Clearance of activity-evoked \(K^+\) transients and associated glia cell swelling occur independently of AQP4; A study with an isoform-selective AQP4 inhibitor

Running title: \(K^+\) clearance and glial swelling occurs independently of AQP4

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Acknowledgements
This study was supported by the Novo Nordisk Foundation (to NM; NNF15OC0017052) and the Lundbeck Foundation (to HSW; R208-2015-2859). Simulations were performed on the Danish e-Infrastructure Cooperation (DeiC) National HPC Center, ABACUS 2.0 at the University of Southern Denmark.

Word Count
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Abstract
The mammalian brain consists of 80% water, which is continuously shifted between different compartments and cellular structures by mechanisms that are, to a large extent, unresolved. Aquaporin 4 (AQP4) is abundantly expressed in glia and ependymal cells of the mammalian brain and has been proposed to act as a gatekeeper for brain water dynamics, predominantly based on studies utilizing AQP4-deficient mice. However, these mice have a range of secondary effects due to the gene deletion. An efficient and selective AQP4 inhibitor has thus been sorely needed to validate the results obtained in the AQP4−/− mice to quantify the contribution of AQP4 to brain fluid dynamics. In AQP4-expressing Xenopus laevis oocytes monitored by a high-resolution volume recording system, we here demonstrate that the compound TGN-020 is such a selective AQP4 inhibitor. TGN-020 targets the tested species of AQP4 with an IC50 of ~3.5 μM, but displays no inhibitory effect on the other AQPs (AQP1-AQP9). With this tool, we employed rat hippocampal slices and ion-sensitive microelectrodes to determine the role of AQP4 in glia cell swelling following neuronal activity. TGN-020-mediated inhibition of AQP4 did not prevent stimulus-induced extracellular space shrinkage, nor did it slow clearance of the activity-evoked K⁺ transient. These data, obtained with a verified isoform-selective AQP4 inhibitor, indicate that AQP4 is not required for the astrocytic contribution to the K⁺ clearance or the associated extracellular space shrinkage.

Key words: AQP4, inhibitor, TGN-020, [K⁺]o clearance, glia cell swelling
Main points:

- TGN-020 is an efficient and isoform-specific inhibitor of AQP4-mediated osmotic water permeability.
- AQP4 is not required for shaping the stimulus-evoked K⁺ transient or the associated extracellular space shrinkage in rat hippocampus

Abbreviations:

aCSF, artificial cerebrospinal fluid; DAB, 1,4-dideoxy-1,4-imino-D-arabinitol; ECS, extracellular space; MCT, monocarboxylate transporters; NBCe1, electrogenic Na⁺/bicarbonate cotransporter 1; TMA, tetramethylammonium; TEA, tetraethylammonium; TGN-020, 2-(nicotinamide)-1,3,4-thiadiazole; AQP, aquaporin.

Introduction

The mammalian brain consists of 80% water, which is dispersed between the intra- and extracellular fluid of brain cells, the latter in continuum with the cerebrospinal fluid (CSF; mainly in the cerebral ventricles and the subarachnoid space), in addition to the fluid content of the vasculature. The cerebral water homeostasis is anticipated to be tightly controlled to ensure stable...
cell volume and intracranial pressure within the physiological range. However, the molecular mechanisms governing the transmembrane movement of water within the central nervous system (CNS) remain largely unresolved. Water can cross cell membranes via water channels (aquaporins; AQP) and cotransport proteins (MacAulay & Zeuthen, 2010). Of the large family of AQPs expressed in the majority of cell types in the mammalian body, AQP4 is abundant in the brain; more specifically in the astrocytic endfeet bordering the vasculature and the pia in addition to the ependymal lining bordering the cerebral ventricles (Nagelhus & Ottersen, 2013). Such prominent localization at the interfaces between blood-brain and CSF-brain, led to a proposed role of AQP4 as an important gatekeeper of brain water entry and exit, in addition to its potential involvement in both the glia-mediated K⁺ buffering and the parallel glia cell swelling taking place during neuronal activity (Jin, Zhang, Binder, & Verkman, 2013; Manley, Binder, Papadopoulos, & Verkman, 2004; Nagelhus et al., 1999; Nagelhus & Ottersen, 2013; Papadopoulos & Verkman, 2007; Strohschein et al., 2011). With the lack of a specific inhibitor of AQP4, these studies were all based on AQP4 knock-out mice (AQP4⁻/⁻). While these mice are deficient in AQP4 expression, it has come to light that several other molecular changes occurred as a secondary effect to the knock-out strategy: (i) These mice have 28% enlarged extracellular space (ECS) in the CNS, (ii) reduced glutamate transporter (GLT-1) expression and thereby presumably delayed glutamate clearance, (iii) increased brain water content, (iv) enhanced astrocytic gap junctional coupling, and (v) a possible impairment of the Na⁺/K⁺-ATPase activity (Haj-Yasein, Vindedal, et al., 2011; Strohschein et al., 2011; Yao, Hrabetova, Nicholson, & Manley, 2008; Zeng et al., 2007). It thus remains unclear if the observed phenotype originated from the reduced osmotic water permeability of the astrocytic membrane or from one of the (un)known secondary effects.

Therefore, a specific inhibitor of AQP4 would be a beneficial tool to include alongside the knock-out strategy. It has, however, proven challenging to develop such an inhibitor, not for want of trying. During the last decade, a few compounds were brought forward as potential inhibitors of AQP4; e.g., tetraethylammonium (TEA), acetazolamide, antiepileptic drugs, and bumetanide (and the derivative AqB013) (Detmers et al., 2006; Huber, Tsujita, Kwee, & Nakada, 2009; Migliati et al., 2009; Tanimura, Hiroaki, & Fujiyoshi, 2009; Verkman, Smith, Phuan, Tradtrantip, & Anderson, 2017), but all of these pharmacological agents were later demonstrated to not be effective blockers.
of AQP4-mediated water permeability (Abir-Awan et al., 2019; Brandt et al., 2018; Sogaard & Zeuthen, 2008). The molecule TGN-020 has been shown to bind to AQP4 (Huber, Tsujita, & Nakada, 2009; Nakamura et al., 2011), act as an inhibitor (Huber, Tsujita, & Nakada, 2012), and promote better outcome in mouse models of ischemic stroke (Igarashi, Huber, Tsujita, & Nakada, 2011; Pirici et al., 2017). TGN-020 may thus be the long awaited AQP4 inhibitor, provided sufficient efficiency and specificity. In the present study, we demonstrate the potency and isoform specificity of TGN-020 as an AQP4 inhibitor and subsequently employ this pharmacological tool to determine the role of AQP4 in activity-evoked K+ clearance and associated extracellular shrinkage in rat hippocampus.

Materials and Methods

Ethical approval
The experiments were performed according to the guidelines of the Danish Veterinary and Food administration (Ministry of Environment and Food) and approved by the animal facility at the Faculty of Health and Medical Sciences, University of Copenhagen. The animal experiments conform to the principles and regulations described in (Grundy, 2015). The surgical protocol, by which the oocytes were retrieved, was approved by The Danish National Committee for Animal Studies.

Experimental procedures - rats
Experiments were performed on male and female rats (Sprague-Dawley, Janvier Labs, France) at P21-P28, housed in the animal facility at the Faculty of Health and Medical Sciences, University of Copenhagen. The rats were anaesthetized using gaseous 2-Bromo-2-Chloro-1,1,1-Trifluoroethane (halothane) (Sigma-Aldrich, Germany, B-4388) prior to decapitation.

Experimental procedures - RNA preparation and heterologous expression in Xenopus laevis oocytes
cDNA encoding rat AQP1-9 (mouse and human AQP4 were also employed) were subcloned into the oocyte expression vectors pXOOM or BS(SK-), linearized downstream from the poly-A
segment, and in vitro transcribed using T7 mMessage machine according to manufacturer’s instructions (Ambion, Austin, TX). cRNA was extracted with MEGAclear (Ambion, Austin, TX) and micro-injected into defolliculated Xenopus laevis oocytes: 10 ng RNA/oocyte. Xenopus laevis frogs were obtained from Nasco (Fort Atkinson, WI) or oocytes were purchased from EcoCyte Bioscience (Germany). Oocytes were collected under anaesthesia (2 g/L Tricain, 3-aminobenzoic acid ethyl ester, Sigma-Aldrich, Germany, A-5040), and the preparation of defolliculated oocytes was carried out as described in (Fenton et al., 2010) and the oocytes were kept in Kulori medium ((in mM): 90 NaCl, 1 KCl, 1 CaCl₂, 1 MgCl₂, 5 HEPES (pH 7.4)) for 3-4 days at 19°C prior to experiments.

Volume recordings of oocytes - Oocytes were placed in an experimental recording chamber, perfused with various solutions, and volume measurements were performed as previously described in (Zeuthen, Zeuthen, & Macaulay, 2007). Briefly, the oocytes were placed in a small chamber with a glass bottom and perfused with solutions of interest. The volume of the oocytes was viewed from below via a long distance objective (x4), and micrographs were captured continuously with a high resolution recording system (with a high signal to noise ratio) based on a CCD camera at a rate of 25 images/s (Zeuthen, Belhage, & Zeuthen, 2006). The perfusion solution consisted of (in mM): 50 NaCl, 2 KCl, 1 MgCl₂, 1 CaCl₂, 10 HEPES, 100 mM mannitol (Tris buffered pH 7.4, 220 mOsm). Hyposmotic solutions were subsequently made by the removal of mannitol (∆50 mM mannitol) with resulting osmolarity of 170 mOsm. The osmotic water permeability was determined by $L_p = (J_v)/(A \times \Delta \pi \times V_w)$, where $J_v$ is the initial water flux during an osmotic challenge, $A$ is the membrane surface area (nine times the apparent area due to membrane folding (Zampighi et al., 1995), $\Delta \pi$ is the osmotic challenge, and $V_w$ is the partial molal volume of water (18 cm³/mol). Osmolarities of all solutions were verified with an accuracy of 1 mOsm with an osmometer Type 15 (Löser Messtechnik, Berlin, Germany). TGN-020 (Sigma-Aldrich, Germany, SML0136) was dissolved in DMSO (stock solution of 20 µM; controls exposed to vehicle (DMSO) only) and its effect on volume changes were recorded after 5 and 10 min of TGN-020 treatment with the oocytes serving as own controls. For long-term application the oocytes were incubated at 19 °C for 60 min.
with 20 μM of TGN-020. Determination of TGN-020-mediated effects on oocytes expressing the different AQP isoforms was carried out in a researcher-blinded fashion.

**Brain slices and solutions**

Following decapitation, the brain was quickly removed and placed into ice-cold cutting solution containing (in mM): 87 NaCl, 70 sucrose, 2.5 KCl, 0.5 CaCl₂, 25 NaHCO₃, 1.1 NaH₂PO₄, 7 MgCl₂, and 25 D-glucose, equilibrated with gaseous 95% O₂, 5% CO₂. Oblique sagittal (transverse) hippocampal slices (400 µm) were cut with a Campden Vibrating Microtome (7000SMZ-2, Campden Instruments, UK). Slices were transferred to the standard artificial cerebrospinal fluid (aCSF) solution containing (in mM): 124 NaCl, 3 KCl, 2 CaCl₂, 25 NaHCO₃, 1.1 NaH₂PO₄, 2 MgCl₂ and 10 D-glucose, and equilibrated with 95% O₂, 5% CO₂ (pH 7.4 at the experimental temperature of 33-34°C) and left to recover at 34°C for 30 min and then kept at room temperature.

