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Astrocyte aquaporin mediates a tonic water efflux maintaining brain homeostasis

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Abstract

Brain water homeostasis provides not only physical protection, but also determines the diffusion of chemical molecules key for information processing and metabolic stability. As a major type of glia in brain parenchyma, astrocytes are the dominant cell type expressing aquaporin water channel. However, how astrocyte aquaporin contributes to brain water homeostasis in basal physiology remains to be understood. We report that astrocyte aquaporin 4 (AQP4) mediates a tonic water efflux in basal conditions. Acute inhibition of astrocyte AQP4 leads to intracellular water accumulation as optically resolved by fluorescence-translated imaging in acute brain slices, and *in vivo* by fiber photometry in moving mice. We then show that the tonic aquaporin water efflux maintains astrocyte volume equilibrium, astrocyte and neuron Ca^{2+} signaling, and extracellular space remodeling during optogenetically induced cortical spreading depression. Using diffusion-weighted magnetic resonance imaging (DW-MRI), we observed that *in vivo* inhibition of AQP4 water efflux heterogeneously disturbs brain water homeostasis in a region-dependent manner. Our data suggest that astrocyte aquaporin, though bidirectional in nature, mediates a tonic water outflow to sustain cellular and environmental equilibrium in brain parenchyma.

Significance statement

Our brain is immersed, thus protected, in a water environment. It ensures intra- and extracellular molecular diffusion, which is vital for brain function and health. Brain water homeostasis is maintained by dynamic water transport between different cell types. Astrocytes are a main type of glial cell widely distributed in brain parenchyma, expressing the bidirectional aquaporin water channel. Here we show that in basal conditions, aquaporin channel mediates a tonic water efflux from astrocytes. This mechanism maintains astrocyte volume stability, activity-gated brain parenchyma remodeling and brain water homeostasis. Our finding sheds light on how astrocytes regulate water states in the brain, and will help to understand brain allostasis in specific life contexts.

eLife assessment

Using in vitro and in vivo experiments, the authors show that astrocytes swell after inhibition of aquaporin 4 (AQP4) with TGN-020, which is indicative of tonic water efflux from these cells under physiological conditions. Though potentially **valuable**, the study is currently **incomplete** due to possible off-target effects of TGN-020, limited mechanistic information underlying the detected effects, and potential limitations of some of the adopted experimental techniques. These findings can be especially relevant for cortical spreading depression in ischemic stroke or seizure and to get a comprehensive understanding of neuron-astrocyte interactions.

Introduction

Every aspect of brain function relies on the delicately maintained water environment. It supports brain structural stability and molecular diffusion, laying the ground for information processing, metabolite shuttling and adaptation to living environments (Kimelberg, 2004). Water is the fundamental constituent of the cerebrospinal fluid infiltrating into the central nervous system and the interstitial fluid distributed in brain parenchyma (Agnati et al., 2017, Brinker et al., 2014). Brain fluid transport is suggested to support the diffusion of energetic fuels like glucose and lactate to warrant the metabolic circumstances in the parenchyma, and is also implicated in the clearance of waste molecules from the brain as described in the glymphatic system (Abbott et al., 2018, Louveau et al., 2017). In addition, brain water diffusion is the basis for diffusion-weighted magnetic resonance imaging (DW-MRI) for structural and functional neuroimaging (Le Bihan et al., 2006), and for clinical diagnostics widely applied for neurological diseases such as ischemia, brain tumor and edema (Gaddamanugu et al., 2022, Le Bihan & Iima, 2015).

Water equilibrium in the brain is maintained by dynamic transport between different cell entities. Aquaporin is a family of transmembrane channels facilitating bidirectional water flow, with aquaporin 4 (AQP4) being the main subtype expressed in the central nervous system. In the brain, AQP4 is predominantly expressed in astrocytes (Papadopoulos & Verkman, 2013, Xiao et al., 2023) that are a major type of glial cell in the parenchyma (Barres, 2008). This feature enables astrocytes to well balance osmotic oscillations imposed by the transmembrane transport of ions, metabolites, and signaling molecules, all mediated by water. Dysregulation in astrocyte aquaporin water transport is implicated in pathological scenarios such as cerebral edema and neuroinflammation (Verkman et al., 2011). Valuable insights have been obtained on the function of astrocyte aquaporin in specific physiopathological conditions including osmotically evoked volume responses, ischemia and neurodegenerative diseases (Verkman et al., 2017). Meanwhile, how astrocyte aquaporin contributes to brain water homeostasis in basal physiology has remained elusive. A better understanding will aid to discern astrocytic regulation of cerebral water states, thereby the potential maladaptations in neurological disorders.

Here, combining *in vivo* chemical targeting, optical and MRI imaging of water dynamics, we report that AQP4 sustains a tonic water efflux from brain astrocytes. This mechanism was found to be critical in maintaining astrocyte volume and signaling stability, as well as the extracellular space remodeling in mouse cortex during optogenetically induced cortical spreading depression. Using DW-MRI, we observed that acute inhibition of astrocyte water efflux *in vivo* heterogeneously alters water diffusion across brain regions. Our finding suggests that aquaporin acts as a water export route in astrocytes in physiological conditions, so as to counterbalance the constitutive

intracellular water accumulation caused by constant transmitter and ion uptake, as well as the cytoplasmic metabolism processes. This mechanism hence plays a necessary role in maintaining water equilibrium in astrocytes, thereby brain water homeostasis.

Results

Astrocyte aquaporin mediates a tonic water efflux

To optically follow *in situ* astrocyte water transport, we performed fluorescence intensity-translated (FIT) imaging. To this end, mouse brain astrocytes were chemically labeled *in vivo* with a highly water-diffusible and astrocyte-specific red fluorescent dye, sulforhodamine B (SRB) (**Fig. 1A**) (Appaix et al., 2012, Pham et al., 2020). About one hour after its intraperitoneal injection, astrocytic SRB labeling was widely distributed in the mouse cortex, as seen in living acute brain slices (**Fig. 1A**) and validated by colocalization with EGFP-identified astrocytes in slices taken from GFAP-EGFP transgenic mice (**Fig. 1A**). Net water transport across the astrocyte membrane alters cytoplasmic SRB concentration and cellular volume, which can thus be followed by changes in fluorescence intensity when imaged in a fixed field of view. To validate the FIT imaging of astrocyte water transport, we employed wide-field fluorescence microscopy for single-plane time-lapse imaging in acute brain slices in the primary somatosensory (S1) cortex. This approach allowed us to collect fluorescence from both the focal plane and along the axial extensions, thereby imprinting volumetric fluorescence into the single image plane. Indeed, water influx induced by the applicaiton of hypoosmotic solution over different periods proportionally decreased astrocyte SRB fluorescence, which also reflected cell swelling (**Fig. 1B**); whereas water export and astrocyte shrinking upon hyperosmotic manipulation increased astrocyte fluorescence (**Fig. 1B**). Hence, FIT imaging enables real-time recording of astrocyte transmembrane water transport and volume dynamics.

In basal conditions, flat fluorescence time course was recorded in brain slices suggesting equilibrated water transport across the astrocyte membrane (**Fig. 1B**) and volume homeostasis in the brain parenchyma. To inspect a tonic role of aquaporin in astrocyte water transport, we sought to acutely block astrocytic aquaporin channel, aquaporin 4 (AQP4). We used the synthetic compound 2-(nicotinamido)-1,3,4-thiadiazole (TGN-020) that is derived from the condensation of nicotinamide and thiadiazole derivatives (Burnett et al., 2015, Huber et al., 2009), whose specificity for AQP4 has been validated *in vitro* by ion channel heterologous expression system (Toft-Bertelsen et al., 2021) and *in vivo* using the AQP4 knockout (KO) mice (Harrison et al., 2020, Igarashi et al., 2013). This approach guaranteed the functional pinpointing of astrocyte aquaporin under physiological conditions, while avoiding the chronic compensations caused by genetic tools and mouse models that have been reported to alter brain water content, volume and extracellular architecture potentially confounding the functional readouts (Binder et al., 2004, Gomolka et al., 2023, Haj-Yasein et al., 2011, MacAulay, 2021, Yao et al., 2015).

AQP4 is a bidirectional channel facilitating passive water transport along the osmotic gradient (Papadopoulos & Verkman, 2013). As an osmotic equilibrium is maintained in the brain parenchyma in basal state, there might not be net water transport across AQP4. However, we observed in cortical slices that acute inhibition of astrocyte AQP4 by TGN-020 caused a gradual decrease in SRB fluorescence intensity, indicating an intracellular water accumulation (**Fig. 1C**). This observation suggests that astrocyte aquaporin mediates a tonic water efflux; its blocking causes intracellular water accumulation and swelling (**Fig. 1C**, *right*). To corroborate this observation *in vivo*, we performed fiber photometry recording in moving mice (**Fig. 1D**). An optical fiber was implanted into the mouse S1 cortex to follow fluorescence signals of local astrocyte population post the intraperitoneal injection of SRB (**Fig. 1D**, *left*). After about one hour, when astrocytes were well labeled, either saline or TGN-020 was injected intraperitoneally. While no significant effect was observed with the saline control, TGN-020 induced a decrease in

