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Hypothermia increases aquaporin 4 (AQP4) plasma membrane abundance in human primary cortical astrocytes via a calcium/ transient receptor potential vanilloid 4 (TRPV4)- and calmodulin-mediated mechanism

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Running title: Hypothermia and AQP4 in human astrocytes

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Abstract

Human aquaporin 4 (AQP4) is the primary water channel protein in brain astrocytes.

Hypothermia is known to cause astrocyte swelling in culture, but the precise role of AQP4 in this process is unknown. Primary human cortical astrocytes were cultured under hypothermic (32°C) or normothermic (37°C) conditions. AQP4 transcript, total protein and surface localized protein were quantified using RT-qPCR, sandwich ELISA with whole cell lysates, or cell-surface biotinylation followed by ELISA analysis of the surface-localized protein, respectively. Four-hour mild hypothermic treatment increased the surface localization of AQP4 in human astrocytes to $155 \pm 4\%$ of normothermic controls, despite no change in total protein expression levels. The hypothermia-mediated increase in AQP4 surface abundance on human astrocytes was blocked using either calmodulin antagonist (trifluoperazine; TFP); TRPV4 antagonist, HC-067047 or calcium chelation using EGTA-AM. The TRPV4 agonist (GSK1016790A) mimicked the effect of hypothermia compared with untreated normothermic astrocytes. Hypothermia led to an increase in surface localization of AQP4 in human astrocytes through a mechanism likely dependent on the TRPV4 calcium channel and calmodulin activation. Understanding the effects of hypothermia on astrocytic

AQP4 cell-surface expression may help develop new treatments for brain swelling based on an in-depth mechanistic understanding of AQP4 translocation.

Introduction

Hypothermia is known to have specific effects on the blood brain barrier (BBB), including changes in blood flow, metabolism, water homeostasis and excitotoxicity, as well as signaling profiles leading to changes in apoptosis and cell survival (Yenari & Han, 2012). Hypothermia was reported to be neuroprotective in stroke patients (Yenari & Hemmen, 2010) but in traumatic brain injury (TBI), a therapeutic role for hypothermia is controversial; different clinical trials have reported both positive and negative outcomes on patient recovery (Andrews *et al.*, 2015; Lazaridis & Robertson, 2016). Cytotoxic oedema, which is a feature of stroke and TBI, occurs when water enters the CNS via astrocytes at the BBB, predominantly through the aquaporin 4 (AQP4) channel and knockout of AQP4 is protective against cytotoxic oedema development in various animal models (Verkman *et al.*, 2006). The specific effects of hypothermia on AQP4 expression in human astrocytes are not known. AQP4 expression in rat astrocytes has been reported to increase after 2h in response to transarterial (cold saline) hypothermia infusion in a middle cerebral artery occlusion (MCAO) model (Kurusu *et al.*, 2016). In a rat model of thrombin-induced vasogenic oedema, AQP4 protein levels increased after 24h, peaking at 48h (Gao *et al.*, 2015). Conversely, hypothermia-induced reduction in cytotoxic cerebral oedema, in a porcine model of cardiac arrest/resuscitation, was concomitant with a reduction in AQP4 protein levels after 24 h (Zhao *et al.*, 2012). Additionally, mild hypothermia was reported to cause a significant astrocyte volume increase to $107.3 \pm 0.4\%$ (mean \pm SEM) of control, after 30 min at 32°C (Plesnila *et al.*, 2000).

Many factors have been reported to alter AQP4 abundance and subcellular localization, including vasopressin, histamine and astrocytic glutamate (Conner *et al.*, 2013). We recently described a mechanism of short-term AQP4 relocalization during tonicity-driven cell swelling in rat astrocytes (Kitchen *et al.*, 2015). To our knowledge, hypothermia has not been previously investigated as a trigger of AQP4 relocalization.

In addition to AQP4, the stretch-activated transient receptor potential vanilloid 4 calcium channel (TRPV4) is known to be essential in cell volume regulation: a TRPV4-mediated increase in Ca^{2+} influx has been suggested to have an effect on the AQP-mediated cell volume regulation following osmotic stress and the activity and localization of TRPV4 was often dependent on aquaporin expression (Benfenati *et al.*, 2011). They proposed a TRPV4/AQP4 complex at the astrocyte membrane was an essential component of an osmosensory system that couples osmotic stress to downstream signaling pathways (Benfenati *et al.*, 2011).

