Hydrocephalus: the role of cerebral aquaporin-4 channels and computational modeling considerations of cerebrospinal fluid

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Aquaporin-4 (AQP4) channels play an important role in brain water homeostasis. Water transport across plasma membranes has a critical role in brain water exchange of the normal and the diseased brain. AQP4 channels are implicated in the pathophysiology of hydrocephalus, a disease of water imbalance that leads to CSF accumulation in the ventricular system. Many molecular aspects of fluid exchange during hydrocephalus have yet to be firmly elucidated, but review of the literature suggests that modulation of AQP4 channel activity is a potentially attractive future pharmaceutical therapy. Drug therapy targeting AQP channels may enable control over water exchange to remove excess CSF through a molecular intervention instead of by mechanical shunting. This article is a review of a vast body of literature on the current understanding of AQP4 channels in relation to hydrocephalus, details regarding molecular aspects of AQP4 channels, possible drug development strategies, and limitations. Advances in medical imaging and computational modeling of CSF dynamics in the setting of hydrocephalus are summarized. Algorithmic developments in computational modeling continue to deepen the understanding of the hydrocephalus disease process and display promising potential benefit as a tool for physicians to evaluate patients with hydrocephalus.

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AQUAPORIN-4 (AQP4) channels are transmembrane proteins that facilitate water transport in the brain. AQP4 channels are present throughout the brain, specifically concentrated in the end-feet of astrocytes and in the glia limitans interna and externa.^{15,53,92} They passively respond to osmotic gradients and play roles in fluid secretion, cell migration, brain edema, metabolism, and many aspects of cell homeostasis.¹³⁵ In addition to their role in brain water transport, AQP4 channels have also been increasingly linked to clearance of ions, metabolites, and even soluble proteins.⁵⁷ In the brain, AQP4 channels have been implicated in a variety of pathological processes, including traumatic brain injury, ischemic injury, vasogenic and cytotoxic cerebral edema, and hydrocephalus.^{64,65,105,110,129}

In hydrocephalus, impaired fluid homeostasis leads to CSF accumulation in the ventricular system, which causes

a variety of progressive or abrupt symptoms in patients. AQP4 channels are implicated in the pathophysiology of hydrocephalus, but their function is not sufficiently understood as a therapeutic target to manage hydrocephalus. The current treatment of choice for hydrocephalus is often limited to mechanical diversion of excess CSF. However, these strategies have many inherent complications. Ventriculoperitoneal shunts have a 5-year complication rate of 32%, and an overall failure rate (requiring shunt revision) of 46.3%.^{109,139}

For these reasons, modulating activity of AQP4 channels (to target CSF clearance) is an attractive therapeutic target for potential future pharmacological treatment of hydrocephalus. It is imperative that the complex mechanisms of brain water homeostasis be elucidated to appropriately develop these therapeutic molecular targets.^{3,8} AQP channels have a firmly established role in cerebral water ho-

ABBREVIATIONS AQP1 = aquaporin-1; AQP4 = aquaporin-4; AP-1 = activating protein-1; BBB = blood-brain barrier; CK-2 = casein kinase-2; ICP = intracranial pressure; mGluR-I = group I glutamate receptor; PKA, PKC, PKG = protein kinase A, C, G.

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FIG. 1. An illustration of hypothesized water transport in normal conditions via AQP4 channels in astroglial cells. The astrocyte is reconstructed from a stack of confocal images (using Mimics reconstruction software).

meostasis; in fact, RNAi knockdown of AQP4 was shown to lead to a 50% reduction in water diffusion at a cellular level as measured by diffusion-weighted imaging (via apparent diffusion coefficient measurements).^{10,45,53,91,121}

Therefore, we have attempted to summarize relevant knowledge of molecular mechanisms of water exchange through AQP4 channels. We begin with an analysis of the current research on the relationship of AQP4 to hydrocephalus, followed by an overview of AQP4 isoforms, promoters, transcription factors, and posttranslational modification. Based on this molecular review of aquaporin channels, we have summarized research on AQP4 as a therapeutic target. We review prior efforts and now present new concepts on improving computational modeling of hydrocephalus as a route to improve diagnosis.

Aquaporin-4 and Hydrocephalus

Hydrocephalus is characterized by an accumulation of CSF in brain ventricles, which may be caused by either CSF overproduction or impaired CSF absorption. In either case, AQP water channels may be involved in the pathogenesis of the disease due to their role in fluid homeostasis. Aquaporin-1 (AQP1) channels are found on epithelial cells in the choroid plexus, and serve in CSF secretion. AQP4, AQP-5, and AQP-9 channels are localized on astrocytes and ependymal cells.¹¹ More specifically, AQP4 is present on astrocytic end-feet processes (adjacent to brain vasculature, the blood-brain barrier [BBB] and blood-CSF barriers), glia limitans interna (astrocyte/ependyma

bordering brain and subarachnoid CSF), and glia limitans externa (astrocyte bordering brain and ventricular CSF).¹¹

Bloch and Manley proposed a transparenchymal pathway of water clearance into the cerebral vasculature in obstructive hydrocephalus, observing an increase in water content in the parenchymal extracellular space in *Aqp4*-null mice in addition to the increase in lateral ventricle size in these mice.¹⁵ This evidence suggests that AQP4 channels play a role in water clearance in obstructive hydrocephalus from both the ventricle and brain extracellular spaces. Figures 1 and 2 illustrate a hypothesis of AQP4 function in cerebral water transport in normal conditions and in hydrocephalus; water flux is shown with arrows. The astrocyte is reconstructed from a stack of confocal images (using Mimics reconstruction software). AQP4 channels appear to be implicated in brain homeostasis and in central plasma osmolarity regulation.¹¹

AQP4-mediated water transport may be employed in hydrocephalus to remove excess CSF, as shown in Fig. 2. The location-specific distribution of AQP4 around “tissue interfaces that are close to CSF and thus water (ventricles, subarachnoid space)” along with the distribution of AQP4 adjacent to the BBB indicates that these channels play a role in water regulation physiology applicable to hydrocephalus.³⁷ Mobasher et al. used protein and tissue microarrays to show abundant human tissue AQP4 expression in the cerebral cortex, cerebellar cortex, ependymal cell layer, hippocampus, and spinal cord, but lower levels in the choroid plexus, white matter, and meninges; these findings

FIG. 2. An illustration of hypothesized water transport in hydrocephalic conditions, allowing for clearance of excess CSF from the ventricular system via AQP4 channels in astroglial cells.

indicate that AQP4 may play an active role in regulating water and ion concentrations in the CSF.^{37,86}

Currently, the majority of existing studies of AQP4 channels and hydrocephalus use experimental animal models. From most studies surveyed, AQP4 channels are found to be upregulated in response to hydrocephalus. However, currently both the specific molecular pathways and the threshold at which upregulation of AQP4 channels occur have not been elucidated.

