Astroglial endfeet exhibit distinct Ca\textsuperscript{2+} signals during hypoosmotic conditions

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Abstract
Astrocytic endfeet cover the brain surface and form a sheath around the cerebral vasculature. An emerging concept is that endfoot control blood–brain water transport and drainage of interstitial fluid and waste along paravascular pathways. Little is known about the signaling mechanisms that regulate endfoot volume and hence the width of these drainage pathways. Here, we used the genetically encoded fluorescent Ca\textsuperscript{2+} indicator GCaMP6f to study Ca\textsuperscript{2+} signaling within astrocytic somata, processes, and endfeet in response to an osmotic challenge known to induce cell swelling. Acute cortical slices were subjected to artificial cerebrospinal fluid with 20% reduction in osmolarity while GCaMP6f fluorescence was imaged with two-photon microscopy. Ca\textsuperscript{2+} signals induced by hypoosmotic conditions were observed in all astrocytic compartments except the soma. The Ca\textsuperscript{2+} response was most prominent in subpial and perivascular endfeet and included spikes with single peaks, plateau-type elevations, and rapid oscillations, the latter restricted to subpial endfeet. Genetic removal of the type 2 inositol 1,4,5-triphosphate receptor (IP3R2) severely suppressed the Ca\textsuperscript{2+} responses in endfeet but failed to affect brain water accumulation in vivo after water intoxication. Furthermore, the increase in endfoot Ca\textsuperscript{2+} spike rate during hypoosmotic conditions was attenuated in mutant mice lacking the aquaporin-4 anchoring molecule dystrophin and after blockage of transient receptor potential vanilloid 4 channels. We conclude that the characteristics and underpinning of Ca\textsuperscript{2+} responses to hypoosmotic stress differ within the astrocytic territory and that IP3R2 is essential for the Ca\textsuperscript{2+} signals only in subpial and perivascular endfeet.

KEYWORDS
AQP4, aquaporin-4, astrocytes, glia, glymphatic, interstitial fluid, TRPV4

1 | INTRODUCTION

Being encased in the rigid skull the brain is critically dependent on mechanisms for volume control, both at the cellular and organ level. Brain swelling, as seen in stroke, traumatic injury, and many other neurological conditions, can develop into a life-threatening condition with elevated intracranial pressure and compromised cerebral blood flow. Tight junctions between vascular endothelial cells are essential components of the blood–brain barrier which normally prevents fluid entering the brain from blood (Hladky & Barrand, 2014). Within the brain, astrocytes serve important roles in water and volume homeostasis (Stokum, Gerzanich, & Simard, 2016).
The discovery that glial endfoot membranes at the brain–blood and brain–cerebrospinal fluid (CSF) interfaces in rodents and humans are packed with aquaporin-4 (AQP4) water channels (Eidsta, Enger, Hans-son, Eide, & Nagelhus, 2017; Nagelhus et al., 1998; Nielsen et al., 1997) changed our view on water transport in the brain (Nagelhus & Ottersen, 2013; Papadopoulos & Verkman, 2013). However, the pathophysiological and physiological roles of AQP4 are complex and still poorly understood. Gene knockdown studies have indicated that AQP4 exacerbates cytotoxic brain edema but reduces vasogenic brain edema (Verkman, Anderson, & Papadopoulos, 2014). At the cellular level, AQP4 increases the kinetics of osmotic swelling and boosts intracellular Ca²⁺ signals that may be necessary for efficient volume regulation (Benfenati et al., 2011; Jo et al., 2015; Mola et al., 2016; Thane et al., 2011), possibly through interaction with the transient receptor potential vanilloid isoform 4 (TRPV4), a swelling-sensitive cation channel (Benfenati et al., 2011; Jo et al., 2015; Toft-Ber-telsen, Krizaj, & MacAulay, 2017).

Recently, it was shown that AQP4 water channels facilitate removal of water (Haj-Yasein et al., 2011) and solutes (Iliff et al., 2012) from the brain parenchyma. A glio-vascular — glymphatic pathway — was proposed to drain excess ISF and waste products, similarly to the lymph system in other organs and tissues. Even though several aspects of the glymphatic hypothesis are controversial (Holter et al., 2004; Male C57BL/6J wildtype (Charles River), B6Ros.Cg-Dmdmdx-3Cv/J (mdx²Cv; The Jackson Laboratory, Bar Harbor, ME; Cox, Phelps, Chapman, & Chamberlain, 1993), and Itrp2⁻/⁻ mice (Li, Zima, Sheikh, Blatter, & Chen, 2005), 10–28 weeks old, were used for the experiments. The animals were allowed ad libitum access to food and water. Experimental groups contained at least four animals. All procedures were approved by the national animal use and care committee (Norwegian Food Safety Authority).