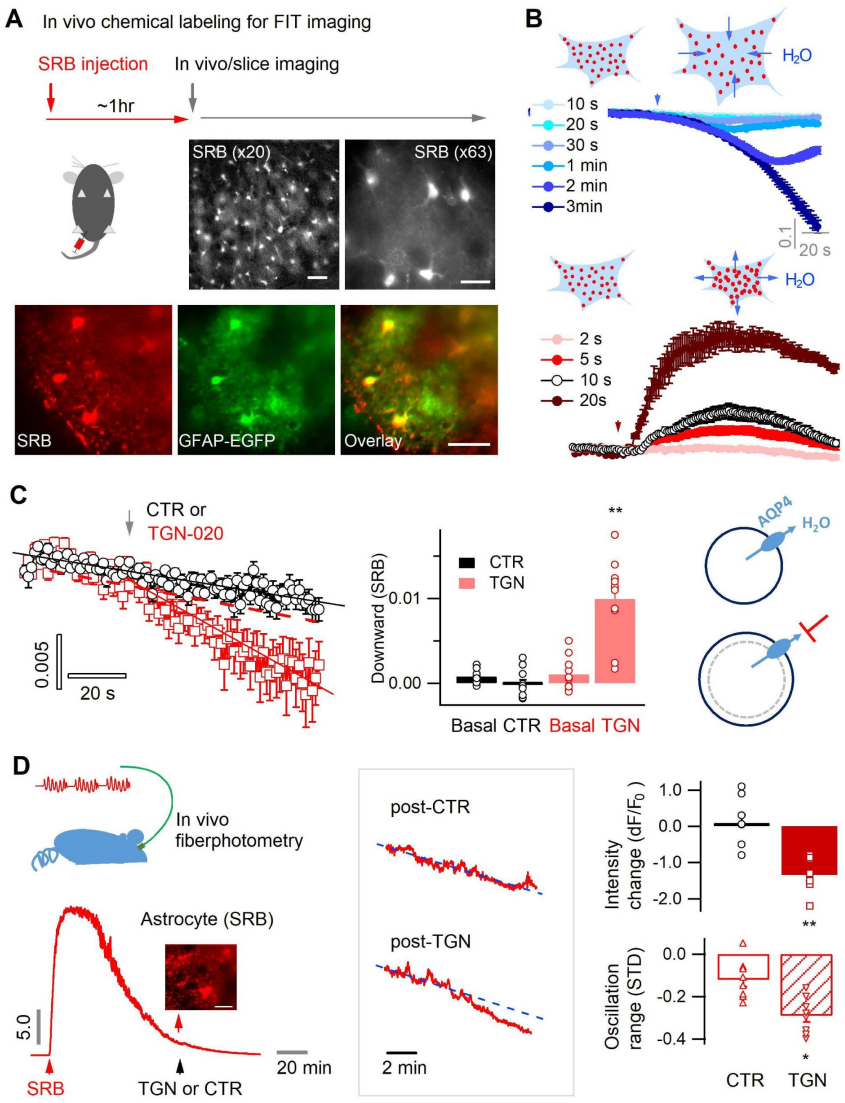

Fig. 1.

Astrocyte aquaporin mediates a tonic water efflux. (**A**) *In vivo* chemical labelling of astrocytes. Sulforhodamine B (SRB, 10 mg/ml) was intraperitoneally injected in awake mice (10 µl/g). Monochrome images show representative astrocyte labeling in living acute brain slices from cortex under low (×20; scale bar, 50 µm) and high magnification (×63; scale bar, 20 µm) by epifluorescence. *Below*, SRB labeling was confirmed to be astrocyte-specific in acute brain slices of the astrocyte reporter line GFAP-EGFP, where light sheet imaging was used to gain optical sectioning (Materials and methods; Fig. S1). Scale bar, 50 µm. (**B**) Optical imaging of astrocyte water transport in acute brain slices. Transmembrane water transport was triggered with hypo- and hypertonic solution, inducing water inflow and outflow that were respectively reflected by SRB fluorescence decrease and increase (expressed as dF/F_0). The hypo- or hypertonic solution was applied to slices over different lengths of duration displayed in colors corresponding to the time courses of SRB fluorescence (n = 52 astrocytes, 4 mice). (**C**) Acutely blocking astrocyte AQP4 with TGN-020 caused intracellular water accumulation and swelling. *Left*, while no effect was seen under CTR condition (vehicle only, n = 23 astrocytes), TGN-020 (20 µM) significantly decreased astrocyte SRB fluorescence (n = 30, 6 mice). Imaging was performed in acute brain slices of layer II/III S1 cortex. *Middle*, the downward slope was compared between the periods before and after the application of TGN-020. *Right*, illustration shows astrocyte aquaporin sustaining a tonic water efflux. Its blockade causes water accumulation and cell swelling. (**D**) *In vivo* validation of the effect of TGN-020 application on astrocyte water homeostasis. *Left*, fiber photometry was used for real-time recording of SRB fluorescence of astrocyte population in S1 cortex in freely moving mice, with saline (CTR) or TGN-020 being intraperitoneally injected when SRB was trapped in astrocytes. Fiber photometry recording shows that *in vivo* SRB injection resulted in rapid entry into mouse cortex and, in about one hour, led to astrocyte labeling (inset scale bar, 50 µm). *Middle*, example response to saline and TGN-020. Relative to CTR, TGN caused a decrease in astrocyte SRB fluorescence and its oscillation range (n = 8 recordings per condition, 5 mice).

astrocyte SRB fluorescence and a reduction in its oscillation range (**Fig. 1D**, *right*), mirroring an intracellular dilution of fluorescent molecules. This observation confirms that acute inhibition of astrocyte aquaporin leads to intracellular water accumulation thereby cell swelling.

Aquaporin inhibition perturbs astrocyte and neuron signaling

Astrocyte volume equilibrium not only determines brain architectural stability, but also associates with dynamic cellular signals. Astrocyte swelling has been shown to alter intracellular Ca^{2+} signaling (Benfenati et al., 2011, Eilert-Olsen et al., 2019). We performed Ca^{2+} imaging to confirm that acute aquaporin inhibition induces astrocyte swelling and then Ca^{2+} oscillation. The genetically encoded Ca^{2+} sensor GCaMP6f was selectively expressed in astrocytes by crossing the Glast-Cre^{ERT2} and $GCaMP6^{floxP}$ mouse lines (Herrera Moro Chao et al., 2022, Pham et al., 2020). Because astrocyte Ca^{2+} signals often occur in local domains, we adapted a light sheet microscope (Pham et al., 2020) for wide-field optical sectioning so as to image them with high signal-to-noise ratio (Fig. S1). We first confirmed that water accumulation, therefore astrocyte swelling, induced by hypotonic solution enhances the intracellular Ca^{2+} signal (**Fig. 2A**). Then we followed astrocyte Ca^{2+} in isotonic control condition while blocking astrocyte AQP4 with TGN-020, and observed that Ca^{2+} signaling was similarly augmented following the acute aquaporin inhibition (**Fig. 2B**). This result confirms that inhibiting astrocyte tonic water efflux leads to intracellular water accumulation and swelling, which causes alterations in astrocytic Ca^{2+} signaling. In addition, astrocyte swelling has been reported to induce the release of neuroactive molecules such as glutamate thereby influencing nearby neuron activity (Fiacco et al., 2007, Yang et al., 2019). Disrupting AQP4 tonic water outflow would not only cause astrocyte swelling but may also influence the activity of neighboring neurons. We then imaged Ca^{2+} signals as a surrogate for neuronal activity in cortical somatostatin (SST) interneurons which exhibit high excitability (Karagiannis et al., 2021) and express both ionotropic and metabotropic glutamate receptors (Cauli et al., 2000), rendering them ideal to sense astrocyte-released signaling molecules. We expressed GCaMP6f in SST interneurons by crossing homozygous SST-Cre (Taniguchi et al., 2011) and homozygous $GCaMP6^{floxP}$ mice. As expected, TGN-020 inhibition of astrocyte AQP4 led to a global Ca^{2+} elevation in SST interneuron populations (**Fig. 2C**). These observations support that astrocyte aquaporin mediates a tonic water efflux, contributing to maintain both the volume and signaling homeostasis.

Aquaporin water efflux regulates astrocyte volume response

We then examined the role of aquaporin water efflux in astrocyte volume response to osmotic changes. The AQP4 blocker TGN-020 or equimolar vehicle was applied throughout the recording, namely being continuously present before and after osmotic challenges. We first followed the evoked water efflux and shrinking induced by hypertonic solution while. As astrocyte AQP4 supports preferentially water efflux, its inhibition would attenuate hypertonicity-imposed water extrusion (**Fig. 3A**). Supporting this, application of TGN-020 slowed down the hypertonicity-induced water efflux and shrinking, reflected by the longer time to peak in the SRB fluorescence time course as compared to control (**Fig. 3B**), though the delay in the initial onset was not significantly prolonged. The maximal increase in astrocyte SRB fluorescence was lower in the presence of TGN-020, suggesting that AQP4 blocking reduced the overall amount of water efflux (**Fig. 3B**).

We next evoked water influx, thereby astrocyte swelling, by hypotonic solution in the presence or absence of TGN-020 (**Fig. 3C**). In contrast to the effects on hypertonicity-evoked water efflux, AQP4 acute inhibition was observed to accelerate both the initial water accumulation and the swelling rate in astrocytes, reflected by their earlier onset in SRB fluorescence decrease and faster reaching to the plateau value relative to the control condition (**Fig. 3D**). This observation cross-validates the role of astrocyte aquaporin in supporting water efflux, whereby its blockade facilitates the initial accumulation of the evoked water influx (**Fig. 3C**). In contrast, the late-stage