**Ion-sensitive microelectrodes and electrophysiological recordings in slices**

Electrophysiological recordings were carried out in a submerged-type recording chamber (Brain Slice Chamber 1, Scientific Systems Design, Digitimer Ltd, UK) at an experimental temperature of 33-34°C and a continuous superfusion at a flow rate of 2.2 ml/min. Recordings were performed within stratum radiatum of the CA1 region. High-frequency stimulation was delivered by a concentric bipolar tungsten electrode (TM33CCNON, World Precision Instruments, UK) inserted into the stratum radiatum in the vicinity (≤ 300 µm) of the recording site. Stimulation trains (22 V at 20 Hz, each pulse of a duration of 80 μs, for 3 sec) were delivered to the slice to activate the Schaffer collaterals. Stimulation trains were delivered at 10 min intervals and involved the pharmacological compounds 1,4-dideoxy-1,4-imino-D-arabinitol (DAB, synthesized by Novo Nordisk A/S, Copenhagen, Denmark, prepared as stock of 500 mM in dH₂O) or TGN-020 (Sigma-Aldrich, Germany, SML0136) with at least three consecutive control recordings prior to washing in compounds. The resulting extracellular field potentials were recorded with thin-walled filamented glass capillary microelectrodes (GC150TF-7.5, Harvard Apparatus, MA) pulled to resistances of 15-25 MΩ when filled with the standard solution (see above). This electrode served as reference signal for the ion-sensitive microelectrodes. Ion-sensitive microelectrodes were prepared from thin-
walled non-filamented glass capillaries (GC150T–7.5, Harvard Apparatus, MA) pulled to obtain a tip diameter in the range of 1-2 µm (Voipio et al., 1994). The capillaries were then silanized internally with gaseous N,N-dimethyltrimethylsilylamine (Sigma-Aldrich, Germany, 41716,) and baked at 190°C for 20 min prior to being backfilled. The electrodes were backfilled with a solution depending on the type of measurement, containing either 150 mM tetramethylammonium (TMA+) chloride (for extracellular space volume measurements), or 150 mM NaCl, 3 mM KCl (for extracellular K+ measurements). The tip of the capillary was afterwards filled with a short column of either TMA+/K+-sensitive liquid membrane solution (50 mg/ml potassium tetrakis (4-chlorophenyl) borate (Sigma-Aldrich, Germany, 60591) in 1,2-dimethyl-3-nitrobenzene (Sigma-Aldrich, Germany, 40870)). For experiments with TMA+, 1.5 mM TMA-Cl was included in the test solution. Note that the TMA+/K+ membrane is highly sensitive to quartenary ions, such as TMA+, however in the absence of such ions it becomes selective to K+ ions, and can therefore be used for both measurements depending on the backfilling solution.

The tips of the ion-sensitive electrode and the reference electrode were placed within a few microns at the exact same depth in the core of the slice. Close distance was ensured via Sensapex micromanipulators (SMX series, Sensapex, Finland), which provide precise µm x,y,z coordinates, by placing the electrode tips closely together above the slice and afterwards moving into the tissue maintaining this narrow distance aided by the x,y,z coordinates. The ion-sensitive signal and the field potential signal were both recorded via an ION-01M amplifier and headstage (NPI electronics, Germany). Online deduction of the field potential signal from that of the ion-sensitive electrode provided the traces employed for analysis. To ensure the quality of the experiments, the resistance of the electrode was tested several times throughout the experiments. If the resistance deviated >~15% from the original value (indicating e.g. change in tip diameter or alterations in the tip liquid membrane column), the experiment was discarded. All recorded signals were filtered at 250 Hz, sampled at 500 Hz and stored for off-line analysis with WinEDR (courtesy of Dr. John Dempster, University of Strathclyde, Glasgow, UK) and GraphPad Prism 7.0. At the end of the experiment, each ion-sensitive microelectrode was thoroughly calibrated in order to translate the signal in mV to either the % volume change or the K+ concentration, for a detailed description, see (Larsen, Stoica, & MacAulay, 2019).
Glycogen measurements in primary cultures of rat astrocytes

Cortical rat astrocytes were cultured from dissected cerebral cortices of P8 rat pups. The dissected cortices were dissociated mechanically by passing the tissue through an 80 µm nylon sieve into Dulbecco’s modified Eagle’s medium (Sigma-Aldrich, Germany, D-5030) containing an additional 6 mM D-glucose, 2.5 mM L-glutamine, 26.2 mM NaHCO₃, 100,000 IU/L of penicillin and 20% foetal bovine serum (Sigma-Aldrich, Germany, A-4503). Single cells were generated by trituration with a syringe equipped with a steel cannula and plated in T25 cell culture flasks. The foetal bovine serum concentration was sequentially reduced to 15 % and 10 % in the second and third week of culture, respectively. 0.25 mM dB-cAMP (Sigma-Aldrich, Germany, D-0627) was added to the culture medium in the third week of culture to obtain morphologically differentiated (Su, Haworth, Dempsey, & Sun, 2000) astrocytes. The astrocytes were kept at 37°C with an atmosphere of 5% CO₂ and were used for experiments three weeks after plating.

Primary cultures of astrocytes were pre-incubated for 2 hours in aCSF with 10 mM glucose. The cells were washed in PBS prior to incubation for 45 min in aCSF with either 10 mM glucose, in the absence of glucose, or in the absence of glucose but in the presence of 1 mM DAB to inhibit glycogenolysis. The incubation was terminated by a wash with cold PBS and cells were transferred to -80 °C before extraction on ice with 70 % ethanol. Control levels of glycogen were obtained from cells extracted immediately after the pre-incubation step. The ethanol extract was centrifuged at 4 °C, 20,000 g for 20 minutes. Pellet was dissolved in 100 µL ddH₂O and homogenized prior to glycogen and protein determination. The glycogen was quantified in a coupled enzymatic assay by measurement of the production of NADPH in the conversion of glucose-6-phosphate to 6-phosphogluconolactone as previously described (Brown, Tekkok, & Ransom, 2003). Briefly, the homogenized cell pellet was resuspended in an acetate buffer (5 mM, pH 5) containing 10 U/ml amyloglucosidase (Sigma-Aldrich, Germany, A7420) and incubated at 37 °C for 1.5 h prior to centrifugation at 20,000 g for 10 min and transfer of the supernatant to a microtiter plate. The cofactors NADP (0.16 mM), ATP (0.81 mM) and magnesium (MgCl₂; 12.1 mM) were added in an alkaline solution (37.1 mM Tris base; 0.007% sodium azide; 13.8 mM HCl) to each well. The reaction was initiated by the addition of hexokinase (1.52 U/ml, 11426362001 Roche, Germany).
and glucose-6-phosphate dehydrogenase (Sigma-Aldrich, Germany, G-6378, 0.54 U/ml). The fluorescence was measured by SpectraMax i3X (Molecular Devices, San Jose, CA, USA) prior to addition of the enzymes to obtain a background measurement, and after 30 min incubation at 37 °C, using 350 and 470 nm as excitation and emission wavelengths, respectively.

**Molecular Dynamics Simulations (MD)**

The initial structure of rAQP4 was obtained from PDB id 2zz9 (www.pdb.org) (Tani et al., 2009). The protein tetramer was inserted in a DOPC lipid bilayer measuring 12.25 nm x 12.25 nm using CHARMM-GUI (Jo, Kim, Iyer, & Im, 2008), and was hydrated with water and 150 mM KCl. The binding energies are ~ 60 kcal/mole, almost the strength of a covalent binding, and much stronger than a streptavidin-biotin binding. The final system contained 340 DOPC molecules, and measured 12.25 x 12.25 x 9.7 nm. The system was energy minimized and equilibrated while slowly releasing backbone restraints on the protein atoms. After equilibration, the simulation of pure AQP4 inside the bilayer was run for 200 ns. The initial force field parameters for TGN-020 were obtained from www.parachem.org (Vanommeslaeghe et al., 2010). Four TGN-020 molecules with different initial orientations were placed on top of the AQP4 in the above system. The system containing protein, lipids and TGN-020 was energy minimized and equilibrated in the same manner, but keeping the TGN-020 atoms restrained during the equilibration period. The simulation was then run for 200 ns with TGN-020. All simulations were performed using GROMACS (Van Der Spoel et al., 2005). Standard MD protocols for equilibration and production runs were used, as reported elsewhere (Yamamoto et al., 2019).

**Data analysis and statistics**

All data are given as mean ± SEM. Statistical tests were performed by use of GraphPad Prism 7.0 (GraphPad Software Inc., La Jolla, CA, USA). Statistical significance was tested with Student’s t-test or one-way ANOVA with Tukey’s multiple comparison post hoc tests as indicated in figure legends. P values < 0.05 were considered statistically significant. The equation \( y = \frac{100}{1 + 10^\lambda((\log IC_{50} - x) \times \text{Hill slope})} \) was used to fit the IC\(_{50}\) curves for each individual experiment, which are shown as summarized. For experiments employing brain slices the number of
experiments, n, signifies individual brain slices and is mentioned in the Result section, whereas the number of animals from which these slices have been obtained is included in the figure legends. In experiments with *Xenopus* oocytes, n refers to number of oocytes, obtained from at least three different batches of oocytes.

**Results**

**TGN-020-mediated inhibition of AQP4**

To evaluate TGN-020’s effect on the AQP4-mediated water permeability, rat AQP4 was heterologously expressed in *Xenopus laevis* oocytes. The most prominent isoform of AQP4 in the brain, called M23 due to its translation initiation site at methionine at position 23 in the primary structure of AQP4, was employed for these experiments (Amiry-Moghaddam & Ottersen, 2003). The osmotic water permeability of the oocytes was determined following introduction of an abrupt hyposmotic challenge (-50 mOsm), during continuous recording of the oocyte volume changes by a high-resolution volume recording system using a CCD camera (see Fig. 1A for experimental setup). This experimental system provides us with a robust read-out of AQP4-dependent water permeability with a negligible background due to the low inherent water permeability of the native oocyte membrane (see Fig. 1B for representative traces and Fig. 1C for osmotic water permeabilities for uninjected oocytes versus AQP4-expressing oocytes, note, control oocytes were exposed to vehicle throughout the study). Exposure to TGN-020 (20 µM, 60 min) completely abolished the AQP4-dependent oocyte swelling following introduction of the osmotic challenge (see Fig. 1B, right panels for representative traces). The water permeability of rAQP4-expressing oocytes (2.66 ± 0.22 × 10⁻³ cm sec⁻¹, n = 9) was reduced to (0.21 ± 0.1 × 10⁻³ cm sec⁻¹, n = 9) in the presence of TGN-020 (Fig. 1C). TGN-020 was equally efficient in inhibiting AQP4 of the other tested species, mouse and human, Fig. 1C. The membrane potential of the AQP4-expressing oocytes was unperturbed by 60 min exposure to TGN-020, verifying that the compound did not reduce the osmotic water permeability of the oocyte by a toxic effect on the cells, Fig. 1D. Such TGN-020-induced reduction in AQP4-mediated water permeability can be observed following either pore block or internalization of the water channel. We verified membrane expression of AQP4 by confocal microscopy of AQP4-expressing oocytes in the absence or presence of TGN-
020. Representative micrographs indicate membrane localization of AQP4 (Fig. 1E), which we analyzed by fluorescent profile plotting, see Fig. 1F for a representative analysis. The membrane abundance of AQP4 was independent of TGN-020 treatment (in arbitrary units; 3.53 ± 0.29, n = 9 in control and 3.71 ± 0.15, n = 9 in TGN-020-treated oocytes), Fig. 1G, suggesting that AQP4 remains in the plasma membrane during exposure to TGN-020. TGN-020 thus appear to inhibit the AQP4-dependent water permeability in a manner distinct from internalization, irrespective of whether the AQP4 was of rat, mouse, or human origin.

**TGN-020 is an isoform-specific AQP4 inhibitor.**
To determine whether the TGN-020 inhibition was selective towards AQP4, oocytes expressing AQP1-9 were exposed to TGN-020 and their water permeability assessed with introduction of a hyposmotic gradient (-50 mOsm). The water permeability of the oocytes expressing the different isoforms cannot be directly compared, as each oocyte may express different amounts of aquaporin. Therefore, the AQP-mediated water permeability was determined with or without TGN-020 across the oocytes expressing the different isoforms. TGN-020 (shown in red in Fig. 2) reduced the water permeability exclusively in oocytes expressing AQP4, representative traces shown in Fig. 2A, and summarized water permeabilities illustrated in Fig. 2B, n = 9-10 of each. TGN-020 thus selectively and efficiently blocks the AQP4 isoform within the group of AQPs most abundantly expressed in cell membranes of mammals.

**TGN-020 exerts an acute blocking effect on AQP4.**
With the observation that TGN-020 selectively blocks AQP4 after prolonged treatment (60 min), we demonstrated the efficacy of the blocker within a shorter time frame. To use each oocyte as its own control to determine time-dependency of an inhibitor effect, we initially performed a time-control experiment to verify sequentially recorded water permeabilities of an identical magnitude in the AQP4-expressing oocytes, at times 0 (control), 5, and 10 min, see representative volume traces in Fig. 3A and summarized data in Fig. 3B, n = 9. Upon acute application of TGN-020 to the recording chamber we observed a decreased AQP4-mediated water permeability at t = 5 min, with complete inhibition at 10 min, Fig. 3C, with summarized data in Fig. 3D (TGN-020 groups are
shown in red). It should be noted, that the 5 min delay in inhibition most likely reflects the speed at which the compound washes into the chamber rather than a slow interaction between the aquaporin and the inhibitor. With the experimental paradigm of 10 min exposure to the compound (i.e. the oocyte was exposed to TGN-020 for 10 min prior to introducing the osmotic challenge), we determined the IC₅₀ for TGN-020 for rAQP4 with inclusion of different TGN-020 concentrations (IC₅₀ of ~ 3.62 ± 0.74 μM, n = 7), Fig. 3E. TGN-020 therefore appears to be an efficient blocker of AQP4-mediated water permeability by a direct action on the water channel.