After inducing acute hydrocephalus in rats with kaolin, Skjolding et al. found that the spatiotemporal distribution of AQP4 channels significantly increases in the days and weeks following induction, suggesting that modifications in AQP4 distribution are part of an important neurodefensive function.¹¹⁹ Mao et al. used a kaolin injection model to study rats with severe hydrocephalus over a longer time course. They also observed that AQP4 mRNA was significantly upregulated in the parietal cerebrum and hippocampus within 4 weeks after induction of hydrocephalus, but noted that channel expression does not increase significantly until rats become 7 weeks old (but then stays elevated at the 9-month marker).^{37,81} This indicates that a possible “time-sensitive and pressure or ECF-fluid-adaptive” mechanism may exist due to transparenchymal flow of CSF in hydrocephalus.^{37,81}

Following induction of hydrocephalus in rats by L- α -lysophosphatidylcholine stearyl injection, Tourdias et al. detected upregulation of periventricular AQP4 in hydrocephalic rats that was strongly correlated with both CSF

volume and periventricular apparent diffusion coefficient. Specifically, AQP4 was first mainly located on astrocyte end-feet, but was later distributed over the whole membrane of astrocytes.¹³⁰ These results also suggest that upregulated AQP4 expression is a physiological adaptation to induced hydrocephalus, possibly to aid and facilitate removal of excess water.

In rats with congenital hydrocephalus, Paul et al. found increased expression of AQP4 at the brain-CSF interfaces as well as surrounding astrocyte end-feet and subpial layers in hydrocephalic animals, which also suggests similar compensatory mechanisms to regulate choroidal CSF secretion and parenchymal fluid absorption.¹⁰⁰ However, AQP4 concentration may not always directly relate to severity of ventricle enlargement in rats with congenital hydrocephalus, as demonstrated by Shen et al. They observed that AQP4 was highly expressed even if rats did not display gross ventriculomegaly (suggesting either a compensatory pathway development or secondary modification in CSF circulation).¹¹⁶

Feng et al. experimented with *Aqp4*-knockout mice, which produced a sporadic 9.6% rate of encephalomegaly (triventricular hydrocephalus with elevated intracranial pressure [ICP]) by the age of 6 weeks, linking AQP4 to the development of congenital hydrocephalus in mice.³⁴ Bloch et al. found accelerated progression of kaolin-induced hydrocephalus in *Aqp4*-knockout mice when compared with controls, with a significantly higher lateral ventricle volume and ICP measurements as early as 3 days after

injection, suggesting that the lack of AQP4 channels is related to the development of hydrocephalus.¹⁴ AQP4 has also been shown to be upregulated after ischemia, but in *Aqp4*-knockout mice, there is reduced edema formation seen following focal cerebral ischemia, which also suggests a role of AQP4 channels in other aspects of brain water homeostasis.¹¹

Hydrocephalus is a disorder characterized by ventricular enlargement, which could arise from different etiologies (congenital or secondary forms) caused by distinct mechanisms at the molecular level. The role of AQP4 may differ depending on the type of hydrocephalus. Excess ventricular fluid accumulation may be caused by CSF overproduction, impaired reabsorption due to obstruction or inflammation, or a combination of both mechanisms. In *Aqp1*-knockout, *Aqp4*-knockout, and wild-type mice, Igarashi et al. found that following intravenous injection of H₂¹⁷O, water entry into the brain is conducted through AQP4 channels, not AQP1, and that the major site of brain water entry may be the Virchow-Robins space.⁵⁶ In certain types of hydrocephalus, AQP4 channels may be involved in the overproduction of CSF. In these forms of hydrocephalus where overproduction of CSF is implicated, inhibition of AQP4 may be therapeutically desired. It is important to understand the molecular mechanism of hydrocephalus to best design therapies to facilitate the clearance of fluid.

Clearly, numerous studies have elucidated that a link exists between AQP4 channels, brain water balance, and hydrocephalus. The CSF cycle commences at AQP channels in the choroid (i.e., AQP1) and may have a terminus at the BBB interface linking capillary fluid exchange to interstitial fluid through AQP4 channels.^{95,96} This CSF route may also be active in the developing fetal brain, in which the arachnoid villi are not yet developed.⁴⁴ Iliff et al. proposed that CSF cycles through the brain interstitial space, traveling across perivascular spaces and effectively functioning as a sink for brain extracellular solutes, which are removed from cells via AQP4 channels.⁵⁷ According to their hypothesis, AQP4 channels can clear water and solutes from CSF and brain parenchyma. In *Aqp4*-knockout mice, bulk flow-dependent clearance of interstitial solutes decreased by approximately 70%.⁵⁷ Iliff et al. posited that the role of AQP4-dependent solute clearance serves as a glial-vascular pathway, which they described as the “glymphatic system.”⁵⁷ Quantifying water fluxes across AQP4 channels in the glymphatic pathway (and thus in brain water balance) would be central to exploring the potential of AQP4 channel modulation as a critical step toward molecular treatment options for hydrocephalus. Increasing AQP channels to facilitate CSF clearance to venous drainage, or decreasing AQP channels in areas of CSF production, may both be reasonable strategies for helping to restore and normalize ventricular volumes in patients with hydrocephalus.

Aquaporin-4 Isoforms, Orthogonal Arrays, and Water Permeability

AQP4 has 3 known isoforms in rats (M1, M23, and Mz), and 2 isoforms in human beings (M1 and M23).^{26,112} Following stimulus, astrocytes can alter the ratio of dif-

ferent AQP4 isoforms through alternative promoter use to regulate membrane water permeability.¹⁰³ The *Aqp4* gene encodes for 2 different mRNAs with different translation-initiating methionines (M1 or M23); however, both isoforms are expressed in AQP4-expressing tissue.¹³⁶ The existence of multiple AQP4 isoforms has been associated with distinct translational efficiencies, organ-specific expression, developmental regulation, formation of square arrays on cellular membranes, and different water conduction rates.^{5,25,35,39,137,141} In rats, 6 cDNA isoforms resulting from alternative splicing have been discovered, and 3 protein isoforms (M1, M23, and Mz) were identified.^{87,112} In the brain, the ratio of M23 to M1 isoforms is 7:1, and the quantity of Mz isoform is very scarce.⁹⁰ The Mz isoform is absent in human tissues due to the presence of a stop codon.