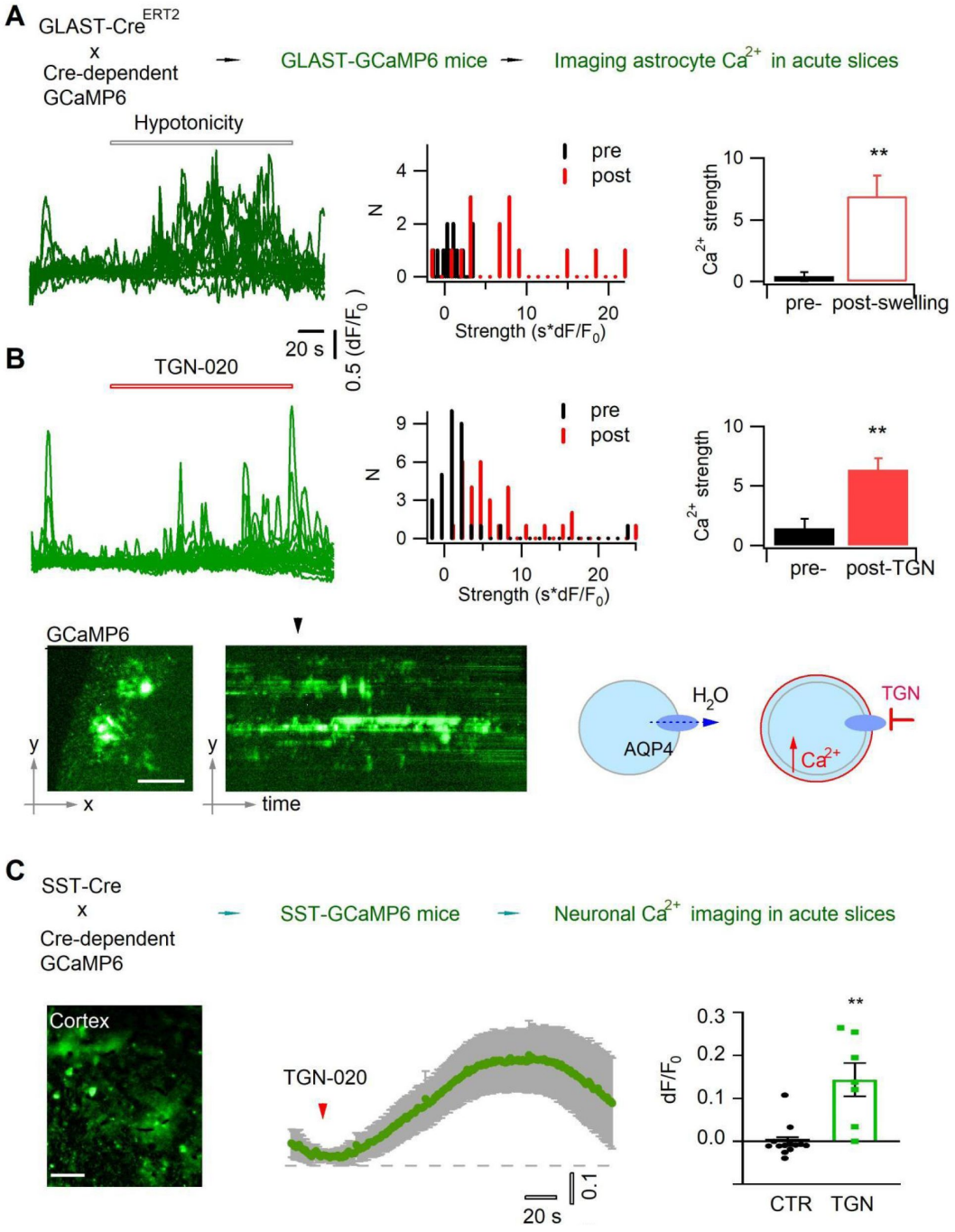

Fig. 2.

Acutely blocking astrocyte aquaporin induces swelling-associated Ca^{2+} oscillation. (**A**) *In vivo* expression in astrocytes of the genetically encoded fluorescent Ca^{2+} indicator GCaMP6 for imaging astrocyte Ca^{2+} in acute brain slices. Light sheet microscopy was used to capture transient Ca^{2+} signals of local regions. As a positive control, astrocyte swelling was induced by hypotonic solution (100 mOsM) that caused Ca^{2+} changes from their homeostatic level. *Left*, representative time courses of swelling-induced Ca^{2+} changes in detected response regions; *middle to left*, the histogram distribution and bar representation showing the signal strengths that were derived from the temporal integral of individual Ca^{2+} time courses normalized per minute, before and after cell swelling (n = 15 astrocytes of three mice). (**B**) Astrocyte Ca^{2+} oscillation caused by acute aquaporin blocking with TGN-020 (n = 31 astrocytes of 5 mice), due to the inhibition of the tonic water efflux that led to astrocyte swelling as illustrated. Time scale and Ca^{2+} rise scale (dF/F0) are the same as in *A*. Scale bar, 50 μm. (**C**) Intercellular effect on SST interneurons of blocking astrocyte aquaporin water efflux. TGN-020 (20 μM) or the equal molar vehicle (CTR) was bath applied to acute cortical slices of SST-GCaMP6 mice (n = 7-12 measurements from 4 mice). Scale bar, 50 μm.

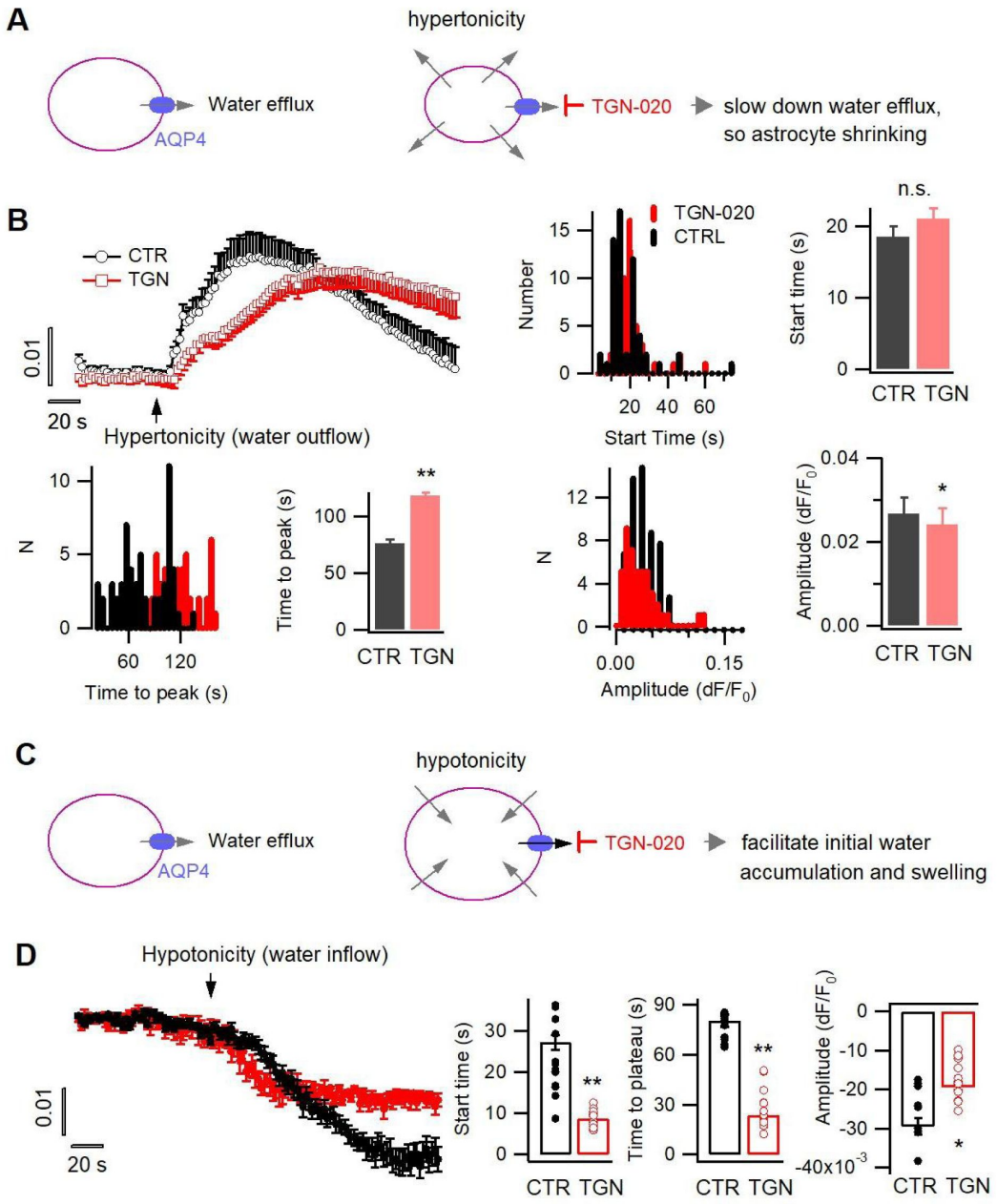

Fig. 3.

Tonic water efflux via aquaporin modulates phasic transmembrane water transport and astrocyte volume response. (**A**) *Left*, in basal condition astrocyte aquaporin mediates a tonic water efflux. *Right*, protocol to induce water outflow from astrocytes, therefore their shrinking, by hypertonic extracellular solution (400 mOsM) in either control condition (CTR) or in the presence of AQP4 inhibitor TGN-020 (20 µM). (**B**) Time courses of astrocyte SRB fluorescence increase upon the phasically induced water outflow, reflecting the occurrence of shrinking. The histograms and bar charts compare the start time, namely the delay between hypertonic solution application and rise in SRB fluorescence, the time to reach the peak of shrinking, and the absolute amplitude of water outflow-induced SRB increase (n = 58 astrocytes for CTR, and 47 astrocytes for TGN-020, four mice). (**C**) *Right*, protocol to trigger water inflow into astrocytes by hypotonic extracellular solution (100 mOsM) in either CTR solution or in the presence of TGN-020 (20 µM). (**D**) Time courses of astrocyte SRB fluorescence decrease caused by water inflow, which also reflects concomitant cell swelling. In contrast to the observation with hypertonicity-induced water outflow and astrocyte shrinking, a reduction was observed for both the start time and the time-to-peak with TGN-020 (n = 12 astrocytes for CTR, and 12 astrocytes for TGN-020, four mice). TGN-020 led to a decrease in the absolute amplitude of astrocyte swelling.

maximum decrease in astrocyte SRB fluorescence was observed to be reduced in the presence of TGN-020 (**Fig. 3D**), reflecting a reduction in the total amount of water accumulation and swelling. As TGN-020 was present prior to hypotonic challenge, it would have slightly swelled astrocytes due to the blockade of tonic water efflux, thereby constraining the range of the further swelling induced the subsequent hypotonicity. Further, while astrocyte AQP4 sustains a water outflow in basal physiology, in conditions when transmembrane water gradient is altered as occurred in hypotonic solutions, the net water flow through AQP4 should be finally dictated by the osmotic gradient due to its bidirectional nature. Therefore, along the imposed hypotonicity, AQP4 started to instruct a water influx; its constant blocking by TGN-020 would have reduced the total amount of water influx and swelling.