This study demonstrates that mild hypothermia, as used therapeutically (32°C), can increase the localization of endogenous AQP4 to the plasma membrane of human primary cortical astrocytes and that this process can be disrupted by inhibition of TRPV4 or calmodulin and by chelation of intracellular calcium. Given the well-established permissive role of AQP4 in cytotoxic oedema development, our data may help to explain why hypothermic intervention in conditions involving cytotoxic oedema is sometimes deleterious and suggest novel hypothermic/pharmacological combination therapies for cytotoxic oedema.

Materials and methods:

Cell culture: Primary human cortical astrocytes (ScienCell) were used following the manufacturer's protocol. All cells were used at passage 4 or below. The cells were incubated in humidified 5% (v/v) CO_2 in air at 37°C or 32°C.

RT-qPCR: Total RNA was isolated using a Qiagen RNeasy plus mini kit (Qiagen), according to the manufacturer's instructions and multi-target RT-qPCR was performed using a StepOnePlus™ Real-Time PCR System (Applied Biosystems) with purified cDNA samples generated using SuperScript™ III Reverse Transcriptase (ThermoFisher Scientific) and QIAquick PCR Purification Kit (Qiagen) to investigate gene expression levels of AQP4 mRNA using Taqman primers (Thermofisher; ID: Hs00242342_m1). *PPIA* (assay ID: Hs99999904_m1) and *CDKN1B* (assay ID: Hs00153277_m1) (Applied Biosystems) were used as control housekeeping genes. Results were analyzed using the $2^{-\Delta\Delta C_t}$ method and presented as relative gene expression normalized to the average cycle threshold for the two housekeeping genes.

ELISA: AQP4 and calmodulin protein levels were measured by sandwich ELISA following the manufacturer's instructions (Abcam) and as described by (Salman *et al.*, 2017), using rabbit polyclonal anti-AQP4 antibody (Abcam, ab46182) and mouse monoclonal anti-AQP4 antibody (Abcam, ab9512) or rabbit polyclonal anti-calmodulin antibody (Abcam, ab38590) with mouse monoclonal anti-calmodulin antibody (Abcam, ab2860). The secondary antibody used in both assays was chicken anti-mouse IgG-HRP antibody (Santa Cruz, sc-2954).

Cell surface biotinylation (CSB): This was performed as described by (Kitchen *et al.*, 2015). Briefly, cell surface proteins were biotinylated using a cell-impermeable amine-reactive biotinylation reagent (EZ-Link Sulfo-NHS-SS-Biotin; Thermo Cat. No. 21331). 96-well Pierce™ NeutrAvidin™ coated plates, (Thermo Scientific; Cat. No. 15129) were used to pull down the biotinylated proteins within the total cell lysate. After washing off unbound proteins, the plate was then incubated with anti-AQP4 antibody (Abcam, ab128906) diluted 1:500 in 0.05% PBS-tween (PBS-T), which bound to the cell-surface biotinylated AQP4 proteins

attached to the avidin coated plate. Anti-AQP4 antibody validation data is provided in supplementary data Figure 1.

Cell Treatments: Hypotonic challenges were achieved by dilution of media with dH₂O to 85 mOsm/kg H₂O; Osmolality was measured using an Osmomat 3000 freezing point depression osmometer (Gonotec). Inhibitors were used at 100x published IC₅₀ values were added 30 minutes prior to initiation and maintained for the duration of each experimental setting. TRPV4 inhibitor (HC-067047) was used at a concentration of 4.8μM; while calmodulin antagonist (trifluoperazine; TFP) was used at a concentration of 127μM. The potent TRPV4 channel agonist GSK1016790A (Sigma) was used at a concentration of 2.1 μM; it was either added 30 minutes before the intervention or at the same time as the intervention. Intracellular calcium chelation was performed using EGTA AM (Invitrogen; E1219) at a concentration of 5 μM following the manufacturer's recommendation. In all cases, cells were at least 92% viable, assessed using CellTiter-Glo[®] Luminescent Cell Viability Assay.