2.1 | Animals

Male C57BL/6J wildtype (Charles River), B6Ros.Cg-Dmdmdx-3Cv/J (mdx²Cv; The Jackson Laboratory, Bar Harbor, ME; Cox, Phelps, Chapman, & Chamberlain, 1993), and Itrp2⁻/⁻ mice (Li, Zima, Sheikh, Blatter, & Chen, 2005), 10–28 weeks old, were used for the experiments. The animals were allowed ad libitum access to food and water. Experimental groups contained at least four animals. All procedures were approved by the national animal use and care committee (Norwegian Food Safety Authority).

2.2 | Plasmid constructs and virus transduction

The pAAV-GFAP-GCaMP6f was constructed as described before (Enger et al., 2015; Tang et al., 2009). For virus infections, the mice were anesthetized with a mixture of zolazepam (188 mg/kg), tiletamine (188 mg/kg), xylazine (4.5 mg/kg), and fentanyl (26 μg/kg) before viruses were stereotactically injected. The injections were done in somatosensory cortex bilaterally at the following stereotactic coordinates relative to Bregma: anteroposterior −0.8 mm, lateral 2.0 mm. During each injection, 0.28–0.35 μL of purified rAAV was delivered at 0.5 mm depth relative to the cortical surface.

2.3 | Slice preparation

Acute cortical slices were prepared from adult mice 2–6 weeks after virus transduction. Mice were euthanized with isoflurane (Baxter). The brains were removed and submerged into gassed (95% O₂ and 5% CO₂) ice-cold cutting solution containing (in mM): 124 NaCl, 2 KCl, 1.25 KH₂PO₄, 2 MgSO₄, 1 CaCl₂, 26 NaHCO₃, and 12 glucose. Coronal cortical slices of 350μm thickness were cut with a vibriscaler (Leica VT1200). In the resting chamber and in the baseline period of the experiments, slices were exposed to isosmotic artificial CSF (aCSF) containing (in mM): 124 NaCl, 2 KCl, 1.25 KH₂PO₄, 2 MgSO₄, 2 CaCl₂, 26 NaHCO₃, and 12 glucose (pH 7.3). The temperature in the resting chamber and during recordings was kept at 31°C.

2.4 | Two-photon Ca²⁺ imaging

Slices were let to rest for 1 hr before they were transferred to a recording chamber (2 mL) where they were held in place by a horseshoe-shaped platinum wire and continuously perfused with aCSF. The GCaMP6f fluorescence was imaged by a two-photon laser scanning microscope (model Ultima; Prairie Technologies) with a 25x, 1.05 numerical aperture, water-immersion objective (XPLN 25×WMP, Olympus), using 900 nm laser pulses for excitation. The laser was used either a Chameleon Vision II (Coherent) or a Mai Tai DeepSee (Spectra Physics). Depending on the expression level of the fluorescent indicator and the tissue depth at which imaging was performed, the laser power applied ranged from 5 to 25 mW (measured by LaserCheck, Coherent). Images were collected from cortical layers I–III at least 40 μm below the section surface. After imaging in normal aCSF, the perfusion solution was switched to hypoosmotic aCSF, in which the concentration of NaCl was reduced from 124 to 93 mM, thus reducing the osmolality by 20% (verified by freezing-point depression). In a subset of experiments (data in Supporting Information) the NaCl was reduced to give 10 or 5% reduction in aCSF osmolality. Time series were taken with frame rates ranging from 1 to 3 Hz. In some experiments, 1 μM of the TRPV4 channel antagonist HC067047 (2-methyl-1-[3-(4-morpholinyl) propyl]-5-phenyl-N-[3-(trifluoromethy1)phenyl]-1H-pyrole-3-carboxamide; Tocris Bioscience, stock solution 10 mM in DMSO), was added to both the normosmotic and the −20% hypoosmotic aCSF.
2.5 | Image analysis

Time series of fluorescence images were imported to Fiji ImageJ (Fiji). Regions of interest (ROIs) were selected manually based on cell morphology from ΔF/F images and SD images (see Section 2.7). Fluorescence was measured in four different astrocytic compartments: somata, fine processes, perivascular endfeet, and subpial endfeet. ROIs over the fine processes were sampled at least 5 μm, and maximum 30 μm, away from the soma. Larger branches were avoided. For perivascular endfeet, the minimum distance between ROIs was set to 10 μm to avoid that the same endfoot was measured in two different ROIs. The distinction between different subpial endfeet was based on morphology.