**Molecular Dynamics Simulations of TGN-020 binding to AQP4.**

To characterize the binding pocket in rAQP4, we implemented molecular dynamics simulations of TGN-020 docked close to four pores of AQP4 (shown in yellow, blue, green and red) with different initial orientations (Fig. 4 A-B). During the sampling window, TGN-020 penetrated into the pore (but never fully permeated) and came to rest in different regions along the pore. Final snapshots of the simulation of TGN-020 bound to an AQP4 tetramer (Fig.4 C-D) illustrate that TGN-020 does not remain bound to the same part of the pore at the used timescale, but to several different regions along the pore. Much longer sampling times may be required for all TGN-020 molecules to find the same binding site on each AQP4 monomer. Molecular dynamics simulations illustrate the water pathway of AQP4 (depicted in red in Fig. 4E (side view) and Fig. 4G (top view)). With inclusion of TGN-020, this water pathway is blocked (Fig. 4F (side view) and Fig. 4H (top view)). These data, in support of the findings from the experimental approach, suggest that the TGN-020 inhibits AQP4-mediated water permeability by a direct pore-blocking effect.

**Acetazolamide and bumetanide are not inhibitors of AQP1- or AQP4-mediated water permeability**

Acetazolamide (Aze) and bumetanide (Bum) have been proposed as inhibitors of AQP1 and AQP4 (Huber, Tsujita, & Nakada, 2009). To test a putative blocking effect of these compounds in our sensitive volume assay, we monitored the osmotically-induced oocyte swelling of AQP1- and AQP4-expressing oocytes exposed to these compounds (1 mM Bum; 100 μM Aze)). Representative volume traces of AQP1-expressing oocytes exposed to an abrupt hyposmotic gradient are illustrated in Fig. 5A-B (time control traces (A) and with Aze application (B)). Summarized water
permeabilities with and without exposure to Aze (Fig. 5C) or Bum (Fig. 5D) illustrate no blocking effects on the water permeabilities of either of these AQPs upon treatment with acetazolamide or bumetanide. Thus, these two compounds are not efficient blockers of these two water channels.

**AQP4 does not contribute to stimulus-evoked ECS shrinkage or K⁺ clearance in acute rat hippocampal slices**

Gliarial AQP4 has been proposed to be involved in clearance of K⁺ from the extracellular space following neuronal activity, and/or permissive for the associated glial cell swelling (Jin et al., 2013; Nagelhus & Ottersen, 2013; Strohschein et al., 2011). With TGN-020 as an efficient blocker of AQP4-mediated water permeability, we determined the contribution of AQP4 to the shaping of the neuronal activity-evoked K⁺ transient in the extracellular space and the associated glial cell swelling. To this end, we employed acute hippocampal slices from rat (P21-28) with electrical stimulation of the CA1 Schaffer collaterals. We monitored the K⁺ transients with K⁺-sensitive microelectrodes and the relative changes of the extracellular space volume (∆ECS) with tetramethylammonium (TMA⁺)-sensitive microelectrodes upon bath application of TMA⁺. This technical approach has previously been validated by demonstration of roles for different K⁺- and H₂O-transporting mechanisms in these processes (Larsen et al., 2014; Larsen, Holm, Vilsen, & MacAulay, 2016; Larsen & MacAulay, 2017). Electrical stimulation of the slice gave rise to field potentials and caused transient [K⁺]₀ increases of ~6-7 mM. The shape of the stimulus-evoked K⁺ transient was unaffected by bath application of the AQP4 inhibitor, TGN-020 (20 μM, 60 min), see representative trace in Fig. 6A. The peak [K⁺]₀ (92 ± 3 % of control, p = 0.064; panel Ai) and the [K⁺]₀ decay constant (97 ± 4 % of control, p = 0.162; panel Aii) were unaffected by blockage of AQP4, n = 6 slices. The associated stimulus-induced increase in TMA⁺ concentration represents a robust read-out of the extracellular space shrinkage (~4-5 % in this experimental series), Fig. 6B. The activity-evoked extracellular space shrinkage was not significantly diminished by the presence of TGN-020 (20 μM, 60 min, 90 ± 6 % of control, p = 0.154; n = 7 slices), see Fig. 6B for representative traces and panel Bi for summarized data. These results suggest that AQP4 does not contribute to shaping the stimulus-induced K⁺ clearance or the associated glial volume dynamics.
The activity-evoked ECS shrinkage does not occur as a consequence of elevated osmotic pressure in the astrocytes following K⁺-mediated glycogen breakdown.

Upon neuronal activity and the associated [K⁺]₀ elevation, K⁺-induced glycogen breakdown occurs in the neighboring astrocytes (Dietzel, Heinemann, Hofmeier, & Lux, 1982). This event leads to an increased number of osmotically active particles in the astrocytes, and thereby creates an osmotic driving force. It has been hypothesized that this elevated osmotic pressure would lead to AQP4-dependent osmotic water flux into the astrocytes, which would promote the observed activity-evoked extracellular space shrinkage due to glia cell swelling. To reveal whether conventional osmotic water transport underlies the activity-evoked glia cell swelling, we determined the effect on stimulus-evoked extracellular space shrinkage following inhibition of the glycogen phosphorylase (with 1 mM 1,4-dideoxy-1,4-imino-D-arabinitol (DAB) (Walls et al., 2008)) in rat hippocampal slices, Fig. 7A. The ECS shrinkage was not significantly altered upon exposure to DAB (45 min, purple trace in Fig. 7A (representative traces) and panel Ai for summarized data, n=5). An additional pilot experiment with a prolonged (~90min) exposure to DAB like-wise failed to affect the volume dynamics (cyan trace in Fig. 7A). Acute brain slices may temporarily lose the astrocytic glycogen stores during the slicing procedure, and these reappear approximately 1h post-slicing with full restoration at 3h (Fiala et al., 2003). The brain slices employed for this experimental series were allowed to rest for 2-3h prior to experiments (n = 5). The ability of DAB to prevent glycogen breakdown is well established in murine astrocytes (Walls et al., 2008). To verify its action on the rat enzyme and, in addition, obtain a positive control of the inhibitor, we tested the ability of DAB to prevent glycogen breakdown in primary cultures of rat astrocytes. The rat astrocytes were pre-incubated with 10 mM glucose for 2 hours prior to the assay initiation to obtain a high level of glycogen at t = 0. The glycogen levels of the cultured rat astrocytes were determined in control conditions (t = 0 min) and after 45 min incubation with either absence of glucose (to induce glycogen breakdown), with high glucose in the medium (10 mM to prevent glycogen breakdown), or in the absence of glucose but presence of DAB. Fig. 7B illustrates the efficient prevention of glycogen breakdown in the presence of 1 mM DAB. These results indicate that osmolyte production (and subsequent osmotic water flow into glia cells) induced by glycogen phosphorylase-mediated...
glycogen breakdown does not contribute to stimulus-evoked extracellular space shrinkage in rat hippocampus.

**DISCUSSION**

With validation of TGN-020 as an efficient and isoform-specific inhibitor of AQP4, we here demonstrate that astrocytic AQP4-dependent osmotic water permeability is not required for activity-evoked K⁺ clearance or the associated extracellular space shrinkage. AQP4 is expressed, in a polarized fashion, in the paravascular astrocytic endfeet (Nielsen et al., 1997), which together with its ability to conduct bi-directional flux of water according to the imposed osmotic gradient (Meinild, Klaerke, & Zeuthen, 1998), has led to its suggested implication in a range of different (patho)physiological processes involving shifts of water between different compartments and cellular structures in the mammalian brain (Nagelhus & Ottersen, 2013). AQP4 has thus been suggested to act as the gatekeeper of brain water entry during brain edema formation in connection with several experimental animal disease models, glial K⁺ clearance during neuronal activity and the associated cell swelling, and the glial water influx sustaining the proposed convectional flow of fluorescent dyes through the brain parenchyma of the glymphatic hypothesis (Nagelhus & Ottersen, 2013; Papadopoulos & Verkman, 2013; Plog & Nedergaard, 2018). These experimental findings were all based on AQP4 knock-out mice, which were later demonstrated to have secondary changes in basic parameters, such as extracellular space size, brain water content, expression of glutamate transporters and connexins, etc. (Haj-Yasein, Vindedal, et al., 2011; Strohschein et al., 2011; Yao et al., 2008; Zeng et al., 2007), which may have introduced confounding elements in the experimental regime. To validate these findings, extensive searches for AQP4 inhibitors have been conducted (Detmers et al., 2006; Huber, Tsujita, Kwee, et al., 2009; Migliati et al., 2009; Tanimura et al., 2009; Verkman et al., 2017), but mostly failed (Abir-Awan et al., 2019; Brandt et al., 2018; Sogaard & Zeuthen, 2008). We here illustrate that bumetanide (a well-established NKCC inhibitor) does not inhibit AQP1 or AQP4 (Brandt et al., 2018), as otherwise proposed (Migliati et al., 2009), and that acetazolamide has no effect on the water permeability of AQP1 or AQP4, as otherwise proposed (Gao et al., 2006; Huber, Tsujita, & Nakada, 2009). While we cannot pin-point the reasons for the repeated discrepancies between inhibitor efficiencies in the aquaporin field, it is reasonable to
suspect that the origin thereof lies within the experimental approach. To obtain the osmotic water permeability of an AQP-expressing cell, the osmotic gradient must be introduced at a faster rate than the rate of cell volume change and the data acquisition must be sufficiently fast to obtain several data points in at the initial linear part of the cell volume change. These basic technical requirements are occasionally neglected in the field of AQP-mediated water permeability and the lack of which may provide a confounding effect.

TGN-020 was initially screened for binding to and blocking AQP4 using in vitro and in silico methods (Huber, Tsujita, & Nakada, 2009). Subsequently, the compound was proposed as a blocker of AQP4 in an ischemic cerebral edema disease model (Igarashi et al., 2011), following a successful development of a PET ligand for AQP4 imaging based on TGN-020 (with less binding observed in brain slices obtained from APQ4−/− mice)(Nakamura et al., 2011). In our sensitive cell volume measuring set-up, we here verified that TGN-020 efficiently blocked osmotic water permeation through AQP4 expressed in the *Xenopus laevis* oocytes. The inhibition was evident in both rat, mouse, and human versions of AQP4 and occurred with an IC₅₀ of ~3.5 µM (in agreement with (Huber, Tsujita, & Nakada, 2009)). AQP4 remained localized to the plasma membrane during the 60 min exposure to TGN-020, which suggests that the inhibitor-mediated reduction in water permeability of the AQP4-expressing oocytes was not due to internalization of AQP4. We therefore propose that TGN-020 mediates its effect on AQP4 by a direct action on the channel. However, the choice of experimental system does not allow for fast measurements of the kinetics of TGN-020 binding to AQP4, so we cannot exclude that TGN-020 acts on AQP4 via some indirect manner. Most importantly, TGN-020 did not block any of the other tested aquaporins (AQP1-9), and can therefore be considered an isoform-specific inhibitor of AQP4 (at least within the group of aquaporins mostly expressed in the mammalian plasma membranes). Molecular dynamics simulations supported binding of TGN-020 to AQP4 and subsequent blockage of the water-permeating pore. Our findings differ from previous docking calculations, which indicated that TGN-020 possibly interacted with residues D69, M70, 173, F77, V145, I205 and R216 (Huber, Tsujita, & Nakada, 2009). The differences most likely arise from the different methodologies used in the two in-silico methods. The docking energies previously reported (Huber, Tsujita, & Nakada,
Molecular dynamics simulations, like those employed in the present study, take into account the full dynamics of the protein, water, ions, and ligand, and the effect of water dynamics on ligand-binding is inherently included. However, much longer simulations than the one conducted in this study are required to accurately predict the optimal ligand-binding site.