Statistical analysis

All data are presented as a fold-change normalized to the experimental control.

RT-qPCR expression, ELISA and cell surface biotinylation data were found to be nonparametric in distribution using the Shapiro-Wilk test. A Kruskal–Wallis analysis with a Conover–Inman *post hoc* test was therefore used to identify significant differences ($p \leq 0.05$) using StatsDirect 3 software. Additionally, data have been reproduced as box-and-whisker plots and are provided collectively in supplementary Figure 2.

Results

RT-qPCR and ELISA of AQP4 following hypothermia in primary human astrocytes

There was a modest increase in AQP4 mRNA levels in cultured human primary astrocytes (measured using RT-qPCR) following 4h mild hypothermia (32°C) compared with control cells grown at 37°C (Figure 1A): the increase was $156\% \pm 16\%$; $p = 0.035$ ($n=4$). However, Figure 1B shows that this small increase in transcript abundance was not translated into a change in protein levels (measured by ELISA), which were not significantly different ($p > 0.05$).

AQP4 plasma membrane abundance using cell surface biotinylation following hypothermia in primary human astrocytes

Cell-surface AQP4 expression after 4h mild hypothermia (32°C) was compared to cells at 37°C using an AQP4 biotinylation assay (as described in our previous study (Kitchen *et al.*, 2015)). The results show that despite there being no change in total AQP4 protein levels (Figure 1B), AQP4 levels *at the cell surface* increased to $155 \pm 4\%$ ($p < 0.0001$), of control values following hypothermia (Figure 2); the modest decrease in surface localization of the control membrane protein, amino acid transporter EAAT1, indicates that the hypothermia-induced increase in AQP4 plasma membrane localization is not a global response to hypothermia associated with all membrane proteins.

Hypotonicity is known to cause cellular swelling and increase in AQP4 surface localization and was used a positive control. Figure 2 shows that hypotonicity significantly increases the AQP4 levels at the cell surface to $192 \pm 11\%$ ($p < 0.0001$) of control values with no significant difference in EAAT1 levels ($p=0.579$).

Validation of calmodulin- and TRPV4- dependent mechanism in AQP4 translocation following hypothermia in primary human astrocytes

We previously identified a regulatory mechanism for AQP4 surface localization in an *in vitro* model using rat astrocyte cell swelling in response to reduced extracellular osmolality (Kitchen *et al.*, 2015). Calmodulin was a key regulator of this process and extracellular Ca^{2+} was essential to mediate the translocation. We therefore measured the localization of AQP4 following inhibition of either calmodulin (using TFP); TRPV4 (using HC-067047) or calcium (using EGTA-AM); Figure 3 shows that all the investigated inhibitors blocked relocalization of AQP4 in human primary astrocytes following 32°C hypothermia. We have previously shown no effect of either TFP or removal of extracellular calcium on the initial (*i.e.* pre- swelling-induced relocalization) distribution of AQP4 in rat astrocytes (Kitchen *et al.*, 2015) suggesting TRPV4 and calmodulin inhibition are disrupting this hypothermic relocalization phenomenon.

The data in Figure 3 indicated that calmodulin activation is required for AQP4 relocalization. Therefore, we assessed calmodulin protein levels under the same hypothermic conditions. Our data showed that 4h mild hypothermia (32°C) significantly increased calmodulin expression to $158 \pm 5\%$ ($p < 0.0001$) compared with cells grown at 37°C ($n=4$).

The involvement of TRPV4 was investigated further using the TRPV4 agonist, GSK1016790A. Figure 4 shows that treating astrocytes with GSK1016790A causes a significant increase in AQP4 surface expression, $145 \pm 10\%$ ($p=0.0042$) compared to control levels, similar to what was observed after hypothermia. Interestingly, there was no additive effect of TRPV4 agonist and hypothermia. Agonist was added 30 minutes before hypothermia in order to follow the same experimental protocol used for the antagonists. We speculated that this 'pre-treatment' may have caused internalization of TRPV4 (which has

been reported for GSK1016790A) (Shibasaki *et al.*, 2015), leading to absence of TRPV4 at the surface for activation by hypothermia. To test this, we repeated the experiment applying the TRPV4 agonist and hypothermia simultaneously. In this case, an additive effect of TRPV4 agonism and hypothermia was seen, with AQP4 surface expression increasing to $183 \pm 4\%$ ($p=0.0003$) of control, significantly higher than either TRPV4 agonist alone ($p=0.0039$) or hypothermia alone ($p=0.025$). The same additive effect was observed following hypotonic intervention: AQP4 surface expression was increased to $189 \pm 10\%$ ($p=0.045$) with TRPV4 agonist pre-treatment, and $223 \pm 5\%$ ($p=0.0253$) with simultaneous treatment.