AQP4 channels exist in the endoplasmic reticulum and Golgi membranes as tetramers, but are seen in large square arrays in the plasma membrane.¹¹¹ AQP4-channel tetramers assemble into higher-order aggregates termed orthogonal arrays (or square arrays) through the interaction of critical residues on adjacent tetramers.³⁹ The formation of AQP4 oligomers on the plasma membrane is a membrane curvature-dependent process that involves yet unknown factors on the plasma membrane. Most likely, the assembly of AQP4 into oligomers occurs prior to expression on the plasma membrane, but not in the endoplasmic reticulum or the Golgi membrane. Given that the size of transport vesicles for proteins traveling through the secretory pathway generally falls between 40 and 70 nm,¹³² AQP4 channels are probably transported in vesicles as tetramers.¹¹¹

Square arrays are approximately 30–150 nm in size, as observed in electron micrographs of astroglial end-foot membranes.¹⁰⁷ The 3 isoforms differ in their ability to form orthogonal arrays on cellular membranes. The M23 isoform forms large square arrays, the M1 isoform forms small or unstable square arrays, and the Mz isoform does not form arrays.^{39,107,112} The expression of M23 and M1 isoforms in different ratios can control square array size.³⁹ The interference of M23-M23 interaction by a residue at the N-terminus of M1 prevents the formation of stable arrays between M1-M23 or M1-M1 interactions.¹²⁴ The functions of these orthogonal arrays are currently an active area of research. The size of square arrays influences the water conductivity of AQP4 channels. The single-channel water permeability of M23 is approximately 8 times greater than that of M1, likely due to the ability of M23 to form stable square arrays.¹¹⁸ It has been further suggested that the larger the array, the more it is able to accommodate a higher water flux through the membrane induced by an osmotic gradient, consistent with the speculation that AQP4 could play an adhesive role at gap junctions.⁴⁸

At the end-foot cellular membrane near the perivascular space, the anchoring of AQP4 requires α -syntrophin, a member of the dystrophin complex. Syntrophin-null mice show normal overall levels of AQP4 in the brain, but have a 90% reduction in AQP4 immunolabeling in the end-foot near the perivascular space.^{7,89} However, only a 50% reduction is observed in end-feet opposing the pial mem-

brane.⁷ It is possible that molecules other than α -syntrophin are required to maintain the polarization and anchoring of AQP4 near the end-foot membrane opposing pia. The difference in anchoring mechanisms of the different cellular AQP4 pools may indicate a differential regulation of AQP4 membrane expression during neurological injury, where AQP4 anchoring in certain end-foot structures may be preserved while in others it is disrupted to control the region-specific dynamics of water clearance.

Because AQP4 anchoring in different domains of the astrocyte membrane is achieved by different molecular complexes, the spatially heterogeneous expression patterns of AQP4 in the postinjury brain (such as end-feet around the BBB versus pial end-feet) are likely achieved through differential regulation of these membrane pools through distinct signaling pathways.⁷ Loss of AQP4 at the end-feet opposing the BBB has been found after ischemia, also supporting the idea of cell region-specific anchoring mechanisms.⁶⁸ However, this role of AQP4 array in intercellular adhesion is generally debated.^{38,143}

Alternative Promoters and Transcription Factors

The use of alternative promoters of the *Aqp4* gene and the differential activation of these promoters during pathological processes may have implications in the regulation of brain water permeability in disease processes, including hydrocephalus. As previously mentioned, numerous studies have shown upregulation of AQP4 expression in hydrocephalic states. Regulatory mechanisms of AQP4 and its role in neurological diseases were recently reviewed by Hsu et al.⁵³ Transcription factors capable of activating the *Aqp4* gene may play an important role in the regulation of AQP4 expression in neurological disorders; in oxidative stress, it has been elucidated that gene nuclear factor (erythroid-derived 2)-like 2 (Nrf2) activates AQP4.^{131,144} Umenishi and Verkman studied the promoter regions associated with exon 0 and exon 1 of the human *Aqp4* gene, and found that the M1 isoform is encoded starting from exon 0, and the M23 isoform from exon 1.¹³¹ Exon 1 has stronger transcriptional activity than exon 0. However, promoter activity of exon 0 has stronger relative activity in brain-derived cells than kidney-derived cells, indicating an organ-specific regulation.

For the M1 isoform, the exon 0 promoter contains 4 TATA boxes, 5 CCAAT boxes, activating protein-1 (AP-1), Sp1, and E-box elements.¹³¹ AP-1 is the primary target of the p38 mitogen-activated protein kinase signaling pathway, which has been confirmed to upregulate AQP4.⁹⁷ On the other hand, E-box is known to have strong inhibition activities on *Aqp4* transcription.¹³¹ In the mouse *Aqp4* gene, an astrocyte-specific enhancer has been identified upstream of exon 0.¹ This enhancer is associated with the Pit-1/Oct/Unc-86 transcription factor family, and is probably responsible for the high expression of AQP4 in brain astrocytes compared with other cell types. However, the roles of the rest of the binding sites in the promoter are unknown. CCAAT-enhancer binding proteins belong to the acute response genes, however, and their effect on AQP4 expression is not known.

For the M23 isoform, the exon 1 promoter contains 1 TATA box, AP-1, AP-2, and E-box elements.¹³¹ A possible binding site for NF- κ B transcription factor in exon 1 promoter construct has been found, and interleukin-1-induced NF- κ B activation has been confirmed to upregulate AQP4.⁵⁹ AP-2 is a developmentally regulated transcription factor that participates in embryogenesis, and may be a potential regulator of AQP4 in the developing brain.¹³⁸ The expression of both M1 and M23 AQP4 isoforms increased in the postnatal periods of rats.¹³⁷ Moreover, AQP4 expression in the hippocampus increases gradually from P9 through 3 weeks and 6 weeks in a laminar-specific pattern.⁵² The possible role of AP-2 in AQP4 developmental regulation remains to be elucidated.