Neuronal activity in the central nervous system associates with elevated $K^+ ([K^+]_o)$ in the extracellular space. The $[K^+]_o$ is rapidly buffered, initially by the nearby astrocytes by mechanisms predominantly involving the glia-specific $K^+$-sensitive $\alpha2\beta2$ isoform combination of the Na$^+$/K$^+$-ATPase, which is kinetically geared to respond to increased $[K^+]_o$ and the associated membrane depolarization (Ransom et al., 2000; D'Ambrosio et al., 2002; Larsen et al., 2014; Stoica et al., 2017). A smaller fraction of the $K^+$ may be removed from the extracellular space by means of the Kir4.1-mediated spatial buffering of $K^+$ (Chever, Djukic, McCarthy, & Amzica, 2010; Haj-Yasein, Jensen, et al., 2011; Larsen & MacAulay, 2014; Newman, 1986), in a manner that has been suggested to require a parallel AQP4-mediated water transport into the glial cell (Jin et al., 2013; Nagelhus et al., 1999; Nagelhus & Ottersen, 2013). With this isoform-specific AQP4 inhibitor, we have in the present study, by use of ion-sensitive microelectrodes, documented that the dynamics of hippocampal stimulus-evoked $[K^+]_o$ transients are unperturbed by blockage of AQP4. Taken together with biophysical considerations of ionic driving forces and earlier reports obtained with AQP4$^{-/-}$ mice (Haj-Yasein et al., 2015; Haj-Yasein et al., 2012; Strohschein et al., 2011), we here cement the notion that AQP4-mediated osmotic water permeability of the perisynaptic astrocytic membrane does not influence the rate of $K^+$ removal from the extracellular space following neuronal activity.

The activity-evoked $K^+$ transient is paralleled by shrinkage of the extracellular space, which is mostly assigned to astrocytic swelling (Dietzel et al., 1980; Connors et al., 1982; Ransom et al., 1985). The astrocytic AQP4 has been assumed to underlie glial volume changes (Nagelhus et al., 1999; Nagelhus & Ottersen, 2013), but was not required for activity-evoked extracellular shrinkage in AQP4$^{-/-}$ mice (Haj-Yasein et al., 2012) or for glia cell swelling during cortical spreading...
depression (Rakers, Schmid, & Petzold, 2017; Rosic et al., 2019). Here, activity-evoked extracellular space shrinkage (quantified by TMA\(^+\)-sensitive microelectrodes) was unperturbed by the presence of TGN-020, illustrating that AQP4-mediated osmotic water permeability was not required for the astrocytic swelling promoting (at least part of) the extracellular space shrinkage. As an additional manner of documenting that conventional osmotic water transport did not underlie the astrocytic cell swelling, activity-evoked glycogen breakdown was inhibited in order to prevent formation of osmotically active glucose-6-phosphate (Wender et al., 2000; Choi et al., 2012; Brown & Ransom, 2015). Such inhibition of glycogen phosphorylase did not affect the stimulus-evoked ECS shrinkage in hippocampal slices. Our results thus suggest that stimulus-induced ECS shrinkage in brain slice experiments does not occur via conventional AQP4-mediated osmotically-driven cell swelling following build-up of osmotic particles, such as those originating from activity-evoked glycogen break-down.

The extracellular space shrinkage had for decades been presumed to occur as a function of the astrocytic clearance of [K\(^+\)], or glutamate (Kofuji & Newman, 2004; Nagelhus et al., 2004; MacAulay & Zeuthen, 2012). However, inhibition of either of the astrocytic K\(^+\) and glutamate-transporting proteins; Kir4.1, NKCC1, KCC, and glutamate transporters failed to prevent the ECS shrinkage (Haj-Yasein et al., 2011; Larsen et al., 2014; Larsen & MacAulay, 2017). We recently demonstrated the pH-regulating cotransporters of bicarbonate and lactate (NBCe1 and MCTs), presumably via their ability to cotransport water (MacAulay & Zeuthen, 2010), as molecular mechanisms underlying parts of the stimulus-evoked extracellular space shrinkage, and by extension, astrocytic cell swelling (Florence et al., 2012; Larsen & MacAulay, 2017). These transporters get activated not by the K\(^+\) transient itself, but by the membrane depolarization associated with it, as well as by the activity-evoked alkaline transient (Theparambil et al., 2014; Theparambil & Deitmer, 2015; Theparambil et al., 2015) and the K\(^+\)-induced elevation of cellular metabolism (Wender et al., 2000; Choi et al., 2012; Brown & Ransom, 2015; Waitt et al., 2017). While this cotransporter-mediated astrocytic cell swelling accounts for approximately half of the volume transients, the remaining half remains unresolved, but as here demonstrated, does not occur via AQP4-mediated osmotically-driven water flux.
In conclusion, we here present TGN-020 as an efficient and isoform-specific inhibitor of AQP4-mediated osmotic water permeability. With this tool, we illustrate that AQP4 is not required for the dynamics of the stimulus-evoked K⁺ transient in rat hippocampus, nor for the associated astrocytic cell swelling.

**Competing interests**
The authors declare that there are no competing interests in this work.

**Author contributions**
All experiments were conducted at The University of Copenhagen, Denmark, in the laboratories of N.M. (Department of Neuroscience) and H.S.W. (Department of Drug Design and Pharmacology). Dynamic simulations were run at the University of Southern Denmark (Department of Physics, Chemistry and Pharmacy) by H.K. N.M., T.L.T.B., B.R.L., S.K.C., H.K. and H.S.W conceived and designed the study. T.L.T.B., B.R.L., H.S.W and S.K.C. performed the experiments. T.L.T.B., B.R.L., S.K.C., and H.K. analyzed the data. All authors contributed to the writing of the manuscript and are accountable for all aspects of the work and all persons designated as authors qualify for the authorship, and all those who qualify for authorship are listed. All authors have read and approved the final version of the manuscript.

**Data availability**
We confirm that all relevant data from this study are available from the first author upon request.

**References**


NKCC1 but is more potent to enhance phenobarbital's anti-seizure efficacy. *Neuropharmacology*, 143, 186-204. doi:10.1016/j.neuropharm.2018.09.025


**Figure legends**

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Figure 1. TGN-020 directly blocks different species of AQP4. (A) Schematic of the volumetric setup used to determine the water permeability of the AQP-expressing oocytes. (B) Representative volume traces of un.injected (top) or rAQP4-expressing (bottom) oocytes without (left) or with (right) pretreatment with 20 µM TGN-020 for 60 min prior to recordings. A hyposmotic challenge (-50 mOsm) was abruptly introduced where marked with the black bar. (C) Summarized water permeabilities of oocytes expressing AQP4 from different species (r, m, h) without or with TGN-020 treatment (shown in red) (n = 9 of each). (D) Oocyte membrane potentials without and with TGN-020 treatment (as above) were recorded to verify that the compound was not toxic for the oocytes (n = 9 of each). (E) Immunocytochemistry with anti-AQP4 antibodies of AQP4-expressing oocytes (without or after 60 min preincubation with TGN-020). No staining was observed in uninjected oocytes, data not shown and (Toft-Bertelsen, Krizaj, & MacAulay, 2017). (F) Profile plot of confocal images from (E), A.U.; arbitrary units. (G) Summarized membrane fluorescence of AQP4-expressing oocytes without and after treatment with TGN-020. ANOVA followed by Tukey's multiple comparison tests or Student’s t-test (G) was used as statistical test. ***: p 0.001; NS: not significant.

Figure 2. TGN-020 is an isoform-selective AQP4 inhibitor. (A) Volume traces from an uninjected oocyte and AQP1-9-expressing oocytes challenged with a hyposmotic gradient (-50 mOsm; indicated by black bars above the traces) without or with TGN-020 treatment (red traces). (B) Summarized water permeabilities of oocytes expressing the nine different AQPs in the absence and presence of TGN-020 (shown in red) (20 µM, 60 min, n = 9 of each). ANOVA followed by Tukey's multiple comparison tests was used as statistical test. ***: p 0.001; NS: not significant.

Figure 3. TGN-020 exerts an acute inhibitory effect on AQP4. (A) A representative time control experiment from an uninjected oocyte and an AQP4-expressing oocyte repeatedly challenged with a hyposmotic gradient (-50 mOsm; indicated by a black bar, t = 0 min (control), t = 5 min, and t = 10 min). (B) Summarized water permeabilities at t = 0 min (control), t = 5 min, and t = 10 min from oocytes expressing AQP4 from different species (n = 9 of each). (C) A representative volume trace from a rAQP4-expressing oocyte challenged with a hyposmotic gradient (-50mOsm, indicated by a
black bar) before (left trace) and after 20 μM of TGN-020 for 5 or 10 min (right; indicated in red).  
(D) Summarized water permeabilities for AQP4-expressing oocytes at t = 0 min (control) and after exposure to TGN-020 for t = 5 min and t = 10 min (n = 9 of each). (E) rAQP4-expressing oocytes exposed to different concentrations of TGN-020, data were fitted with Graphpad Prism using nonlinear regression analysis to obtain the IC50 from the mean of the independent regression analyses (n = 7). ANOVA followed by Tukey's multiple comparison tests was used as statistical test. ***: p 0.001; NS: not significant.

Figure 4. Blocking of AQP4 by TGN-020 in molecular dynamics simulations. Initial (A-B) and final (C-D) top and side view snapshot of TGN-020 docked (A-B) or bound (C-D) to an AQP4 tetramer. Water and lipids have been eliminated for clarity. In at least one of the tetramers, TGN-020 enters the pore and blocks it, while no TGN-020 permeation through the channel was detected.  
(E, G) A representative side (E) and top (G) view of a simulation snapshot of a single monomer of AQP4 without TGN-020 (the water pathway through the pore is shown in red). (F) and (H) present top and side views of a simulation snapshot (200 ns) of a single monomer of AQP4, where water access through the channel is blocked by TGN-020 (shown as orange sticks). Plausible amino acid residues that interact with different molecules of TGN-020 in the four monomers have been highlighted.

Figure 5. Acetazolamide and bumetanide are not inhibitors of AQP1 or AQP4. (A-B) Representative volume traces from an AQP1-expressing oocyte repeatedly challenged with a hyposmotic gradient (-50 mOsm; indicated by a black bar) without (A) and with (B) exposure to acetazolamide (Aze, 100 μM). (C) Summarized water permeabilities of AQP1- or AQP4-expressing oocytes exposed to acetazolamide (n = 9 of each). (D) Summarized water permeabilities of AQP1- or AQP4-expressing oocytes exposed to bumetanide (Bum, 1 mM, n = 12-14 of each). ANOVA followed by Tukey's multiple comparison tests was used as statistical test; NS: not significant.
Figure 6. AQP4 does not contribute to activity-evoked K⁺-clearance and associated extracellular space shrinkage in hippocampal brain slices. Electrical stimulation of the Schaffer collaterals (3 sec at 20 Hz, marked by a black bar) in rat hippocampal slices promoted the neuronal activity leading to the [K⁺]₀ transient and associated extracellular space shrinkage monitored by ion-sensitive microelectrodes. (A). Stimulus-evoked changes in [K⁺]₀ prior (black trace) and post (red trace) exposure to TGN-020 (20 μM, 60 min) are shown as representative traces. Summary of the stimulus-evoked peak [K⁺]₀ (Ai) and [K⁺]₀ decay constants (Aii; the blue lines mark the linear section used for decay constant quantification; r² ≥ 0.99 for all experiments) before and after TGN-020, n = 6 slices from 5 animals. (B). Representative traces of the stimulus-evoked changes of the extracellular space (black: control; red: in the presence of TGN-020; 20 μM, 60 min), ΔECS summarized in (Bi). Statistical significance was tested with Student’s paired t-test; NS: not significant.

Figure 7. Inhibition of glycogen phosphorylase with DAB does not affect activity-evoked ECS dynamics. A. Representative traces of stimulus-induced changes in the ECS prior (as in Fig. 6) before (control; shown in black) and after (purple) exposure to 1 mM DAB (45 min). The cyan trace represents a single experiment with 90 min exposure of DAB. Inset: Schematic illustrating a prevented breakdown of glycogen by DAB. Ai. The stimulus-evoked change in the ECS in the presence of DAB (45 min) was normalized to its control trace and summarized (111.1 ± 7.1 % of control, n = 5 slices from 4 animals). B. Primary cultures of rat astrocytes were utilized to test the efficiency of DAB to prevent glycogen breakdown. Astrocytes were incubated for 45 min with either no glucose, 10 mM glucose, or no glucose in the presence of 1 mM DAB prior to determination of the cellular glycogen content. The control cells were sampled prior to incubation (t = 0 min). Statistical significance was tested with either Student’s paired t-test (Ai) or one-way ANOVA with Tukey’s multiple comparison post hoc tests (B). *; p < 0.05, ** p < 0.01, NS: not significant.
Clearance of activity-evoked $K^+$ transients and associated glia cell swelling occur independently of AQP4; A study with an isoform-selective AQP4 inhibitor

Running title: $K^+$ clearance and glial swelling occurs independently of AQP4

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Acknowledgements
This study was supported by the Novo Nordisk Foundation (to NM; NNF15OC0017052) and the Lundbeck Foundation (to HSW; R208-2015-2859). Simulations were performed on the Danish e-Infrastructure Cooperation (DeiC) National HPC Center, ABACUS 2.0 at the University of Southern Denmark.