There were no significant changes in EAAT1 surface expression levels following the agonist/inhibitors in figures 3 and 4 (data not shown).

Discussion

Hypothermia affects many physiological mechanisms of the brain and has a widely-recognised therapeutic potential in the reduction of early stage (cytotoxic) oedema (Previch *et al.*, 2016). This oedema can form when astrocytes have reduced ion homeostasis and increased intracellular tonicity, which is followed by water influx via osmosis. This in turn is thought to activate the mechanosensitive calcium channels (mainly TRPV4) leading to an increase in calcium influx (Benfenati *et al.*, 2011) enhancing calmodulin activity, including calmodulin-mediated AQP4 surface relocalization (Verkman *et al.*, 2014; Vella *et al.*, 2015). The proposal that hypothermia influences important cellular signalling mechanisms is supported by recent work showing that hypothermia/TRPV4 mediated effects on intracellular signaling (Zou *et al.*, 2015).

To our knowledge this is the first demonstration of changes to AQP4 surface localization levels in response to hypothermic treatment of human primary cortical astrocytes. We have also demonstrated that inhibiting calmodulin (using either the antagonist, TFP or the TRPV4 inhibitor, HC-067047) or intracellular calcium (using EGTA-AM), blocks the hypothermia-mediated increase in AQP4 surface expression in human astrocytes. Interestingly, it has been shown that TRPV4 activity is inhibited by cold (temperature < 34 °C) in mouse neurons (Shibasaki *et al.*, 2015). It is also well-established that TRPV4 opens in response to cell swelling, but the calcium signalling responses are different in neurons (fast, inactivating currents) and glia (slow currents, with sustained $[Ca]^{2+}_i$ elevation) (Ryskamp *et al.*, 2014). In astrocytes, where hypothermia induces cell swelling, it is not obvious whether TRPV4 would be activated by mechanical swelling, inactivated by cold, or at intermediate activity, depending on the exact temperature and cell volume. Our data suggest that TRPV4 retains at least some activity in astrocytes at 32 °C, as the AQP4 relocalization observed in response to hypothermia is blocked by a TRPV4 antagonist and recapitulated by a TRPV4 agonist under normothermic (37 °C) conditions. The absence of any additive effect between pre-treatment with TRPV4 agonist and its level at the time of hypothermic intervention, could be due to receptor internalization; which could explain the additive effect seen when cells are exposed to the intervention and the agonist at the same time.

It is thought that AQP4 deletion/knockdown is protective and AQP4 overexpression is harmful in animal models of early brain oedema (Verkman *et al.*, 2006). We suggest that some of the deleterious effects of hypothermic intervention in oedema that have been reported (Lazaridis & Robertson, 2016) may be mediated by increased AQP4 surface expression *via* a TRPV4/calmodulin-dependent relocalization mechanism. Further work showing inhibition of human astrocytic swelling in the presence of calmodulin and/or TRPV4 inhibitors would further support the role of AQP4 in astrocytic swelling in stroke and traumatic injury.

Separating the mechanisms involved in the beneficial and damaging effects of hypothermic intervention may allow us to further refine the clinical value of hypothermia for oedema prevention following stroke or TBI. In the future, further, co treatment with putative AQP4 inhibitors targeting the sub-cellular relocalization pathway would allow exploitation of the neuroprotective effects of hypothermia whilst mitigating any harmful effects.

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Competing Interest

The authors do not have any competing interests.

Author Contributions

MMS performed all laboratory work, and initial data analysis, contributed to study design and helped draft the manuscript. MTC, ACC, RMB, MNW and PK conceived the study, participated in its design and coordination, assisted in data and statistical analysis, and co-wrote the manuscript with the help of JEB. All authors read and approved the final manuscript.