Astrocytes are widely distributed throughout the brain parenchyma, orchestrating water transport and volume homeostasis (Ochoa-de la Paz & Gulias-Canizo, 2022). Water outflow via astrocyte AQP4 may play a role in the structural remodeling of parenchyma at the global level. We then induced general cell swelling by triggering cortical spreading depression (CSD) (Holthoff & Witte, 1996, Zhao et al., 2019). To be orthogonal to the pharmacological control of astrocyte aquaporin, the CSD depolarization wave was initiated by optogenetically stimulating ChR2-expressing glutamatergic pyramidal cells in the cortical slices of Emx1-Cre/Ai32ChR2 mice (Gorski et al., 2002, Madisen et al., 2012), termed Opto-CSD (Chung et al., 2019). As cell swelling increases the transmittance of infrared light, we imaged Opto-CSD by the intrinsic optical signal (IOS) derived from infrared illumination (Holthoff & Witte, 1996, MacVicar & Hochman, 1991), which was spectrally distinct from ChR2 photoactivation (detailed in Methods). The IOS displayed transient increase across cortical layers during the Opto-CSD (**Fig. 4A-C**), exhibiting propagating kinetics characteristic of spreading depression (Chung et al., 2019, Holthoff & Witte, 1996). The biphasic starting of IOS coincided with a sharp response in the extracellular potential indicating the CSD initiation (Fig. S2A). IOS signal contains a first peak reflecting the rapid CSD response (**Fig. 4C**, *b*) followed by a prolonged phase of general cellular swelling (**Fig. 4C**, *c*). By combining fluorescence imaging of SRB-labelled astrocytes, whose spectrum is separated from the IOS infrared signal, we observed that astrocytes swelling (i.e., decrease in SRB fluorescence) paralleled CSD swelling (Fig. S3). Consistent with our observation on astrocyte volume response (**Fig. 3D**), when pre-incubating slices with TGN-020 to block AQP4 tonic water outflow, the initiation of both the CSD and general swelling was accelerated while their maximum amplitude reduced (**Fig. 4D-E**; Fig. S2B). Blocking action potentials with tetrodotoxin (TTX) only reduced the amplitude of the initial CSD response while the effect on general swelling is insignificant (**Fig. 4D-E**). We further followed the swelling of individual SRB-labelled astrocytes during Opto-CSD. In consistence with the result obtained from hypotonic challenge (**Fig. 3D**), the presence of TGN-020 reduced the peak amplitude of astrocyte swelling (i.e., the maximal SRB fluorescence decrease; **Fig. 4F**). As a post hoc adaptation to the transiently induced Opto-CSD, astrocyte swelling is followed by a shrinking to recover cellular volume to the homeostatic level, which was recorded as a rebound in SRB fluorescence intensity. We noted that TGN-020 presence significantly restrained the recovery of SRB fluorescence back to the baseline (**Fig. 4F**), showing that AQP4 inhibition hinders astrocyte water efflux therefore the shrinking.

Collectively, our data show that astrocyte AQP4 sustains a tonic water outflow regulating the cellular volume response and the general cell swelling of the parenchyma.

Tonic astrocyte water transport underlies brain homeostasis

Water homeostasis sets the basis for molecular diffusion in the brain, which instructs neurotransmitter availability, ion recycling and metabolite trafficking. We then examined *in vivo* the role of astrocyte aquaporin outflow in brain water diffusion using DW-MRI (Le Bihan & Iima, 2015). It uses the diffusion of water molecules to generate contrast that can be quantified by the apparent diffusion coefficient (ADC) in the nerve tissue (Beaulieu, 2002, Le Bihan, 2014). We used a 7T MRI to image global brain water diffusion in mice lightly anesthetized with medetomidine that normalized the animals under the sedative state. Astrocyte AQP4 outflow was

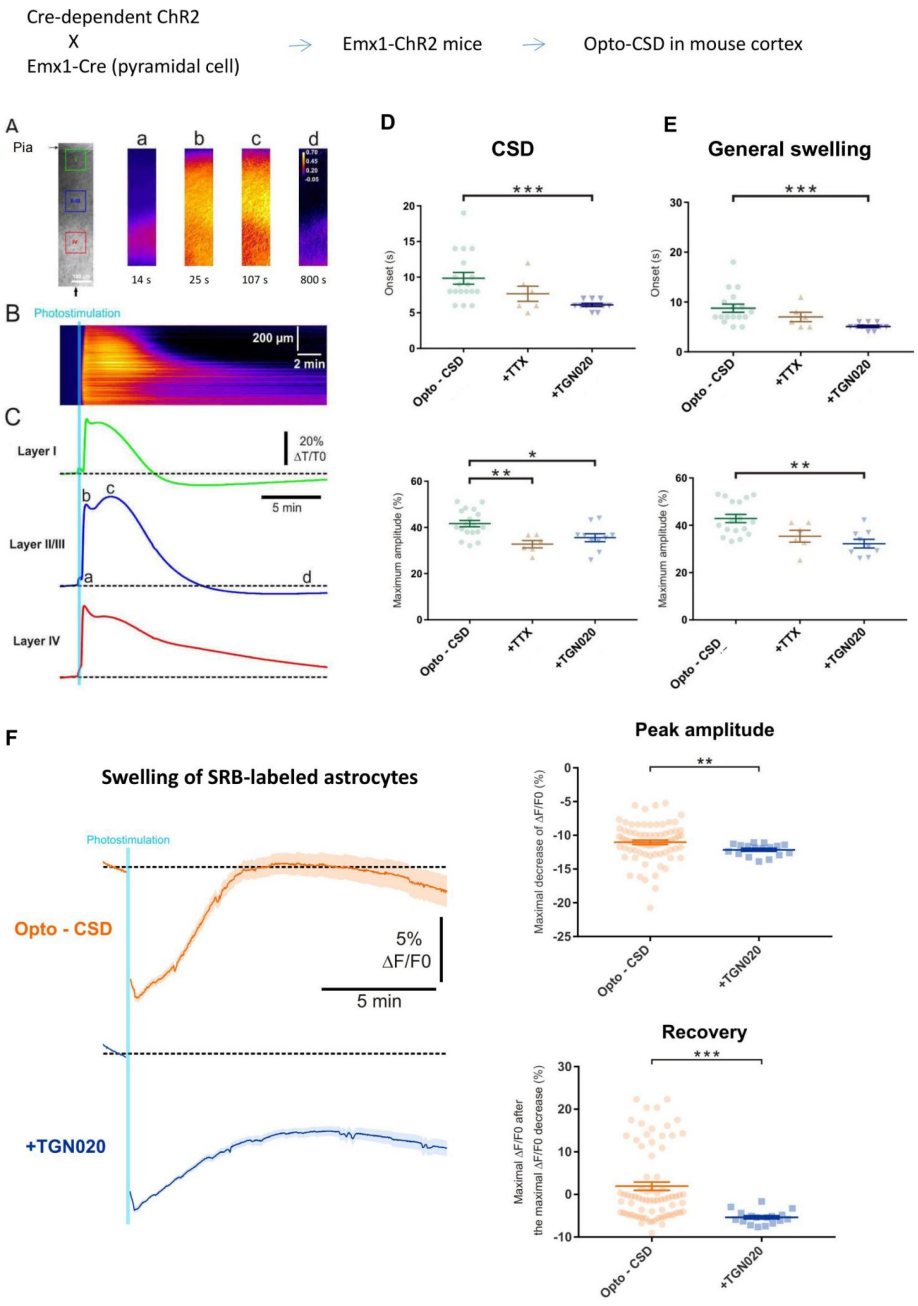

Fig. 4.

AQP4-mediated tonic water outflow regulates global swelling in cortical parenchyma. CSD-associated general swelling was induced by photostimulating ChR2-expressing pyramidal cells in acute cortical slices, and recorded by imaging intrinsic optical signal (IOS) with infrared illumination. (**A**) Representative recording. The pia surface is on the upper side; green, blue and red squares correspond to regions of interest in Layers I, II-III and IV, respectively. Transmittance signals are represented in pseudocolor images at different time points post photostimulation. Scale bar, 100 µm. (**B-C**) Kymograph and time courses showing the IOS changes following photoactivaiton, derived from the radial line of interest indicated by a black arrow in *A*. The light blue line indicates the 10-s photostimulation that increases the infrared transmittance signal (dT/T_0) across cortical layers, as also illustrated by the time courses; *a*, *b*, *c* and *d* correspond to the time points depicted in *A*. After the onset delay (*a*), the first (*b*) and second (*c*) peak of IOS are characteristic of the CSD and a prolonged general swelling, respectively. (**D-E**) TGN-020 (20 µM) inhibition of AQP4 reduced the initial onset and the maximal amplitude of both the CSD and general swelling (n = 6 - 18 measurements from 13 mice per condition) in layer II/III cortex. Inhibiting spiking activity with TTX (1 µM) only affected the amplitude of the initial CSD response. (**F**) Astrocytes swelling, reflected by SRB fluorescence decrease, monitored in control condition (n = 75 astrocytes) and in the presence of TGN-020 (n = 17 astrocytes) in layer II/III cortex.

acutely perturbed by TGN-020 applied via intraperitoneal injection. Brain water diffusion was mapped every 5 min before and after TGN or saline (as control) administration (**Fig. 5A**). Each acquisition sweep was performed with three b values (0, 250, 1800) that accounted for ADC, so to fit the exponential curve (**Fig. 5B**). Compared to the control saline, TGN-020 increased basal water diffusion within multiple regions including the cortex, hippocampus and the striatum in a heterogeneous manner (**Fig. 5C**). The temporal features in water diffusion change appeared to be also different: an early elevation followed by a reversible tendency was observed for cortical areas, a rapid and long-lasting elevation for the striatum while a delayed and transient increase for the hippocampus (**Fig. 5D**). The *in vivo* neuroimaging results confirm that the tonic water efflux from astrocyte aquaporin contributes to maintain the homeostasis of brain water diffusion, and also suggest spatiotemporal heterogeneities in brain water handling.