Word Count

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Competing Interests: 12
References: 1833
Footnotes: 0
Figure Legends: 1088
Total word count: 9596

Number of Pages: 24
Number of Figures: 7
Number of Tables: 0

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Abstract

The mammalian brain consists of 80% water, which is continuously shifted between different compartments and cellular structures by mechanisms that are, to a large extent, unresolved. Aquaporin 4 (AQP4) is abundantly expressed in glia and ependymal cells of the mammalian brain and has been proposed to act as a gatekeeper for brain water dynamics, predominantly based on studies utilizing AQP4-deficient mice. However, these mice have a range of secondary effects due to the gene deletion. An efficient and selective AQP4 inhibitor has thus been sorely needed to validate the results obtained in the AQP4−/− mice to quantify the contribution of AQP4 to brain fluid dynamics. In AQP4-expressing *Xenopus laevis* oocytes monitored by a high-resolution volume recording system, we here demonstrate that the compound TGN-020 is such a selective AQP4 inhibitor. TGN-020 targets the tested species of AQP4 with an IC50 of ~3.5 μM, but displays no inhibitory effect on the other AQPs (AQP1-AQP9). With this tool, we employed rat hippocampal slices and ion-sensitive microelectrodes to determine the role of AQP4 in glia cell swelling following neuronal activity. TGN-020-mediated inhibition of AQP4 did not prevent stimulus-induced extracellular space shrinkage, nor did it slow clearance of the activity-evoked K⁺ transient. These data, obtained with a verified isoform-selective AQP4 inhibitor, indicate that AQP4 is not required for the astrocytic contribution to the K⁺ clearance or the associated extracellular space shrinkage.

**Key words:** AQP4, inhibitor, TGN-020, [K⁺]₀ clearance, glia cell swelling
Main points:
- TGN-020 is an efficient and isoform-specific inhibitor of AQP4-mediated osmotic water permeability.
- AQP4 is not required for shaping the stimulus-evoked K⁺ transient or the associated extracellular space shrinkage in rat hippocampus

Abbreviations:
aCSF, artificial cerebrospinal fluid; DAB, 1,4-dideoxy-1,4-imino-D-arabinitol; ECS, extracellular space; MCT, monocarboxylate transporters; NBCe1, electrogenic Na⁺/bicarbonate cotransporter 1; TMA, tetramethylammonium; TEA, tetraethylammonium; TGN-020, 2-(nicotinamide)-1,3,4-thiadiazole; AQP, aquaporin.

Introduction
The mammalian brain consists of 80% water, which is dispersed between the intra- and extracellular fluid of brain cells, the latter in continuum with the cerebrospinal fluid (CSF; mainly in the cerebral ventricles and the subarachnoid space), in addition to the fluid content of the vasculature. The cerebral water homeostasis is anticipated to be tightly controlled to ensure stable cell volume and intracranial pressure within the physiological range. However, the molecular mechanisms governing the transmembrane movement of water within the central nervous system (CNS) remain largely unresolved. Water can cross cell membranes via water channels (aquaporins; AQP) and cotransport proteins (MacAulay & Zeuthen, 2010). Of the large family of AQPs expressed in the majority of cell types in the mammalian body, AQP4 is abundant in the brain; more specifically in the astrocytic endfeet bordering the vasculature and the pia in addition to the ependymal lining bordering the cerebral ventricles (Nagelhus & Ottersen, 2013). Such prominent localization at the interfaces between blood-brain and CSF-brain, led to a proposed role of AQP4 as an important gatekeeper of brain water entry and exit, in addition to its potential involvement in both the glia-mediated K⁺ buffering and the parallel glia cell swelling taking place during neuronal activity (Jin, Zhang, Binder, & Verkman, 2013; Manley, Binder, Papadopoulos, & Verkman, 2004; Nagelhus et al., 1999; Nagelhus & Ottersen, 2013; Papadopoulos & Verkman, 2007; Strohschein et al., 2011). With the lack of a specific inhibitor of AQP4, these studies were all based on AQP4 knock-out mice (AQP4⁻/⁻). While these mice are deficient in AQP4 expression, it has come to light
that several other molecular changes occurred as a secondary effect to the knock-out strategy: (i) These mice have 28% enlarged extracellular space (ECS) in the CNS, (ii) reduced glutamate transporter (GLT-1) expression and thereby presumably delayed glutamate clearance, (iii) increased brain water content, (iv) enhanced astrocytic gap junctional coupling, and (v) a possible impairment of the Na⁺/K⁺-ATPase activity (Haj-Yasein, Vindedal, et al., 2011; Strohschein et al., 2011; Yao, Hrabetova, Nicholson, & Manley, 2008; Zeng et al., 2007). It thus remains unclear if the observed phenotype originated from the reduced osmotic water permeability of the astrocytic membrane or from one of the (un)known secondary effects.

Therefore, a specific inhibitor of AQP4 would be a beneficial tool to include alongside the knock-out strategy. It has, however, proven challenging to develop such an inhibitor, not for want of trying. During the last decade, a few compounds were brought forward as potential inhibitors of AQP4; e.g., tetraethylammonium (TEA), acetazolamide, antiepileptic drugs, and bumetanide (and the derivative AqB013) (Detmers et al., 2006; Huber, Tsujita, Kwee, & Nakada, 2009; Migliati et al., 2009; Tanimura, Hiroaki, & Fujiyoshi, 2009; Verkman, Smith, Phuan, Tradtrantip, & Anderson, 2017), but all of these pharmacological agents were later demonstrated to not be effective blockers of AQP4-mediated water permeability (Abir-Awan et al., 2019; Brandt et al., 2018; Sogaard & Zeuthen, 2008). The molecule TGN-020 has been shown to bind to AQP4 (Huber, Tsujita, & Nakada, 2009; Nakamura et al., 2011), act as an inhibitor (Huber, Tsujita, & Nakada, 2012), and promote better outcome in mouse models of ischemic stroke (Igarashi, Huber, Tsujita, & Nakada, 2011; Pirici et al., 2017). TGN-020 may thus be the long awaited AQP4 inhibitor, provided sufficient efficiency and specificity. In the present study, we demonstrate the potency and isoform specificity of TGN-020 as an AQP4 inhibitor and subsequently employ this pharmacological tool to determine the role of AQP4 in activity-evoked K⁺ clearance and associated extracellular shrinkage in rat hippocampus.

Materials and Methods

Ethical approval

The experiments were performed according to the guidelines of the Danish Veterinary and Food administration (Ministry of Environment and Food) and approved by the animal facility at the Faculty of Health and Medical Sciences, University of Copenhagen. The animal experiments conform to the principles and regulations described in (Grundy, 2015). The surgical protocol, by
which the oocytes were retrieved, was approved by The Danish National Committee for Animal Studies.

Experimental procedures - rats
Experiments were performed on male and female rats (Sprague-Dawley, Janvier Labs, France) at P21-P28, housed in the animal facility at the Faculty of Health and Medical Sciences, University of Copenhagen. The rats were anaesthetized using gaseous 2-Bromo-2-Chloro-1,1,1-Trifluoroethane (halothane) (Sigma-Aldrich, Germany, B-4388) prior to decapitation.

Experimental procedures - RNA preparation and heterologous expression in Xenopus laevis oocytes
cDNA encoding rat AQP1-9 (mouse and human AQP4 were also employed) were subcloned into the oocyte expression vectors pXOOM or BS(SK-), linearized downstream from the poly-A segment, and in vitro transcribed using T7 mMessage machine according to manufacturer’s instructions (Ambion, Austin, TX). cRNA was extracted with MEGAclear (Ambion, Austin, TX) and micro-injected into defolliculated Xenopus laevis oocytes: 10 ng RNA/oocyte. Xenopus laevis frogs were obtained from Nasco (Fort Atkinson, WI) or oocytes were purchased from EcoCyte Bioscience (Germany). Oocytes were collected under anaesthesia (2 g/L Tricain, 3-amino-benzoic acid ethyl ester, Sigma-Aldrich, Germany, A-5040), and the preparation of defolliculated oocytes was carried out as described in (Fenton et al., 2010) and the oocytes were kept in Kulori medium ((in mM): 90 NaCl, 1 KCl, 1 CaCl2, 1 MgCl2, 5 HEPES (pH 7.4)) for 3-4 days at 19°C prior to experiments.

Volume recordings of oocytes - Oocytes were placed in an experimental recording chamber, perfused with various solutions, and volume measurements were performed as previously described in (Zeuthen, Zeuthen, & Macaulay, 2007). Briefly, the oocytes were placed in a small chamber with a glass bottom and perfused with solutions of interest. The volume of the oocytes was viewed from below via a long distance objective (×4), and micrographs were captured continuously with a high resolution recording system (with a high signal to noise ratio) based on a CCD camera at a rate of 25 images/s (Zeuthen, Belhage, & Zeuthen, 2006). The perfusion solution consisted of (in mM): 50 NaCl, 2 KCl, 1 MgCl2, 1 CaCl2, 10 HEPES, 100 mM mannitol (Tris buffered pH 7.4, 220 mOsm). Hyposmotic solutions were subsequently made by the removal of mannitol (Δ50 mM mannitol)
with resulting osmolarity of 170 mOsm. The osmotic water permeability was determined by \( L_p = (J_v)/(A \times \Delta \pi \times V_w) \), where \( J_v \) is the initial water flux during an osmotic challenge, \( A \) is the membrane surface area (nine times the apparent area due to membrane folding (Zampighi et al., 1995), \( \Delta \pi \) is the osmotic challenge, and \( V_w \) is the partial molal volume of water (18 cm\(^3\)/mol). Osmolarities of all solutions were verified with an accuracy of 1 mOsm with an osmometer Type 15 (Löser Messtechnik, Berlin, Germany). TGN-020 (Sigma-Aldrich, Germany, SML0136) was dissolved in DMSO (stock solution of 20 µM; controls exposed to vehicle (DMSO) only) and its effect on volume changes were recorded after 5 and 10 min of TGN-020 treatment with the oocytes serving as own controls. For long-term application the oocytes were incubated at 19 °C for 60 min. with 20 µM of TGN-020. Determination of TGN-020-mediated effects on oocytes expressing the different AQP isoforms was carried out in a researcher-blinded fashion.

**Brain slices and solutions**

Following decapitation, the brain was quickly removed and placed into ice-cold cutting solution containing (in mM): 87 NaCl, 70 sucrose, 2.5 KCl, 0.5 CaCl\(_2\), 25 NaHCO\(_3\), 1.1 NaH\(_2\)PO\(_4\), 7 MgCl\(_2\), and 25 D-glucose, equilibrated with gaseous 95% O\(_2\), 5% CO\(_2\). Oblique sagittal (transverse) hippocampal slices (400 µm) were cut with a Campden Vibrating Microtome (7000SMZ-2, Campden Instruments, UK). Slices were transferred to the standard artificial cerebrospinal fluid (aCSF) solution containing (in mM): 124 NaCl, 3 KCl, 2 CaCl\(_2\), 25 NaHCO\(_3\), 1.1 NaH\(_2\)PO\(_4\), 2 MgCl\(_2\) and 10 D-glucose, and equilibrated with 95% O\(_2\), 5% CO\(_2\) (pH 7.4 at the experimental temperature of 33-34°C) and left to recover at 34°C for 30 min and then kept at room temperature.