Posttranslational Modification

Astrocytes regulate their membrane water permeability through AQP4 channels by employing transcriptional, translational, and posttranslational control mechanisms. Neurological disease processes such as hydrocephalus may involve the dysregulation of AQP4 signaling pathways—a product of a combination of transcriptional events and pathways, as depicted in a signaling map by Hsu et al.⁵³ After the translation process, phosphorylation of several amino acid residues on the polypeptide chain is required for the correct trafficking, expression, and membrane targeting of AQP4, as well as for its assembly into higher-order arrays. The phosphorylation of AQP4 can effectively and rapidly change membrane water permeability through either endocytosis of the water channels or the disruption of square array formation. In terms of pharmacological interventions, targeting AQP4 posttranslational mechanisms, such as phosphorylation, will probably result in a rapid and transient effect compared with transcriptional or translational regulation strategies. Here, we briefly summarize posttranslational modification of the AQP4 polypeptide and its effect on the function and subcellular localization of AQP4.

Immediately after the emergence of the polypeptide from the ribosomes, the translocon Sec61 α inserts the 6 membrane domains sequentially into the endoplasmic reticulum membrane.¹¹³ After the translation event, the transition through the Golgi apparatus requires the phosphorylation of AQP4 by casein kinase-2 (CK-2) at several amino acid residues in the C-terminal domain. AQP4 is constitutively phosphorylated, and a quadruple-substitution mutant of M1 AQP4 at Ser²⁷⁶, Ser²⁸⁵, Thr²⁸⁹, and Ser³¹⁶, which prevents its phosphorylation by CK-2, accumulates inside the Golgi apparatus. This evidence strongly suggests that the phosphorylation of these residues is required for the Golgi transition of AQP4.⁶¹ Leaving the Golgi apparatus, AQP4 proteins localize to the cell membrane. Confirmed AQP4 phosphorylation sites and their effects on AQP4 trafficking are shown in Fig. 3, built upon an AQP polypeptide adapted from Ratelade and Verkman.¹⁰⁸

The phosphorylation of AQP4 at different amino acid residues can alter the single-channel water permeability (as shown in the left column of Fig. 3). Gunnarson et al. found that water permeability is increased through the phosphorylation of Ser¹¹¹ by the activation of group I glu-

FIG. 3. Confirmed AQP4 phosphorylation sites and their effects on AQP4, as depicted on an AQP polypeptide. Structure adapted from Ratelade and Verkman 2012.

tamate receptors (mGluR-I).⁴⁶ The activation of mGluR-I can be induced by endogenous glutamate or by an agonist.⁴⁶ mGluR-I stimulation causes the release of Ca²⁺ from intracellular stores, which leads to nitric oxide production and the activation of protein kinase G (PKG), which phosphorylates Ser¹¹¹.⁴⁶ Silberstein et al. found that a phosphomimetic M23-Ser^{111E} forms 2.5-times larger square arrays compared with wild-type M23 and possesses a 1.5-fold increase in single-channel water permeability.¹¹⁸

However, Assentoft et al. did not find an increase in water permeability in oocytes transfected with AQP4 with a phosphomimetic Ser¹¹¹ site or in oocytes transfected

ed with AQP4 and treated with protein kinase A (PKA) or PKG activators.⁹ They also did not detect evidence of Ser¹¹¹ phosphorylation *in vivo* after treating animals with an mGluR1/5 analog.⁹ Their evidence argues against the regulation of AQP4 water permeability via Ser¹¹¹ phosphorylation. Because astrocytic Ca²⁺ waves are known to propagate through the astrocyte syncytium via interconnected gap junctions, it is possible that Ca²⁺ signaling is a fast-acting and long-range control mechanism of astrocytic membrane water permeability. Deletion of AQP4 channels has also been found to reduce Ca²⁺ signaling.¹²⁸

Following AQP4 expression on the cellular membrane,

posttranslational regulation mechanisms allow a rapid alteration of water permeability. A transient and rapid alteration of cell water permeability can be accomplished by the endocytosis of AQP4 channels expressed on the membrane. AQP4 is endocytosed through interactions of the tyrosine motif at the C-terminus with adaptor protein-2 complex in clathrin-coated pits.⁸⁰ AQP4 endocytosis was reduced by 80% when either the Tyr²⁷⁷ was replaced with an alanine, or when Val²⁸⁰ was replaced with a serine. The tyrosine motif YMEV at the AQP4 C-terminus directly interacts with the adaptor protein-2 clathrin-adaptor complex to mediate endocytosis. A majority of the endocytosed AQP4 is directed to the lysosomes for degradation, and this lysosomal targeting of AQP4 is increased when Ser²⁷⁶ is phosphorylated by CK-2.⁸⁰

A recent study showed that hypotonicity induces a rapid membrane localization of AQP4 through a PKA-calmodulin signaling mechanism, which involves the Ser²⁷⁶ phosphorylation site.⁶⁶ Interestingly, Ser²⁷⁶ phosphomimetic mutation is not sufficient to increase AQP4 membrane translocation, but the mutation of Ser²⁷⁶ to alanine blocks hypotonicity-induced translocation, indicating that Ser²⁷⁶ phosphorylation is necessary but not sufficient for AQP4 membrane translocation.⁶⁶ Together with the report that Ser²⁷⁶ phosphorylation increases AQP4 targeting to lysosomes, this evidence could suggest that the phosphorylation of Ser²⁷⁶ on AQP4 is a necessary signal for initiating subcellular targeting of the endosomal pool of AQP4, but the specific subcellular destination of the endosomal AQP4 relies on additional modification signals.

The internalization of AQP4 expressed in *Xenopus* oocytes can be initiated by protein kinase C (PKC) activation, which is at least partially mediated by the phosphorylation of Ser¹⁸⁰.⁸⁸ In PKC-induced internalization, AQP4 remains intact inside its cytosolic compartments without degradation, which can allow the reexpression of AQP4 on the membrane.⁸⁸ The phosphorylation of Ser¹⁸⁰ by PKC activators, including dopamine, has been reported to decrease the water pore permeability without the internalization of AQP4 in pig kidney epithelial cells as opposed to findings by Moeller et al. and Zelenina et al.^{88,142} These 2 studies show that the posttranslational regulation of cell membrane versus internal AQP4 pools are controlled by different mechanisms in mammalian versus oocyte cells. AQP4 upregulation after ischemia is also reversed by PKC activation at the transcriptional level, showing that PKC signaling reduces AQP4-mediated water permeability through both transcriptional and posttranslational mechanisms.⁶⁸ Reversible internalization of membrane AQP4 has also been shown to be triggered by histamine exposure.²⁰

Aquaporin-4 Channels: A Potential Therapeutic Drug Target for Hydrocephalus?