2.6 | Induction of brain edema and measurement of brain water content by the wet/dry weight method

Wildtype and Itpr2−/− mice received buprenorphine (2 mg/kg) prior to induction of brain edema/decapitation. Brain edema was induced by intraperitoneal injection of distilled water (10% of body weight) under brief isoflurane anesthesia (2−3%). A subset of mice received intraperitoneal injection of isotonic saline (10% of body weight). The mice were allowed to wake up in separate cages. Forty minutes after water injection the mice were sacrificed by cervical dislocation. The brain was taken out, weighed and put in vacuum oven (80°C) overnight. After 24 hr, the dried brain was weight. The brain water content in percent was calculated as (wet mass − dry weight) × 100/(wet weight). Baseline control mice received only buprenorphine before the brain was taken out and processed as described above.

2.7 | Statistics

Mean fluorescence was extracted from each ROI and signals were normalized in consecutive 4-min bins by the following formula: ΔF/F0 = (fluorescence − median fluorescence)/median fluorescence. We used spike frequency, spike amplitude, and area under curve (AUC) as measures of Ca2+ activity. A local maximum was defined to be a spike if the following conditions were met: (i) the local maximum was the largest local maximum in between two subsequent local minima; (ii) the peak-to-peak amplitude of the local maximum and a subsequent local minimum was at least 0.5; (iii) the local maximum was above a threshold of ΔF/F = 0.5. Conditions (i) and (ii) ensured that multiple spikes were possible above the threshold of ΔF/F = 0.5, and condition (iii) ensured that a local maximum below ΔF/F = 0.5 was not defined as spike even though it fulfilled conditions (i) and (ii). The data were divided into two bins of 4 min: one describing baseline activity and the other describing the activity when the slices were bathed in hypotonic solution. Mean AUC and mean spike rate for all experiments were plotted as a function of time (Figure 1d). The spike rate was produced by convolving the spike trains with a normalized Gaussian function of width 10 s. The mean AUC was smoothed by convolving with the same function. Based on these graphs and measured time to fill the recording chamber with hypoosmotic solution (less than 1 min 30 s), the experimental bin was set to 5.5−9.5 min. Generalized linear mixed effect models with nested random effects were used to account for this hierarchical structure in the statistical analysis: ROIs were grouped within brain slices, and the brain slices were grouped within mice. For spike frequency, we assumed an underlying Poisson distribution, AUC was assumed to be normally distributed and spike amplitude to be gamma distributed. The statistical analyses were performed in MATLAB (Version R2014a for Mac OSX), by using the fitlme() function for AUC and fitglm() function for spike frequency and amplitude. For all analysis, we used the nested random effects denoted (1|MouseID) + (1|MouseID:SliceID) + (1|MouseID:SliceID:ROIID) in MATLAB’s Wilkinson notation. Here, MouseID is the unique mouse identifier, SliceID is the unique slice identifier, and ROIID is the unique ROI identifier. In Figure 1, the fixed effects were given by two predictors and their interaction term: the categorical sites (soma, processes, perivascular endfeet, and subpial endfeet) and the stimulus (zero or one corresponding to the baseline and experimental bin, respectively). In Figures 2 and 3, the fixed effects were given by three predictors for spikes and AUC: genotype (or channel blocking), sites, and stimulus; with all interaction effects. Spike amplitudes were collected from the experimental bin only, and the genotype and sites were used as predictors. In Figure 4, we used linear regression. Matlab’s fitlme() function was applied with genotype and state (control or hypoosmotic) as predictors. Unless stated otherwise, mean and SEM are shown in the text and figures.

3 | RESULTS

3.1 | Reduction of aCSF osmolarity triggers prominent Ca2+ responses in astrocytic processes and endfeet in wildtype mice