Discussion

Water equilibrium underlies brain function, plasticity and dynamics. Astrocytes are the principal brain cell type expressing aquaporin water channels that are highly implicated in regulating local environments in the parenchyma (Xiao et al., 2023). We show that albeit being a bidirectional channel, astrocyte aquaporin sustains a tonic water outflow in basal states to ensure structural and functional stability, as well as the water homeostasis at the brain level.

The observation of a basal aquaporin water efflux implies there is a constitutive water accumulation in brain astrocytes. As a ubiquitous vehicle for transporting ions, transmitters and metabolites, water enters astrocytes via a wide range of ion channels, transporters and exchangers. For instance, they express Na^+/K^+ and Na^+/HCO^- cotransporters to dynamically regulate intra- and extracellular ion homeostasis (MacAulay, 2021). Standing as a major glial cell type controlling the neuropil environment, astrocytes take up synaptically released K^+ through inwardly rectified K^+ channels and neurotransmitters (e.g., glutamate, GABA) via high-affinity transporters to safeguard synaptic transmission (Dallerac et al., 2018). The transmembrane molecular exchange may lead to continuous water entry into astrocytes. Moreover, astrocytes juxtapose the cerebral vasculature, being a front relay station for brain metabolism. They express glucose transporters and lactate-permeable monocarboxylate transporters facilitating energy substrate uptake and transfer between blood vessels and neuron-glia networks (Cauli et al., 2023). As water is constantly produced during metabolic processes (Bonvento & Bolanos, 2021), astrocyte metabolism would also contribute to the constitutive water accumulation in the cytoplasm. An efficient efflux pathway appears necessary to counterbalance the constitutive water buildup, thereby maintaining astrocyte and brain homeostasis. Our data show that astrocyte AQP4 fulfills such a role by mediating tonic water outflow (Fig. S4), lending functional supports to the previous suggestion (Amiry-Moghaddam et al., 2003). It has been observed that knock out of AQP4 leads to water over accumulation in the brain, likely due to the reduction in water exit from the pial membrane (Haj-Yasein et al., 2011, Vindedal et al., 2016). Our result also echoes that in the principal cells of the kidney collecting duct, AQP4 is suggested to mainly mediate water exit to balance the AQP2-sustained water entry (Noda et al., 2010). Moreover, we observed that inhibiting astrocyte basal water efflux alters Ca^{2+} activities in both astrocytes and neurons, reminiscent of the early finding that inhibition of AQP4 by TGN-020 enhances cerebral blood flow in mouse cortex (Igarashi et al., 2013).

This study also provides mechanistic hints to understand AQP4-relevant pathologies in specific contexts. For instance, astrocyte water accumulation and swelling are implicated in the development of brain edema. Different types of edema have been delineated depending on the pathological contexts, including such as cytotoxic, vasogenic and hydrocephalic subtypes. The presence of AQP4 has been found to ameliorate vasogenic (vascular leak) and hydrocephalic edema (Jeon et al., 2021, Tait et al., 2010), where the excessive water has been suggested to exit the brain via AQP4-dependent route. This is consistent with our observation that astrocyte

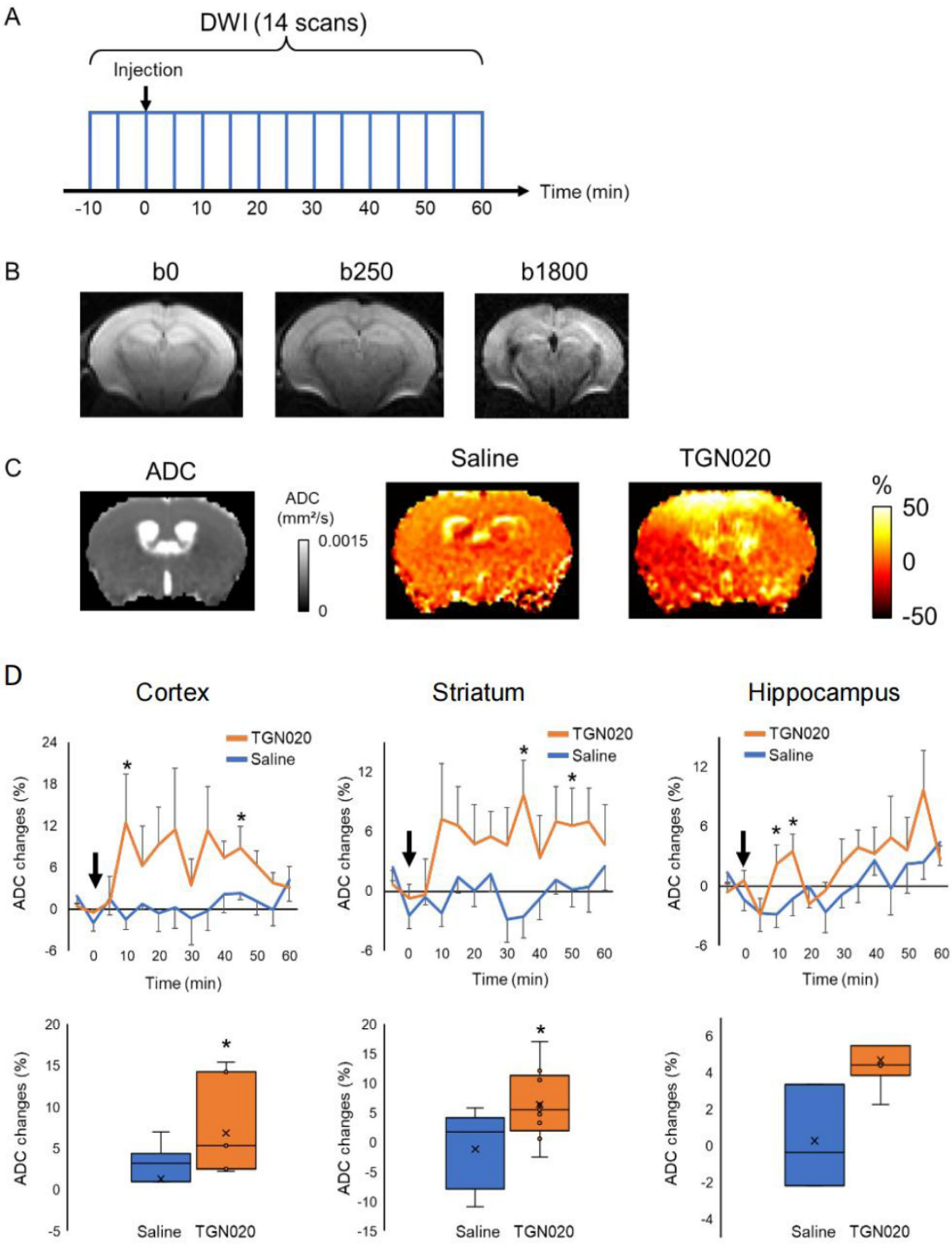

Fig. 5.

Perturbing the tonic water efflux via astrocyte aquaporin alters brain water homeostasis. *In vivo* DW-MRI (7T) was employed to map water diffusion in the entire brain scale following the acute inhibition of astrocytic AQP4 (TGN-020, intraperitoneal injection, 200 mg/kg), with paralleled control experiments performed with saline injection. (**A**) Experimental protocol for DW-MRI. Saline or TGN020 was injected at 10 min after the start of acquisition. DWI was acquired every 5 min. (**B**) Representative image obtained at three different b values to derive the water diffusion rates. (**C**) *Left*, brain water diffusion rate was mapped by the calculation of apparent diffusion coefficient (ADC). *Right*, representative images illustrating the relative changes of ADC at 60 min after injection of saline or TGN-020. (**D**) Time courses depicting the temporal changes in ADC in the cortex, striatum, and hippocampus, revealing the regional heterogeneity. Arrowhead indicates the injection of saline or TGN-020 (n = 10 mice for saline injection, 9 mice for TGN-020).

AQP4 contributes to basal water extrusion. Hence aquaporin facilitators would help to control vasogenic and hydrocephalic edema. Differently, inhibiting AQP4 has been observed to ameliorate cytotoxic edema encountered during cerebral ischemia (Verkman et al., 2011, Tait et al., 2010). Beneficial effects of TGN-020 on edema have been reported in spinal cord injuries (Li et al., 2019), in ischemia (Sun et al., 2022) and in retinal edema in diabetic animal models (Oosuka et al., 2020). These observations suggest that the role of AQP4 in pathological conditions is context-dependent. Indeed, while our results suggest astrocyte AQP4 mediates a water outflow in basal states, the direction of water transport in specific contexts will be determined by the actual transmembrane water gradient. As the origin of edema is variable and water is among the many other ions involved in edema formation (Stokum et al., 2016), astrocyte aquaporin may either be the primary cause for cytosolic water accumulation or secondarily sustain protective water extrusion. In neuromyelitis optica spectrum disorders (NMOSD), an unusual nervous autoimmune disorder often featured with circulating IgG autoantibody against AQP4, the impairment in astrocyte water homeostasis has been observed (Hinson et al., 2012, Lucchinetti et al., 2014). The inhibition of astrocyte AQP4 by autoantibody may initially affect the basal water efflux. Yet chronic pathological evolution could convert astrocytes into reactive states, with either or both the aquaporin expression, intra- and extracellular environments being altered, the role of AQP4 in water transport might be accordingly shifted (Mireles-Ramirez et al., 2022). Additionally, pro-inflammatory cytokines and chemokines during the immunological maladaptation could also contribute to shape astrocyte functions including aquaporin-mediated water transport (Lucchinetti et al., 2014, Mireles-Ramirez et al., 2022). Recent data show that TGN-020 targeting of AQP4 mitigates the inflammation during ischemia-reperfusion injury, by enhancing glymphatic amyloid clearance and inhibiting the ERK 1/2 pathway (Li et al., 2023), suggesting that in pathological conditions, multiple factors could be recruited by astrocyte aquaporin targeting.