Current treatment strategies for hydrocephalus involve mechanical shunting of excess CSF. Surgery and mechanical strategies are effective, but are not without complications. A study of 14,455 individuals revealed a 5-year complication rate of 32% for ventriculoperitoneal shunts, and another study of 1015 individuals founds an overall failure rate (requiring shunt revision) of 46.3%.^{109,139} Del Bigio and

Di Curzio recently published a comprehensive review of current nonsurgical therapeutic options for hydrocephalus, reviewing efficacy of osmotic agents, acetazolamide, ion channel blockers, steroids, and other agents; they concluded that no compelling nonsurgical therapies have yet been developed for hydrocephalus.²⁸ Regarding osmotic agents, Del Bigio concludes that although mannitol is used widely in treatment of an acute “elevated ICP in situations of brain swelling,” it is seldom used in hydrocephalus.²⁸ Some studies have shown that mannitol/urea, which induces hyperosmolarity in the blood and induces water flux from brain to blood, may cause a rebound elevation in ICP when given to children with hydrocephalus.^{28,31,47}

The administration of osmotic agents alone has not been found to be useful. However, in the future, treatments aimed at interfering with CSF production may create non-invasive management options for some types of hydrocephalus. A review by Poca and Sahuquillo concluded that the most suitable current drug therapy for hydrocephalus appears to be acetazolamide (with or without furosemide). These authors also stated that osmotic agents are no longer considered efficacious in the treatment of hydrocephalus.¹⁰⁴ In 1980, Smith and Johanson showed that carbonic anhydrase inhibitors (acetazolamide and benzolamide) interfered with ion transport, decreasing basolateral sodium entry into the choroid epithelium and decreasing the rate of the apical sodium/potassium pump.¹²⁰ Acetazolamide has also been shown to inhibit AQP-mediated water conductance in relation to inhibition of choroid plexus carbonic anhydrase.¹²⁵ In human subjects, acetazolamide treatment has mixed reviews, with some authors noting therapeutic benefit in different case studies over the years; recent studies state that some patients are “responders” to acetazolamide therapy whereas others are “nonresponders.”^{73,24,60,82,83,85,102,123}

Gao et al. have described beneficial aspects of acetazolamide treatment, i.e., marked attenuation of thrombin-induced hydrocephalus in rats (intraventricular hemorrhage model); this also suggests a decreased CSF production mechanism.^{41,42} Poca and Sahuquillo mentioned efficacy with the injection of fibrinolytic therapy directly into the ventricular system to similarly aid posthemorrhagic hydrocephalus management by reducing postshunt complications, such as catheter obstruction.¹⁰⁴ In fact, several recent studies have found that intraventricular tissue plasminogen activator is a safe and effective method for improving CSF shunting, with low risk of bleeding, infection, and other adverse effects; intraventricular tissue plasminogen activator may aid an acute treatment strategy, but by itself it is not a long-term treatment method for hydrocephalus.^{30,106,146}

Currently, existing studies of water channels and hydrocephalus confirm that AQP4 channels are generally upregulated in response to congenital hydrocephalus.²¹ Either facilitating water clearance via AQP4 upregulation or reducing CSF production via AQP1 downregulation are both plausible mechanisms of action to help treat hydrocephalus.^{95,96} AQP channels are directly involved in bulk water movement in the brain, including CSF production and elimination.^{126,133,134,140} Water diffusion has been found to be altered in the brain via *Aqp* gene deletion, RNAi, lead exposure, and numerous other methods.^{10,45,53,91,121} By

using diffusion-weighted imaging to measure changes in water diffusion, Badaut et al. found that RNAi knockdown of AQP4 led to a 50% reduction in water mobility.¹⁰ As a therapeutic target, targeting AQP4 posttranslational mechanisms such as phosphorylation will probably result in rapid and transient changes in AQP expression compared with transcriptional or translational regulation strategies.

Current research on AQP4 channel modulation has shown that a variety of different compounds inhibit AQP4 channels. Via *in vitro* functional assays, arylsulfonamide drugs (antiepileptic agents) have been shown to function as AQP4 inhibitors.^{54,55} Similarly, gold and silver compounds have also been shown to function as AQP inhibitors.⁹³ Migliati et al. found that bumetanide and functional derivatives reduce AQP4 osmotic water flux in AQP4-expressing *Xenopus laevis* oocytes.⁸⁴ Kato et al. found that the general anesthetic propofol (2,6-diisopropylphenol) reversibly and specifically inhibits the osmotic water permeability of AQP4 proteoliposomes in the presence of Zn²⁺.⁶³ De Bellis et al. found that transfection of a human *Aqp4* splice variant exhibits a dominant-negative activity, decreasing AQP4 expression.²⁶

Ambarki et al. found that piroxicam pretreatment in ischemia-reperfusion injury is neuroprotective, with significantly reduced brain edema linked to a downregulation of AQP4 channel expression.⁶ An interesting evaluation would be to note whether the piroxicam effects on focal cerebral ischemia also translate to therapeutic benefit in AQP4 modulation as a treatment for hydrocephalus. Papadopoulos and Verkman highlighted potential benefits of AQP modulation in a variety of brain diseases; they noted that small-molecule AQP4 inhibitor development progress has been slow, stemming from technical issues and “poor druggability” of the AQP channel.^{98,135} They also described AQP4 inhibitors as 4 classes of AQP-targeted small molecules: “cysteine-reactive heavy metal-based inhibitors; small-molecule scaffolds that are reported to inhibit water conductance; small molecules that target the interaction between AQP4 and the NMO autoantibody; and agents that act as chemical chaperones to facilitate the cellular processing of NDI-causing AQP2 mutants.”¹³⁵

In terms of enhancing AQP4 channel function, there are many fewer researched methods. *Aqp* gene transfer is a promising concept, which has been shown to be efficacious in rats, pigs, and finally a small group of patients who were treated with adenoviral-mediated transfer of the *Aqp-1* cDNA as a treatment for radiation-induced salivary hypofunction.^{12,13,43} Similar *Aqp* gene transfer may be adapted as a treatment for hydrocephalus, by increasing the number of AQP4 channels to help mediate water balance in the ventricular system. Although not clear if applicable to the ventricular AQP4, studies have shown increased AQP4 expression secondary to nitric oxide and lipopolysaccharide in neural tissue.^{72,94}

Based on the experiments conducted regarding AQP4 expression, it is possible to conclude that upregulating AQP4 channels may improve water clearance in hydrocephalus. However, it is still relatively unknown what molecular mechanisms drive AQP4 modifications in the brain in hydrocephalus. A recent study by Aghayev et al. discovered that in rats, AQP4 expression is not increased in mild

hydrocephalus (during initial stages), but it is increased in severe hydrocephalus.² If these observations carry over to human beings, this may decrease the efficacy of potential therapeutic AQP4 modulators in early hydrocephalus.