Acute cortical slices prepared from rAAV-GFAP-GCaMP6f transduced mice 2–6 weeks after virus injection were studied with two-photon microscopy under normosmotic and hypoosmotic conditions (Figure 1a). Our strategy to use the human GFAP promoter to drive sensor expression was successful, as GCaMP6f fluorescence was restricted to astrocytes in the region studied (upper half of the cortex; Enger et al., 2015). Ca2+ signals were assessed in manually drawn ROIs, which included astrocytic somata, fine processes, perivascular endfeet, and subpial endfeet (Figure 1b). During baseline conditions (aCSF of 300 mOsm/L, 4 min recording) localized, brief elevations in GCaMP6f fluorescence were observed in all astrocytic compartments. Within 90 s after hypotonic (240 mOsm/L, i.e., 20% reduction from baseline) aCSF was perfused into the slice chamber—a lag that corresponds to the time it takes to fill an empty chamber—the frequency of Ca2+ transients increased in all astrocytic compartments except the soma (Figure 1b,c). In the fine processes, the majority of osmotically evoked Ca2+ signals had a single peak shape, whereas endfeet also displayed plateau-type Ca2+ elevations and rapid Ca2+ oscillations with 3–30 peaks per event (Figure 1b). The latter were confined to subpial endfeet in which they occurred in 18 of 57 (32%) of the Ca2+...
traces. Oscillatory Ca\textsuperscript{2+} responses in subpial endfeet typically displayed ~10 peaks per min and lasted 1–3 min. Such oscillations were not observed at baseline conditions. Also in fine processes and perivascular endfeet did we observe responses with several or multiple peaks, but these peaks were less distinct and occurred at lower frequency (typically ~5 peaks per min).
To reveal the kinetics of the Ca^{2+} responses, we plotted spike rate and AUC as a function of elapsed time (Figure 1d). Based on these plots and the chamber perfusion time, we defined the bin from 5.5 to 9.5 min (i.e., from 1.5 to 5.5 min after the switch to hypoosmotic aCSF) as the response bin to be compared with the baseline bin (each bin lasting 4 min). The plots revealed that the Ca^{2+} responses to hypoosmotic stress lasted only a few minutes and were followed by Ca^{2+} signaling patterns resembling those at baseline conditions. Comparison of spike rate in the two bins confirmed that the −20% hypoosmotic challenge augmented Ca^{2+} signaling in all astrocytic compartments except the soma (Figure 1e; spike rate: somata \( p = .12 \), fine processes \( p < .001 \), perivascular endfeet \( p < .001 \), subpial endfeet \( p < .001 \)). The increase in spike rate was higher in both types of endfeet than in fine astrocytic processes (\( p < .001 \) for both comparisons). The amplitudes of the Ca^{2+} spikes were similar in normosmotic and hypoosmotic conditions in all compartments except perivascular endfeet, in which the amplitude increased during osmotic swelling (Figure 1f; somata \( p = .71 \), fine processes \( p = .86 \), perivascular endfeet \( p = .017 \), subpial endfeet \( p = .13 \)). Analysis of the time integral (AUC) of the Ca^{2+} transients showed that all compartments except the soma (Figure 1e; AUC: somata \( p = .12 \), fine processes \( p < .001 \), perivascular endfeet \( p < .001 \), subpial endfeet \( p < .001 \)). The increase in AUC was higher in both types of endfeet than in fine astrocytic processes (\( p < .001 \) for both comparisons). The amplitudes of the Ca^{2+} spikes were similar in normosmotic and hypoosmotic conditions in all compartments except perivascular endfeet, in which the amplitude increased during osmotic swelling (Figure 1f; somata \( p = .71 \), fine processes \( p = .86 \), perivascular endfeet \( p = .017 \), subpial endfeet \( p = .13 \)). Analysis of the time integral (AUC) of the Ca^{2+} transients showed that all compartments except the soma

![Figure 1](https://example.com/figure1.png)

**Figure 1** Exposure of acute cortical slices from rAAV-GFAP-GCaMP6f transduced adult wildtype mice to hypoosmotic aCSF elicits diverse Ca^{2+} signaling responses within the astrocytic territory. (a) Experimental setup. (b) Two-photon GCaMP6f fluorescence images from wildtype mice during baseline (300 mOsm/L) and experimental (240 mOsm/L) conditions. Upper and lower panels display the cortical surface/cortical layer I and cortical layer II, respectively. SD images for the entire imaging session are shown to the right. Normalized traces of GCaMP6f fluorescence are from ROIs indicated in the images. Dashed vertical line indicates when 240 mOsm/L aCSF was perfused into the slice chamber. Ca^{2+} spikes as indicated with red dots were automatically detected with custom-made software. (c) Merged GCaMP6f fluorescence traces from all experiments with Ca^{2+} spikes indicated for four different astrocytic compartments: somata, fine processes, perivascular endfeet, and subpial endfeet. (d) Time series plots of Ca^{2+} spike rate and area under curve (AUC) for the four astrocytic compartments. A 4-min period starting 90 s after application of 240 mOsm/L aCSF (dashed line) was defined as the experimental bin (gray background color). (e) Histogram showing spike rate for astrocytic somata (bars as in b). (f) Histogram showing amplitude of Ca^{2+} spikes during hypoosmotic conditions in all compartments except perivascular endfeet, in which the amplitude increased during osmotic swelling (Figure 1f; somata \( p = .71 \), fine processes \( p = .86 \), perivascular endfeet \( p = .017 \), subpial endfeet \( p = .13 \)). Analysis of the time integral (AUC) of the Ca^{2+} transients showed that all compartments except the soma

![Figure 2](https://example.com/figure2.png)

