively) in basal brain water content ($p > 0.05$ for both comparisons, unpaired t test).

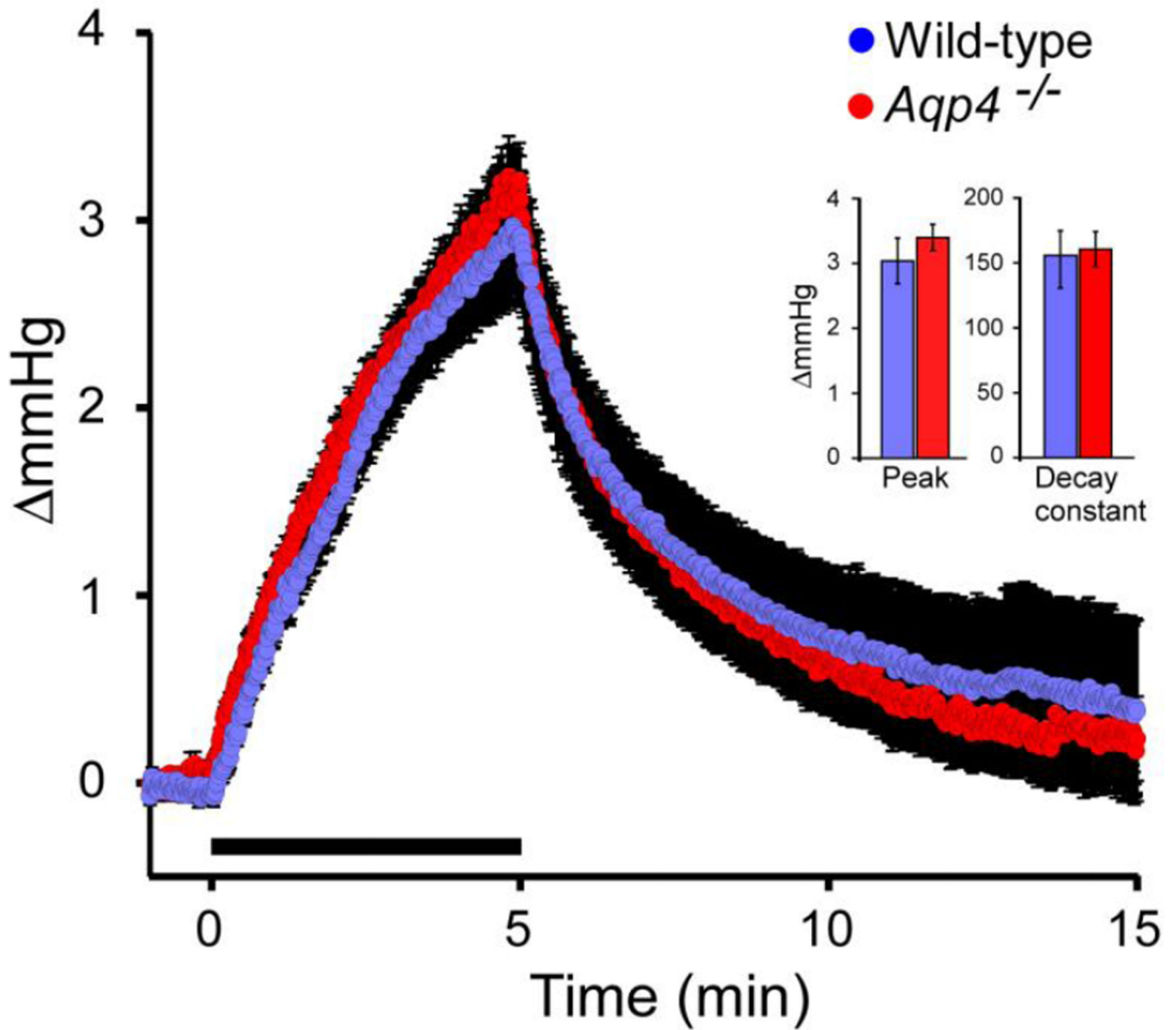


Figure 4. Effect of intracisternal infusion of aCSF on ICP. Infusion of aCSF at 2 μ l/min for 5 min (infusion period indicated by bar) elevates ICP in both *Aqp4*^{-/-} and wild-types. Neither peak ICP increase nor ICP decay differed between the genotypes ($p > 0.05$, unpaired *t* test for both comparisons).