The upregulation of AQP4 near the ventricles in severe hydrocephalus may also be a result of the inflammatory markers implicated in the pathogenesis of hydrocephalus (including tumor necrosis factor- α , interleukin-1 β , and transforming growth factor- β), and not purely a protective mechanism to improve water clearance.^{19,73,127} In addition to the water and ion homeostatic functions of AQP4, it has been suggested that AQP4 functions as a cell adhesion molecule, plays a role in the structural and functional integrity of the ependyma, and modulates size regulation of orthogonal arrays; these factors should be kept in mind when designing targeted therapies for AQP4.^{33,48,75,119}

AQP4 channels have also been found to play a role in regulating adult neurogenesis and stem cell proliferation.^{69,119,145} Another concern related to regulating AQP4 for pharmaceutical uses is that it may have a variety of effects on the body; AQP4 channels are not only located in the CNS, but also in skeletal muscle sarcolemma, the male genital system (seminiferous tubules, seminal vesicles, prostate, and epididymis), the respiratory system (lung and bronchus), the kidney, the gastrointestinal system (parietal cells and salivary glands), and in various other parts of the human body.⁸⁶

However, these challenges are not insurmountable. Many other currently used systemic therapies with a specific target may have numerous systemic effects (e.g., antihistamines, NSAIDs, and so on). Beneficial AQP4 modulators must have minimal neurotoxicity and beneficial pharmacological parameters, and also be able to cross the BBB and be effective medications. If the benefits of therapy are able to overcome side effects, pharmacological agents targeting AQPs may pose a viable route to help treat hydrocephalus, either in conjunction with current surgical methods or as a monotherapy when surgery is contraindicated (Table 1).

Computational Modeling of CSF Transport

The discussion of AQP4 channels and the role of intracranial water shift in hydrocephalus clearly underscores the importance of molecular transport processes for normal homeostasis of brain water and in the disease process of hydrocephalus. Quantifying the impact of AQP channels on brain fluid exchange would help researchers to elucidate the causes of hydrocephalus, its progression, and how to treat it. A quantitative understanding of the production and flow of CSF is vital to gaining more accurate insight into the pathological changes that occur in hydrocephalus. Physiological CSF flow, possibly mediated by the amount and placement of AQP channels, is an important aspect of the ventricular system—from the initial ependymal-cell CSF production in the choroid plexus, surface of the ventricles, and lining of the subarachnoid space, to the eventual travel of the fluid through the third and fourth ventricles, and finally to the subarachnoid space and reabsorption to the vascular system. Physiologically accurate computational modeling of fluid flow in hydrocephalus will help to design bypass strategies in noncommunicat-

TABLE 1. Key points by subtopic

Subtopic	Key Points
AQP4 & hydrocephalus	AQP4 channels are intimately involved in brain water regulation. Numerous studies have shown a link btwn hydrocephalus & secondary changes in AQP4 expression, as well as primary <i>Aqp4</i> knockout leading to accelerated development of hydrocephalus.
AQP4 channel characteristics	A review has been performed of elucidated AQP4 isoforms, orthogonal arrays, alternative promoters, transcription factors, & post-translational modification. Further molecular research on AQP4 channels will supplement the development of drug therapy.
AQP4: a potential therapeutic target for hydrocephalus?	Effective pharmaceutical methods for treatment of hydrocephalus do not exist. AQP4 is an attractive target for drug therapy. Compounds to modulate AQP4 function are under development & face potential limitations.
Computational modeling of CSF transport	Three aspects of computational modeling of hydrocephalus deserve further attention: ICP, ventricular vol, & osmolarity. Accurate computational models of the ventricular system developed from real-time imaging may help improve early diagnosis as well as surgical planning for hydrocephalus.

ing hydrocephalus, as well as complement shunting device placement, and will also help to design how to best restore normal CSF exchange in communicating hydrocephalus.⁷⁷ Many groups have proposed computer models to describe CSF flow dynamics in the ventricular system, showing that it is possible to model the disease process of hydrocephalus.^{6,17,40,49,50,58,77,78}

Previous models of hydrocephalus divided the cranial space into individual compartments, such as ventricles, parenchyma, cerebral blood, and cranial subarachnoid space. Externally imposed changes to fluid exchange between these compartments, such as obstruction of the villi, induce accumulation of the fluid and subsequently lead to CSF accumulation in the ventricular system.^{16,18,78} Compartmental models often incorporate compliance curves that link cranial compartment volume with the ICP.⁷⁸ However, when incorporating intracranial compliance curves, the relationships between pressure and volume are given as inputs, and thus are not predictive. As such, compartmental models based on predefined fluid exchange and cerebral compliance laws merely reproduce known effects, such as enlargement of ventricles and pressure changes, without actually addressing the biochemical changes that occur at the molecular level.

Therefore, simplified compartmental models lacking molecular water-transport aspects can reproduce changes that occur in hydrocephalus (such as ventricular enlargement and pressure changes), but have so far not been successful in improving diagnosis or treatment options. To improve diagnosis of hydrocephalus and its management, a new family of models is needed that addresses the pathology at the molecular level in addition to the macroscopic

changes that are observed clinically. Based on the review of molecular aspects of water transport in the brain, 3 significant aspects appear to deserve more attention in future computational models of hydrocephalus and diseases that involve CSF dynamics. These include ICP, ventricular volume, and osmolarity.

The first subject in need of further clarification is ICP. For physicians, ICP is a well-recognized clinical quantity that can be measured with spinal tap or monitored acutely with pressure sensors.^{36,74,114} In computational models of intracranial dynamics, pressure differences are calculated using the equations of motion for the CSF, the Navier-Stokes equations. A recent review by Linninger et al. summarized models of CSF dynamics and diseases.⁷⁷ Most existing computational models use an incompressible fluid assumption that does not set a specific value for absolute hydrostatic pressure (ICP). Thus incompressibility merely implies a “pressure drop” needed to entertain fluid motion by overcoming viscous frictional losses, but it does not correlate to absolute ICP.