**Ion-sensitive microelectrodes and electrophysiological recordings in slices**

Electrophysiological recordings were carried out in a submerged-type recording chamber (Brain Slice Chamber 1, Scientific Systems Design, Digitimer Ltd, UK) at an experimental temperature of 33-34°C and a continuous superfusion at a flow rate of 2.2 ml/min. Recordings were performed within stratum radiatum of the CA1 region. High-frequency stimulation was delivered by a concentric bipolar tungsten electrode (TM33CCNON, World Precision Instruments, UK) inserted into the stratum radiatum in the vicinity (≤ 300 µm) of the recording site. Stimulation trains (22 V at 20 Hz, each pulse of a duration of 80 µs, for 3 sec) were delivered to the slice to activate the Schaffer collaterals. Stimulation trains were delivered at 10 min intervals and involved the pharmacological compounds 1,4-dideoxy-1,4-imino-D-arabinitol (DAB, synthesized by Novo

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Nordisk A/S, Copenhagen, Denmark, prepared as stock of 500 mM in dH$_2$O or TGN-020 (Sigma-Aldrich, Germany, SML0136) with at least three consecutive control recordings prior to washing in compounds. The resulting extracellular field potentials were recorded with thin-walled filaments glass capillary microelectrodes (GC150TF-7.5, Harvard Apparatus, MA) pulled to resistances of 15-25 MΩ when filled with the standard solution (see above). This electrode served as reference signal for the ion-sensitive microelectrodes. Ion-sensitive microelectrodes were prepared from thin-walled non-filamented glass capillaries (GC150T-7.5, Harvard Apparatus, MA) pulled to obtain a tip diameter in the range of 1-2 µm (Voipio et al., 1994). The capillaries were then silanized internally with gaseous N,N-dimethyltrimethylsilylamine (Sigma-Aldrich, Germany, 41716) and baked at 190°C for 20 min prior to being backfilled. The electrodes were backfilled with a solution depending on the type of measurement, containing either 150 mM tetramethylammonium (TMA$^+$) chloride (for extracellular space volume measurements), or 150 mM NaCl, 3 mM KCl (for extracellular K$^+$ measurements). The tip of the capillary was afterwards filled with a short column of either TMA$^+$/K$^+$-sensitive liquid membrane solution (50 mg/ml potassium tetrakis (4-chlorophenyl) borate (Sigma-Aldrich, Germany, 60591) in 1,2-dimethyl-3-nitrobenzene (Sigma-Aldrich, Germany, 40870)). For experiments with TMA$^+$, 1.5 mM TMA$^-$Cl was included in the test solution. Note that the TMA$^+$/K$^+$ membrane is highly sensitive to quartenary ions, such as TMA$^+$, however in the absence of such ions it becomes selective to K$^+$ ions, and can therefore be used for both measurements depending on the backfilling solution.

The tips of the ion-sensitive electrode and the reference electrode were placed within a few microns at the exact same depth in the core of the slice. Close distance was ensured via Sensapex micromanipulators (SMX series, Sensapex, Finland), which provide precise µm x,y,z coordinates, by placing the electrode tips closely together above the slice and afterwards moving into the tissue maintaining this narrow distance aided by the x,y,z coordinates. The ion-sensitive signal and the field potential signal were both recorded via an ION-01M amplifier and headstage (NPI electronics, Germany). Online deduction of the field potential signal from that of the ion-sensitive electrode provided the traces employed for analysis. To ensure the quality of the experiments, the resistance of the electrode was tested several times throughout the experiments. If the resistance deviated > ~15% from the original value (indicating e.g. change in tip diameter or alterations in the tip liquid membrane column), the experiment was discarded. All recorded signals were filtered at 250 Hz, sampled at 500 Hz and stored for off-line analysis with WinEDR (courtesy of Dr. John Dempster, University of Strathclyde, Glasgow, UK) and GraphPad Prism 7.0. At the end of the experiment,
each ion-sensitive microelectrode was thoroughly calibrated in order to translate the signal in mV to either the % volume change or the $K^+$ concentration, for a detailed description, see (Larsen, Stoica, & MacAulay, 2019).

**Glycogen measurements in primary cultures of rat astrocytes**
Cortical rat astrocytes were cultured from dissected cerebral cortices of P8 rat pups. The dissected cortices were dissociated mechanically by passing the tissue through an 80 µm nylon sieve into Dulbecco’s modified Eagle’s medium (Sigma-Aldrich, Germany, D-5030) containing an additional 6 mM D-glucose, 2.5 mM L-glutamine, 26.2 mM NaHCO$_3$, 100,000 IU/L of penicillin and 20% foetal bovine serum (Sigma-Aldrich, Germany, A-4503). Single cells were generated by trituration with a syringe equipped with a steel cannula and plated in T25 cell culture flasks. The foetal bovine serum concentration was sequentially reduced to 15 % and 10 % in the second and third week of culture, respectively. 0.25 mM dB-cAMP (Sigma-Aldrich, Germany, D-0627) was added to the culture medium in the third week of culture to obtain morphologically differentiated (Su, Haworth, Dempsey, & Sun, 2000) astrocytes. The astrocytes were kept at 37°C with an atmosphere of 5% CO$_2$ and were used for experiments three weeks after plating.

Primary cultures of astrocytes were pre-incubated for 2 hours in aCSF with 10 mM glucose. The cells were washed in PBS prior to incubation for 45 min in aCSF with either 10 mM glucose, in the absence of glucose, or in the absence of glucose but in the presence of 1 mM DAB to inhibit glycogenolysis. The incubation was terminated by a wash with cold PBS and cells were transferred to -80 °C before extraction on ice with 70 % ethanol. Control levels of glycogen were obtained from cells extracted immediately after the pre-incubation step. The ethanol extract was centrifuged at 4 °C, 20,000 g for 20 minutes. Pellet was dissolved in 100 µL ddH$_2$O and homogenized prior to glycogen and protein determination. The glycogen was quantified in a coupled enzymatic assay by measurement of the production of NADPH in the conversion of glucose-6-phosphate to 6-phosphogluconolactone as previously described (Brown, Tekkok, & Ransom, 2003). Briefly, the homogenized cell pellet was resuspended in an acetate buffer (5 mM, pH 5) containing 10 U/ml amylglucosidase (Sigma-Aldrich, Germany, A7420) and incubated at 37 °C for 1.5 h prior to centrifugation at 20,000 g for 10 min and transfer of the supernatant to a microtiter plate. The cofactors NADP (0.16 mM), ATP (0.81 mM) and magnesium (MgCl$_2$; 12.1 mM) were added in an alkaline solution (37.1 mM Tris base; 0.007% sodium azide; 13.8 mM HCl) to each well. The reaction was initiated by the addition of hexokinase (1.52 U/ml, 11426362001 Roche, Germany).
and glucose-6-phosphate dehydrogenase (Sigma-Aldrich, Germany, G-6378, 0.54 U/ml). The fluorescence was measured by SpectraMax i3X (Molecular Devices, San Jose, CA, USA) prior to addition of the enzymes to obtain a background measurement, and after 30 min incubation at 37 °C, using 350 and 470 nm as excitation and emission wavelengths, respectively.

**Molecular Dynamics Simulations (MD)**

The initial structure of rAQP4 was obtained from PDB id 2zz9 (www.pdb.org) (Tani et al., 2009). The protein tetramer was inserted in a DOPC lipid bilayer measuring 12.25 nm x 12.25 nm using CHARMM-GUI (Jo, Kim, Iyer, & Im, 2008), and was hydrated with water and 150 mM KCl. The binding energies are ∼60 kcal/mole, almost the strength of a covalent binding, and much stronger than a streptavidin-biotin binding. The final system contained 340 DOPC molecules, and measured 12.25 x 12.25 x 9.7 nm. The system was energy minimized and equilibrated while slowly releasing backbone restraints on the protein atoms. After equilibration, the simulation of pure AQP4 inside the bilayer was run for 200 ns. The initial force field parameters for TGN-020 were obtained from www.parachem.org (Vanommeslaeghe et al., 2010). Four TGN-020 molecules with different initial orientations were placed on top of the AQP4 in the above system. The system containing protein, lipids and TGN-020 was energy minimized and equilibrated in the same manner, but keeping the TGN-020 atoms restrained during the equilibration period. The simulation was then run for 200 ns with TGN-020. All simulations were performed using GROMACS (Van Der Spoel et al., 2005).

Standard MD protocols for equilibration and production runs were used, as reported elsewhere (Yamamoto et al., 2019).

**Data analysis and statistics**

All data are given as mean ± SEM. Statistical tests were performed by use of GraphPad Prism 7.0 (GraphPad Software Inc., La Jolla, CA, USA). Statistical significance was tested with Student’s t-test or one-way ANOVA with Tukey’s multiple comparison *post hoc* tests as indicated in figure legends. *P* values < 0.05 were considered statistically significant. The equation \( y = \frac{100}{(1 + 10^((\log IC_{50} - x) \times \text{Hill slope}))} \) was used to fit the IC\textsubscript{50} curves for each individual experiment, which are shown as summarized. For experiments employing brain slices the number of experiments, n, signifies individual brain slices and is mentioned in the Result section, whereas the number of animals from which these slices have been obtained is included in the figure legends. In
experiments with *Xenopus* oocytes, n refers to number of oocytes, obtained from at least three different batches of oocytes.

**Results**

**TGN-020-mediated inhibition of AQP4**

To evaluate TGN-020’s effect on the AQP4-mediated water permeability, rat AQP4 was heterologously expressed in *Xenopus laevis* oocytes. The most prominent isoform of AQP4 in the brain, called M23 due to its translation initiation site at methionin at position 23 in the primary structure of AQP4, was employed for these experiments (Amiry-Moghaddam & Ottersen, 2003). The osmotic water permeability of the oocytes was determined following introduction of an abrupt hyposmotic challenge (-50 mOsm), during continuous recording of the oocyte volume changes by a high-resolution volume recording system using a CCD camera (see Fig. 1A for experimental setup). This experimental system provides us with a robust read-out of AQP4-dependent water permeability with a negligible background due to the low inherent water permeability of the native oocyte membrane (see Fig. 1B for representative traces and Fig. 1C for osmotic water permeabilities for uninjected oocytes versus AQP4-expressing oocytes, note, control oocytes were exposed to vehicle throughout the study). Exposure to TGN-020 (20 µM, 60 min) completely abolished the AQP4-dependent oocyte swelling following introduction of the osmotic challenge (see Fig. 1B, right panels for representative traces). The water permeability of rAQP4-expressing oocytes (2.66 ± 0.22 × 10⁻³ cm sec⁻¹, n = 9) was reduced to (0.21 ± 0.1 × 10⁻³ cm sec⁻¹, n = 9) in the presence of TGN-020 (Fig. 1C). TGN-020 was equally efficient in inhibiting AQP4 of the other tested species, mouse and human, Fig. 1C. The membrane potential of the AQP4-expressing oocytes was unperturbed by 60 min exposure to TGN-020, verifying that the compound did not reduce the osmotic water permeability of the oocyte by a toxic effect on the cells, Fig. 1D. Such TGN-020-induced reduction in AQP4-mediated water permeability can be observed following either pore block or internalization of the water channel. We verified membrane expression of AQP4 by confocal microscopy of AQP4-expressing oocytes in the absence or presence of TGN-020. Representative micrographs indicate membrane localization of AQP4 (Fig. 1E), which we analyzed by fluorescent profile plotting, see Fig. 1F for a representative analysis. The membrane abundance of AQP4 was independent of TGN-020 treatment (in arbitrary units; 3.53 ± 0.29, n = 9 in control and 3.71 ± 0.15, n = 9 in TGN-020-treated oocytes), Fig. 1G, suggesting that AQP4 remains in the plasma membrane during exposure to TGN-020. TGN-020 thus appear to inhibit the
AQP4-dependent water permeability in a manner distinct from internalization, irrespective of whether the AQP4 was of rat, mouse, or human origin.

*TGN-020 is an isoform-specific AQP4 inhibitor.*

To determine whether the TGN-020 inhibition was selective towards AQP4, oocytes expressing AQP1-9 were exposed to TGN-020 and their water permeability assessed with introduction of a hyposmotic gradient (-50 mOsm). The water permeability of the oocytes expressing the different isoforms cannot be directly compared, as each oocyte may express different amounts of aquaporin. Therefore, the AQP-mediated water permeability was determined with or without TGN-020 across the oocytes expressing the different isoforms. TGN-020 (shown in red in Fig. 2) reduced the water permeability exclusively in oocytes expressing AQP4, representative traces shown in Fig. 2A, and summarized water permeabilities illustrated in Fig. 2B, n = 9-10 of each. TGN-020 thus selectively and efficiently blocks the AQP4 isoform within the group of AQPs most abundantly expressed in cell membranes of mammals.