**Figure 2** Exposure of acute cortical slices from Itpr2^{−/−} mice to hypoosmotic aCSF reveals different underpinning of swelling-induced Ca^{2+} signals within the astrocytic territory. (a) As in Figure 1c, but traces are from Itpr2^{−/−} mice and somatic Ca^{2+} signals are not shown. (b) Histogram showing relative change in spike rate (i.e., the ratio between spike rate in experimental and baseline conditions) for wildtype (white bars) and Itpr2^{−/−} (gray bars) mice. Astrocytic compartments are denoted as in Figure 1e. (c) Histogram showing amplitude of Ca^{2+} spikes during hypoosmotic conditions in Itpr2^{−/−} and wildtype mice (bars as in b). (d) Histogram showing change in AUC (difference between hypoosmotic and baseline AUC) for Itpr2^{−/−} and wildtype mice (bars as in b). Brackets and \( p \)-values indicate comparisons (relative change in spike rate; spike amplitude; AUC). Number of traces, slices, and animals for each compartment were; soma: 46, 18, and 6, respectively; fine processes: 123, 18, and 6; perivascular endfeet: 52, 14, and 6; subpial endfeet: 71, 15, and 6 (same numbers for all parameters). Number of amplitudes in the hypoosmotic bin; fine processes: 246; perivascular endfeet: 8; subpial endfeet: 22 [Color figure can be viewed at wileyonlinelibrary.com]
responded to hypoosmotic conditions with increased Ca²⁺ signaling (Figure 1g, AUC: somata $p = .38$, fine processes $p < .001$, perivascular endfeet $p < .001$, subpial endfeet $p < .001$) and that the increase was higher in subpial and perivascular endfeet than in fine processes ($p < .001$ for both comparisons, not displayed).

When acute brain slices were exposed to aCSF with only 10 or 5% reduction in osmolarity Ca²⁺ signaling was unaffected in all four astrocytic compartments (Figure S1).

### 3.2 Osmotically induced Ca²⁺ signals in subpial and perivascular astrocytic endfeet rely on IP3R2-mediated Ca²⁺ release from the endoplasmic reticulum

Next, we assessed the origin of the osmotically evoked Ca²⁺ signals in astrocytic processes and endfeet. Itpr2⁻/⁻ mice, which lack the IP3R2 Ca²⁺ release channel in endoplasmic reticulum (Li et al., 2005), were subjected to the same experimental protocol as wildtype mice (Figure 2a). In Itpr2⁻/⁻ mice, Ca²⁺ signals within fine processes increased during hypoosmotic aCSF ($p < .001$ for comparison with baseline for spike rate) and the signaling response was comparable to that of wildtypes (Figure 2b, $p = .46$ and $p = .54$ for comparison for relative change in spike rate and change in AUC, respectively, $p = .007$ for Ca²⁺ spike amplitude during hypoosmotic stress, which was modestly reduced in mutants). In contrast, perivascular and subpial endfeet of Itpr2⁻/⁻ mice showed severely suppressed Ca²⁺ signaling during hypoosmotic stress (Figure 2a), indicating that Ca²⁺ release from the endoplasmic reticulum is essential for the endfoot Ca²⁺ response.

Note that subpial endfeet of Itpr2⁻/⁻ mice showed a much lower Ca²⁺ spike rate than wildtypes both during hypoosmotic and baseline conditions ($p < .001$ for both conditions, not displayed) and that relative change in spike rate failed to differ between genotypes ($p = .19$, $p = .007$ for Ca²⁺ spike amplitude during hypoosmotic stress, which was modestly reduced in mutants). In contrast, perivascular and subpial endfeet of Itpr2⁻/⁻ mice showed severely suppressed Ca²⁺ signaling during hypoosmotic stress (Figure 2a), indicating that Ca²⁺ release from the endoplasmic reticulum is essential for the endfoot Ca²⁺ response.
and after TRPV4 blockage endfeet are attenuated in dystrophin deficient mice (Figure 2b). However, the change in AUC differed clearly between Itpr2−/− and wildtype mice for both subpial (p < .001) and perivascular (p < .001) endfeet (Figure 2d). Itpr2 deletion also reduced the amplitude of the Ca2+ spikes during hypoosmotic conditions in perivascular endfeet, but not in subpial endfeet (p = .029 and p = .24, respectively, for comparison with wildtype, Figure 2c). Notably, the hypoosmotically induced rapid Ca2+ oscillations in subpial endfeet were absent in Itpr2−/− mice.