We observed that acutely blocking aquaporin caused basal water accumulation leading to swelling in astrocytes, and facilitated the initial occurrence of the evoked swelling by hypoosmoticity and during cortical spreading depression. This recalls early observation by electron microscopy that knocking out the anchoring protein alpha-syntrophin to disperse AQP4 clustering induces an enlargement of astrocyte endfeet (Amiry-Moghaddam et al., 2003). It likely reflects astrocyte local swelling caused by the disruption of AQP4-mediated water efflux. Our observation also parallels the report that AQP4 knock out facilitates astrocytes swelling induced by hypoosmotic solution (Murphy et al., 2017). Nevertheless, genetic inactivation of AQP4 also reveals inhibitory effects on astrocyte swelling (Benfenati et al., 2011, Mola et al., 2016, Woo et al., 2018). Such discrepancy might be due to the variable compensations in brain water content and structure integrity during chronic genetic manipulations (Binder et al., 2004, Gomolka et al., 2023, Haj-Yasein et al., 2011, MacAulay, 2021, Yao et al., 2015). In addition, though our data suggest that under basal condition, astrocyte AQP4 sustains a tonic water outflow, in conditions when massive water influx persists as exemplified by the application of hypoosmotic solutions, the direction of water transport of AQP4 would follow the imposed water gradient.

Astrocyte aquaporin modulates brain water transport (MacAulay, 2021), though its role in the glymphatic system is under deliberation (Mestre et al., 2018, Rasmussen et al., 2022, Smith & Verkman, 2019, Smith et al., 2017). Our data suggest that astrocyte aquaporin-mediated outflow helps to maintain local water environment in the brain parenchyma. Moreover, besides affecting water transport, targeting AQP4 also impacts astrocyte volume therefore the size of extracellular space. These two factors would need to be considered when evaluating AQP4 involvement in brain fluid transport.

We show by DW-MRI that water transport by astrocyte aquaporin is critical for brain water homeostasis. When blocking AQP4 with TGN-020, therefore its basal water outflow, we observed spatially heterogeneous elevations of diverse temporal kinetics in brain water diffusion rate. Our current data are derived from 5 min apart acquisitions, providing information over the early phase of AQP4 inhibition therefore extending our early report (Debacker et al., 2020). The

regional heterogeneity likely reflects the various levels of AQP4 expression; its relative enrichment in the cerebral cortex (Clarke et al., 2018) corresponds to the pronounced effect observed here by DW-MRI. An overall increase in brain water diffusion rate was observed when blocking AQP4 water efflux. This treatment causes water accumulation inside astrocytes and their swelling, which reduces the extracellular space but increases the intracellular space. Water diffusion would be enhanced inside astrocytes and decreased in extracellular space, respectively. DW-MRI maps global water diffusion of both intra- and extracellular space. Likely, a net increase in brain water diffusion may reflect its intracellular increase exceeding the extracellular decrease. In addition, convective brain fluid flow has been suggested to be present in the perivascular space (Smith & Verkman, 2019), a scenario extended by the glymphatic system to the extracellular space of the parenchyma (Mestre et al., 2018). In this sense, transiently squeezing extracellular space might increase the rate of the water flow. The current MRI data yet lack sufficient spatial resolution to delineate the spatial compartmentalization of brain fluid flow.

Our study sheds light on the mechanisms by which astrocyte aquaporin contributes to the water environment in brain parenchyma, and will help to understand the processes underlying brain homeostasis and adaptation to life conditions.

Materials and methods

Animals and acute brain slice preparation

Experiments were undertaken in accordance with European Community guiding principles on the care and use of animals (86/609/CEE), and the rules of the host institute. For water transport and volume imaging, coronal slices comprising somatosensory cortex were acutely prepared from C57BL/6 mice of both sexes at ages of 4-6 weeks, unless otherwise indicated. Mice were deeply anesthetized by isoflurane (Axcience) evaporation in a closed plexiglass box. The brain was taken out and placed in a modified artificial cerebrospinal fluid (aCSF) for slicing (in mM: 30 NaCl, 4.5 KCl, 1.2 NaH2PO4, 1 MgCl2, 26 NaHCO3, 10 D-Glucose, and 194 sucrose) maintained at 4 °C during sectioning. The brain was cut into 300-µm thick slices with a vibratome (Leica VT1200S). Brain slices were recovered in standard aCSF (mM: 124 NaCl, 4.5 KCl, 1.2 NaH2PO4, 1 MgCl2, 2 CaCl2, 26 NaHCO3, and 10 D-Glucose) at 37 °C for about 1 h, and the same aCSF was used for brain slice imaging at room temperature. For optogenetic cortical spreading depression (opto-CSD) experiments, 18 to 21 day-old mice were deeply anesthetized with 150 µL of isoflurane (ISOVET, isoflurane, Piramal/healthcare). After the decapitation, the brain was quickly removed and placed into ice-colded oxygenated aCSF containing the following (in mM): NaCl 125; NaHCO3 26; Sucrose 15; Glucose 10; KCl 2,5; CaCl2 2; NaH2PO4 1,25; MgCl2 1; kynurenic acid 1 (nonspecific glutamate receptor antagonist, Sigma-Aldrich). Coronal slices from mouse somatosensory cortex (300µm thick) were cut with a vibratome (VT1000, Leica) and allowed to recover at room temperature for at least 45 min in aCSF saturated with O_2/CO_2 (95%/5%).

In vivo chemical and genetic targeting of astrocytes

Mice of similar age were used also for *in vivo* labeling of astrocytes, where the astrocyte-specific red fluorescent dye sulforhodamine B (10 mg/ml, Sigma) was intraperitoneally injected into awake mice at a dose of 10 µL/g. The genetically encoded Ca^{2+} indicator GCaMP6 was expressed in astrocytes *in vivo* by crossing a Cre-dependent GCaMP6f mouse line (Ai95, The Jackson Laboratory) with an astrocyte-specific Glast-Cre^{ERT2} mouse line (Slezak et al., 2007). Tamoxifen (T5648, Sigma) was dissolved in ethanol 100 % (at 1 mg/250µL) and vortexed until tamoxifen is fully dissolved. Corn oil (sigma) were then added to have a final solution at 10% ethanol and 90% oil. Solution is then heated at 37°c for 15 min and vortexed, and put in sonicator for 15 min to reach transparency, and then aliquoted. All procedures were made under light-proof protocols. Aliquots were stored at -20 °C until use. GCaMP6f/Glast-Cre^{ERT2} mice were injected with tamoxifen at ~3–4 weeks of age, once a day (1 mg) for two consecutive days. Genotyping of the Cre-dependent

GCaMP6f mouse line (Ai95, The Jackson Lab) used the standard primers and polymerase chain reaction protocols provided by the supplier. Genotyping of GLAST-Cre^{ERT2} mouse line was performed using the primers for Cre recombinase (TK139/141) as reported (Slezak et al., 2007). For Ca^{2+} imaging in somatostatin interneurons, homozygous mice B6J.Cg-Sst$^{tm2.1(cre)Zjh}$/MwarJ (SST-Cre, Jackson Laboratory) (Taniguchi et al., 2011) have been crossed with homozygous Ai95$^{GCaMP6f/GCaMP6f}$ mice to obtain heterozygous SST-cre$^{cre/WT}$::Ai95$^{GCaMP6f//WT}$ mice. For opto-CSD, homozygous mice B6;129PEmx1tm1lr/J ((Gorski et al., 2002), Emx1-Cre$^{cre/cre}$, Jackson Laboratory) have been crossed with homozygous mice B6;129SGt(ROSA)26Sortm32.1(CAG-COP4*H134R/EYFP)Hze/J ((Madisen et al., 2012) Ai32$^{ChR2/ChR2}$, Jackson Laboratory) to obtain heterozygous Emx1-cre$^{cre/WT}$::Ai32$^{ChR2/WT}$ mice. All procedures using animals were carried out in strict accordance with French regulations (Rural Code R214/87 to R214/130) and conformed to the ethical recommendations of the European Economic Community (Directive 86/609/EEC) and the National Charter French on ethics in animal experimentation. All protocols were approved by the Charles Darwin ethics committee and submitted to the French Ministry of Education and Research (Approval 2015 061011367540 APAFIS#573-2015061011367547 v1).

Ex vivo fluorescence imaging

Light sheet and epifluorescence imaging were both performed using a wide-field upright microscope (Zeiss Axioskop 50, Germany) equipped with water-immersion objectives. Epifluorescence illumination was provided by a monochromator light source (Polychrome II, TILL Photonics, Germany) directly coupled to the imaging objective via an optical fiber. We used water immersion imaging objectives (ZEISS): ×10 NA0.3 used to have a global view, ×20 NA0.5 used to perform light sheet imaging, and ×63 NA1.0 to image individual astrocytes under epifluorescence configuration so as to collect their whole-cell SRB fluorescence to follow astrocyte water transport and volume dynamics. The green fluorescence of GCaMP6 was separated from the red fluorescence of SRB, by spectrally exclusive double-band filters (Di03-R488/561-t3 and FF01-523/610, Semrock). SRB-labeled astrocytes were excited at 550 nm. Fluorescence signal was collected using a digital electron-multiplying charge-coupled device (EMCCD Cascade 512B, Photometrics).