*TGN-020 exerts an acute blocking effect on AQP4.*

With the observation that TGN-020 selectively blocks AQP4 after prolonged treatment (60 min), we demonstrated the efficacy of the blocker within a shorter time frame. To use each oocyte as its own control to determine time-dependency of an inhibitor effect, we initially performed a time-control experiment to verify sequentially recorded water permeabilities of an identical magnitude in the AQP4-expressing oocytes, at times 0 (control), 5, and 10 min, see representative volume traces in Fig. 3A and summarized data in Fig. 3B, n = 9. Upon acute application of TGN-020 to the recording chamber we observed a decreased AQP4-mediated water permeability at t = 5 min, with complete inhibition at 10 min, Fig. 3C, with summarized data in Fig. 3D (TGN-020 groups are shown in red). It should be noted, that the 5 min delay in inhibition most likely reflects the speed at which the compound washes into the chamber rather than a slow interaction between the aquaporin and the inhibitor. With the experimental paradigm of 10 min exposure to the compound (i.e. the oocyte was exposed to TGN-020 for 10 min prior to introducing the osmotic challenge), we determined the IC₅₀ for TGN-020 for rAQP4 with inclusion of different TGN-020 concentrations (IC₅₀ of ~ 3.62 ± 0.74 μM, n = 7), Fig. 3E. TGN-020 therefore appears to be an efficient blocker of AQP4-mediated water permeability by a direct action on the water channel.
Molecular Dynamics Simulations of TGN-020 binding to AQP4.

To characterize the binding pocket in rAQP4, we implemented molecular dynamics simulations of TGN-020 docked close to four pores of AQP4 (shown in yellow, blue, green and red) with different initial orientations (Fig. 4 A-B). During the sampling window, TGN-020 penetrated into the pore (but never fully permeated) and came to rest in different regions along the pore. Final snapshots of the simulation of TGN-020 bound to an AQP4 tetramer (Fig.4 C-D) illustrate that TGN-020 does not remain bound to the same part of the pore at the used timescale, but to several different regions along the pore. Much longer sampling times may be required for all TGN-020 molecules to find the same binding site on each AQP4 monomer. Molecular dynamics simulations illustrate the water pathway of AQP4 (depicted in red in Fig. 4E (side view) and Fig. 4G (top view)). With inclusion of TGN-020, this water pathway is blocked (Fig. 4F (side view) and Fig. 4H (top view)). These data, in support of the findings from the experimental approach, suggest that the TGN-020 inhibits AQP4-mediated water permeability by a direct pore-blocking effect.

Acetazolamide and bumetanide are not inhibitors of AQP1- or AQP4-mediated water permeability

Acetazolamide (Aze) and bumetanide (Bum) have been proposed as inhibitors of AQP1 and AQP4 (Huber, Tsujita, & Nakada, 2009). To test a putative blocking effect of these compounds in our sensitive volume assay, we monitored the osmotically-induced oocyte swelling of AQP1- and AQP4-expressing oocytes exposed to these compounds (1 mM Bum; 100 μM Aze)). Representative volume traces of AQP1-expressing oocytes exposed to an abrupt hyposmotic gradient are illustrated in Fig. 5A-B (time control traces (A) and with Aze application (B)). Summarized water permeabilities with and without exposure to Aze (Fig. 5C) or Bum (Fig. 5D) illustrate no blocking effects on the water permeabilities of either of these AQPs upon treatment with acetazolamide or bumetanide. Thus, these two compounds are not efficient blockers of these two water channels.

AQP4 does not contribute to stimulus-evoked ECS shrinkage or K⁺ clearance in acute rat hippocampal slices

Glia AQP4 has been proposed to be involved in clearance of K⁺ from the extracellular space following neuronal activity, and/or permissive for the associated glial cell swelling (Jin et al., 2013; Nagelhus & Ottersen, 2013; Strohschein et al., 2011). With TGN-020 as an efficient blocker of AQP4-mediated water permeability, we determined the contribution of AQP4 to the shaping of the neuronal activity-evoked K⁺ transient in the extracellular space and the associated glia cell swelling.
To this end, we employed acute hippocampal slices from rat (P21-28) with electrical stimulation of the CA1 Schaffer collaterals. We monitored the K⁺ transients with K⁺-sensitive microelectrodes and the relative changes of the extracellular space volume (ΔECS) with tetramethylammonium (TMA⁺)-sensitive microelectrodes upon bath application of TMA⁺. This technical approach has previously been validated by demonstration of roles for different K⁺- and H2O-transporting mechanisms in these processes (Larsen et al., 2014; Larsen, Holm, Vilsen, & MacAulay, 2016; Larsen & MacAulay, 2017). Electrical stimulation of the slice gave rise to field potentials and caused transient [K⁺]o increases of ~6-7 mM. The shape of the stimulus-evoked K⁺ transient was unaffected by bath application of the AQP4 inhibitor, TGN-020 (20 μM, 60 min), see representative trace in Fig. 6A. The peak [K⁺]o (92 ± 3 % of control, p = 0.064; panel Ai) and the [K⁺]o decay constant (97 ± 4 % of control, p = 0.162; panel Aii) were unaffected by blockage of AQP4, n = 6 slices. The associated stimulus-induced increase in TMA⁺ concentration represents a robust read-out of the extracellular space shrinkage (~4-5 % in this experimental series), Fig. 6B. The activity-evoked extracellular space shrinkage was not significantly diminished by the presence of TGN-020 (20 μM, 60 min, 90 ± 6 % of control, p = 0.154; n = 7 slices), see Fig. 6B for representative traces and panel Bi for summarized data. These results suggest that AQP4 does not contribute to shaping the stimulus-induced K⁺ clearance or the associated glial volume dynamics.

The activity-evoked ECS shrinkage does not occur as a consequence of elevated osmotic pressure in the astrocytes following K⁺-mediated glycogen breakdown.

Upon neuronal activity and the associated [K⁺]o elevation, K⁺-induced glycogen breakdown occurs in the neighboring astrocytes (Dietzel, Heinemann, Hofmeier, & Lux, 1982). This event leads to an increased number of osmotically active particles in the astrocytes, and thereby creates an osmotic driving force. It has been hypothesized that this elevated osmotic pressure would lead to AQP4-dependent osmotic water flux into the astrocytes, which would promote the observed activity-evoked extracellular space shrinkage due to glia cell swelling. To reveal whether conventional osmotic water transport underlies the activity-evoked glia cell swelling, we determined the effect on stimulus-evoked extracellular space shrinkage following inhibition of the glycogen phosphorylase (with 1 mM 1,4-dideoxy-1,4-imino-D-arabinitol (DAB) (Walls et al., 2008)) in rat hippocampal slices, Fig. 7A. The ECS shrinkage was not significantly altered upon exposure to DAB (45 min, purple trace in Fig. 7A (representative traces) and panel Ai for summarized data, n=5). An additional pilot experiment with a prolonged (~90min) exposure to DAB like-wise failed to affect the...
the volume dynamics (cyan trace in Fig. 7A). Acute brain slices may temporarily lose the astrocytic glycogen stores during the slicing procedure, and these reappear approximately 1h post-slicing with full restoration at 3h (Fiala et al., 2003). The brain slices employed for this experimental series were allowed to rest for 2-3h prior to experiments (n = 5). The ability of DAB to prevent glycogen breakdown is well established in murine astrocytes (Walls et al., 2008). To verify its action on the rat enzyme and, in addition, obtain a positive control of the inhibitor, we tested the ability of DAB to prevent glycogen breakdown in primary cultures of rat astrocytes. The rat astrocytes were pre-incubated with 10 mM glucose for 2 hours prior to the assay initiation to obtain a high level of glycogen at t = 0. The glycogen levels of the cultured rat astrocytes were determined in control conditions (t = 0 min) and after 45 min incubation with either absence of glucose (to induce glycogen breakdown), with high glucose in the medium (10 mM to prevent glycogen breakdown), or in the absence of glucose but presence of DAB. Fig. 7B illustrates the efficient prevention of glycogen breakdown in the presence of 1 mM DAB. These results indicate that osmolyte production (and subsequent osmotic water flow into glia cells) induced by glycogen phosphorylase-mediated glycogen breakdown does not contribute to stimulus-evoked extracellular space shrinkage in rat hippocampus.

DISCUSSION

With validation of TGN-020 as an efficient and isoform-specific inhibitor of AQP4, we here demonstrate that astrocytic AQP4-dependent osmotic water permeability is not required for activity-evoked K⁺ clearance or the associated extracellular space shrinkage. AQP4 is expressed, in a polarized fashion, in the paravascular astrocytic endfeet (Nielsen et al., 1997), which together with its ability to conduct bi-directional flux of water according to the imposed osmotic gradient (Meinild, Klaerke, & Zeuthen, 1998), has led to its suggested implication in a range of different (patho)physiological processes involving shifts of water between different compartments and cellular structures in the mammalian brain (Nagelhus & Ottersen, 2013). AQP4 has thus been suggested to act as the gatekeeper of brain water entry during brain edema formation in connection with several experimental animal disease models, glial K⁺ clearance during neuronal activity and the associated cell swelling, and the glial water influx sustaining the proposed convectional flow of fluorescent dyes through the brain parenchyma of the glymphatic hypothesis (Nagelhus & Ottersen, 2013; Papadopoulos & Verkman, 2013; Plog & Nedergaard, 2018). These experimental findings were all based on AQP4 knock-out mice, which were later demonstrated to have secondary changes
in basic parameters, such as extracellular space size, brain water content, expression of glutamate transporters and connexins, etc. (Haj-Yasein, Vindedal, et al., 2011; Strohschein et al., 2011; Yao et al., 2008; Zeng et al., 2007), which may have introduced confounding elements in the experimental regime. To validate these findings, extensive searches for AQP4 inhibitors have been conducted (Detmers et al., 2006; Huber, Tsujita, Kwee, et al., 2009; Migliati et al., 2009; Tanimura et al., 2009; Verkman et al., 2017), but mostly failed (Abir-Awan et al., 2019; Brandt et al., 2018; Sogaard & Zeuthen, 2008). We here illustrate that bumetanide (a well-established NKCC inhibitor) does not inhibit AQP1 or AQP4 (Brandt et al., 2018), as otherwise proposed (Migliati et al., 2009), and that acetazolamide has no effect on the water permeability of AQP1 or AQP4, as otherwise proposed (Gao et al., 2006; Huber, Tsujita, & Nakada, 2009). While we cannot pin-point the reasons for the repeated discrepancies between inhibitor efficiencies in the aquaporin field, it is reasonable to suspect that the origin thereof lies within the experimental approach. To obtain the osmotic water permeability of an AQP-expressing cell, the osmotic gradient must be introduced at a faster rate than the rate of cell volume change and the data acquisition must be sufficiently fast to obtain several data points in at the initial linear part of the cell volume change. These basic technical requirements are occasionally neglected in the field of AQP-mediated water permeability and the lack of which may provide a confounding effect.

TGN-020 was initially screened for binding to and blocking AQP4 using in vitro and in silico methods (Huber, Tsujita, & Nakada, 2009). Subsequently, the compound was proposed as a blocker of AQP4 in an ischemic cerebral edema disease model (Igarashi et al., 2011), following a successful development of a PET ligand for AQP4 imaging based on TGN-020 (with less binding observed in brain slices obtained from APQ4−/− mice)(Nakamura et al., 2011). In our sensitive cell volume measuring set-up, we here verified that TGN-020 efficiently blocked osmotic water permeation through AQP4 expressed in the *Xenopus laevis* oocytes. The inhibition was evident in both rat, mouse, and human versions of AQP4 and occurred with an IC50 of ~3.5 µM (in agreement with (Huber, Tsujita, & Nakada, 2009)). AQP4 remained localized to the plasma membrane during the 60 min exposure to TGN-020, which suggests that the inhibitor-mediated reduction in water permeability of the AQP4-expressing oocytes was not due to internalization of AQP4. We therefore propose that TGN-020 mediates its effect on AQP4 by a direct action on the channel. However, the choice of experimental system does not allow for fast measurements of the kinetics of TGN-020 binding to AQP4, so we cannot exclude that TGN-020 acts on AQP4 via some indirect manner.
Most importantly, TGN-020 did not block any of the other tested aquaporins (AQP1-9), and can therefore be considered an isoform-specific inhibitor of AQP4 (at least within the group of aquaporins mostly expressed in the mammalian plasma membranes). Molecular dynamics simulations supported binding of TGN-020 to AQP4 and subsequent blockage of the water-permeating pore. Our findings differ from previous docking calculations, which indicated that TGN-020 possibly interacted with residues D69, M70, I73, F77, V145, I205 and R216 (Huber, Tsujita, & Nakada, 2009). The differences most likely arise from the different methodologies used in the two in-silico methods. The docking energies previously reported (Huber, Tsujita, & Nakada, 2009) did not take ligand van-der Waals terms into account. Molecular dynamics simulations, like those employed in the present study, take into account the full dynamics of the protein, water, ions, and ligand, and the effect of water dynamics on ligand-binding is inherently included. However, much longer simulations than the one conducted in this study are required to accurately predict the optimal ligand-binding site.

