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Acetazolamide-induced Decrease Of Apical Fluid Flow In Choroid Plexus Is Independent Of The Concomitant Changes In Aquaporin-1 Expression

2010

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ACETAZOLAMIDE-INDUCED DECREASE OF APICAL FLUID FLOW IN
CHOROID PLEXUS IS INDEPENDENT OF THE CONCOMITANT CHANGES
IN AQUAPORIN-1 EXPRESSION

by

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B.A. University of Florida, 2007

A thesis submitted in partial fulfillment of the requirements
for the degree of Master of Science in Biotechnology
in the Burnett School of Biomedical Sciences
in the College of Medicine
at the University of Central Florida
Orlando, Florida

Fall Term
2010

ABSTRACT

Acetazolamide (AZA), the only drug approved for treatment of hydrocephalus, is effective in only 25-30% of patients while its effect on fluid flow in the choroid plexus (CP) is unknown. The drug reversibly inhibits Aquaporin 4 (AQP4), the most highly expressed 'water pore' in the brain, and it is postulated that it reduces cerebrospinal fluid (CSF) production by modulating AQP1 (mostly found in the apical membrane of the CP). In this study, we sought to elucidate the effect of AZA on AQP1 and fluid flow in CP. Primary CP culture from p10 Sprague-Dawley rats and TRCSF-B cell line were grown on Transwell permeable supports, treated with 100μM AZA or 100μM Vinpocetine (previously shown to increase AQP1 levels), and tested by: a) Fluid assays using TRITC-labeled Dextran to assay direction and extent of fluid flow; b) Immunoblot, Immunocytochemistry (ICC), and RT-PCR for AQP1 expression. Immnoblots and ICC analyses showed that AQP1 protein levels decrease in a delayed manner (lowest at 12 hours) with AZA treatment. The reduction in AQP1 protein was transient and preceded by a reduction in mRNA levels (lowest at 6 hours). Transwell fluid assays indicate a shift in fluid flow at 2 hours, prior to the changes in AQP1 mRNA or protein. Alteration of fluid flow by AZA (in both primary culture and TR-CSFB) is similar to Vinpocetine's effect in primary culture. Together with drug-induced alterations in AQP1 levels, these data suggest independent mechanisms behind fluid flow and AQP1 expression.

I would like to dedicate this achievement to my parents, Shane and Parviz Ameli, who have been absolutely critical to every success I have ever had. Mom and Dad, without you nurturing my strengths and fostering in me the values I need to withstand the difficulties of life, I would be but a shell of what I am. Thank you for proving me wrong when I needed to be put in my place. Thank you for managing my stubbornness. Mostly, thank you for loving me as much as you do.

I would also like to recognize my brother Neema for his support through all my endeavors. He may be younger, but I find his advice and guidance to be invaluable. I would not trade him for the world and I only hope that I can be as good a brother and role model for him as he is for me.

And, finally, I would like to recognize Dr. Jogi V. Pattisapu and Dr. Sic L. Chan for their dedication to the education of young minds. Thank you for your patience in guiding me into lands previously unknown to me. Thank you for recognizing the delicate, but essential difference between when I needed to be pushed forward by force or by encouragement. Biomedical Science is a rigorous craft, and I could not have asked for a better set of mentors. I hope that I will go on to represent you well in this field, and I sincerely plan to keep in touch with you both. I cannot thank you enough. I wish you great successes, even exceeding what you have already achieved.

Thank you to everyone who had any part in molding a kid with big dreams such as myself into someone who holds in his own hands the opportunity to one day see his ideas become reality.

ACKNOWLEDGMENTS

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LIST OF ACRONYMS/ABBREVIATIONS

AQP	Aquaporin
AZA	Acetazolamide
BCA Assay	Bicinchoninic Acid Assay
CA	Carbonic Anhydrase
cAMP	Cyclic Adenosine Monophosphate
cDNA	Complementary Deoxyribonucleic Acid
cGMP	Cyclic Guanine Monophosphate
CP	Choroid Plexus
CSF	Cerebrospinal Fluid
DMEM	Dulbecco's Modified Eagle Medium
FAB	Fluid Assay Buffer
FBS	Fetal Bovine Serum
GLUT1	Glucose Transporter 1
ICC	Immunocytochemistry
P10	10 days postnatal (10 days after birth)
PBS	Phosphate Buffer Saline
PKA	Protein Kinase A
RNA	Ribonucleic Acid
RT-PCR	Reverse Transcriptase Polymerase Chain Reaction
sAC	Soluble Adenylyl Cyclase

SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
shRNA	Small Hairpin Ribonucleic Acid
TEER	Transepithelial Electrical Resistance
TRITC	Tetramethyl Rhodamine Iso-Thiocyanate
TTF-1	Thyroid Transcription Factor 1

INTRODUCTION AND LITERATURE REVIEW

What is Hydrocephalus?

Hydrocephalus is a neurological disorder characterized by an excessive accumulation of fluid in the skull cavity. This phenomenon may be caused by disturbances in both the production and absorption of cerebrospinal Fluid (CSF). A ventricular system that is continuous with the central spinal canal is responsible for the flow of CSF throughout the cranium, which serves many functions: (i) Shielding of buoyant brain tissue from potential damage by acting as a shock absorber, (ii) transport of nutrients and waste in the brain, and (iii) compensation for changes in intracranial blood volume by flowing between the brain and the spinal cord. In normal individuals, CSF is constantly produced to circulate through the brain, serve the above functions, and subsequently reabsorbed into the blood stream with any waste it may have collected. This critical balance is altered in hydrocephalus patients, leading to a buildup of CSF [1].
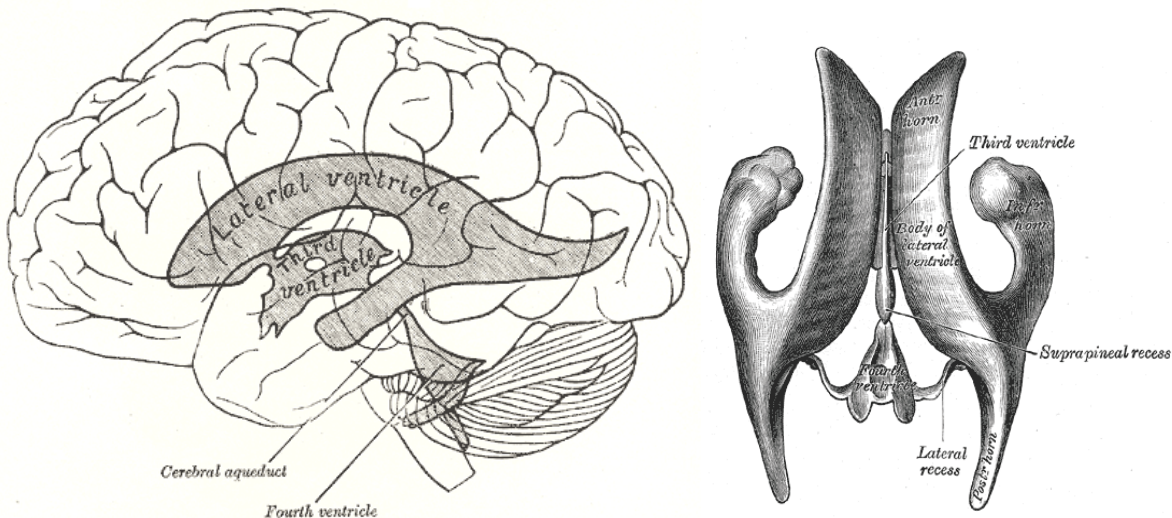
Figure 1 Cerebral Ventricles.
Images depict ventricles as well as connecting aqueducts through which CSF flows throughout the brain. Sources:
http://scienceblogs.com/corpuscallosum/2007/07/small_brain_normal_life.php
http://sites.google.com/site/kidsjudge2009ventricularsystem/project-synopsis

The accumulation of fluid leads to dilation of the ventricles in the brain. Genetic background, age, and tolerance to excess CSF all contribute to the symptoms experienced by affected individuals. In infants, the ventricular dilation can often be seen in the form of a visibly rapid increase in the size of an affected individual's head (head circumstance). In older individuals, the size of the skull changes minimally if at all and hence the accumulation of pressure on the brain can more quickly lead to a multitude of symptoms. In adults and children, symptoms may include: Headache, nausea, papilledema (swelling of optic disk), sunsetting of the eyes, poor coordination/balance, urinary incontinence, developmental retardation (in children), lethargy, drowsiness, irritability, memory loss, in addition to other changes in personality and memory [1].

Forms of Hydrocephalus

There are four general classifications for hydrocephalus: congenital, acquired, ex-vacuo, and normal pressure hydrocephalus. Congenital hydrocephalus is present at birth and is normally caused by genetic malfunction and/or early developmental influences. Acquired hydrocephalus is developed after birth and is generally the result of injury or disease. The two remaining types of hydrocephalus are classified separately as they do not clearly fit into either of the above categories. Ex-vacuo hydrocephalus is often an effect of stroke or traumatic injury and may actually lead to shrinking of brain tissue. Normal pressure hydrocephalus has been seen in patients of all ages but most frequently occurs in the elderly. It may result from a subarachnoid hemorrhage, head trauma, infection, tumor, or complications of surgery, but there are those who have developed this form of hydrocephalus in the absence of all these factors [2].

Hydrocephalus can also be classified as "Communicating" or "Non-communicating." The difference in these classifications relies on whether or not the CSF is able to flow between the ventricles within the brain itself. "Non-communicating" is defined as hydrocephalus involving a obstruction of flow within the brain. A single obstruction is required for this classification. A common form of non-communicating hydrocephalus, and a common cause of hydrocephalus overall, is "aqueductal stenosis" – which refers to the blockage of the *aqueduct of Sylvius* (also: mesencephalic duct, cerebral aqueduct), a passageway between the third and forth ventricles in the center of the brain. "Communicating" Hydrocephalus is as result of insufficient CSF reabsorption without any blockage in fluid flow between portions of the brain. Many causes for this pathology include: Injuries to the intravenous and subarachnoid areas, meningitis, genetic/developmental disorders, and infections/inflammation of the subarachnoid space [1, 2].

Prevalence and Cost

It is difficult to estimate the number of individuals suffering from hydrocephalus in the United States or globally because there are currently no registries or databases to track such statistics. However, by some estimates, roughly 1 in 500 children are suffering from this debilitating disease. "In 2003, hydrocephalus accounted for 0.6% of all pediatric admissions, 1.8% of all pediatric hospital days, and 3.1% of all pediatric hospital charges; in contrast, cystic fibrosis accounted for 0.3%, 0.6%, and 0.9%, respectively" [2]. This cure-less disease is the leading cause for
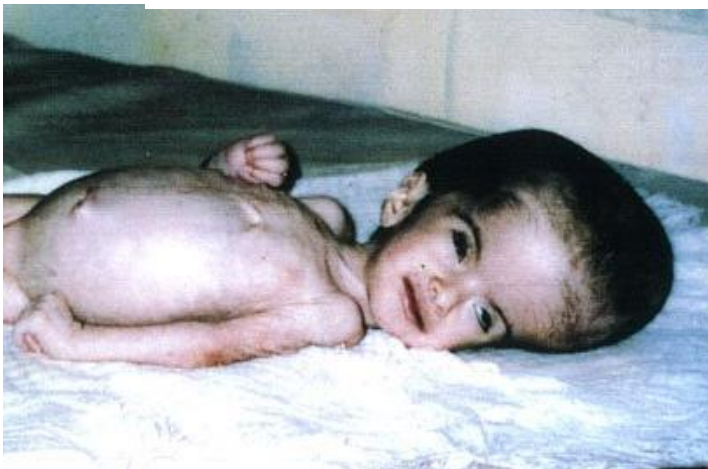
Figure 2 Photograph of an infant with severe untreated hydrocephalus
Source: http://www.pathguy.com/lectures/hydrocephalus.jpg

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brain surgery in American children [1]. In the same year, children with hydrocephalus were

documented to account for $2 billion in healthcare costs. Increasing both the efficacy and cost-

efficiency of hydrocephalus management is of prime concern [2].