3.3 | Osmotically induced Ca2+ signals in astrocytic endfeet are attenuated in dystrophin deficient mice and after TRPV4 blockage

Since AQP4 water channels modulate swelling kinetics and swelling-induced Ca2+ signals (Thrane et al., 2011), we assessed the osmotic GCaMP6f fluorescence response in astrocytic endfeet of mdx2CyC mice (Figure 3a), which lack the endfoot AQP4 anchor dystrophin. In mdx2CyC mutants, the relative Ca2+ spike rate increase was attenuated versus wildtypes in both types of endfeet (Figure 3b, p = .018 and p = .011 for perivascular and subpial endfoot, respectively). For change in AUC only subpial differed between genotypes for (p = .021, Figure 3b). The amplitude of the Ca2+ spikes occurring during hypoosmotic stress did not differ between mdx2CyC and wildtype mice (Figure 3b). While dystrophin removal overall only moderately attenuated the osmotic Ca2+ responses in endfeet, it severely reduced the occurrence of rapid Ca2+ oscillations in subpial endfeet (2 of 45 traces in mutants, i.e., 4 vs. 32% in wildtypes).

Next, we assessed whether TRPV4 channels, which are expressed in astrocytic endfeet and possibly interact with AQP4 (see Section 1), are implicated in the endfoot Ca2+ response to hypoosmotic aCSF. Surprisingly, exposure of wildtype slices to the TRPV4 antagonist HC067047 (1 μM) increased the Ca2+ spike rate in both perivascular and subpial endfeet at baseline, normosomatic conditions (spike rate per min was 0.407 ± 0.1048 vs. 0.0805 ± 0.0281 without antagonist, p = .008, for perivascular endfeet and 0.3723 ± 0.1380 vs. 0.1140 ± 0.0391, p = .01, for subpial endfeet). Blockage of TRPV4 did not prevent the osmotically induced Ca2+ signaling in any of the two endfoot compartments, but the relative spike rate increase was smaller in wildtypes (Figure 3f, p < .001 both for perivascular and subpial endfeet; spike rate per min for the two types of endfeet was 0.677 ± 0.1387 and 0.967 ± 0.2231, respectively). However, neither the amplitude nor change in AUC differed from that of wildtypes (Figure 3g,h). In mice treated with TRPV4 antagonist, rapid Ca2+ oscillations in subpial endfeet were sparse (observed in only 4 of 45 Ca2+ traces, i.e., 9%) during hypoosmotic conditions. Intriguingly, after TRPV4 blockade we observed rapid Ca2+ oscillations in subpial endfeet also during normosmatic conditions (1 of 45 traces) and in perivascular endfoot during hypoosmotic conditions (2 of 51 of traces).

3.4 | Impact of IP3R2-mediated Ca2+ release on brain water accumulation during hypoosmotic stress

To investigate whether IP3R2-mediated Ca2+ signals regulate blood–brain water transport in vivo Itpr2−/− and wildtype mice were subjected to water intoxication, a well-established cytotoxic brain edema model (Manley et al., 2000). The mice were shortly anesthetized with isoflurane during intraperitoneal water injection (10% of body weight) but thereafter kept awake since anesthesia suppresses astrocytic Ca2+ signaling (Thrane et al., 2012). In both genotypes, the brain water content measured by the wet/dry weight method was higher in water-injected than in noninjected mice (p < .001 for both wildtypes and Itpr2−/−), but Itpr2 deletion failed to affect the increase in water content (Figure 4; p = .15). Intraperitoneal injection of isotonic saline (10% of body weight) did not increase brain water content in any of the two genotypes (values for noninjected and saline-injected mice were 78.0% ± 0.08% and 78.0% ± 0.20%, p = .76 for comparison, for wildtype mice, and 77.9% ± 0.05% and 77.8% ± 0.08%, p = .67, for Itpr2−/− mice).

4 | DISCUSSION

In this study, we characterized astrocytic Ca2+ signaling in acute cortical slices exposed to aCSF with 20% reduction in osmolality. We demonstrated that the osmotic challenge elicited Ca2+ signals that differed in frequency, underpinning, and shape within the astrocytic territory. A striking finding was that astrocytic endfeet under the pia mater and around blood vessels showed the most pronounced Ca2+ response to hypoosmotic stress, both with respect to increase in relative spike rate and AUC. One could argue that the difference in Ca2+ response between astrocytic regions reflected unequal osmotic challenge between the superficial and deep brain tissue since the hypoosmotic aCSF was perfused into the slice chamber. However, time plots of Ca2+ spike rate and AUC of the various astrocytic profiles did not reveal apparent differences in the timing of the responses, and perivascular endfeet were sampled also from deep within the tissue. Thus, we find it more likely that the diversity of Ca2+ responses within the astrocytic territory relies on molecular and functional specialization of astrocytic microdomains.
The distinct Ca\textsuperscript{2+} responses of endfeet could rely on faster swelling due to their high density of AQP4 water channels. Notably, AQP4 is reported to speed up swelling and augment intracellular Ca\textsuperscript{2+} signaling during hypoosmotic conditions (Benfenati et al., 2011; Mola et al., 2016; Thrane et al., 2011). A role of AQP4 was supported by the finding that mdx\textsuperscript{Cv} mice, which have 65% less AQP4 in perivascular endfoot membranes (Enger et al., 2012), exhibited attenuated endfoot Ca\textsuperscript{2+} responses. However, other mechanisms might contribute to this finding. Specifically, dystrophin could anchor other membrane proteins involved in volume regulation or transmit mechanical forces to endfoot osmosensors, yet uncharacterized.