A light sheet imaging system was adapted from our previous publication (Pham et al., 2020), where a chamber with a slightly tilted surface was used so as to spare the agarose embedding and allow the light sheet to access to any parts of the brain slice (Fig. S1). Briefly, the in-focus light sheet was introduced laterally from an independent optical module (Alpha3 light sheet add-on, Phaseview, France) equipped with an air objective (Zeiss EC EPIPlan ×10, 0.25NA). This optical module was orthogonal to the upright imaging pathway and coupled via a wavelength combiner (Thorlabs) to two continuous wave lasers 473-nm and 561-nm (CNI, China), for the excitation of GCaMP6 and SRB, respectively. The imaging chamber (20 × 30 × 46 mm width, height and length) was custom-designed using 3D modeling software (Trimble SketchUP), produced by a Mojo 3D printer (Stratasys) where the horizontal slice-supporting surface in the chamber was tilted by 8° along the laser entry direction. In this way, the brain slice could be laid on the slightly tilted surface while being stabilized with a conventional Harp slice anchor grid (Warner Instruments), whereby the laterally generated laser light sheet could reach local regions throughout the brain slices. The chamber was mounted on a motorized PI stage (Physik Instrumente GmbH, Germany) for axial micro-manipulation. Laser excitation and image acquisition were controlled by MetaMorph (Molecular Devices). Fluorescence dynamics of individual astrocytes was analyzed with ImageJ software (NIH). Background averaged from non-fluorescent subregion was subtracted from time-lapse images. Because single astrocytes typically occupy an area of ~50-100 µm in radius (Appaix et al., 2012), regions of interest of 50-µm diameter were delineated surrounding identified astrocyte footprint, to comprise the extended area of individual astrocytes while minimizing interference with neighbors. Time courses extracted from single regions of interest were normalized to the baseline (F0) expressed as dF/F_0.

Dynamic imaging of the extracellular space and astrocytes volume changes

Individual slices were transferred to a submerged recording chamber and perfused with oxygenated aCSF (~2 ml/min) lacking kynurenic acid. Slices were observed using a double port upright microscope (BX51WI, Olympus, Japan) equipped with Dodt gradient contrast optics (DGC; Luigs and Neumann), collimated light emitting device (LED; 780 nm, ThorLabs) as transmitted light sources, ×4 (PlanN, 0.10NA, Olympus), ×20 (UMPlanFL N, 0.50 W, Olympus) or ×40 (LUMPlanF/IR, 0.80 w, Olympus) objectives, and a digital camera (OrcaFlash 4.0, Hamamatsu) attached on the front port of the microscope. The observation with ×4 allows to delimit the cortical layers and to confirm the presence of "barrels" in layer IV. The ×20 objective was used to observe infrared intrinsic optical signals (IOS) (Holthoff & Witte, 1996, MacVicar & Hochman, 1991) of the cortical layers I to IV. The ×40 objective was used to image SRB labelled astrocytes combined with IOS in layers I and II/III.

After one minute of baseline period, a 10-s continuous photostimulation at 470 nm of the $ChR2_{H134R}$ (30% of the maximal power of the LED) was carried out via the epifluorescence port of the microscope using a collimated LED system (CoolLED, PreciseExcite) and a set of multiband filters consisting of an excitation filter (HC 392/474/554/635, Semrock), a dichroic mirror (BS 409/493/573/652, Semrock) and an emission filter (HC 432/515/595/730, Semrock) to observe the induced changes of light transmittance. The infrared and epifluorescence images were acquired each at 1 Hz (exposure 100 ms, binning 2×2) using the Imaging Workbench 6.1 software (Indec Byosystems) for a total duration of 15 min.

In vivo fiber photometry

The optical fiber (200 µm core, NA = 0.37, Neurophotometrics) coupled to a stainless-steel ferrule (1.25 mm) was stereotaxically implanted in mouse S1 cortex (rostro-caudal 0 mm, lateral +/-2.5 mm, vertical -1.2mm), and fixed to the skull with dental cement (SuperBond, Sun medical). Mice were habituated to the cage and fiber photometry setup for 30 minutes. Then, fluorescence was recorded in mobile mice. 5-min baseline was recorded before SRB injection, then SRB was applied to label brain astrocytes via intraperitoneal injection and the fluorescence time course was continuously recorded. To examine *in vivo* the effect on astrocyte water transport of acute AQP4 blocking, either TGN-020 (200 mg/kg; Tocris or MedChemExpress) or saline control was intraperitoneally injected 1 h after SRB injection, a time point when astrocytes were well labeled as confirmed by brain slice imaging.

Fluorescence signals were recorded using a Neurophotometrics fiber photometry system (FP3002). A branched fiber optic patch cord (BFP_200/230/900-0.37_FC-MF1.25, Doric Lenses) connected to the fiber photometry apparatus was attached to the implanted fiber optic cannula using a cubic zirconia sleeve. To record fluorescence signals from SRB, excitation light from 560-nm LED was band-pass filtered, collimated, reflected by a dichroic mirror and focused by a ×20 objective. The excitation light power at the tip of the patch cord was 50 µW. Emitted fluorescence was band-pass filtered and focused on digital camera. Signals were collected at a rate of 1 Hz for SRB and visualized using the open-source software Bonsai 2.4 (*http://bonsai-rx.org*) and analyzed in Igor software (Wavemetrics).

In vivo diffusion-weighted MRI

To test the effect of astrocyte AQP4 acute inhibition on brain water homeostasis, we used diffusion-weighted magnetic resonance imaging (DW-MRI) to calculate the apparent diffusion coefficient (ADC) of tissue water molecules in the brain. MRI experiment was performed with medetomidine anesthesia (0.6 mg/kg/h, i.v.). The mice were injected with either the water-soluble

TGN-020 sodium salt (Key Organics, LR-0041) or saline. For the crossover test, the order of injection of TGN-020 or saline was randomly determined. The respiration was monitored, and the body temperature was maintained at 36 °C throughout the measurement.

The MRI experiments were conducted on a Bruker 7T scanner with a cryogenic radio frequency coil. After 10 min from the start of the scanning, either TGN-020 (200 mg/kg body weight) or Saline was injected. Then, scanning was continued for 60 min. The respiration rate was monitored and was confirmed within the range of 80-104 /min throughout the experiment. Mice were injected with 200 mg/kg of TGN-020. We used a standard diffusion-weighted spin echo EPI sequence, with the following parameters: spatial resolution = 175 × 175 × 500 μm^3/voxel, 3 b-values (b = 0, 250 and 1800 s/mm^2), 6 directions, 1 segment, echo time = 37.1 ms / repetition time = 5769 ms, bandwidth = 300 kHz, δ = 3 ms, Δ = 24 ms, scan time = 5 min. The DWI scan was continued for 70 min (total 14 scans).

The ADC at each time point was calculated from DWI data using DSIstudio (*https://dsi-studio.labsolver.org/*). The percentage change in ADC was then calculated according to the equation: $\Delta ADC_i = (ADC_i / ADC_2 - 1) \times 100$ (%). The ADC_i is the ADC at the i-th timepoint. We define the ADC at the 2^{nd} time-point as the baseline because saline injection was performed after the 2^{nd} DWI scan. The averaged ADC changes within the regions of interests (ROIs) were calculated using a homemade program. The ROIs of the cortex, the hippocampus, and the striatum were created with reference to Allen mouse brain atlas.

Statistics

Experimental data are expressed as mean ± standard error unless otherwise mentioned. The *t*-test was performed for two-group comparisons and significant difference was determined by p values less than 0.05. Mann-Whitney U test was also used to ascertain the significance level, in considering the deviation of the experimental data from the theoretical symmetrical distribution. Bonferroni-Holm correction was used for multiple comparisons. Statistical tests were carried out with Matlab (The MathWorks) and Statistica 6 (Statsoft). The significance levels are shown in figures by $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

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Competing interest

The authors declare no competing financial interests.

Data sharing plans

The authors commit to share data, documentation, and code used in analysis.

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Reviewer #1 (Public Review):

Summary:

Pham and colleagues provide an illuminating investigation of aquaporin-4 water flux in the brain utilizing ex vivo and in vivo techniques. The authors first show in acute brain slices, and in vivo with fiber photometry, SRB-loaded astrocytes swell after inhibition of AQP4 with TGN-020, indicative of tonic water efflux from astrocytes in physiological conditions. Excitingly, they find that TGN-020 increases the ADC in DW-MRI in a region-specific manner, potentially due to AQP4 density. The resolution of the DW-MRI cannot distinguish between intracellular or extracellular compartments, but the data point to an overall accumulation of water in the brain with AQP4 inhibition. These results provide further clarity on water movement through AQP4 in health and disease.

Overall, the data support the main conclusions of the article, with some room for more detailed treatment of the data to extend the findings.

Strengths:

The authors have a thorough investigation of AQP4 inhibition in acute brain slices. The demonstration of tonic water efflux through AQP4 at baseline is novel and important in and of itself. Their further testing of TGN-020 in hyper- and hypo-osmotic solutions shows the expected reduction of swelling/shrinking with AQP4 blockade.

Their experiment with cortical spreading depression further highlights the importance of water efflux from astrocytes via AQP4 and transient water fluxes as a result of osmotic gradients. Inhibition of AQP4 increases the speed of tissue swelling, pointing to a role in the efflux of water from the brain.

The use of DW-MRI provides a non-invasive measure of water flux after TGN-020 treatment.

Weaknesses:

The authors specifically use GCaMP6 and light sheet microscopy to image their brain sections in order to identify astrocytic microdomains. However, their presentation of the data neglects a more detailed treatment of the calcium signaling. It would be quite interesting to see whether these calcium events are differentially affected by AQP4 inhibition based on their cellular localization (ie. processes vs. soma vs. vascular end feet which all have different AQP4 expressions).

The authors show the inhibition of AQP4 with TGN-020 shortens the onset time of the swelling associated with cortical spreading depression in brain slices. However, they do not show quantification for many of the other features of CSD swelling, (ie. the duration of swelling, speed of swelling, recovery from swelling).

Significance:

AQP4 is a bidirectional water channel that is constitutively open, thus water flux through it is always regulated by local osmotic gradients. Still, characterizing this water flux has been challenging, as the AQP4 channel is incredibly water-selective. The authors here present important data showing that the application of TGN-020 alone causes astrocytic swelling, indicating that there is constant efflux of water from astrocytes via AQP4 in basal conditions. This has been suggested before, as the authors rightfully highlight in their discussion, but the evidence had previously come from electron microscopy data from genetic knockout mice.