Choroid Plexus and CSF Flow

In order to improve treatment of hydrocephalus, we must first gain better understanding of it as a

disease. This involves the identification of the relevant anatomical structures on which we should

focus our molecular investigations. Being a disorder of fluid accumulation, many would agree

that one should focus hydrocephalus research on understanding the mechanisms of CSF

production, absorption, and flow in the brain [3-5].
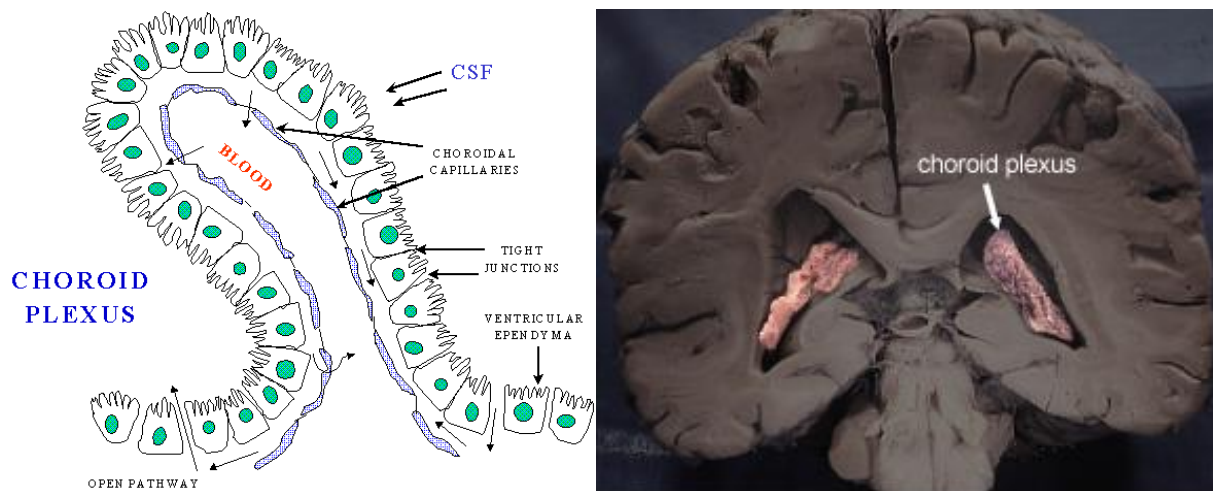
Figure 3 Images of the Choroid Plexus
Sources: http://www.daviddarling.info/encyclopedia/C/choroid_plexus.html
http://www.sci.uidaho.edu/med532/choroid.htm

The choroid plexus (CP) is comprised of modified ependymal cells that serve as the barrier

between the blood and the CSF [6]. The CP can most commonly be identified via the presence of

transthyretin (TTR), a transporter of thyroxine and retinol, or Kir 7.1, an inward-rectifying

potassium ion channel [7, 8]. CP cells are thought to play an essential role in CSF formation [9].

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The flow of CSF in the brain begins in the lateral and third ventricles. The CP in these areas produces CSF that flows into the fourth ventricle through the cerebral aqueduct. The CP in the fourth ventricle contributes to CSF volume and the fluid flows through the foramina outward into the cisterna magna and other large cisterns. From the cisterns, the CSF flows posteriorly to the subarachnoid space around the spinal cord as well as upward into the cerebral hemispheres. Later, the fluid flows outward to the arachnoid villi and into the venous blood of the superior sagittal sinus [10]. Most commonly, hydrocephalus is caused by blockage of the cerebral aqueduct ("aqueductal stenosis"). Hence, the CSF produced by the lateral and third ventricles is of particular importance as this fluid would be upstream of the blockage, leading to the build-up of pressure. The CP of these ventricles is thought to produce CSF via the expression of aquaporin proteins, namely in the form of Aquaporin 1 [9].

Aquaporin Proteins
In the late 1990's, Peter Agre came across an unknown protein in immunoblots of the Rh blood antigen. After repeated contamination of his samples by this protein, he eventually identified it as the long-sought cellular water pore. In 2003, he shared the Nobel Prize in Chemistry with Robert MacKinnon for the discovery and initial characterization of aquaporin (AQP) proteins [11].

AQP proteins are widely considered "the plumbing system for cells" [12]. These integral membrane proteins form pores in cell membranes that are capable of mediating the transport of water, ions, and small non-polar molecules [13]. AQPs are expressed in many cell types, especially those with an essential function involving the transport of water [14]. The brain is

known to differentially express 6 isoforms of AQPs: AQP1, AQP3, AQP4, AQP5, AQP8, AQP9 [15-19]. In the brain, three AQPs have been extensively characterized (AQP1, 4, 9). While AQP4 is predominant brain aquaporin, AQP1 is of particular interest for its exceptional abundance in CP cells [20, 21]. The high degree of AQP1 expression in the CP apical border may indicate an essential role in CSF production (and perhaps absorption) [22].

AQP proteins, although thought to account for the bulk of fluid flow in many tissues, are typically driven by a shift in osmotic balance resulting from parallel transport of ions via pumps and channels [23]. It is also of note that current scientific interest is shifting away from a purely osmotic model of cellular water flow. Recent data implicates cotransporters and uniports, such as the Glucose Transporter 1 (GLUT1), as significant players in the transport of water independent of osmotic gradients [24].

As discussed above, aquaporin proteins have been shown to be linked with many disorders and disease states. Hence, understanding the physiological function of aquaporin proteins is imperative to the treatment of many neurological disorders [3, 4, 25, 26]. The CSF-producing CP is of particular interest in Hydrocephalus and other fluid disorders. The apical membrane of CP cells contain the majority of the brain's AQP1 protein [27, 28]. This localization of AQP1 was confirmed in our model via confocal microscopy (Fig 8C). Due to the high density of AQP1 specific to the CP, these channel proteins are likely involved fluid formation. One study showed that CSF production in AQP1-null mice is reduced by approximately 25% [3, 4]. In low-gravity

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situations, decreased AQP1 expression levels in rat CP correlated with reduced CSF production [29].

Beyond Hydrocephalus, the study of aquaporin proteins is critical to the future scientific understanding of many bodily processes and disease states. Despite their seemingly simple cellular role of passive water transport, AQP proteins have proven to be involved in countless aspects of molecular physiology. Among other functions, AQP proteins are essential to the production of aqueous humor in the eyes, blood filtration in the kidneys, and differentiation of neuroblasts and epidermoblasts in *Drosophila* via interaction with BIB [23, 30].

AQPs have also been implicated in numerous pathologies. Upregulation of aquaporins has been documented to enhance brain edema and protect against seizures and epilepsy [26, 31, 32]. Increases in AQP4 are seen in astrocytic brain tumors and have been shown to decrease with radiation and chemotherapy [33-35]. AQP 1 is upregulated in the early stages of Alzheimer's disease and has also been correlated with malformations in cortical development [36-38].

Modulating Cerebral Fluid Flow, with Focus on Hydrocephalus: Shunt, Acetazolamide, and Vinpocetine

The most common treatment for hydrocephalus involves neurosurgical implantation of a cerebral shunt in the patient's brain. Based upon location of the blockage and other patient-specific factors, the neurosurgeon determines the best ventricle of the brain in which to insert the shunt and also the best location for transport of the excess fluid (most commonly the abdomen, but sometimes the heart or lungs). This dangerous procedure may cause brain damage itself and is not guaranteed to work [39].
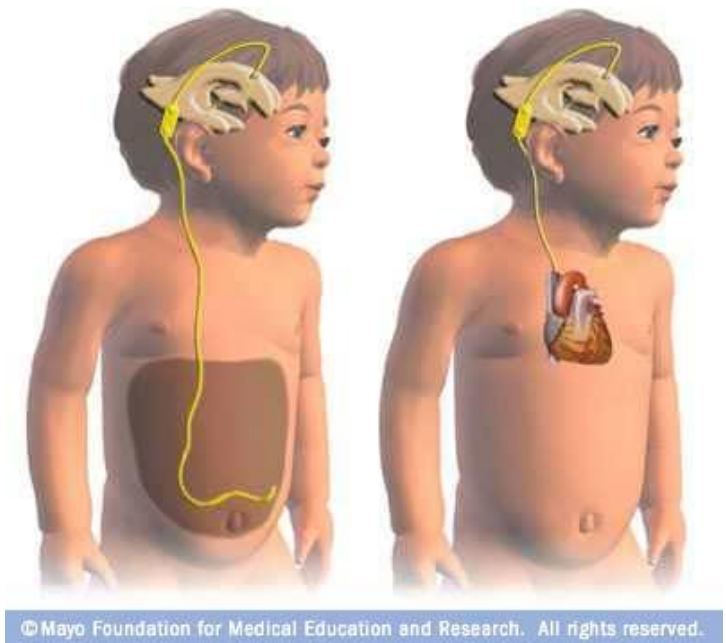
Figure 4 Images of Shunts in Hydrocephalic Children
Images depict a child with a shunt placed into the cranium as well as possible locations to which excess CSF could be shunted. Sources: http://www.rarenyheter.com/?p=1392 http://www.doctors.ly/forums/showthread.php?t=25340&page=8

Roughly one half of all shunts fail within 2 years, leading to repeated surgery to replace the device [40]. Depending on many factors ranging from the surgeon's experience and the duration of surgery to the patient's state of health, implanted shunts may become infected at rates as high

as 12.9% or 27% (estimates vary), with all infections being considered life-threatening [41, 42]. The shunt is attached to a valve that may or may not be programmable. Valves often become obstructed and need to be watched carefully to avoid overdrainage, which can complicate treatment by leading to other disorders [41]. Although the risk of complications from these procedures has improved significantly, the shunt treatment has remained largely unchanged since its development in the late 1950's [39, 40]. In 2010, 50 years after the shunt was first developed, the major advancements offered to hydrocephalus patients are improved construction materials for shunts and programmable valves [43].

Notably, Acetazolamide (AZA, Diamox) is a carbonic anhydrase inhibitor that has considerable therapeutic effects in many, but not all hydrocephalus patients [3, 4]. It is used as a diuretic and in the treatment of glaucoma, idiopathic intracranial hypertension and seizures [5, 43, 44]. AZA has also been shown to reduce CSF production by as much as 50% in some cases of hydrocephalus [45]. In these cases, AZA is proposed to reduce CSF production by inhibiting AQP1 [46, 47]. However, AZA is ineffective on purified AQP1 protein reconstituted into liposomes, although inhibition of AQP4 was observed [48]. Nonetheless, AZA also decreases CSF production in AQP1-null mice [3].The goal of this study was to further elucidate the relationship between AZA treatment and AQP1 in the CP of Sprague Dawley rats. Little is known regarding the mechanism of this drug in hydrocephalus patients. Identifying the proteins affected by AZA in the CP could provide new targets for future treatments of brain fluid disorders and answer questions regarding the inefficacy of this drug in the majority of hydrocephalus patients [49].
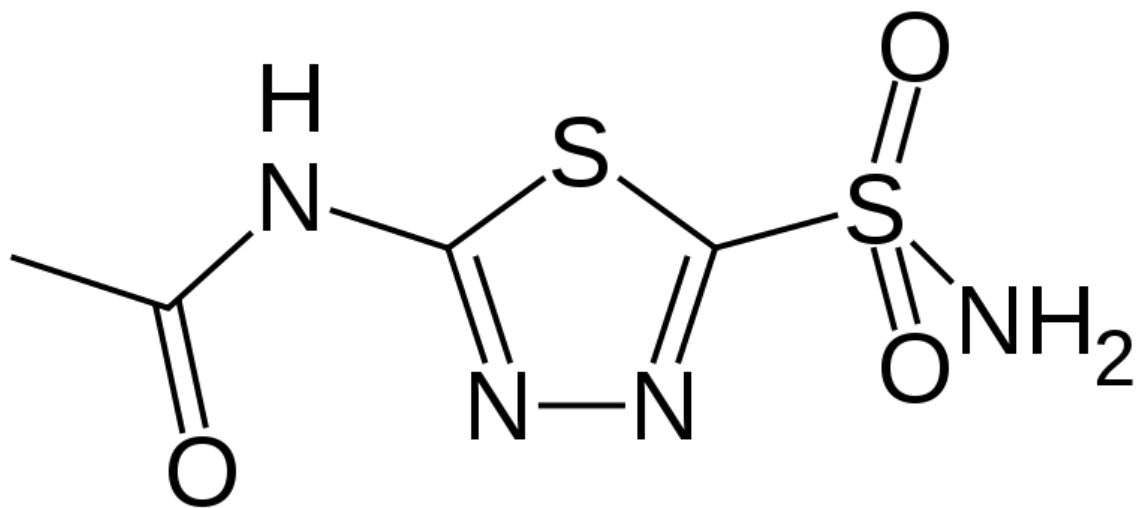
Figure 5 Acetazolamide Structure
Source: http://commons.wikimedia.org/wiki/File:Acetazolamide_skeletal.svg