The osmotically induced Ca\textsuperscript{2+} signals in perivascular and subpial endfeet were severely suppressed in IP3R2-deficient mice indicating that the responses in these domains depend on Ca\textsuperscript{2+} release from the endoplasmic reticulum. Despite this fact, Ca\textsuperscript{2+} influx across the plasma membrane could also contribute. Notably, TRPV4 nonselective cation channels expressed in glial endfeet (Benfenati et al., 2007; Dunn, Hill-Eubanks, Liedtke, & Nelson, 2013) were found to mediate Ca\textsuperscript{2+} signaling responses to hypotonic stimuli (Benfenati et al., 2011; Jo et al., 2015; Ryskamp et al., 2014). While it was proposed that TRPV4-AQP4 interactions "turbocharge" astroglial sensitivity to small osmotic gradients (Iuso & Krizaj, 2016), a recent study concluded that TRPV4 reacted to volume changes rather than osmotic changes (Toft-Bertelsen et al., 2017). Application of a TRPV4 antagonist to our acute brain slices surprisingly increased astrocytic Ca\textsuperscript{2+} signaling in baseline, normosmotic conditions, which complicates the assessment of TRPV4's role in hypoosmotically induced Ca\textsuperscript{2+} signals. However, TRPV4 channels were not essential for the endfoot Ca\textsuperscript{2+} responses, since TRPV4 blockage only attenuated the increase in Ca\textsuperscript{2+} spike rate and failed to affect the increase in AUC.

The osmotic Ca\textsuperscript{2+} response within fine astrocytic processes was insensitive to Itpr2 deletion, in contrast to the situation in endfeet. This finding was not surprising since the most delicate astrocytic processes are too thin to accommodate endoplasmic reticulum (Patrushev, Gavrilov, Turlapov, & Semyanov, 2013). Thus, it is likely that the intracellular Ca\textsuperscript{2+} increase in fine processes during hypoosmotic stress relies on Ca\textsuperscript{2+} entry across the plasma membrane. In larger astrocytic processes Ca\textsuperscript{2+} release from mitochondria, known to occur during metabolic activity (Agarwal et al., 2017), could also explain the increase in Ca\textsuperscript{2+} signaling since volume regulation consumes energy (Olson, Sankar, Holtzman, James, & Fleischhacker, 1986).

Hypoosmotically induced Ca\textsuperscript{2+} signals within the astrocytic territory also differed in shape. Whereas the Ca\textsuperscript{2+} transients in fine processes typically had a single peak, endfoot Ca\textsuperscript{2+} responses also included plateau-type Ca\textsuperscript{2+} elevations and rapid Ca\textsuperscript{2+} oscillations. The latter were confined to subpial endfeet and there occurred in one third of the profiles. The rapid Ca\textsuperscript{2+} oscillations in subpial endfeet were critically dependent on IP3R2 receptors but also showed some dependency on TRPV4 and dystrophin. Thus, the unique oscillatory Ca\textsuperscript{2+} signaling pattern in subpial endfeet—which to our knowledge has never been described before—is likely to be triggered by rapid water entry through AQP4 channels, swelling-induced Ca\textsuperscript{2+} influx from the extracellular space via TRPV4 channels and Ca\textsuperscript{2+} induced Ca\textsuperscript{2+} release from the endoplasmic reticulum mediated by IP3R2 receptors.

Surprisingly, the astrocytic cell bodies did not respond to the hypoosmotic challenge in the present experiments. In a previous study using bulk loading of the synthetic Ca\textsuperscript{2+} dye Rhod2 AM, we reported that astrocytic cell bodies responded to osmotic swelling with Ca\textsuperscript{2+} spikes both in vitro and in vivo (Thrane et al., 2011). However, in that study acute cortical slices were obtained from mouse pups and the experiments performed at room temperature. It is possible that the somatic Ca\textsuperscript{2+} response in the immature animals relies on autocrine signaling pathways that are developmentally regulated. Furthermore, the in vivo experiments on adult mice revealed that the osmotically induced Ca\textsuperscript{2+} responses in astrocytic cell bodies occurred in the late phase of brain swelling, that is, ~30–45 min after intraperitoneal injection of water (Thrane et al., 2011). Furthermore, Ca\textsuperscript{2+} spikes occurring during advanced edema could rely on ischemia, which is known to elicit astrocytic Ca\textsuperscript{2+} signals (Duffy & Macvicar, 1996).