AQP4 expression has been linked with the glymphatic circulation of cerebrospinal fluid through perivascular spaces since its rediscovery in 2012 [1]. Further studies of aging[2],

genetic models[3], and physiological circadian variation[4] have revealed it is not simply AQP4 expression but AQP4 polarization to astrocytic vascular endfeet that is imperative for facilitating glymphatic flow. Still, a lingering question in the field is how AQP4 facilitates fluid circulation. This study represents an important step in our understanding of AQP4's function, as the basal efflux of water via AQP4 might promote clearance of interstitial fluid to allow an influx of cerebrospinal fluid into the brain. Beyond glymphatic fluid circulation, clearly, AQP4-dependent volume changes will differentially alter astrocytic calcium signaling and, in turn, neuronal activity.

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(2) Kress, B.T., et al., Impairment of paravascular clearance pathways in the aging brain. Ann Neurol, 2014. 76(6): p. 845-61.
(3) Mestre, H., et al., Aquaporin-4-dependent Glymphatic Solute Transport in the Rodent Brain. eLife, 2018. 7.
(4) Hablitz, L., et al., Circadian control of brain glymphatic and lymphatic fluid flow. Nature Communications, 2020. 11(1).

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Reviewer #2 (Public Review):

Summary:

The paper investigates the role of astrocyte-specific aquaporin-4 (AQP4) water channel in mediating water transport within the mouse brain and the impact of the channel on astrocyte and neuron signaling. Throughout various experiments including epifluorescence and light sheet microscopy in mouse brain slices, and fiber photometry or diffusion-weighted MRI in vivo, the researchers observe that acute inhibition of AQP4 leads to intracellular water accumulation and swelling in astrocytes. This swelling alters astrocyte calcium signaling and affects neighboring neuron populations. Furthermore, the study demonstrates that AQP4 regulates astrocyte volume, influencing mainly the dynamics of water efflux in response to osmotic challenges or associated with cortical spreading depolarization. The findings suggest that AQP4-mediated water efflux plays a crucial role in maintaining brain homeostasis, and indicates the main role of AQP4 in this mechanism. However authors highlight that the report sheds light on the mechanisms by which astrocyte aquaporin contributes to the water environment in the brain parenchyma, the mechanism underlying these effects remains unclear and not investigated. The manuscript requires revision.

Strengths:

The paper elucidates the role of the astrocytic aquaporin-4 (AQP4) channel in brain water transport, its impact on water homeostasis, and signaling in the brain parenchyma. In its idea, the paper follows a set of complimentary experiments combining various ex vivo and in vivo techniques from microscopy to magnetic resonance imaging. The research is valuable, confirms previous findings, and provides novel insights into the effect of acute blockage of the AQP4 channel using TGN-020.

Weaknesses:

Despite the employed interdisciplinary approach, the quality of the manuscript provides doubts regarding the significance of the findings and hinders the novelty claimed by the authors. The paper lacks a comprehensive exploration or mention of the underlying molecular mechanisms driving the observed effects of astrocytic aquaporin-4 (AQP4) channel

inhibition on brain water transport and brain signaling dynamics. The scientific background is not very well prepared in the introduction and discussion sections. The important or latest reports from the field are missing or incompletely cited and missconcluded. There are several citations to original works missing, which would clarify certain conclusions. This especially refers to the basis of the glymphatic system concept and recently published reports of similar content. The usage of TGN-020, instead of i.e. available AER-270(271) AQP4 blocker, is not explained. While employing various experimental techniques adds depth to the findings, some reasoning behind the employed techniques - especially regarding MRI - is not clear or seemingly inaccurate. Most of the time the number of subjects examined is lacking or mentioned only roughly within the figure captions, and there are lacking or wrongly applied statistical tests, that limit assessment and reproducibility of the results. In some cases, it seems that two different statistical tests were used for the same or linked type of data, so the results are contradictory even though appear as not likely - based on the figures. Addressing these limitations could strengthen the paper's impact and utility within the field of neuroscience, however, it also seems that supplementary experiments are required to improve the report.

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Reviewer #3 (Public Review):

Summary:

In this manuscript, the authors propose that astrocytic water channel AQP4 represents the dominant pathway for tonic water efflux without which astrocytes undergo cell swelling. The authors measure changes in astrocytic sulforhodamine fluorescence as the proxy for cell volume dynamics. Using this approach, they perform a technically elegant series of ex vivo and in vivo experiments exploring changes in astrocytic volume in response to AQP4 inhibitor TGN-020 and/or neuronal stimulation. The key finding is that TGN-020 produces an apparent swelling of astrocytes and modifies astrocytic cell volume regulation after spreading depolarizations. Additionally, systemic application of TGN-020 produced changes in diffusion-weighted MRI signal, which the authors interpret as cellular swelling. This study is perceived as potentially significant. However, several technical caveats should be strongly considered and perhaps addressed through additional experiments.

Strengths:

(1) This is a technically elegant study, in which the authors employed a number of complementary ex vivo and in vivo techniques to explore functional outcomes of aquaporin inhibition. The presented data are potentially highly significant (but see below for caveats and questions related to data interpretation).

(2) The authors go beyond measuring cell volume homeostasis and probe for the functional significance of AQP4 inhibition by monitoring Ca2+ signaling in neurons and astrocytes (GCaMP6 assay).

(3) Spreading depolarizations represent a physiologically relevant model of cellular swelling. The authors use ChR2 optogenetics to trigger spreading depolarizations. This is a highly appropriate and much-appreciated approach.

Weaknesses:

(1) The main weakness of this study is that all major conclusions are based on the use of one pharmacological compound. In the opinion of this reviewer, the effects of TGN-020 are not consistent with the current knowledge on water permeability in astrocytes and the relative contribution of AQP4 to this process.

Specifically: Genetic deletion of AQP4 in astrocytes reduces plasmalemmal water permeability by ~two-three-fold (when measured a 37oC, Solenov et al., AJP-Cell, 2004). This is a significant difference, but it is thought to have limited/no impact on water distribution. Astrocytic volume and the degree of anisosmotic swelling/shrinkage are unchanged because the water permeability of the AQP4-null astrocytes remains high. This has been discussed at length in many publications (e.g., MacAulay et al., Neuroscience, 2004; MacAulay, Nat Rev Neurosci, 2021) and is acknowledged by Solenov and Verkman (2004).

Keeping this limitation in mind, it is important to validate astrocytic cell volume changes using an independent method of cell volume reconstruction (diameter of sulforhodamine-labeled cell bodies? 3D reconstruction of EGFP-tagged cells? Else?)

(2) TGN-020 produces many effects on the brain, with some but not all of the observed phenomena sensitive to the genetic deletion of AQP4. In the context of this work, it is important to note that TGN-020 does not completely inhibit AQP4 (70% maximal inhibition in the original oocyte study by Huber et al., Bioorg Med Chem, 2009). Thus, besides not knowing TGN-020 levels inside the brain, even "maximal" AQP4 inhibition would not be expected to dramatically affect water permeability in astrocytes.

This caveat may be addressed through experiments using local delivery of structurally unrelated AQP4 blockers, or, preferably, AQP4 KO mice.

(3) This reviewer thinks that the ADC signal changes in Figure 5 may be unrelated to cellular swelling. Instead, they may be a result of the previously reported TGN-020-induced hyphemia (e.g., H. Igarashi et al., NeuroReport, 2013) and/or changes in water fluxes across pia matter which is highly enriched in AQP4. To amplify this concern, AQP4 KO brains have increased water mobility due to enlarged interstitial spaces, rather than swollen astrocytes (RS Gomolka, eLife, 2023). Overall, the caveats of interpreting DW-MRI signal deserve strong consideration.

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Author response:

We sincerely thank the editors and reviewers for the rigorous evaluation of our work and the precious time invested. The positive comments resonate with our endeavor to explore the intrinsic role of astrocyte aquaporin in brain water homeostasis. Meanwhile, we very appreciate the constructive suggestions of the reviewers to consolidate this study. Here is the provisional response, which briefly outlines our acknowledgement of the reviewers' suggestions:

To Reviewer #1:

- Imaging data will be examined and collected to determine whether AQP4 inhibition has differential effects on astrocyte calcium signals in terms of cellular locations.
- New analysis will be performed for CSD swelling data to provide additional kinetic information.
- The mentioned original papers are important, and will be included in the revision.

To Reviewer #2:

We agree, a careful revision will improve and better position the study.

- Echoing Reviewer #1, the introduction and discussion will be strengthened with current scientific contexts, while paying attention to the important advances in glymphatic system. The limits of the study mentioned in the reviews will be stated.
- The use of TGN-020 was based on its validation by wide range of ex vivo and in vivo studies. AER-270(271) was nicely introduced by Farr et al., 2019 (PMID: 30738082). Its validation in vivo in AQP4 KO mice, and the comparison to TGN-020, is reported in a very recent study (Giannetto et al., 2024 - PMID: 38363040) that provides valuable insights.
- The description of specific methodologies, including the DW-MRI, will be reinforced. The presentation of experiments and statistical analysis will be refined.

To Reviewer #3:

- Solenov et al., 2004 (PMID: 14576087) used the calcein quenching assay and KO mice convincingly showing AQP4 is a functional water channel in cultured astroctyes. AQP4 deletion reduced both astrocyte water permeability and the absolute amplitude of swelling over comparable time, and also slowed down cell shrinking, which overall parallels our results from acute AQP4 blocking. Yet in Solenovr's study, the time to swelling plateau was prolonged in AQP4 KO astrocytes, differing from our data of acute blocking. This difference may be due to compensatory mechanisms in chronic AQP4 KO, or reflect the different volume responses in cultured astrocytes from brain slices/in vivo results as noted previously (e.g., Risher et al., 2009 - PMID: 18720409). As suggested, methods for volume recordings will be examined.
- It is an important point that TGN-020 partially blocks AQP4, implying the actual functional impact of AQP4 per se might be stronger than what we observed. TGN provides a means to acutely probe AQP4 function in situ, still we agree, its limitation needs be acknowledged.
- As also pointed by Reviewer #2, the description and interpretation of DW-MRI data will be improved.