What is needed are more complete molecular or biochemical models to accurately quantify absolute pressure changes caused by tissue deformations such as ventricular enlargement; compression of the cerebral arterial, capillary, and venous blood pools; or expansions of the spinal subarachnoid space. Such models require fluid–structure interaction between CSF and brain matter, while simultaneously incorporating blood flow and CSF interactions.²² More fundamental models addressing compressibility and deformations between fluid and solid compartments will create true predictions of ICP rise subject to external influences, by correlating physical and molecular aspects of intracranial water exchanges and CSF dynamics. These predictions will provide relevant information, so that ICP changes in the CSF and its effects on cerebral blood flow and brain tissue can be explained.

A second question concerns the impact of ICP and compressibility on volume changes of intracranial compartments. It is not clear how ICP rise leads specifically to an increase in the size of the ventricles, rather than modifications in other spaces in the CSF system such as the subarachnoid space. In Hakim’s original “water-filled sponge” hypothesis of the brain (see article by Clarke and Meyer), ICP rise was seen to induce a mechanical wall deformation leading to the enlargements of the ventricles.²³ However, work by Linninger et al. and Penn et al. showed that ICP differences are small, and it is unlikely that mechanical deformation alone causes ventricular enlargement in hydrocephalus.^{76,101}

Uncertainty also pertains to the exact location of spatial displacement needed to accommodate ventricular enlargement. It is well known that ventriculomegaly is a symptom of hydrocephalus. However, it is poorly understood which spaces are displaced when ventricles enlarge. One option is that the extracellular space in the parenchyma diminishes. Work by Del Bigio and colleagues supports the finding that the extracellular volume fraction is smaller in hydrocephalic animals when compared with normal specimens.^{27,29,117} However, the experimental techniques to measure minimal changes rely on tissue fixation, which is prone to altering the extracellular space. Therefore, it is

not clear whether the extracellular space volume fraction truly shrinks after ventricular enlargement. It would be invaluable to further investigate possible alterations of extracellular space in hydrocephalus, for example, by using specialized integrated optical imaging methods developed by Nicholson⁶² or by diffusion-tensor imaging methods.^{62,115}

Another theory regarding hydrocephalic CSF accumulation in the ventricles is that volume compensation occurs by expansion of the cortical surfaces, thus reducing the cranial subarachnoid space available to the CSF. Accordingly, the CSF volume that is needed to enlarge ventricles would merely shift from the cranial subarachnoid space to the ventricles. This process could take effect without the need for actual CSF accumulation or changes to CSF production or reabsorption. Figure 4 illustrates the space relationships. Figure 4A, C, and E shows a normal human cortex generated from medical images. The model shown by Fig. 4B, D, and F was generated artificially by expanding the cortical surface by 1 mm from the actual cortical surface (a 1-mm-level set increase in size).

This modest expansion of the cortical surface substantially reduces the CSF volume occupied by the cranial subarachnoidal space. The cortical surface shifts in the hypothetical expansion are close to the image resolution threshold, so it may be difficult to detect these in a patient's MR image. Figure 4 also shows that these changes in the subarachnoid space may easily be dismissed as anatomical variability between patients. Distance maps were created to precisely quantify the volume difference due to a 1-mm cortical expansion. Two different "masks" (shown in Fig. 5) were entered into an algorithm to calculate the volume difference of 185.9 ml between the normal and the expanded cortical surfaces. Thus, the 1-mm shift in cortical surface corresponded to a total of 185.9 ml of CSF volume change.

From a volumetric point of view, this shows that the entirety of a substantial ventricular enlargement could be accommodated by small expansions of the cortical surface. If this were true in hydrocephalus, the expansion of CSF volume may not solely constitute an accumulation of water pressing on neural tissue, but also a redistribution of CSF from the cranial subarachnoid space to ventricular spaces. Whether the expansion of the ventricle causes extracellular space reduction/compression, or redistribution of water volume from the cranial subarachnoid space to the ventricular system, is currently poorly explored. Advances in medical imaging and segmentation algorithms are expected to clarify which final volume shifts occur in hydrocephalus, thus allowing us to calculate ventricular volume changes for patients.

The third area of interest in hydrocephalus pertains to the effect of osmolarity. It has been shown in experiments that the change of CSF osmolarity alone may be sufficient to alter ventricular size. Two groups have shown that artificial change of osmolarity in ventricular CSF by hyperosmolar infusion into the ventricular system is followed by ventricular enlargement.^{67,70,71} If these experiments prove to be valid, it would suggest that ventricular expansion can occur merely by changing the osmolarity, without the specific need for pressure changes or physiological infusion of water.

FIG. 4. Volume changes depicted by 3D reconstruction of MR images, following a 1-mm shift of the cortical surface. **A and B:** A 1-mm shift in the cortical surface is not grossly perceptible on the comparison transverse section. **A, C, and E:** Cortical surface reconstruction of a volunteer. **B, D, and F:** Cortical surfaces following a uniform 1-mm shift of the cortical surface.

This evidence suggests that osmolarity changes affect intracranial water shifts via the secondary flux of water through the AQP water channels, which are known to operate by means of osmolar gradients. A transiently hyperosmolar CSF would then be implicated in hydrocephalus, because it is established that the choroid plexus secretion of sodium and chloride may cause the development of hydrocephalus.¹²²

This contribution of osmolarity and thus AQP water channels to hydrocephalus should not be ignored in the current understanding of hydrocephalus, and thus needs to be accounted for in computational models of hydrocephalus. Whereas experimental evidence generates new data about hydrocephalus disease progression, computational models currently lag behind in introducing these phenomena into a quantitative biochemical and biomechanical model to explain the molecular aspects of hydrocephalus. A first attempt to quantify osmolarity and fluid exchange in the brain was described recently.¹⁷

FIG. 5. A hypothetical ventricular expansion is shown on transverse and coronal sections following a 1-mm cortical shift. A 3D binary mask of the cortex has an isotropic voxel size of $1 \times 1 \times 1$ mm. **A:** A 3D binary mask prior to 1-mm cortical shift. After computing the distance map, the voxels with an intensity ≥ 1 (i.e., 1 mm from the cortical surface) are added, and a new mask is created. **B:** The total volume increase amounted to 185.9 ml. This change in size means that ventricular enlargement may be accommodated via small expansions of the cortical surface. **C:** The “Boundary Difference” shows the 1-mm increase in cortical surface between the “Normal” and “Dilated” brain. This small shift is hardly detectable in MRI scans due to limited image resolution.