Brain water entry and exit must occur across subpial and perivascular endfeet which form the outermost layers of neural tissue and are positioned next to the fluid compartments draining the brain. Do endfoot Ca\textsuperscript{2+} signals regulate brain water transport across the blood–brain and brain–CSF interfaces? During acute hypoosmotic conditions the blood–brain water entry is facilitated by endfoot AQP4 water channels (Amiry-Moghaddam et al., 2004; Haj-Yasein et al., 2011; Manley et al., 2000), which most likely are not regulated by channel gating (Assentoft, Larsen, & MacAulay, 2015). However, astrocytic Ca\textsuperscript{2+} signals have been implicated in export of osmolytes and water during cell volume regulation (Hoffmann et al., 2009). Given that hypoosmotically induced Ca\textsuperscript{2+} signals in endfeet rely on IP3R2 receptors we tested whether Itpr2−/− and wildtype mice differed in brain water accumulation during water intoxication, an in vivo model of brain edema in which mice are injected with distilled water intraperitoneally. To minimize animal discomfort, we used an osmotic challenge that was lower (~10% reduction in plasma osmolarity) than in our in vitro model (~20% reduction in perfused aCSF osmolarity). We found that IP3R2-deficient mice showed similar increase in brain water content as wildtype animals. Additional in vitro experiments revealed that aCSF with only 10% reduction in osmolarity failed to elicit astrocytic Ca\textsuperscript{2+} signals. Thus, it seems that osmotic changes must be of a certain magnitude or occur sufficiently fast to elicit astrocytic Ca\textsuperscript{2+} responses. It is likely that our in vivo brain edema model was associated with osmolarity changes that did not meet these criteria.

The osmolarity reduction within the slice that triggered astrocytic Ca\textsuperscript{2+} signals must have been substantially less than 20%, since astrocytes responded already when an equal amount of ~20% hypoosmotic aCSF and normosmotic aCSF had been mixed in the slice chamber (see Section 3). The precise kinetics of osmolarity changes within the slice under our experimental conditions is hard to estimate, also due to the onset of cell volume regulatory mechanisms with release of osmolytes and the fact that the extracellular space comprises of tunnels and sheets of different size (Kinney et al., 2013). Whether the osmotic changes in our in vitro model mimic those in vivo remains an open question.
Accumulating evidence suggests that Ca\(^{2+}\) signals play a role in glial cell volume regulation (Benfenati et al., 2011; Jo et al., 2015). Inadvertent swelling of astrocytic endfeet must be detrimental for glycophytic function, as it would shrink both gaps between endfeet and the paravascular compartment, both of which are postulated drainage routes (Iliff et al., 2012). At the same time, dynamic volume changes of endfeet could constitute a mechanism for regulating the efficacy of interstitial waste clearance, for example, during the sleep–awake cycle. Proper volume control of astrocytic endfeet is also necessary for normal cerebral perfusion. Endfoot swelling may compress capillaries or have indirect effects on vascular tone by releasing vasocoactive molecules.

Astrocitic Ca\(^{2+}\) signals increase not only during osmotic swelling but also following electrical stimulation and application of neurotransmitters (Haustein et al., 2014; Tang et al., 2015). While a plethora of transporters and channels as well as G-protein coupled receptors are considered important for activity-dependent Ca\(^{2+}\) signals (Shigetomi, Patel, & Khakh, 2016), few investigators have addressed the possibility that swelling could trigger the response. The large surface area to volume ratio of the delicate astrocytic processes in neuropil (Hama, Arii, Katayama, Marton, & Ellisman, 2004) should make them particularly prone to swelling during glutamate uptake, which imposes an osmotic load and cellular water entry. It is tempting to speculate that also spontaneous microdomain Ca\(^{2+}\) signals, which are largely IP3R2-independent (Srinivasan et al., 2015), reflect signaling during astrocyte volume adjustment.

In conclusion, our study has demonstrated that astrocytic endfeet display osmotically induced Ca\(^{2+}\) signals that differ quantitatively, qualitatively, and mechanistically from those in other astrocytic microdomains. The endfoot Ca\(^{2+}\) signals—including the unique rapid Ca\(^{2+}\) oscillations in subpial endfeet—should be further explored as regulators of water transport and cell volume in health and disease.

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CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

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