Vinpocetine
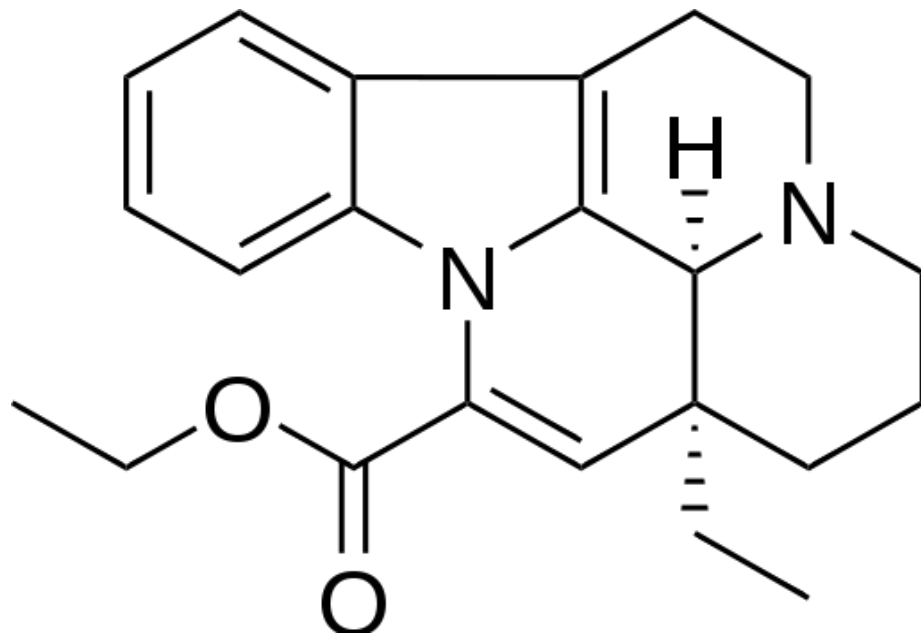
Figure 6 Vinpocetine Structure
Source: http://en.wikipedia.org/wiki/File:Vinpocetine.svg

Vinpocetine is a nootropic semisynthetic derivative of Vincamine, a peripheral vasodilator

produced by the periwinkle plant thought to augment the flow of blood to the brain [50].

Vinpocetine is available by prescription in Japan and Europe, but is sold primarily as a dietary

supplement in the United States. Although it has been reported to exhibit neuroprotective effects,

improve memory, and enhance blood flow to the brain, properly designed studies of Vinpocetine

remain lacking [51-53]. Our lab has previously shown that Vinpocetine gradually increases the

expression of AQP1 at the apical surface of rat choroid plexus cells [22]. Previous data from our

lab showed a decrease in AQP1 in hydrocephalic rats and it was postulated that the effect of

vinpocetine on AQP1 could lead to therapeutic effects for hydrocephalus patients. Hence this

drug was included in our study as a comparison for Acetazolamide, a pharmaceutical with

known therapeutic effects in the treatment of hydrocephalus.

My contributions to this project consisted of assisting Leena Paul in completing experiments,

while simultaneously learning the above techniques. This experience served as impetus for my

studies of Acetazolamide and AQP1 in the Choroid Plexus and provided valuable data in

analysis of the subsequent results. This drug was included in our studies as a control against

which we could compare our results, for both the expression of AQP1 and the apical fluid flow.

Hypothesis

Because AQP1 is also highly expressed in tissues known to regulate water transport such as the

eyes, it has been shown to be essential to the production of aqueous humor in AQP null mice

(exhibiting hindered production of ocular fluid) [54]. We propose that the high concentration of

AQP1 in the CP implicate it as an essential player in CP function – namely, the production (and perhaps absorption) of CSF. AQP1 protein expression has also been shown to be upregulated in injured astrocytes, which have a tendency to retain water – showing a correlation between AQP1 expression and fluid retention [55].

The main objective of this study was whether AZA may impact AQP1 CP expression in apical fluid flow. We hypothesized that AZA operates in the treatment of brain edema by decreasing the levels of functional AQP1 protein in CP cells, a phenomenon related but unessential to the change in fluid flow.

METHODS

Animals

All protocols were approved by the Institutional Animal Care Committee. Sprague Dawley (SD) rats were purchased from Charles River Laboratories (Cary, North Carolina). Ten-day old (p10) pups were used for this experiment. In most instances, pregnant mothers were purchased and birthed pups were maintained at our institution under 12-hour day-night cycles with free access to food, water, and maternal feedings. In a few instances, young pups were purchased and maintained under the same conditions described above. Roughly one fourth of the animals were purchased and delivered as litters of 7-day or 8-day old (p7 or p8) pups – these animals were maintained under the same conditions as those above.

Tissue Culture

Dissection/Primary Culture

Sprague Dawley rats were between p8 and p11 upon dissection, with nearly all pups at p10 (n=120). They were anesthetized using isoflurane-soaked cotton balls in a closed container for roughly 3-5 minutes and then quickly decapitated to minimize suffering. All steps of culture from the decapitation onward were done under sterile conditions in the tissue culture hood. While holding the head at the nose with a large pair of tweezers, a small scissor was used to cut the skin from the base of the skull to the forehead. The skin of the head was then pulled back and the scissors were used to severe any connective tissue preventing full exposure of the skull. Starting with a puncture at the base of the skull, the scissors were then used to cut the skull to the forehead creating two flaps of bone on either side of the head. While still grasping the head with

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the large tweezers, a pair of small tweezers was used to pull the skull flaps laterally away from the midline until they detached from the head in order to fully expose the brain. The small tweezers were then used to pry and lift the brain out of the skull and into a dish of sterile 1X PBS. This was done carefully to ensure the brain remained intact and that the cerebellum was not severed in any way.

Using a set of 2 extra-fine tweezers, the brain was dissected under the hood in the sterile PBS. It was folded open from the longitudinal fissure to expose the inner ventricles. The extra-fine tweezers were then used to reach under the lateral ventricles, just inside the arching line of the hippocampus, to obtain the choroid plexus. The Y-shaped thread-like tissue was pulled from this area – most often, in one swift motion that removed nearly the entire structure. This was done for both lateral ventricles. It is of importance to note that the choroid plexus is not attached to the brain by any fastidious means and should be quite readily removed if grasped properly and not torn by aggressive handling. After removal of the lateral choroid plexus, the cerebellum and associated portion of the brain stem were detached from the brain. This detached piece was then turned so that the dorsal portion was facing upward. The cerebellum and brain stem were separated enough for a pair of tweezers to reach in and pull out the choroid plexus of the fourth ventricle. This was done with care as with the lateral ventricles in an attempt to remove the entire structure. Again, if done properly, the choroid plexus of the fourth ventricle should also be removed with significant ease. The choroid plexus of the fourth ventricle has a feathery appearance, with many protrusions along a thin thread-like structure and is fairly easy to identify, even with the naked eye, when the brain stem and cerebellum are pulled apart. Upon removal, all

tissues to be used for culture were maintained in 10% Fetal Bovine Serum (FBS) Culture Medium (DMEM:F12 with 10 % FBS, 4mM glutamine, 5µg/ml insulin, 10U/ml Penicillin, 0.1mg/ml streptomycin, 10ng/ml EGF, 5µg/ml Insulin, 5µg/ml Transferrin, 5ng/ml Sodium selenite) supplemented with 4µM Arabinoside Cytosine.

Transwells and 24-well dishes were both coated with Laminin for primary culture. The stock solution of laminin was diluted 1:25 (40µg/µl) in sterile 1X PBS (Sterilized by autoclave). This dilution was used for coating. Volumes of 50-100µl were used to coat transwell membranes and 100-150µl used to coat each well of 24-well dishes. TRCSF-B cell line was grown on commercially coated collagen plates or transwell membranes coated with 25µg/µl collagen by the researcher (1:50 dilution of stock).

All dishes were exposed to UV light in a tissue culture hood for 5-10 minutes prior to addition of substrate. Coating took place for at least 3 hours at room temperature, but most often occurred overnight at room temperature. Dishes were wrapped with aluminum foil sterilized with 70% ethanol and kept in the tissue culture hood under UV light during coating. Solutions of substrate dilutions were often reused 3-5 times with storage at -20° Celsius in between usage.

When dissection was complete, the extracted tissue was transferred in the media to a 15ml conical tube and allowed to settle to the bottom of the container. The excess media was removed and 4-6 ml of 0.25% Trypsin solution with phenol red was added at room temperature (roughly 2-3 times the volume of the tissue). The amount of trypsin was estimated with reference to the

amount of tissue obtained – usually, 10-12 animals were dissected in a single sitting. The tube was then incubated at 37° C 15-20 minutes with occasional agitation by flicking and swirling. A Pasteur pipet was utilized and the tissue was titrated in order to break up the clumps – this was done carefully to homogenize the tissue as much as possible, while also avoiding damage to the cells as they were released into solution. Pasteur pipetting was done repeatedly in alternation with 5-minute periods of additional incubation at 37° C until the tissue was significantly digested as evidenced by minimal or nonexistent clumps of tissue. At this point, the media took on a cloudy look from the cells released into the solution. Importantly, the addition of phenol red causes the trypsin to go from a reddish color to more of an orange as the reaction continues. The tissue should be sufficiently digested to move forward before this orange color is reached, which may require significant time Pasteur pipetting. However, best results are obtained via gentle Pasteur pipetting as opposed to prolonged chemical digestion of tissue.

Once the solution of tissue and media achieved sufficient homogeneity, the solution was passed through a 100μm cell strainer. An additional 3-5ml of 10% FBS culture medium was also passed through the strainer to obtain any loose cells that remained stuck on the strainer as well as to stop the trypsin reaction. The strained solution was then centrifuged at 1200 rpm (285 rcf) for 5 minutes [56, 57]. The entire media/trypsin solution was aspirated off to leave only a clump of cells at the bottom of the tube. Although it varied depending on the pellet size, the cells were usually resuspended in 1-2 ml of 10% FBS culture medium supplemented with arabinoside cytosine. The cells were most often resuspended with a Pasteur pipet until homogeneity was achieved. This solution was used for cell counting.

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To count the cells, 10µl of the freshly agitated cell resuspension was added to 10µl of 0.4%

Trypan Blue and mixed by gentle pipetting. A 15µl portion of this solution was used to count the

cells with a standard haemocytometer and a hand-held cell counter. The following formula was

used to calculate the cell density of the resuspension:

(Average cells counted) (Dilution of resuspension) (ml of resuspension medium) (10,000)

This gave the total number of cells obtained, which ranged from roughly 0.4-1.0 million

cells/pup dissected depending on the efficacy of the dissection. The cells were then diluted to a

density of 1000 cells/µl using 10% FBS culture medium supplemented with arabinoside

cytosine. The cells were then seeded at a density of 300,000 cells/well (300µl) for transwell

dishes and 600,000 cells/well (600µl) in 24-well dishes. For transwell dishes, the 300µl of cell-

containing medium was placed in the apical chamber while 600µl of the same medium without

cells was placed in the basolateral chamber.

Dishes of cells were placed in a tissue culture incubator set to 37° Celsius and 5% CO_2. Media

was changed every 1-2 days and medium older than 10 days was not used for culture. The

dissection was done entirely under aseptic conditions and all tools were freshly washed and

autoclaved for each day of dissection.

Cells were maintained at 37° C and 5% CO_2 on laminin-coated tissue cultureware, and medium was changed every 24-48 hours. Primary culture was mostly grown in 10% FBS (DMEM:F12 with 10 % FBS, 4mM glutamine, 5µg/ml insulin, 10U/ml Penicillin, 0.1mg/ml streptomycin, 10ng/ml EGF, 5µg/ml Insulin, 5µg/ml Transferrin, 5ng/ml Sodium selenite). For the first 4 days of culture only, the medium was also supplemented with 4µM Arabinoside Cytosine to reduce fibroblast contamination.

Arabinoside Cytosine (Cytarabadine, Ara-C) is an antimetabolic agent that damages the viability of actively dividing cells. Its rapid conversion into cytosine arabinoside triphosphate allows for its integration into cellular DNA during S-phase of the cell cycle and subsequent inhibition of the actions of both DNA and RNA polymerases. Due to the nature of its mechanism, Ara-C is especially effective against rapidly dividing cells that undergo mitosis often. Because Choroid Plexus cells are slowly dividing cells, the addition of Ara-C to our culture medium for the initial period of culture allows for reduction in the population of fast-growing cells such as fibroblasts that commonly contaminate CP cultures. Arabinoside cytosine was included in the 10% FBS primary culture medium for the first 4 days in culture. After this point, 10% FBS primary culture medium as described above was utilized.

TRCSF-B Cell Line Culture
The TRCSF-B, Choroid Plexus stable cell line established from transgenic rats infected with temperature sensitive simian virus 40 large T-antigen gene, was kindly provided by Dr. Terasaki [58]. These cells were maintained at 34° C and 5% CO_2 on collagen-coated tissue

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cultureware according to guidelines published by Terasaki [58]. Media was changed every 24-48 hours as needed.

RT-PCR

Choroid Plexus primary culture was grown in laminin-coated 24-well dishes according to the culturing methods described above. Cells were treated with either 1x Fluid Assay Buffer (FAB) alone or with 100μM AZA (Sigma, A6011) supplemented. RNA was harvested with 200μl of RLT buffer (Qiagen) with 2-mercaptoethanol at 1, 3, 6, 12 and 24 hours. Baseline wells were treated with an equal volume of vehicle and harvested at 24 hours.

Following the RNAeasy kit (Qiagen, Maryland) instructions RNA was isolated involving "On-Column DNase Digestion with the RNase-Free DNase Set". RNA was quantified via nanodrop and cDNA were prepared with iScript cDNA synthesis kit (Bio-Rad). The resulting cDNA was utilized for qRT-PCR expression analysis of Aquaporin 1 mRNA with primers described below. 18S was employed as an internal control.

AQP1 Forward   5'- CCCTCTTCGTCTTCATCAGC -3'

AQP1 Reverse   5'- GTTGAGGTGAGCACCACTGA -3'

18S Forward   5'- GTAACCCGTTGAACCCCATT -3'

18S Reverse   5'- CCATCCAATCGGTAGTAGCG -3'

Immunoblot

Harvest of Protein:

Cells were treated with FBS media Control, 1x FAB (baseline sample) or 100 μM AZA

(dissolved in water – Sigma, A6011) in 1x Fluid Assay Buffer for 6, 12, and 24 hours. Cells were

washed with sterile 1x PBS and lysed on the plate with appropriate volume of 1x Lamelli Buffer

and a cell scraper, followed by scraping with the tip of the pipet used to transfer harvested

protein to a microcentrifuge tube. However, cells grown on transwell membranes were harvested

by trypsinization. The trypsin cell suspension was then pipetted off and transferred to a

microcentrifuge tube.

For trypsinized cells, the microcentrifuge tube was then spun at 10,000 rpm for 5 minutes at

room temperature. The fluid was carefully aspirated such that the pellet was undisturbed and an

appropriate volume of 1x lamelli buffer was added (usually between 20-100μl depending on the

size of the pellet). The pellet was resuspended and lysed by brief vortex followed by 3-5 quick

pulses of a sonicator at half power. Samples obtained via scraping were also briefly sonicated.

Protein Quantification:

Bicinchoninic Acid Assay (BCA, Pierce; Rockford, Illinois) was used to quantify protein

concentration during SDS-PAGE. Samples were brought to room temperature (from the -20° C if

stored, or from 37° C if freshly digested) and passed through a 20-guage syringe 5-10 times to

loosen and minimize any clumps that may have formed during storage. 200μl of BCA reagent

was incubated with 5 μl of each sample in a clear 96-well plate for 30 minutes at 37° C. Samples

were diluted prior to incubation if necessary for readings to fall into sensitivity range of BCA assay.

SDS-PAGE Analysis:
Equal amounts of protein (10-20μg) were separated on an SDS-PAGE gel at 100 volts. The gel was then transferred onto a nitrocellulose membrane by wet transfer at 90 volts for 70 minutes in cold transfer buffer with constant stirring for equal dissipation of heat. Transfer of molecular weight ladder was used as confirmation of successful procedure. When standardizing the procedure, Coomassie staining of the remaining acrylamide gel was performed to ensure most of the protein was transferred onto the membrane.

Protein Blotting:
Membranes were blocked for 1 hour in 5% non-fat milk in PBS-T (0.05% Tween-20) at room temperature. Blots were then incubated in 1:5000 dilution of Aquaporin 1 primary antibody (Alpha Diagnostics, Rabbit anti-Rat) in blocking solution overnight at 4 Celsius. Blots were then washed 3 times for 5-7 minutes (15-20 minutes total) in PBS-T. Blots were then incubated in a 1:5000 dilution of Goat anti-Rabbit HRP secondary antibody (Santa Cruz Biotechnology , sc-2004) in blocking solution for 1 hour at room temperature. Membranes were then washed 4 times in PBS-T totaling 20-30 minutes. After washing, blots were developed using the Supersignal West Pico Chemiluminescent Substrate (Thermo Scientific). Blots were incubated in the dark with substrate for 5 minutes and then developed in a dark room with a standard developing machine.

β-Actin blots followed the same general protocol as the Aquaporin 1 blots. Primary β-Actin antibody (Sigma, A1978; Saint Louis, Missouri) was mouse anti-rat 1:10,000 in blocking solution for 1 hour at room temperature and secondary antibody was goat anti-mouse (Santa Cruz Biotechnology, sc-2020) 1:10,000 in blocking solution for 1 hour at room temperature. All washing and developing steps were similar.

Lentivirus shRNA Knockdown of AQP1

Lentivirus containing AQP1 shRNA (sc-156108-V) and Cop-GFP control lentivirus (sc-108084) was obtained from Santa Cruz Biotechnology. CP rat primary culture cells were plated at a concentration of 2 million cells/well in 1-2 ml of complete culture medium in 6-well dishes and allowed to grow to near confluence over a 24-hour period. On the following day, the media was replaced with a fresh stock of complete medium (DMEM/F12) supplemented with 5µg/ml Polybrene (Santa Cruz Biotechnology, sc-134220). The Lentivirus particles (stored at -80°C) were thawed at room temperature and gently mixed before use. Lentivirus was then pipetted in 10µl or 20µl aliquots into the appropriate wells. The plates were gently swirled and placed into an incubator (37°C, 5% CO_2) for infection. Infected cells were harvested at 24, 48, and 72 hours and assayed by AQP1 knockdown via immunoblot analysis. Fluorescent microscopy was used to image the sister cultures infected with Cop-GFP lentivirus. Results show significant portion of cells were infected (Cop-GFP), but no visible knockdown of AQP1 (immunoblot).

Assessment of Confluency

Cells were seeded onto pre-coated transwell membranes and allowed to grow to confluence.

Primary culture was seeded at 300,000 cells/transwell and TRCSF-B cells were seeded at

100,000 cells/transwell. Confluence of monolayers was assessed by a combination of the Lucifer

Yellow passage assay as well as Trans-Epithelial Electrical Resistance (TEER) measurements made with an EVOm2 machine (World Precision Instruments). The threshold TEER resistance measurement for transwell membranes to be considered confluent was determined first by obtaining TEER values and then by

Figure 7 TEER Measurement
Source: http://www.itqb.unl.pt/labs/nutraceuticals-and-delivery/facilities-and-services-1

comparing those values with the results of Lucifer Yellow Assay of the same membranes.

Once confluency was determined, the cultures were given complete culture medium

(DMEM/F12) supplemented with 0.5% FBS to allow AQP proteins to obtain polarity (as

previously described) and the fluid assay was performed the following day [56, 57].

Lucifer Yellow Assay

Lucifer yellow (LY) is a fluorescent dye that is transported across cell layers by passive

paracellular transport. Transport of this dye can be assessed via spectrofluorometric analysis.

Cells are grown on a transwell membrane in appropriate medium to what is suspected to be a confluent monolayer. A 100ug/ml working solution of LY in HBSS is made. The medium is aspirated and the cells and well are washed twice with sterile 1x PBS or HBSS, and 600ul of HBSS is added to the basolateral chamber while 100ul of the LY working solution is added to the apical chamber.

The cells are then stored under normal conditions in a tissue culture incubator for 1 hour. The fluid in the basolateral chambers are each removed and measured in triplicates (485nm excitation, 530nm emission). An average of the fluorescent readings is plugged into the following equation to calculate the percent passage of the LY dye:

% LY Passage = $\dfrac{\text{RFU (test)} - \text{RFU (blank)}}{\text{RFU (donor)} - \text{RFU (blank)}}$ X 100

RFU blank = Fluorescence of Blank (HBSS)
RFU test = Fluorescence of LY in experimental basolateral fluid (as a result of passage)
RFU donor = Fluorescence of original LY working solution.

A percent passage below 3% is considered sufficiently confluent while values greater than 4% are indicative of leaky monolayers.

Fluid Assay

Fluid assay was done following procedure by Hakvoort, et al. [56]. Fluid assay was performed in 1x Fluid Assay Buffer (15mM NaHCO3, 15mM HEPES, 0.5mM Na_2HPO4, 0.5mM NaH_2PO4,

17.5mM Glucose, 122mM NaCl, 4mM KCl, 1mM CaCl$_2$, 1mM MgCl$_2$, 5µg/ml Insulin, pH 7.3).

Baseline wells were vehicle-treated cells used to define the baseline fluid flow. For baseline

wells, Fluid Assay Buffer was supplemented with only 100µM TRITC-Dextran (Sigma, T1287)

while experimental wells also contained 100µM AZA (Sigma, A6011) or 100µM Vinpocetine

(Sigma, V6383). Both the apical and basolateral chambers of the transwell membranes received

350µl of the appropriate Fluid Assay Buffer solution.

After treatment, both the apical and basolateral fluids were harvested at 2 hours, 6 hours, 12

hours, and 24 hours and read in a spectrofluorometer (Cary Eclipse with corresponding software)

for TRITC content (excitation: 542nm, emission: 572nm). Baseline wells were harvested at 2

hours. The 2 hour timepoint was chosen for harvest in baseline wells because this was the

timepoint at which the cells were most significantly affected in the experimental wells and thus

the timepoint as which a baseline was of greatest interest. All fluids were measured in triplicate

and averaged. These averages were used to calculate fluid flow according to the equation:

$$\mathbf{V_{sec} = ((CFD_i - CFD_a) \div CFD_i) \times V_0}$$

Where:

V_{sec} = Volume of fluid secreted in ul/hr

CFD_i= Initial fluorescent reading of TRITC-dextran solution

CFD_a = Final fluorescent reading of TRITC-dextran in the apical solution

V_0 = Initial volume of fluid applied to both sides of transwell membrane (350µl)

Immunocytochemistry

After fluid assay and TRITC measurements, transwell membranes were washed with excess 1x PBS and fixed for 10 minutes at room temperature (RT) with 4% paraformaldehyde. After subsequent washing, membranes were blocked with 10% goat serum with 0.1% Triton X-100 in 1X PBS for 45 minutes at RT. Membranes were then incubated with a 1:200 dilution of primary antibody (Aquaporin 1, Alpha Diagnostics, AQP11-A; Kir 7.1, Santa Cruz Biotechnology, sc-22440) in 5% goat serum in 1x PBS for overnight at 4º C. Membranes were washed three times before incubation with a 1:200 dilution of secondary antibody (Goat Anti-Rabbit Alexa Fluor 488 or Goat Anti-Mouse Alexa Fluor 568). Nuclear staining was done with Hoescht 33362 for 10 minutes at RT. After a final washing, transwell membranes were mounted onto coverslips for subsequent imaging using fluorsave (Calbiochem, 345789).

Immunofluorescent staining was observed with a Nikon 80i fluorescent and IP Labs (BD Biosciences) imaging software. For quantification, digitized immunofluorescent images were acquired using Qimaging Retiga 2000 SVGA FAST 1394 cooled digital camera system with1600 x 1200 pixel array with 12-bit, 20 MHz digitization camera mounted on a NIKON 80i Research Upright Microscope and then analyzed with IP lab software (BD Biosciences- Bio-imaging). For each culture, 1 transwell membrane was probed for each time point, and 4 random fields were analyzed from each transwell membrane. Total area of pixel intensity was quantified with the automated measurement tools in IP lab software. The total density was averaged and expressed as normalized, corrected values.

Confocal microscopy was performed by Leena Paul and Srinu Chigurupati. These data were included as a complement to the Vinpocetine immunoblot data, which I assisted in obtaining. Confocal microscopy was utilized to confirm AQP1 localization to apical brush border in cultured CP (Fig 8C).

RESULTS

Vinpocetine Treatment increases apical AQP1 expression

Experiments probing the effect of Vinpocetine on Choroid Plexus (CP) cells were performed to

confirm the proper performance of experimental methods (as compared to previous

experiments). CP cells harvested from tissue from the brains of p8-p11 SD rats were cultured and

grown to confluence. They were then treated with 100μM Vinpocetine. Immunoblots of

Vinpocetine-treated cells harvested at 1, 3, 6, 12, and 24 hours were compared to immunoblot of

vehicle-treated control. Results were normalized to β-Actin protein (Fig 8A). Densitometric

analysis revealed a gradual increase in AQP1 levels with significant and sustained increases from

6 to 24 hour (Fig 8B). Immunocytochemistry of treated CP cells also revealed an increase in

AQP1 levels which appears to be highly localized to the apical membrane of choroid plexus cells

(Fig 8C).
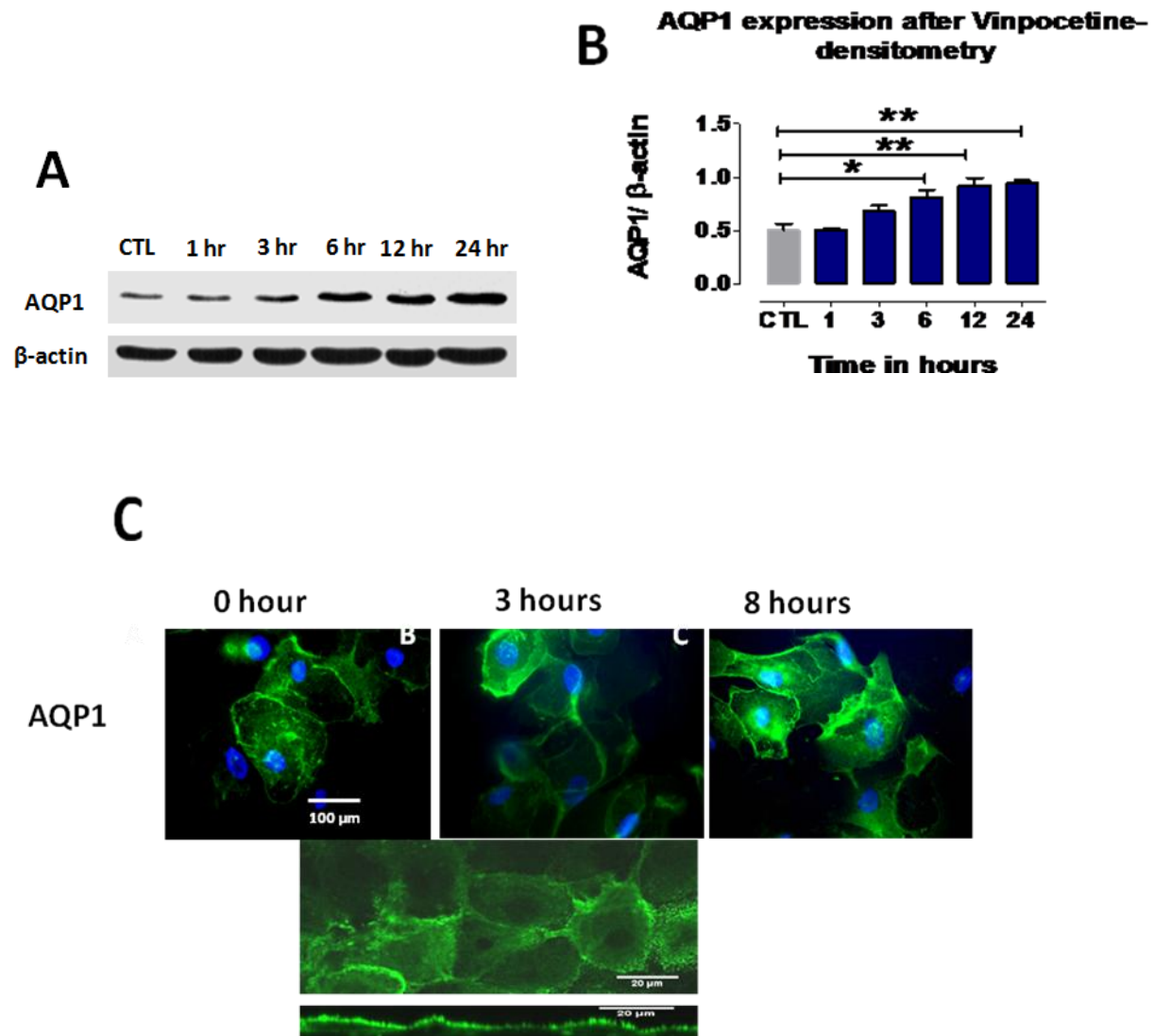
Figure 8 AQP1 and Vinpocetine
(A) Immunoblots revealed a gradual increase in AQP1 protein levels as a result of Vinpocetine treatment of CP primary culture. (B) Densinometric quantification of AQP1 and Vinpocetine immunoblots determined that increases in AQP1 were significant beginning at 6 hours. (C) Immunocytochemistry in Vinpocetine-treated CP primary culture revealed an increase in AQP1 immunnofluorescence over time. Additionally, confocal microscopy illustrated AQP1 to be located to the apical membrane of CP primary culture cells, as expected.

AZA-treated CP Culture Displays a Decrease in Both AQP1 protein and mRNA levels

Immunoblot and RT-PCR were utilized to determine the effect of AZA treatment on AQP1

expression. Choroid Plexus cells harvested from tissue extracted from the brains of p10 SD rats

were cultured to confluence and treated with 100µM AZA. Immunoblots of treated cells

harvested at 2, 6, 12, and 24 hours displayed a decrease and subsequent return of AQP1 protein

expression, as compared to baseline and normalized to β-Actin (Figs 9A, 9B). This trend implies

an effect of AZA treatment on the expression of AQP1. RT-PCR using AQP1 primers, with 18S

as a loading control, were performed on AZA-treated primary culture and illustrated a trend in

mRNA expression similar to that seen in both the immunoblot, with the changes in mRNA

expression being temporally consistent with the changes in AQP1 protein levels (mRNA

minimum at 6 hours and protein minimum at 12 hours). These results suggest that the previously

observed changes in protein expression were mediated by changes in the levels of AQP1 mRNA.

This implies that AZA affects the expression of AQP1 through a mechanism that leads to a

decrease in AQP1 mRNA, as detected by our primers (Fig 9C).

Interestingly, a rebound phenomenon was seen at 24 hours in both immunoblots and RT-PCR,

with a notable increase in mRNA levels and a modest increase in protein. This rebound

expression pattern in AQP mRNA is similar to that seen in rodent studies involving a decrease in

AQP1 in low-gravity situations before subjects are returned to normal gravity and AQP1 levels

normalize [29]. It may be reasonably suggested that re-expression of AQP1 after knockdown

commonly follows this pattern.
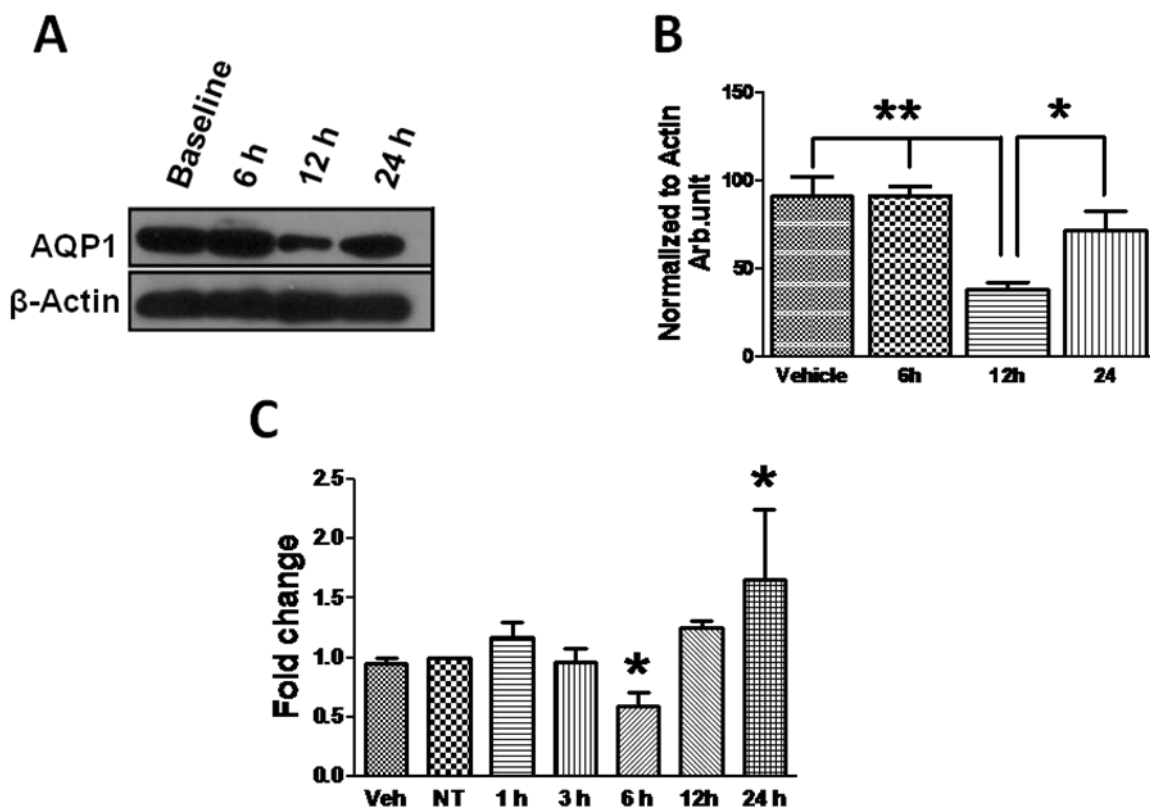
Figure 9 AQP1 protein and mRNA expression levels in AZA-treated CP primary culture
(A) Immunoblot of AQP1 expression, normalized to β-Actin. (B) Densinometric Analysis of AQP1 immunoblot. Decrease of AQP1 protein at 12 hours is significant. (C) RT-PCR of AQP1 mRNA levels, normalized to 18S (Veh = Vehicle; NT = Nontreated).

Immunocytochemistry of AZA-treated CP Culture Corroborated RT-PCR and Immunoblot trends of AQP1 expression

Immunoblots and ICC of AQP1 in AZA-treated CP cells revealed a continual decrease in protein

level over 12 hours, after which it returns to baseline levels by 24 hours (Figs 9A-9B, 10A-10B).

Digital densitometric quantification of AQP1 pixel intensity corroborated previous observed

trends, which indicated significant changes at 6 and 12 hours (Fig 10B). Images also revealed

that the resurgence of AQP1 immunoreactivity as evidenced by an increase of AQP1 punctate staining in the cytoplasm, further suggesting that this was likely due to increased mRNA expression as observed previously. Interestingly, a distinct change in the AQP1 expression pattern was also clear upon AZA treatment. The cells appear to lose a continuous network of AQP1 protein spanning over many cells. Instead, the protein localizes to the plasma membranes in a manner that outlines the individual cell borders (Fig 10A). This change may merely be an intermediate step in the re-expression of AQP1 after knockdown, but further experiments are needed to determine the significance of this change in expression pattern.

At 24 hours, ICC images of AZA-treated primary culture showed an increase in punctate AQP1 immunoreactivity within the cell. Earlier timepoints (0, 2 hrs) demonstrate AQP1 immunoreactivity with little or no puncta, while later images clearly show AQP1 puncta as protein levels return (6, 12, 24 hrs). In combination with immunoblot and RT-PCR results, the ICC data suggest the presence of immature or newly translated AQP1 as protein levels climb after reduction (Fig 10A). RT-PCR and immunoblots were used to determine if the effect of AZA on AQP1 is mediated at the level of transcription and translation. Results demonstrated a maximum decrease in AQP1 mRNA levels at 6 hours that corresponded temporally to the illustrated decreases in AQP1 protein levels at 12 hours (Fig 9C). It has previously been shown that AZA, while able to inhibit AQP4, does not affect AQP1 protein [48]. Our results are consistent with previous data, suggesting the effect of AZA on AQP1 does not involve direct interaction of drug with protein.
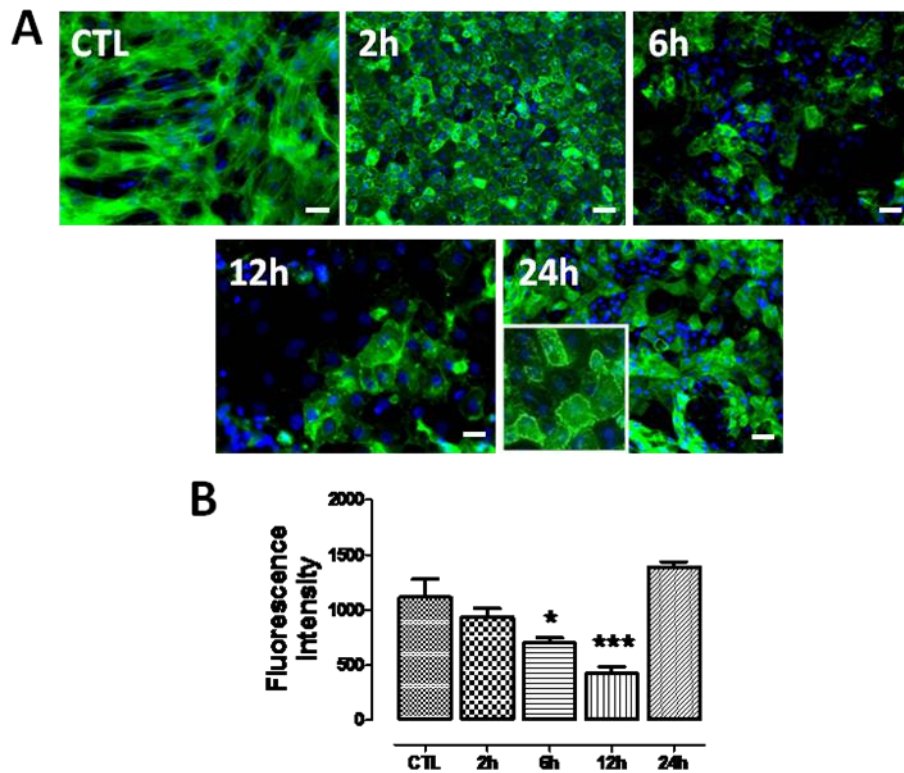
Figure 10 Immunocytochemistry of AQP1 in AZA-treated CP Primary Cultures
(A) AQP1 levels shown (green) along with Hoescht nuclear stain (blue). Gradual decrease and return of AQP1 protein is observed. Insert of 24h timepoint displays punctate of resurging AQP1 and strong localization to cell membranes. (B) Quantfication of AQP1 fluorescent intensity in ICC. Decrease in AQP1 at 6 and 12 hours are significant. Scale bar=20μm.

Development of an AQP1-Deficient Negative Control: Unsuccessful Implementation of Lentivirus Containg AQP1 shRNA

Preceding functional assays of drug effect on fluid flow in the choroid plexus, an AQP1-deficient

negative control was obtained. After unsuccessful knockdown of AQP1 in the CP primary

culture via lentivirus shRNA (Fig 11A) despite successful transduction of virus (Fig 11B), efforts

were shifted toward the TR-CSFB CP cell line (Fig 12). Immunoblots and immunocytochemistry

of AQP1 levels in TR-CSFB cells revealed minimal or undetectable levels of protein and hence this cell line was used as our negative control for the AQP1 rich CP primary culture (Figs 12A, 12B).

In order to determine the effect of the altered AQP1 expression on the fluid flow, it was necessary to develop control cells deficient in AQP1 for comparison. Prior to functional assays of fluid flow, focus was on acquisition of an AQP1-deficient negative control for the AQP1-rich CP primary culture cells. Initial focus was on the use of Lentivirus loaded with AQP1 shRNA (Santa Cruz Biotechnology). Cells were grown in a 6-well dish and treated with high (20μl/well) or low (10μl/well) titrations of lentivirus and efficiency of AQP1 knockdown was observed via immunoblot at 24, 48, and 72 hours. Control cells were transduced using the lower titration of 10μl of Cop-GFP lentivirus control vector and harvested at 72 hours. A lower titration of control lentivirus was utilized to show the level of transduction in the wells with less lentivirus. The CoP-GFP control transduction efficient, determined via fluorescent microscopy, was shown to be sufficient and hence it was determined that, even at the lower concentrations, the number of cells transduced with lentivirus was significant enough to produce an effect in the population (Fig 11B). Results indicate nearly non-existent knockdown of AQP1 protein (Fig 11A).
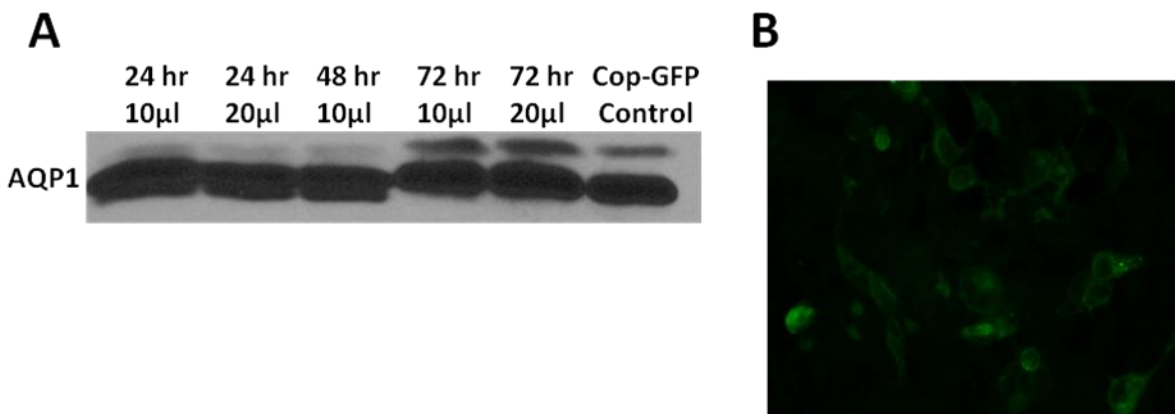
Figure 11 Lentivirus shRNA knockdown of AQP1 in CP Primary Culture
(A) Immunoblots of AQP1 in CP primary culture infected with titrations of lentivirus loaded with AQP1 shRNA. Results show no desirable change in AQP1 expression. (B) Image of cells infected with lentivirus loaded with Cop-GFP nucleic acid. A significant number of cells expressed GFP and were hence successfully infected by the lentivirus.

TR-CSFB CP Cell Line was Shown to be and Sufficient AQP1-deficient Control

As a substitute for the failed AQP1 lentivirus knockdown in primary culture, the TR-CSFB CP cell line was considered as an AQP1-deficient negative control. Experimentation went forward with this cell line after immunoblots of TR-CSFB extract (up to 500μg protein loaded) revealed naturally undetectable levels of AQP1 (Fig 12A). Immunocytochemistry of TR-CSFB corroborated the immunoblot results, showing nearly undetectable expression of AQP1 protein (Fig 12B).
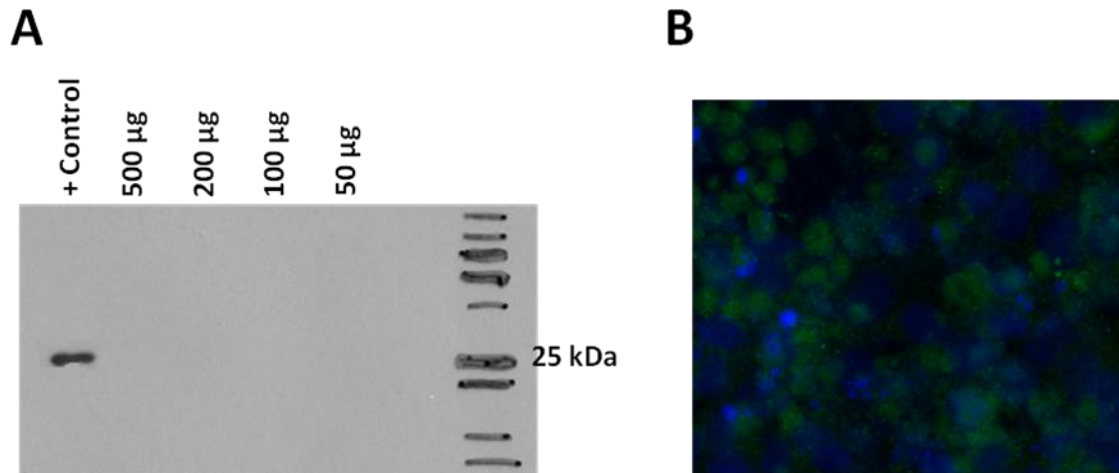
Figure 12 AQP1 levels in TR-CSFB CP cell line
(A) Immunoblot of AQP1 in TR-CSFB lysate, with positive control. Results reveal that AQP1 is expressed at undetectable levels, even with 500μg of protein loaded. (B) Immunocytochemistry of AQP1 in the TR-CSFB with AQP1 in green and DAPI nuclear stain in blue. Images show little AQP1 expression as settings were brightened to visualize AQP1.

Extracted Cells for Primary Culture were Confirmed to be CP Cells via Kir 7.1 Staining

Immunocytochemistry was performed to ensure that dissection for primary culture yielded

sufficient CP enrichment and also to make certain that the TR-CSFB cell line had not been

contaminated with other cells. The potassium channel Kir 7.1 is a known CP cell marker and was

hence used in this analysis [8]. Results indicate that primary culture is sufficiently enriched with

CP cells, as prescribed by CP culture guidelines (80% or greater enrichment is acceptable) (Per

Raymond Franklin, M.D./Ph.D., Head of the ORMC Pathology Department) (Fig 13A). The TR-

CSFB cell line also expressed sufficient levels of Kir 7.1 (Fig 13B).
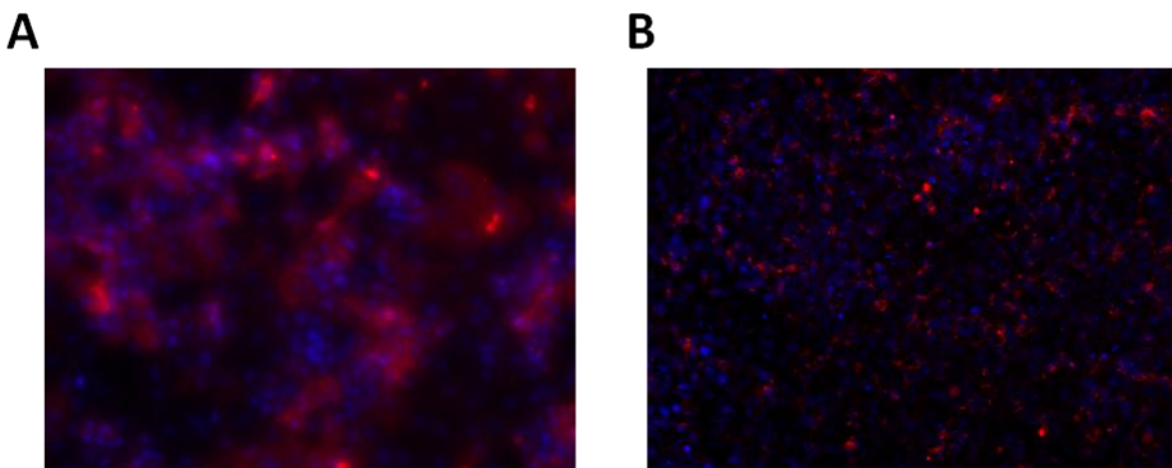
Figure 13 Kir 7.1 staining
(A) Kir 7.1 immunocytochemistry in primary culture showed significant reactivity and thus sufficient enrichment with CP cells. (B) Kir 7.1 staining in TR-CSFB cell line also showed sufficient enrichment with CP and hence no significant contamination of our cell line culture.

The TEER Measurement was Established as an Effective Method to Measure Confluency of Cell Cultures

Prior to functional characterization by fluid flow assay, it is critical to establish the confluency of

monolayers; leaky membranes may be a result of insufficient confluence and would lead to

unwanted fluid flow. Hence, it was essential to establish a methodology to achieve this goal. It is

widely accepted that the best method to establish confluency without harming the cells or the

monolayer is the use of TEER measurements. Prior to employing the EVOM TEER machine,

one must establish a minimum resistance reading to consider sufficiently confluent in ones

experimental system. To establish this value, the Lucifer Yellow Assay was performed and

results were compared with TEER values. TEER values were obtained for both primary culture

cells and TR-CSFB cells grown in transwell permeable supports when cells appeared confluent

via light microscopy. These values were compared to results of the subsequent Lucifer Yellow

Assay on the same membranes. Those TEER values consistently corresponding with confluence as determined by the Lucifer Yellow Assay were established as minimum resistances for determination of confluence. A sample of the obtained data is shown (Fig 14B).

It was suggested that the change in AQP1 expression patterns observed in the ICC images of AZA-treated CP cells could be manifestations of a morphological change in the cells that could compromise the monolayer such that it could lead to leakage of in the membrane (Fig 10A). Thus, we also investigated the effect of this potential morphological change on the integrity of the monolayer. To assay this, monolayers were determined to be confluent via TEER measurement and subsequently treated with AZA. TEER measurements were repeated at 1, 3, 6, and 12 hours after drug treatment to determine if any significant change in confluence occurred. It was determined that the observed morphological changes did not compromise the integrity of the monolayers in a significant manner – a sample of this data is provided (Fig 14C).
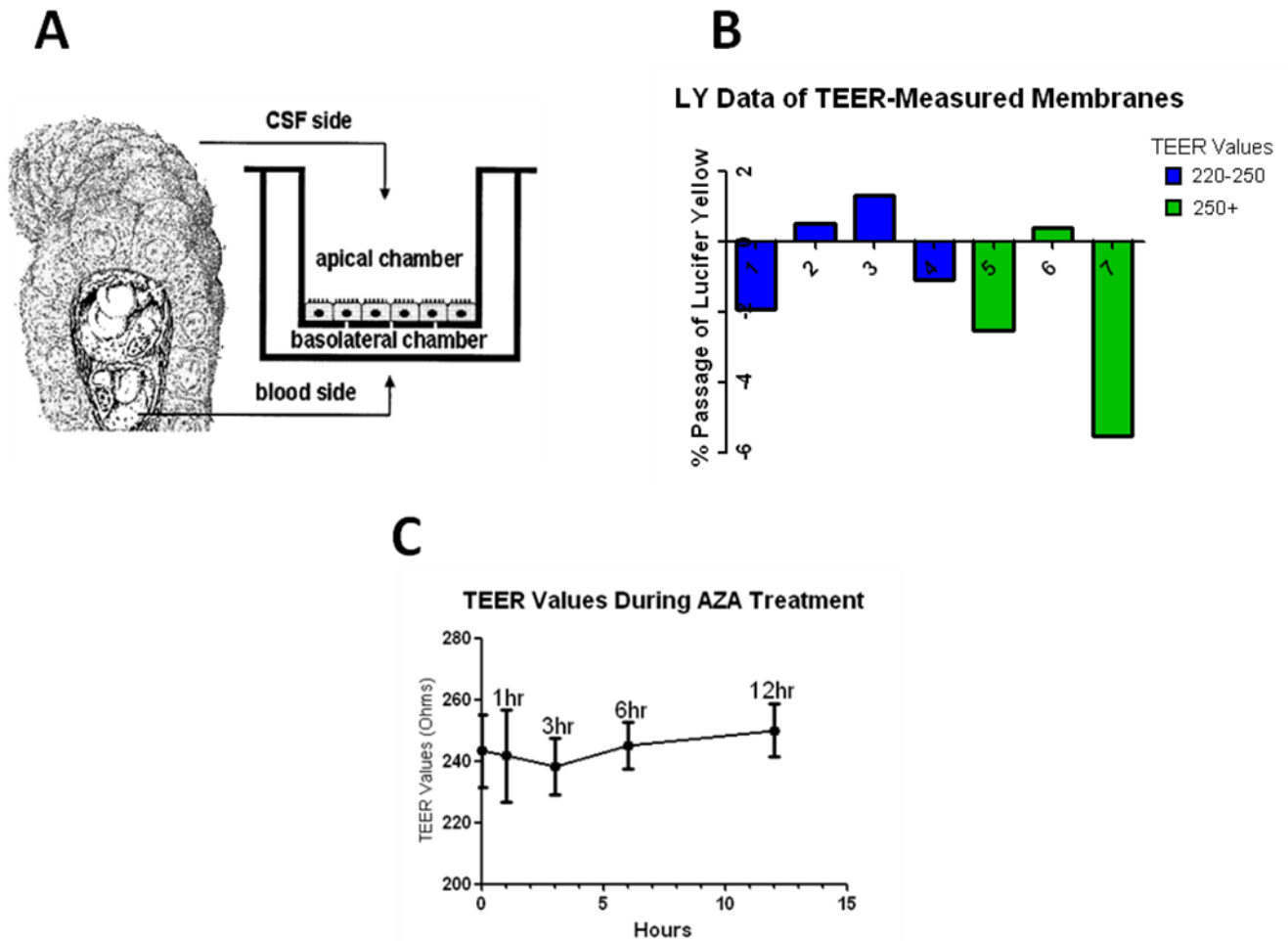
Figure 14 Preparation for Fluid Assays
(A) Chorid plexus cultures were grown in monolayer on transwell membranes to emulate the layer of ependymal cells that form the Blood-CSF Barrier. (B) Transepithelial Electrical Resistance values were standardize by systematic comparison with the Lucifer Yellow Assay. Monolayers with TEER values greater than 220 ohms were considered to be confluent. (C) The change in cell morphology observed in ICC data (Figure 10) created concern that AZA treatment may disturb the integrity of the monolayer. TEER values were continually taken after AZA-treatment began and compared with original TEER values prior to treatment. Although some fluctuation was observed, results show no significant variation in the obtained TEER values, indicated that AZA treatment does not harm the integrity of the CP monolayers.

**<u>Fluid Flow Shifts in AZA-treated CP cultures Precede and Mirror Expression Patterns
Observed in AQP1</u>**

Fluid assay with AZA was performed on confluent transwells (as determined by TEER

measurements). Both primary CP culture and TRCSF-B cell line demonstrated an immediate

functional change as evidenced by fluid transport away from the apical chamber at 2 hours

followed by a normalization of fluid flow as it again neared baseline levels (Figs 15A). Although

the shift in fluid flow preceded the decrease in AQP1 protein, the effects are of a similar trend

and, at this point, it was suggested the fluid flow may be a delayed effect of the trend in AQP1

expression.

Fluid assay was also performed in primary culture with Vinpocetine as well to confirm

implementation of methodology consistent with experiments performed by pervious lab

members. Interestingly, the results of this fluid assay again follow the same trend as those seen in

both of the AZA fluid assays – primary culture and TR-CSFB (Fig 15C). A similar trend in fluid

flow was observed with Vinpocetine despite the drug's opposite effect on AQP1 expression –

AZA leads to decrease in AQP1, while Vinpocetine leads to an increase in AQP1.

Primary CP cells grown in our transwell model of CSF production yielded an AZA-induced

functional change in fluid flow at 2 hours (Fig 15A). This decrease in apical flow represents a

decrease in CSF production, but occurs at earlier timepoints than the decreases in both AQP1

mRNA and protein. Notably, the apical fluid flow was always decreased at 2 hours and the

corresponding basolateral fluid flow was not necessarily equal or opposite in nature, suggesting

retention of fluid by CP cells during AZA treatment, which is in accordance with the changes in

cellular appearance seen in ICC data. Notably, AZA has previously been shown to increase cell volume and water retention [59]. AQP1 protein expression has also been shown to be upregulated in injured astrocytes, which have a tendency to retain water – suggesting a possible role of AQP1 in this retention [55].

The TRCSF-B cell line expressed very low levels of AQP1 protein, and was therefore used as a negative control for AQP1-rich CP primary culture cells (Fig 12). Transwell studies of confluent TRCSF-B cells demonstrated similar fluid flow characteristics (as seen in primary culture) despite the lack of AQP1 protein, suggesting that the observed effects of AZA are independent of AQP1 expression (Fig 15B).

Furthermore, the fluid assay done with Vinpocetine actually also showed a similar trend in fluid flow as the fluid assay done with AZA (Fig 15C). This is despite the increase in AQP1 illustrated by our earlier data (Fig 1). Both AZA and Vinpocetine have been suggested as pharmaceuticals of interest in the treatment of hydrocephalus and such thought appears to be justified by the similar decrease in fluid flow observed via treatment with both drugs. However, when these fluid flow similarities are juxtaposed with the distinct differences in the effect of these drugs on AQP1 expression, the data implies that the alteration of fluid flow and change in AQP1 protein levels are independent of each other.
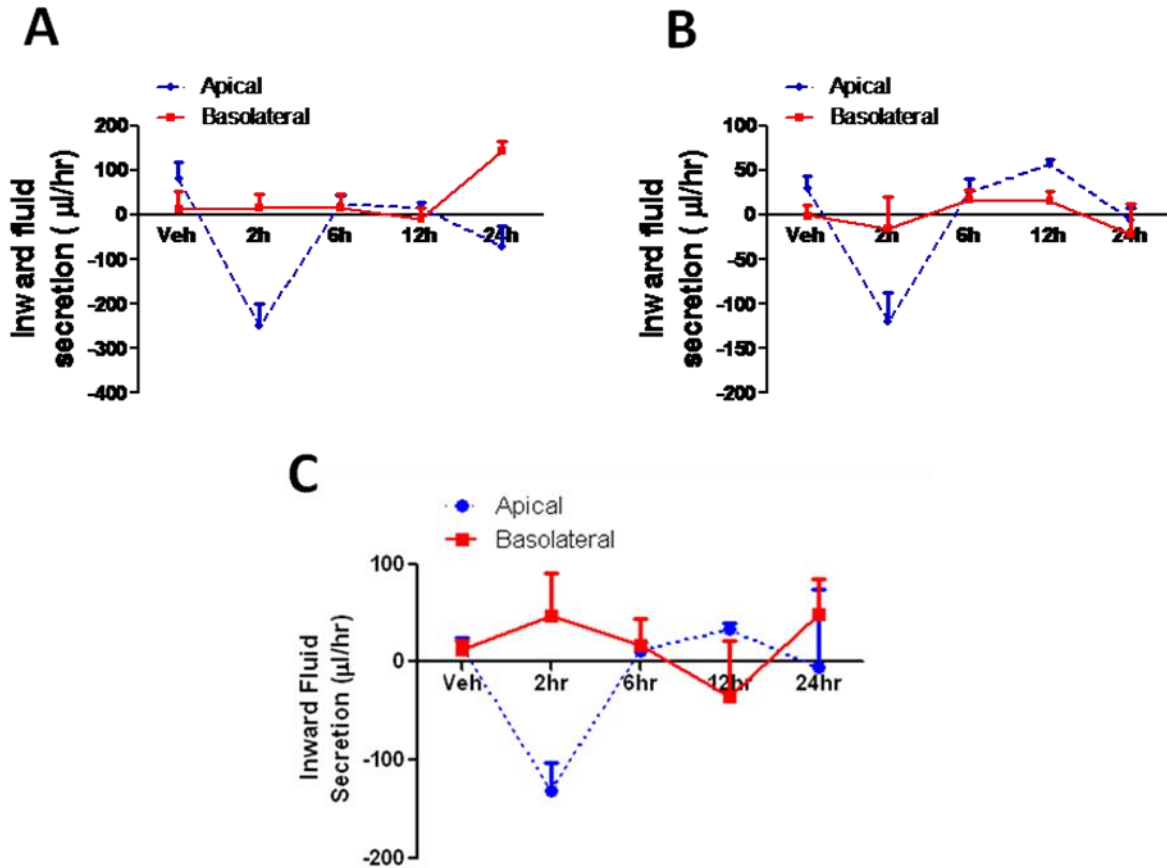
Figure 15 TRITC-Dextran Fluid Assay of drug-treated Confluent Monolayers.
(A) Fluid assay of CP Primary culture shows an AZA-induced decrease in apical fluid flow at 2 hours. (B) Fluid assay of AZA-treated TRCSF-B cell line culture displays a similar trend to that seen in primary culture. (C) Fluid assay of Vinpocetine-treated CP primary culture cells also show a similar trend to the above fluid assays, despite the increase in AQP1 we have shown to be induced by Vinpocetine and the decrease in AQP1 we have shown to result from AZA treatment. These results appear to indicate that the change in fluid flow observed from both Vinpocetine and AZA treatment are independent of AQP1, despite the effects these drugs have on the expression of this protein.

DISCUSSION

Controversy exists in the literature regarding the effect of AZA on AQP1 expression, as well as the relationship between the expression of AQP1 and the apical fluid flow in choroid plexus cells. Some researchers claim AQP1 plays a vital role in the CP fluid flow mechanism, while others insist it is unrelated [3, 46, 47]. These seemingly contradictory findings may be reconciled with our findings of early fluid flow changes and delayed AQP1 alterations upon treatment with AZA. Our data suggests that changes in AQP1 expression is affected by AZA treatment, but is also not directly responsible for CP apical fluid flow alterations as previously suggested [3, 4, 49]. To our knowledge, we are the first to show that AZA treatment leads to changes in AQP1 expression and alters apical fluid flow, while also determining that these occurrences are independent of each other.

A recent study by Tresguerres et al. (2009) demonstrates soluble adenylyl cyclase (sAC) is activated by the presence of bicarbonate ion [60]. Adenylyl cyclase forms cyclic AMP (cAMP), which causes significant increases in the levels of AQP1, presumably through activation of PKA [61-63]. In studies of sAC and the localization of renal vacuolar H+-ATPase, treatment using AZA and the sAC-inhibitor KH7 were shown to have similar effects, implying that the action of AZA is similar to the action of KH7. Moreover, in H+-ATPase experiments, the addition of 6-MB-cAMP negated the effects of both treatments, suggesting sAC deactivation as an effect of AZA treatment [64]. Furthermore, Thyroid Transcription Factor-1 (TTF-1) is known to be co-

expressed with AQP1 in rat CP and also known to enhance the transcription of the AQP1 gene [65]. In lung, cAMP activates TTF-1 via phosphorylation mediated by PKA [66].

When combining all these findings with our data, it may be reasonable to hypothesize that AZA acts through adenylyl cyclase, and subsequently PKA and TTF-1, to affect the expression of AQP1 in a manner independent of fluid flow. Because Acetazolamide is a known inhibitor of carbonic anhydrase (CA), the enzyme responsible for conversion of carbonic acid to bicarbonate [5], it is likely that the AZA-mediated inhibition of CA leads to a decrease in the levels of bicarbonate which leads to a reduction in adenylyl cyclase activation, followed by an decrease in AQP1 protein levels mediated by PKA and TTF-1 (Fig 16) [60, 65, 66].

Although literature would suggest that AZA most likely acts through the adenylyl cyclase pathway discussed above, it is important to recognize that other mechanisms may also be involved in the observed changes in AQP1 expression and fluid flow. The variations in fluid flow could be related to the altered activation of adenylyl cyclase by a second pathway that is also initiated by adenylyl cyclase activation.

Alternatively, the fluid flow changes may result from some other effect of AZA treatment on CP cells, such as shifting of ion concentrations – it has previously been shown that shifts in the concentrations of sodium and hydrogen ions alter fluid flow [59, 67]. Such a phenomenon would be consistent with the characterization of AQP proteins as passive diffusers of water in the presence of changes in osmotic balance since, in such cases, the ion gradient would be the

driving force behind the flow of fluid [23], although this depiction of AQP proteins is not unanimously accepted.

Interestingly, there is controversy regarding the ability of AQP proteins to channel ions in addition to water. In one study, cGMP was shown to affect the ion-channeling abilities of AQP1 [68-71]. Although this effect of cGMP could not be replicated by other groups [72-77], such an effect could significantly affect the fluid flow dynamics of CP cells, and hence may be worthy of study.

In our ICC data, later time points show the re-expression of AQP1 protein to correspond to the appearance of AQP1 immunoreactive puncta. The presence of puncta implies that newly synthesized AQP1 protein is present in endosomes after the initial decrease in AQP1 protein. Because PKC has been shown to mediate AQP1 trafficking in a manner dependent upon tonicity and microtubule dynamics [78], future studies may investigate the relation of PKC activation levels on expression of newly synthesized AQP1 in the context of AZA-treatment of CP cells.

Future studies should focus on precise elucidation of the effects of AZA treatment on adenylyl cyclase activation, cAMP/PKA, cGMP/PKC, and fluid flow, with minor focus on AQP proteins and their relationship to these mechanisms. Understanding the exact mechanisms of AZA treatment on choroid plexus cells may lead to new therapeutic targets and development of improved drugs development to treat cerebral edema and other fluid disorders, such as hydrocephalus.

To further study the effect of AZA on AQP1 in more detail, immunocytochemistry should be performed on drug-treated CP cells in the absence of permeabilizing agents. This will allow for assessment of the effect of drug treatment on the surface expression of AQP1. In conjunction, studies could be done to determine if decreases in visualized AQP1 are mediated by proteolytic of endocytic pathways. This could be done by ICC evaluating the colocalization of AQP1 protein with endosome markers such as Rab5 to determine the extent of endosome-mediated decreases in AQP1 surface expression.

In the future, it may also be advantageous to establish an effective method of AQP1 knockdown as a more appropriate negative control to AQP1-rich CP cell (in place of TR-CSFB cell line). Toward this goal, lentivirus transduction with higher virus titers, smaller wells, or a different target sequence may be implemented before conclusions regarding the efficacy of lentivirus-mediated AQP1 knockdown are made.

Conclusions

AZA treatment of choroid plexus monolayers decreases the apical fluid flow and leads to an associated fall and subsequent resurgence in AQP1 expression at both the protein and RNA levels. This drug-induced change in AQP1 expression may be mediated by adenylyl cyclase, cAMP, PKA, and TTF-1 (Fig 16); while the shift in fluid flow may result from shifts in ion concentrations (perhaps mediated by cGMP).

Future studies should focus on further solidifying the relationship between AZA, AQP1, and apical fluid flow. Successful elucidation of these mechanisms behind the AZA-induced functional change in CP fluid flow could provide new drug targets in the treatment of fluid disorders in the brain (including hydrocephalus) as well as other organs.
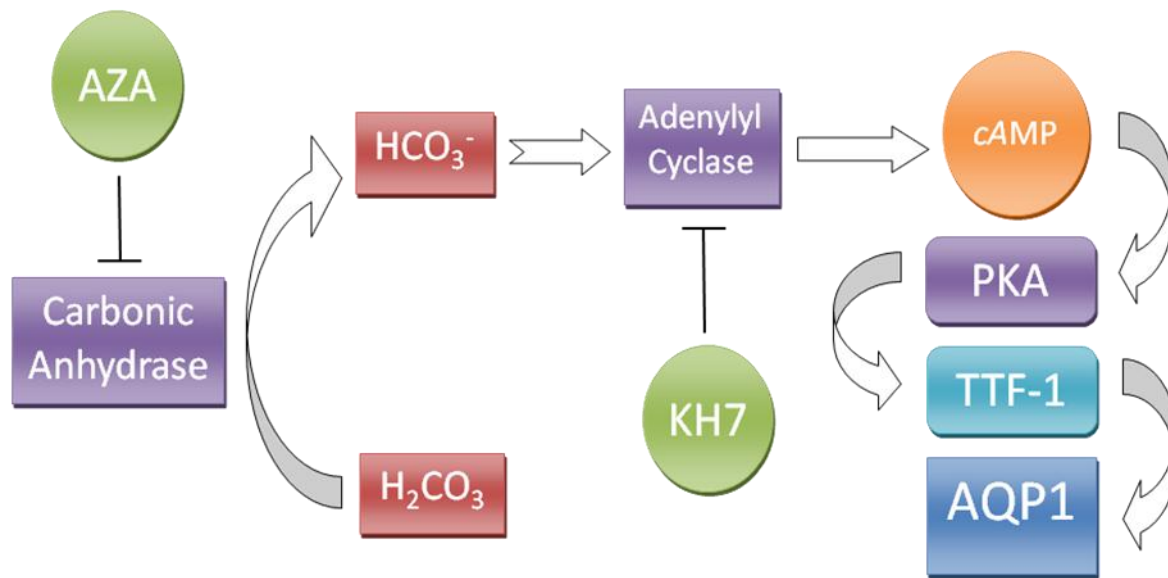
Figure 16 Proposed mechanism of AZA effect on AQP1 expression based upon current literature

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