Neuronal activity in the central nervous system associates with elevated K⁺ ([K⁺]ₒ) in the extracellular space. The [K⁺]ₒ is rapidly buffered, initially by the nearby astrocytes by mechanisms predominantly involving the glia-specific K⁺-sensitive α2β2 isoform combination of the Na⁺/K⁺-ATPase, which is kinetically geared to respond to increased [K⁺]ₒ and the associated membrane depolarization (Ransom et al., 2000; D'Ambrosio et al., 2002; Larsen et al., 2014; Stoica et al., 2017). A smaller fraction of the K⁺ may be removed from the extracellular space by means of the Kir4.1-mediated spatial buffering of K⁺ (Chever, Djukic, McCarthy, & Amzica, 2010; Haj-Yasein, Jensen, et al., 2011; Larsen & MacAulay, 2014; Newman, 1986), in a manner that has been suggested to require a parallel AQP4-mediated water transport into the glial cell (Jin et al., 2013; Nagelhus et al., 1999; Nagelhus & Ottersen, 2013). With this isoform-specific AQP4 inhibitor, we have in the present study, by use of ion-sensitive microelectrodes, documented that the dynamics of hippocampal stimulus-evoked [K⁺]ₒ transients are unperturbed by blockage of AQP4. Taken together with biophysical considerations of ionic driving forces and earlier reports obtained with AQP4⁻/⁻ mice (Haj-Yasein et al., 2015; Haj-Yasein et al., 2012; Strohschein et al., 2011), we here cement the notion that AQP4-mediated osmotic water permeability of the perisynaptic astrocytic membrane does not influence the rate of K⁺ removal from the extracellular space following neuronal activity.
The activity-evoked K⁺ transient is paralleled by shrinkage of the extracellular space, which is mostly assigned to astrocytic swelling (Dietzel et al., 1980; Connors et al., 1982; Ransom et al., 1985). The astrocytic AQP4 has been assumed to underlie glial volume changes (Nagelhus et al., 1999; Nagelhus & Ottersen, 2013), but was not required for activity-evoked extracellular shrinkage in AQP4⁻/⁻ mice (Haj-Yasein et al., 2012) or for glia cell swelling during cortical spreading depression (Rakers, Schmid, & Petzold, 2017; Rosic et al., 2019). Here, activity-evoked extracellular space shrinkage (quantified by TMA⁺-sensitive microelectrodes) was unperturbed by the presence of TGN-020, illustrating that AQP4-mediated osmotic water permeability was not required for the astrocytic swelling promoting (at least part of) the extracellular space shrinkage. As an additional manner of documenting that conventional osmotic water transport did not underlie the astrocytic cell swelling, activity-evoked glycogen breakdown was inhibited in order to prevent formation of osmotically active glucose-6-phosphate (Wender et al., 2000; Choi et al., 2012; Brown & Ransom, 2015). Such inhibition of glycogen phosphorylase did not affect the stimulus-evoked ECS shrinkage in hippocampal slices. Our results thus suggest that stimulus-induced ECS shrinkage in brain slice experiments does not occur via conventional AQP4-mediated osmotically-driven cell swelling following build-up of osmotic particles, such as those originating from activity-evoked glycogen break-down.

The extracellular space shrinkage had for decades been presumed to occur as a function of the astrocytic clearance of [K⁺]₀ or glutamate (Kofuji & Newman, 2004; Nagelhus et al., 2004; MacAulay & Zeuthen, 2012). However, inhibition of either of the astrocytic K⁺ and glutamate-transporting proteins; Kir4.1, NKCC1, KCC, and glutamate transporters failed to prevent the ECS shrinkage (Haj-Yasein et al., 2011; Larsen et al., 2014; Larsen & MacAulay, 2017). We recently demonstrated the pH-regulating cotransporters of bicarbonate and lactate (NBCe1 and MCTs), presumably via their ability to cotransport water (MacAulay & Zeuthen, 2010), as molecular mechanisms underlying parts of the stimulus-evoked extracellular space shrinkage, and by extension, astrocytic cell swelling (Florence et al., 2012; Larsen & MacAulay, 2017). These transporters get activated not by the K⁺ transient itself, but by the membrane depolarization associated with it, as well as by the activity-evoked alkaline transient (Theparambil et al., 2014; Theparambil & Deitmer, 2015; Theparambil et al., 2015) and the K⁺-induced elevation of cellular metabolism (Wender et al., 2000; Choi et al., 2012; Brown & Ransom, 2015; Waitt et al., 2017). While this cotransporter-mediated astrocytic cell swelling accounts for approximately half of the
volume transients, the remaining half remains unresolved, but as here demonstrated, does not occur via AQP4-mediated osmotically-driven water flux.

In conclusion, we here present TGN-020 as an efficient and isoform-specific inhibitor of AQP4-mediated osmotic water permeability. With this tool, we illustrate that AQP4 is not required for the dynamics of the stimulus-evoked K⁺ transient in rat hippocampus, nor for the associated astrocytic cell swelling.

**Competing interests**
The authors declare that there are no competing interests in this work.

**Author contributions**
All experiments were conducted at The University of Copenhagen, Denmark, in the laboratories of N.M. (Department of Neuroscience) and H.S.W. (Department of Drug Design and Pharmacology). Dynamic simulations were run at the University of Southern Denmark (Department of Physics, Chemistry and Pharmacy) by H.K. N.M., T.L.T.B., B.R.L., S.K.C., H.K. and H.S.W conceived and designed the study. T.L.T.B., B.R.L., H.S.W and S.K.C. performed the experiments. T.L.T.B., B.R.L., S.K.C., and H.K. analyzed the data. All authors contributed to the writing of the manuscript and are accountable for all aspects of the work and all persons designated as authors qualify for the authorship, and all those who qualify for authorship are listed. All authors have read and approved the final version of the manuscript.

**Data availability**
We confirm that all relevant data from this study are available from the first author upon request.

**References**


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**Figure legends**

**Figure 1. TGN-020 directly blocks different species of AQP4.** (A) Schematic of the volumetric setup used to determine the water permeability of the AQP-expressing oocytes. (B) Representative volume traces of uninjected (top) or rAQP4-expressing (bottom) oocytes without (left) or with (right) pretreatment with 20 µM TGN-020 for 60 min prior to recordings. A hyposmotic challenge (-50 mOsm) was abruptly introduced where marked with the black bar. (C) Summarized water permeabilities of oocytes expressing AQP4 from different species (r, m, h) without or with TGN-020 treatment (shown in red) (n = 9 of each). (D) Oocyte membrane potentials without and with TGN-020 treatment (as above) were recorded to verify that the compound was not toxic for the oocytes (n = 9 of each). (E) Immunocytochemistry with anti-AQP4 antibodies of AQP4-expressing oocytes (without or after 60 min preincubation with TGN-020). No staining was observed in uninjected oocytes, data not shown and (Toft-Bertelsen, Krizaj, & MacAulay, 2017). (F) Profile plot of confocal images from (E), A.U.; arbitrary units. (G) Summarized membrane fluorescence of AQP4-expressing oocytes without and after treatment with TGN-020. ANOVA followed by Tukey's multiple comparison tests or Student’s t-test (G) was used as statistical test. ***: p 0.001; NS: not significant.
**Figure 2. TGN-020 is an isoform-selective AQP4 inhibitor.** (A) Volume traces from an uninjected oocyte and AQP1-9-expressing oocytes challenged with a hyposmotic gradient (-50 mOsm; indicated by black bars above the traces) without or with TGN-020 treatment (red traces). (B) Summarized water permeabilities of oocytes expressing the nine different AQPs in the absence and presence of TGN-020 (shown in red) (20 µM, 60 min, n = 9 of each). ANOVA followed by Tukey's multiple comparison tests was used as statistical test. ***: p 0.001; NS: not significant.

**Figure 3. TGN-020 exerts an acute inhibitory effect on AQP4.** (A) A representative time control experiment from an uninjected oocyte and an AQP4-expressing oocyte repeatedly challenged with a hyposmotic gradient (-50 mOsm; indicated by a black bar, t = 0 min (control), t = 5 min, and t = 10 min). (B) Summarized water permeabilities at t = 0 min (control), t = 5 min, and t = 10 min from oocytes expressing AQP4 from different species (n = 9 of each). (C) A representative volume trace from a rAQP4-expressing oocyte challenged with a hyposmotic gradient (-50 mOsm, indicated by a black bar) before (left trace) and after 20 µM of TGN-020 for 5 or 10 min (right; indicated in red). (D) Summarized water permeabilities for AQP4-expressing oocytes at t = 0 min (control) and after exposure to TGN-020 for t = 5 min and t = 10 min (n = 9 of each). (E) rAQP4-expressing oocytes exposed to different concentrations of TGN-020, data were fitted with Graphpad Prism using nonlinear regression analysis to obtain the IC$_{50}$ from the mean of the independent regression analyses (n = 7). ANOVA followed by Tukeys's multiple comparison tests was used as statistical test. ***: p 0.001; NS: not significant.

**Figure 4. Blocking of AQP4 by TGN-020 in molecular dynamics simulations.** Initial (A-B) and final (C-D) top and side view snapshot of TGN-020 docked (A-B) or bound (C-D) to an AQP4 tetramer. Water and lipids have been eliminated for clarity. In at least one of the tetramers, TGN-020 enters the pore and blocks it, while no TGN-020 permeation through the channel was detected. (E, G) A representative side (E) and top (G) view of a simulation snapshot of a single monomer of AQP4 without TGN-020 (the water pathway through the pore is shown in red). (F) and (H) present top and side views of a simulation snapshot (200 ns) of a single monomer of AQP4, where water access through the channel is blocked by TGN-020 (shown as orange sticks). Plausible amino acid residues that interact with different molecules of TGN-020 in the four monomers have been highlighted.
Figure 5. Acetazolamide and bumetanide are not inhibitors of AQP1 or AQP4. (A-B) Representative volume traces from an AQP1-expressing oocyte repeatedly challenged with a hyposmotic gradient (-50 mOsm; indicated by a black bar) without (A) and with (B) exposure to acetazolamide (Aze, 100 μM). (C) Summarized water permeabilities of AQP1- or AQP4-expressing oocytes exposed to acetazolamide (n = 9 of each). (D) Summarized water permeabilities of AQP1- or AQP4-expressing oocytes exposed to bumetanide (Bum, 1 mM, n = 12-14 of each). ANOVA followed by Tukey's multiple comparison tests was used as statistical test; NS: not significant.

Figure 6. AQP4 does not contribute to activity-evoked K⁺-clearance and associated extracellular space shrinkage in hippocampal brain slices. Electrical stimulation of the Schaffer collaterals (3 sec at 20 Hz, marked by a black bar) in rat hippocampal slices promoted the neuronal activity leading to the [K⁺]ₒ transient and associated extracellular space shrinkage monitored by ion-sensitive microelectrodes. (A). Stimulus-evoked changes in [K⁺]ₒ prior (black trace) and post (red trace) exposure to TGN-020 (20 μM, 60 min) are shown as representative traces. Summary of the stimulus-evoked peak [K⁺]ₒ (Ai) and [K⁺]ₒ decay constants (Aii; the blue lines mark the linear section used for decay constant quantification; r² ≥ 0.99 for all experiments) before and after TGN-020, n = 6 slices from 5 animals. (B). Representative traces of the stimulus-evoked changes of the extracellular space (black: control; red: in the presence of TGN-020; 20 μM, 60 min), ΔECS summarized in (Bi). Statistical significance was tested with Student’s paired t-test; NS: not significant.

Figure 7. Inhibition of glycogen phosphorylase with DAB does not affect activity-evoked ECS dynamics. A. Representative traces of stimulus-induced changes in the ECS prior (as in Fig. 6) before (control; shown in black) and after (purple) exposure to 1 mM DAB (45 min). The cyan trace represents a single experiment with 90 min exposure of DAB. Inset: Schematic illustrating a prevented breakdown of glycogen by DAB. Ai. The stimulus-evoked change in the ECS in the presence of DAB (45 min) was normalized to its control trace and summarized (111.1 ± 7.1 % of control, n = 5 slices from 4 animals). B. Primary cultures of rat astrocytes were utilized to test the efficiency of DAB to prevent glycogen breakdown. Astrocytes were incubated for 45 min with either no glucose, 10 mM glucose, or no glucose in the presence of 1 mM DAB prior to determination of the cellular glycogen content. The control cells were sampled prior to incubation (t
= 0 min). Statistical significance was tested with either Student’s paired t-test (Ai) or one-way ANOVA with Tukey’s multiple comparison post hoc tests (B). *; p < 0.05, ** p < 0.01, NS: not significant.