The incorporation of osmotic gradients and water exchange through AQP channels helps drive the ultimate shifts in fluid that form the basis of the disease process of hydrocephalus. Osmolarity differences also link the CSF compartment to the vascular system. A significant improvement in current hydrocephalus models concerns the incorporation of the cerebral vasculature. There is experimental evidence that has demonstrated blood perfusion changes in hydrocephalus.^{32,79,99} However, quantitative models to correlate ICP shifts to changes in blood perfusion are in their infancy. An example of using current image-segmentation techniques for automatic reconstruction of subject-specific cerebrovascular models is shown in Fig. 6. It depicts variations in shape, volume, and articulation of arterial and venous vascular trees between different subjects. Automatic recognition methods of subject-specific cerebrovascular trees from MR images were used to reconstruct and evaluate the subject-specific angioarchitecture.⁵¹ Modeling vascular structures in the brain may allow for the incorporation of realistic representations of the cerebral circulation, and especially the capillary bed, in models of hydrocephalus. Real-time subject-specific computational analysis allows the creation of a “brain atlas” for patients, which can play a vital role to help guide diagnosis and treatment for neurological diseases.

A review of the molecular perspective of hydrocephalus points toward the fact that future computational models should integrate molecular water transport shifts and their

impact on the well-known ventricular deformation and accumulation of CSF. Combining molecular perspectives with clinical knowledge may help advance the treatment and diagnosis of hydrocephalus. In obstructive hydrocephalus, current treatment involves restoring normal CSF flow, either by removing the obstructive lesion or by creating a bypass of the obstruction.⁷⁷ In primary malabsorptive hydrocephalus, such as in malabsorption at arachnoid villi, shunting of CSF from the brain has been shown to be life-saving in a disease process that was fatal as little as 65 years ago.⁷⁷ The integration of ICP, ventricular volume, and the relationship of osmolarity to molecular AQP4 pathology is difficult to intuitively align with clinical enlargement of the ventricles, and observed changes in volume and pressure parameters. Use of computational models may potentially help clinicians to better design bypass strategies in non-communicating hydrocephalus, complement shunt device placement in communicating hydrocephalus, and aid surgical planning for disorders of CSF flow.

Conclusions

AQP4 channels play an important role in CSF homeostasis and may be a significant factor in diseases of CSF flow. Current effective treatment options for hydrocephalus involve mechanical shunting of CSF, which has many inherent limitations. Mechanical shunts reduce ICP by direct removal of volume. Today, viable and reliable phar-

FIG. 6. Image-segmentation techniques are shown for automatic reconstruction of subject-specific cerebrovascular models.

maceutical methods to control the volume of CSF are limited. In the future, it may be possible for molecular shunts to directly modify the flux of brain water through AQP4 channels or by secondarily modifying transcellular osmotic gradients to shift fluid flow.

Ideally, development of a molecular shunt would harness the body's own compensatory upregulation of AQP4 channels to help activate reabsorption of CSF from the ventricles without the need for external fluid diversions. Therefore, modulating the activity of AQP4 channels is an attractive therapeutic target for potential molecular treatment of hydrocephalus. It is imperative that the complex

mechanisms of brain water homeostasis continue to be elucidated to appropriately explore these therapeutic molecular targets. Further work into clarifying AQP4 isoforms, promoters, transcription factors, and posttranslational modification methods will help identify the most appropriate therapeutic targets for AQP4 channel modulation.

There are many challenges for the design of therapies that create molecular shunts for hydrocephalus. These include difficulty in creating accurate water-permeation assays, a low hit rate for identification of AQP inhibitors, the small size and pore diameter of AQP channels, and minimal change to AQP channel function with mutations, sug-

gesting that binding of inhibitors must occur deep within the AQP channel.¹³⁵ However, these challenges are not insurmountable. If efficacious AQP4 modulators are created with minimal neurotoxicity and beneficial pharmacological parameters, they may become useful for treating hydrocephalus at the molecular level without the need for surgical intervention. Further research is needed to identify agents that modulate water flux through AQP4 channels. Pharmacological agents targeting AQPs may pose a viable alternative to help treat hydrocephalus in conjunction with current surgical methods or as a monotherapy when surgery is contraindicated.

More complete models of CSF transport with consideration to absolute ICP, volume, osmolarity, and water flux will help us continue to improve our understanding of the molecular aspects of this complex disease. Although it is clear that ICP is a vital quantity, current computational models are limited in predicting true absolute ICP rise. There is a mismatch between ICP as a clinical quantity and absolute pressure predictions in models, because existing computational models do not account for molecular water exchange at the production or reabsorption level or at the interface between the capillary bed and the interstitial fluid. Moreover, there are no hydrocephalus models that account for osmolarity gradients. Computational models that are able to quantify absolute pressure, volume, and osmolarity changes in line with new evidence are expected to better elucidate the disease process of hydrocephalus.

Although absolute ICP cannot be determined solely by imaging studies, ventricular volume is more accessible. By leveraging volume data through image-segmentation techniques to reconstruct patient MR images into computational models, potential benefits may be derived to promote earlier and more accurate diagnosis of diseases involving CSF-filled spaces. Elevated volume of the ventricular system is a common characteristic of hydrocephalus in numerous types, regardless of measured ICP. High-precision reconstructions may allow direct comparison of the current size of the ventricular system with previous states of ventricular volumes.

Metrics and shape changes may aid clinicians in determining if the patient is at baseline or beginning to accumulate excess CSF. More precise imaging for diagnosis may potentially allow for earlier treatment of patients with new-onset hydrocephalus. In addition, accurate 3D computational models may help surgical planning for shunt placement. Computational modeling has the potential to demonstrate a variety of clinical benefits, both as a functional tool for physicians to aid diagnosis and treatment of their patients with hydrocephalus, as well as to help clinicians better understand the pathophysiology of the complex molecular interactions of water exchange that occur in hydrocephalus.

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Disclosures

The authors report no conflict of interest concerning the materials or methods used in this study or the findings specified in this paper.

Author Contributions

Conception and design: Linninger, Desai, Mehta. Acquisition of data: Linninger, Desai, Hsu, Schneller. Analysis and interpretation of data: Desai, Hsu, Schneller, Hobbs. Drafting the article: Desai, Hsu, Mehta. Critically revising the article: Linninger, Desai, Hsu, Hobbs, Mehta. Reviewed submitted version of manuscript: Desai, Hsu, Hobbs, Mehta. Approved the final version of the manuscript on behalf of all authors: Linninger. Statistical analysis: Mehta. Administrative/technical/material support: Linninger, Schneller. Study supervision: Desai.

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