Data Accessibility

All relevant data are within the paper and its Supporting Information files were made publicly available at: DOI 10.6084/m9.figshare.5293672

Abbreviations

AQP4 aquaporin 4. ELISA (enzyme-linked immunosorbent assay). TBI Transient Brain Injury. EAAT1 Excitatory Amino Acid Transporter 1. TRPV4 transient receptor potential vanilloid 4.

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Figure 1

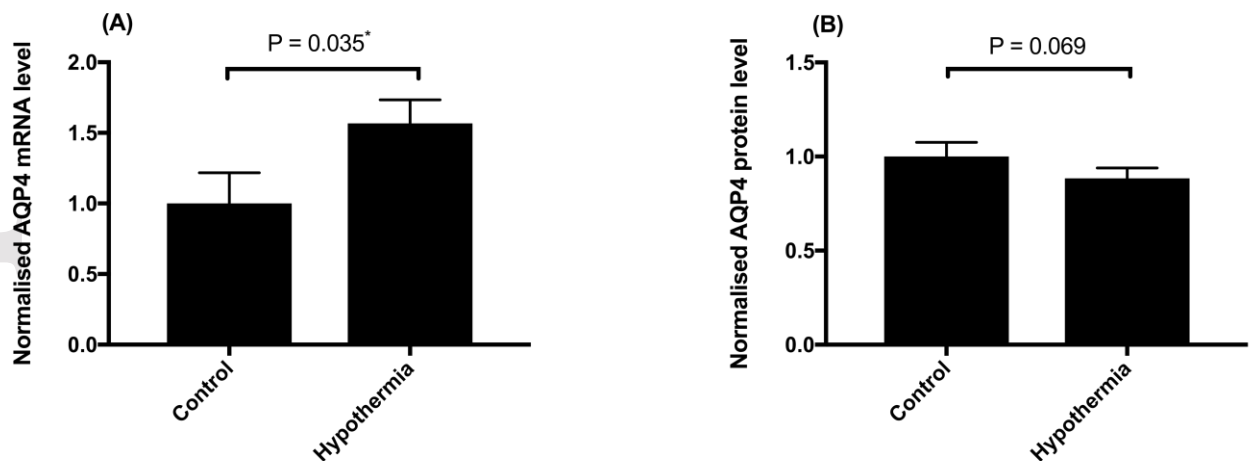


Figure 2

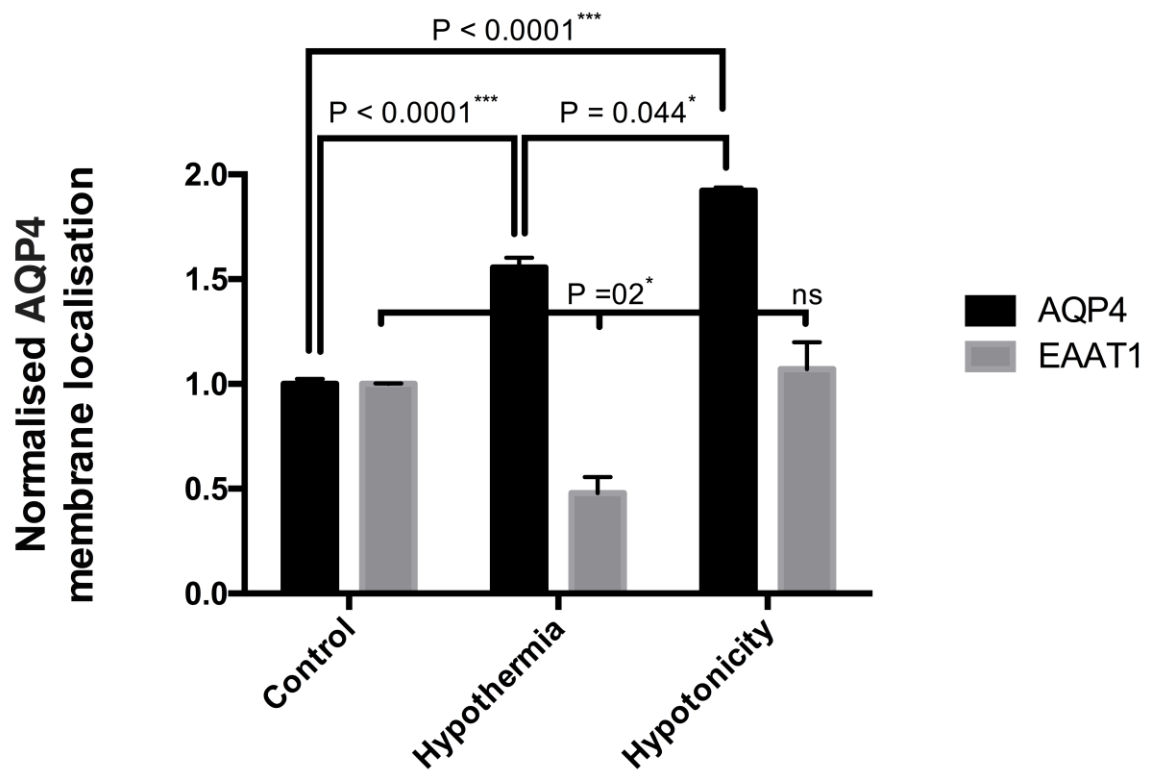


Figure 3

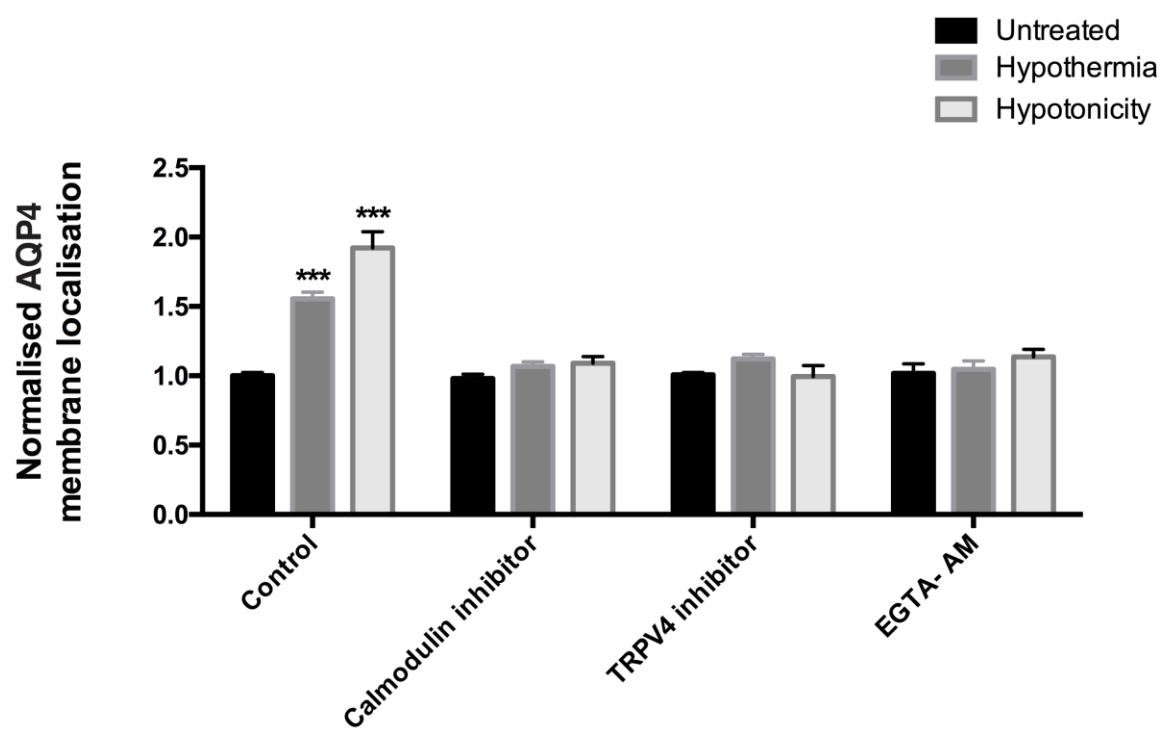


Figure 4:

