Drug development in targeting ion channels for brain edema
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Cerebral edema is a pathological hallmark of various central nervous system (CNS) insults, including traumatic brain injury (TBI) and excitotoxic injury such as stroke. Due to the rigidity of the skull, edema-induced increase of intracranial fluid significantly complicates severe CNS injuries by raising intracranial pressure and compromising perfusion. Mortality due to cerebral edema is high. With mortality rates up to 80% in severe cases of stroke, it is the leading cause of death within the first week. Similarly, cerebral edema is devastating for patients of TBI, accounting for up to 50% mortality. Currently, the available treatments for cerebral edema include hypothermia, osmotherapy, and surgery. However, these treatments only address the symptoms and often elicit adverse side effects, potentially in part due to non-specificity. There is an urgent need to identify effective pharmaceutical treatments for cerebral edema. Currently, ion channels represent the third-largest target class for drug development, but their roles in cerebral edema remain ill-defined. The present review aims to provide an overview of the proposed roles of ion channels and transporters (including aquaporins, SUR1-TRPM4, chloride channels, glucose transporters, and proton-sensitive channels) in mediating cerebral edema in acute ischemic stroke and TBI. We also focus on the pharmacological inhibitors for each target and potential therapeutic strategies that may be further pursued for the treatment of cerebral edema.

Keywords: cerebral edema; ischemic stroke; traumatic brain injury; ion channels; transporters

INTRODUCTION
Cerebral edema complicates severe central nervous system (CNS) injuries, and is considered one of the pathological hallmarks of traumatic brain injury (TBI) and excitotoxic injury such as stroke [1–3]. Cerebral edema is the leading cause of death within the first week of stroke, with up to 80% mortality in severe cases [4, 5]. It also accounts for up to 50% mortality in TBI [1]. Given the rigidity of the skull, any increase in intracranial fluid due to edema can significantly raise intracranial pressure (ICP) and compromise perfusion [6]. Physiological ICP ranges between 5 and 15 mmHg; however acute brain injury may elevate this value to over 20 mmHg. If left unchecked, this elevated ICP can compress the surrounding brain tissue and lead to ischemia, further accumulation of the edema fluid, brain herniation, and eventually death [6, 7]. Patients who survive from cerebral edema often experience long-term consequences such as chronic encephalopathy, and vascular dementia [8]. Currently available treatments for cerebral edema include hypothermia and osmotherapy. The most commonly used osmotic agents are mannitol and hypertonic saline. Both are osmotic diuretics and can temporarily reduce ICP by generating an osmotic gradient across the blood–brain barrier (BBB) that draws excess water into the vasculature [9]. The potential adverse effects of these osmotic agents include acute renal failure, hypotension, hemolysis, and congestive heart failure [10]. They may also provoke a rebound elevation in ICP [10]. Surgical procedures, including drainage of cerebrospinal fluid (CSF) and decompressive craniotomy, are also frequently performed [7, 11, 12]. Unfortunately, these current treatments are only symptomatic, nonspecific, and often cause significant complications [13]. As there is no effective therapeutic intervention or drug for cerebral edema, continued pharmaceutical research in this field including the discovery and screening of pharmacological agents is of critical importance.

First described by Klatzo et al. in 1967, cerebral edema may be classified as cytotoxic or vasogenic based on the status of the BBB [2] (Fig. 1). Cytotoxic edema, also known as oncotic cell swelling, manifests minutes after acute brain injury. As a premorbid process following the activation of ion channels, cells experience an acute influx of cations, mainly Na⁺. Cation influx, in turn, drives Cl⁻ influx [14, 15]. These resultant changes in osmotic gradients immediately translate to water influx from the interstitial compartment to the intracellular compartment, causing cells to swell. Cell blebbing, thus, is a key characteristic of cytotoxic edema. The process of cytotoxic edema takes place in all cell types in the brain, including neurons, glial cells, endothelial cells, and is particularly prominent in astrocytes. Since cell volume regulation is often compromised in cases of brain injury, sustained unregulated ion channel activation and subsequent water influx often lead to oncotic cell death [3, 16]. It should be noted that while cytotoxic edema resembles a redistribution of water, it does not increase brain water content or cause brain swelling by itself. Rather, it initiates ionic and vasogenic edema, which lead to brain swelling. As the influx of Na⁺, Cl⁻, and water persists, the ionic constituents and the volume of the interstitial compartment start to deplete. This is due to the fact that the intracellular compartment is much larger than the...
interstitial compartment, which only comprises ~12% of the total volume [14, 15]. Cytotoxic edema thereby generates a new Na\(^+\) gradient, that can potentially serve as a driving force for the subsequent formation of ionic edema [17].

Ionic edema was only recently classified as a distinct subtype of cerebral edema. It is defined as brain swelling due to water influx from an external fluid source in the presence of an intact BBB [16]. The development of ionic edema is an extracellular process, and is thought to be mediated by ion channels and transporters of endothelial cells. It is essentially a two-step process, during which solutes and water are first transported into endothelial cells via the abluminal membrane, and subsequently transported out via the abluminal membrane [14, 18]. As endothelial cells are key components of the neurovascular unit, dysregulation of these cells may cause an early transient leakage of the BBB. Acute cerebrovascular insults that generate cytotoxic and ionic edema early-on (<24 h), are also known to develop secondary vasogenic edema after 2–4 days. In fact, this biphasic edema response is often seen in ischemic stroke and TBI [8]. A very recent study suggested that the immediate source of Na\(^+\) and water-mediated ionic edema in acute brain injuries could also arise from CSF, rather than being entirely from the vascular compartment [19]. CSF is thought to transport along the penetrating perivascular spaces into the brain parenchyma, and this process is facilitated by aquaporins expressed at the perivascular astrocytic endfeet. This glymphatic system may have a major role in developing ionic edema.

Vasogenic edema, but not ionic edema, includes the breakdown of the BBB and the extravasation of serum proteins such as albumin and immunoglobulin G from the capillaries into the interstitium [14]. BBB hyperpermeability not only favors the influx of salt and proteinaceous fluid, either directly or through pinocytic vesicles, but also promotes the infiltration of circulating leukocytes, which has been speculated to promote the clearance of cellular debris after injuries [20]. Aside from oncotic cell death in endothelial cells and astrocytes, which normally maintain BBB integrity, multiple proteins have been proposed to be upregulated to facilitate vasogenic edema and BBB breakdown. These proteins include matrix metalloproteinase (MMP), substance P, vascular endothelial growth factor, thrombin, and pro-inflammatory cytokines such as tumor necrosis factor, interleukins (IL)-6, and IL-1β [21–26]. Together, they mediate neuroinflammation and further degradation of tight junctions, exacerbating cerebral edema. Astrocytes and endothelial cells may experience oncotic cell death and retraction, respectively. Figure created with BioRender.com.

Fig. 1 Status of the blood–brain barrier (BBB) at different phases of cerebral edema. In cytotoxic edema (also known as oncotic cell swelling), cells experience an acute influx of solutes (black dots), mainly Na\(^+\) and Cl\(^-\). Changes in osmotic gradients translate to water influx (blue arrow) from the interstitial compartment to the intracellular compartment. Cytotoxic edema is particularly prominent in astrocytes. Ionic edema is defined as brain swelling due to water influx from an external fluid source in the presence of an intact BBB. Solute and water influx are mediated by ion channels and transporters of endothelial cells. Upregulation of ion channels and transporters also occurs in astrocytes. Astrocyte swelling may lead to the release of excitatory amino acids (EAA). Vasogenic edema includes the breakdown of the BBB and the extravasation of serum proteins such as albumin and immunoglobulin G (IgG). The transport of solutes and proteinaceous fluid may occur directly (large blue arrow) or through pinocytic vesicles. Multiple factors, including metalloproteinase (MMP), substance P, vascular endothelial growth factor (VEGF), thrombin, and pro-inflammatory cytokines such as tumor necrosis factor (TNF), interleukins (IL)-6, and IL-1β are involved. Together, they mediate neuroinflammation and further degradation of tight junctions, exacerbating cerebral edema. Astrocytes and endothelial cells may experience oncotic cell death and retraction, respectively.
as the mitochondria. However, prolonged Ca\(^{2+}\) uptake by the mitochondria affects the mitochondrial membrane potential, leading to impaired ATP synthesis and reactive oxygen species production. The excessive Ca\(^{2+}\) is then pumped back into the cytoplasm, and Ca\(^{2+}\) overload triggers the activation of various downstream excitotoxic cascades [31]. The overall ionic imbalance and its related events can work both independently to initiate BBB dysfunction and synergistically to propagate each other’s actions and exacerbate BBB breakdown.

Ion channels play important roles in both glutamatergic and non-glutamatergic mechanisms in cerebral ischemia and stroke. Although glutamate receptor-triggered excitotoxicity is traditionally thought to be the central mechanism in acute brain injuries including stroke, recently, more attention has been drawn to non-glutamatergic mechanisms as the clinical trials of anti-excitotoxic therapies have not reached the expected benefits in stroke patients [31–33]. These alternative non-glutamatergic mechanisms can also lead to ionic imbalance and eventually neuronal cell death. Various ion channels have been implicated in cerebral ischemia and stroke. These ion channels include neuronal ATP-sensitive potassium (K\(_{ATP}\) (Kir6.2/SUR1)) channels, transient receptor potential (TRP) channels, acid-sensing ion channels (ASICs), hemichannels, volume-regulated anion channels (VRACs), and other ion exchangers and nonselective cation channels [34–40] (Fig. 2). The critical roles of ion channels in stroke-related complications such as cerebral edema have also been investigated and confirmed in many studies.

To date, we still lack effective pharmacological agents in attenuating cerebral edema formation and progression despite significant mortality and clinical complications. A crucial step in developing novel and effective therapies is to first understand the molecular and cellular mechanisms of cerebral edema. Ion channels currently represent the third-largest target class in drug development and are critical mediators throughout the stages of cerebral edema as well as stroke. Thus, pharmacological agents that target or modulate ion channel functions have the therapeutic potential to attenuate cerebral edema in acute brain injuries (Table 1). This review aims to (1) summarize the proposed roles of ion channels in mediating cerebral edema in acute ischemic stroke and TBI, as well as the current progress and challenges in discovering ion channel inhibitors; and (2) discuss the potential pharmacological strategies in the drug development for cerebral edema.

**AQUAPORIN (AQP)**

AQP are specialized cell membrane proteins that are permeable to water molecules [41]. Each member of the AQP family has a molecular weight of ~30 kDa and consists of six transmembrane (TM) helices, two opposing short helices, and intracellular carboxyl (C) and amino (N) termini [41]. The two short helices contain the signature triplet amino acid NPA (Asn-Pro-Ala) motif, which facilitates the movement of water [41].

AQPs are organized in homotetramers, where each AQP has four water channels that function independently. This organization also gives rise to a central pore, of which ions and/or gases can flow through depending on the specific AQP subtype [41]. Water flows through AQPs bidirectionally depending on the osmotic gradients. Among the 13 mammalian AQP members, AQP1, -4, and -9 are the most abundant subtypes expressed in the brain [41]. AQP1 is mainly expressed in epithelial cells of the choroid plexus and contributes to CSF production and regulation. AQP4 is the most abundant water channel found throughout the brain and is intensively studied in the context of acute brain injuries [41]. AQP4 is highly expressed at astrocytic endfeet, where it closely contacts the cerebral vascular compartment at the BBB [41, 42]. In acute brain injuries, however, astrocytic AQP4 expression can reorganize from being highly polarized at endfeet to widely distributed throughout cell bodies and the fine astrocytic

**Fig. 2** Schematic depiction of the main channels and transporters that have been implicated in facilitating cerebral edema and excitotoxicity during moderate and severe acute brain injuries. Arrows indicate the direction of transport. Note that KCC2 and NCX are more likely to operate in their reverse modes under conditions of moderate and severe injury, and CIC direction of rectification varies between family members. The respective expression of these channels and transporters depends on the cell type and stages of cerebral edema. Figure created with BioRender.com.
processes [43, 44]. Interestingly, most of the neurons in the CNS do not express functional AQP5s, suggesting the water influx in neurons is mediated by other transport mechanisms [45, 46]. A low level of AQP4 expression has been detected in endothelial cells, as well as ependymal cells lining the ventricles.

Both TBI and ischemic stroke yield distinct patterns of AQP4 activation [47–50]. In mouse models of transient middle cerebral artery occlusion (tMCAO), AQP4 expression was found to be rapidly upregulated in astrocytes immediately after the onset of stroke, and the upregulation was correlated temporally with the extent of edema [50]. This correlation was only observed in AQP4, but not AQP1 or AQP9. In addition, a second peak of maximal brain swelling at 48 h post-ischemia coincided with the second peak of AQP4 expression [50]. AQP4 mRNA was also maximally upregulated and corresponded to brain swelling in the peri-infarcted cortex 3 days after focal MCAO in rats, as monitored by MRI [48]. Consistent with these findings, AQP4−/− mice showed a significant reduction in mortality, infarction, and brain edema following both transient and permanent MCAO (pMCAO), with the BBB remaining intact [51–53]. AQP4−/− mice also exhibited improvement in long-term neurobehavioural outcomes [51–53]. Similar protective effects of AQP4 deficiency were reported even in a severe, global ischemia model [54].

In contrast, reductions in AQP4 expression at the perivascular astrocytic endfoot have been demonstrated in rat and mouse models of tMCAO [43, 55]. AQP4 mRNA was downregulated at 24 h after pMCAO [56], and AQP4−/− mice showed higher mortality, post-ischemic inflammation, and brain atrophy following tMCAO [57]. These contradictory findings may be explained by the severity of the injury, prompting the hypothesis that under severe insults, damaged brain cells are not able to sufficiently synthesize new AQP4 proteins.

In various models of acute TBI, genetic ablation of AQP4 in mice reduces ICP, cell death, water accumulation, astrogliaosis, and lesion volume [58, 59]. Consistent with these findings, small-interfering RNA (siRNA) targeting AQP4 resulted in reduced edema formation, neuronal cell death, and reduced TBI in rats [60]. The downregulation of AQP4 expression in response to experimental brain trauma has also been reported [49, 61]. A significant decrease in AQP4 expression, correlating with increased brain water content, was reported 48 h post injury in a rat model of TBI [61]. In another study, both AQP4 immunolabelling and AQP4 mRNA were reduced in the edematous cortex along with impaired BBB integrity [49].

Although few human studies have been conducted, AQP4 expression was found to be elevated in brain specimens of

### Table 1. Pharmacologic agents targeting ion channels and transporter in cerebral edema.

| Name of the compound | Ion channel/ transporter | Effects on cerebral edema in animal models of Ischemic stroke | TBI | Clinical trails | Reference |
|----------------------|--------------------------|-------------------------------------------------------------|-----|----------------|-----------|
| Aza                  | AQP4, nonselective       | Controversial effects                                       | Reduce cerebral edema | –            | [68–71]   |
| TGN-020              | AQP4, nonselective       | Reduce cerebral edema                                       | Has not been evaluated | –           | [72, 73] |
| AER-270 and AER-271  | AQP4, selective          | Reduce cerebral edema                                       | No effect               | Phase I, II (Aeromics Inc), results pending | [75–77] |
| Aquaporumab          | Specific monoclonal AQP4 antibody | Has not been evaluated                                         | Has not been evaluated | –           | [84]     |
| Gilbenclamide        | SUR1-TRPM4, nonselective | Reduce cerebral edema                                       | Reduce cerebral edema | Phase I, II (Remedy Pharmaceutical Inc), reduces edema and mortality, improves functional outcomes in stroke patients | [96–100] |
| Bumetanide           | Selectively inhibit NKCC1 at low doses; inhibit KCC2 and AQP4 | Reduce cerebral edema                                       | Reduce cerebral edema | –           | [78–80, 116] |
| VU0463271            | KCC2, Selective          | Has not been evaluated                                       | Has not been evaluated | –           | [145–147] |
| Tamoxifen and DCPIB  | VRAC, nonselective       | Has not been evaluated                                       | Has not been evaluated | –           | [162, 163] |
| T16ainh-A01 and CaCCinh-A01 | TMEM16A, selective | Has not been evaluated                                       | Has not been evaluated | –           | [177]     |
| CFTRinh-172          | CFTR, selective          | Has not been evaluated                                       | Has not been evaluated | –           | [190–192] |
| SEA0400              | Primarily inhibits the reverse mode of NCX1, low affinity for NCK2 | Has not been evaluated                                       | Has not been evaluated | –           | [219, 222] |
| Phlorizin            | SGLT                     | Reduce cerebral edema                                       | Has not been evaluated | –           | [231]     |
| Amiloride            | ASIC, NHE                | Has not been evaluated                                       | Has not been evaluated | –           | [37, 249] |
| Psalmotoxin-1 (PcTx1)| ASIC, selective          | Has not been evaluated                                       | Has not been evaluated | –           | [37, 247, 248] |
| HOE-642              | NHE, selective           | Reduce cerebral edema                                       | Has not been evaluated | –           | [243, 252, 253] |
patients with ischemic stroke [62]. A more recent postmortem study however, has suggested that the changes in perivascular AQP4 expression are regional-specific, and significantly differ between gray and white matter [55]. CSF from patients with severe TBI showed a significantly elevated level of AQP4 and brain specimens of TBI patients revealed an increase in AQP4 expression from 15 h to 8 days post injury [63, 64].

Aside from injury severity, conflicting results in AQP4 expression following acute brain injuries can also be explained by its dual function, namely the early-on deleterious role in edema formation and the later beneficial role in edema resolution [65]. In fact, increased AQP4 expression was observed to be associated with edema resolution, measured over time using MRI [47, 50, 60]. Moreover, this increase in AQP4 was mainly distributed at the perivascular astrocytic endfeet and the glia limans, which suggests the potential role of AQP4 in facilitating edematous fluid elimination through the subarachnoid space [47, 50]. The dual function of AQP4, therefore, suggests that AQP4 inhibitors may be beneficial in reducing cerebral edema if administrated at early stages after the brain injury. In addition, it cannot be excluded that some astrocyte function could be altered in AQP4−/− mice, for example, the ability of glutamate reuptake could be affected due to a reduced level of glutamate transporter GLT-1 [66]. Furthermore, altered K+ reuptake by astrocytes has also been reported in AQP4−/− mice, which may contribute to the neuroprotection observed in AQP4−/− animals [65, 67].

Heavy metals, such as mercury and zinc have been known to directly bind to AQP subtypes and inhibit AQP-mediated water transport in vitro. However, the therapeutic potential of using heavy metals in managing cerebral edema is largely limited due to their nonspecific and potentially toxic properties. In the past decade, targeting AQP4 by small molecule inhibitors has become the main focus of pharmacological research, and several small molecule inhibitors have been identified. Studies by Huber et al. identified a series of arylsulphonamides, antiepileptics, and small molecules that can inhibit AQP4-mediated water transport in hAQP4-expressing Xenopus oocytes [68]. Among these compounds, the carbonic anhydrase inhibitor Acetazolamide (AZA), which is commonly used for reducing intraocular pressure in glaucoma, exerts the most potent and dose-dependent inhibition (IC50 = 0.86 μM) against AQP4 [68]. This inhibitory effect of AZA on AQP4-mediated water conductance has been both confirmed and completely refuted in other in vitro studies [69, 70]. A very recent study has shown the in vivo protective effects of AZA against cerebral edema in a mouse model of TBI. Treatment of AZA prevents the reorganization of AQP4 and attenuates cytotoxic edema in both the cortex and hippocampus [71]. A novel AQP4 inhibitor TGN-020 was later identified by Huber et al. with an IC50 of 3 μM. Administration of TGN-020, both pre- and post-MCAO, reduced cerebral edema, cortical infarction, gliosis, and albumin effusion in rodents [72, 73]. Crystal structures of RaQ4 bound to AZA and TGN-020 have been revealed by in silico studies [68, 74]. The residues of rAQP4 responsible for the binding sites of these two compounds are quite conserved to hAQP4, suggesting their potential inhibitory effects on hAQP4 [68, 74]. However, their effects on AQP4 are not selective, as ACA and TGN-020 may additionally inhibit AQP1.

The phenylbenzamides AER-270 (IC50 = 0.42 μM) and its phosphonate produg derivative AER-271, are small molecule AQP4 inhibitors that have been most recently identified. AER-271 has a >5000-fold enhanced water solubility, and can be converted by phosphatasors to AER-270 in vivo. A very recent study showed that AER-271 selectively and effectively reduced AQP4-mediated cerebral edema and improved neurological outcome in rodent models of both tMCAO and water intoxication [75]. However, despite of having a trend in reducing ICP and contusion volume, AER-271 seems to be ineffective in reducing cerebral edema in various TBI models [76, 77]. Currently, a Phase-I clinical trial of AER-271 has been completed by Aeromics Inc. OH, USA. A Phase-II trial evaluating AER-271 in cerebral edema prevention in acute ischemic stroke was supposed to begin in 2019. Updates regarding the trial progress have yet to be released.

The loop diuretics bumetanide and its derivatives have also been proposed to inhibit AQP4. Bumetanide has been shown to reduce brain edema following tMCAO via reducing AQP4 expression [78, 79]. However, bumetanide also exerts inhibitory effects on the sodium–potassium–chloride cotransporter (NKCC), the potassium-chloride cotransporter (KCC), and AQP1 of the choroid plexus epithelium, thus its beneficial effects on edema could be due to inhibition of these other targets [79, 80]. Other compounds including phorbol myristate acetate, a PKC activator, and vasopressin 1A receptor antagonist, SR49059, have also been suggested to reduce ischemic and trauma-induced edema by modulating AQP4, yet the complete mechanisms of their actions remain unclear [81–83]. The specific monoclonal AQP4 antibody Aquaporumab has been shown in vivo to reduce neumyelitis optica, an autoimmune demyelinating disease, but it has yet to be evaluated in ischemic stroke or TBI [84]. A more recent in vivo study has revealed that targeting AQP4 subcellular localization, rather than directly targeting the channel activity, significantly reduced cell-surface expression of AQP4 and AQP4-mediated CNS edema, which offers an alternative approach for anti-edematous therapy [85].

To conclude, the variations in AQP4 expression observed in different in vivo studies depend on the degree of injury severity, the pathological models, and the dual nature of AQP4 function. Small molecule AQP4 inhibitors, with defined target-specific and time-specific pharmacological properties, may serve as a potential treatment for cerebral edema. The therapeutic effects of AER-271 are yet to be evaluated in clinical trials. Regardless, many reports have served as “proof of principle” studies, highlighting the important role of AQP4 in cerebral edema. The discovery of selective AQP4 inhibitor(s) will be a critical goal in drug development.

SUR1-TRPM4

The ion channel complex sulfonylurea receptor 1—TRP melastatin 4 (SUR1-TRPM4) was first discovered in reactive astrocytes obtained from the hypoxic core by Chen et al. [86]. This ion channel complex is formed by two subunits, namely, the regulatory subunit SUR1 and the pore-forming subunit TRPM4. SURs belong to the superfamily of ATP-binding cassette (ABC) transporters, which use the energy derived from ATP hydrolysis to mediate a variety of cellular functions [87]. SUR1 and SUR2 are unique among ABC transporters in that they serve as ion channel regulators [87]. Under physiological conditions, SUR1 is expressed in neurons, but not in astrocytes or endothelial cells. Contrary to what is seen under physiological conditions, brain tissue isolated from rat ischemic core shows an upregulation of SUR1 protein and mRNA in neurons, astrocytes, and endothelial cells [88]. Upregulation of SUR1 was also observed in rat cortical impact TBI model, with a doubling of the SUR1 gene expression 6 h post TBI [89]. Clinically, SUR1 expression is elevated in the CSF of TBI patients and is undetectable in healthy controls. SUR1 expression in these patients correlates with an increased risk of cerebral edema [90]. Single nucleotide polymorphisms of ABCC8 have also been associated with a higher risk of edema development in TBI patients [91]. Although SUR1 acts as the regulatory subunit of both ATP-sensitive K+ (KATP) channels and the nonselective cation channel NCX3, the upregulation of SUR1 and resultant cerebral edema in acute brain injuries were proposed to be associated with its regulation of NCX3, but not of KATP [88, 89]. This is further confirmed in a very recent study using highly specific antisense oligodeoxynucleotides [92].
TRPM4 is a Ca^{2+}-activated, nonselective, monocation channel. TRPM4 alone is a functional ion channel, whereas SUR1 cannot translocate to the cell membrane without binding to a pore-forming subunit [93]. Reciprocally, SUR1 doubles TRPM4's affinity for Ca^{2+}-calmodulin, and its sensitivity to intracellular Ca^{2+} when they co-assemble as heteromers. Unlike most of the ion channels and transporters implicated in cerebral edema, SUR1-TRPM4 channels are expressed de novo only after acute CNS injuries [94]. The expression of functional SUR1-TRPM4 occurs approximately 3-h post MCAO in the ischemic core, and 8-h post MCAO in the penumbra [88].

ATP depletion or a nanomolar change of Ca^{2+} concentration rapidly activates SUR1-TRPM4 [86, 94]. In acute brain injuries, activation of SUR1-TRPM4 by increased intracellular Ca^{2+} initially reduces the driving force of Ca^{2+} influx [94]. Sustained SUR1-TRPM4 activation under conditions of severe ATP depletion, however, can lead to Na^{+} influx and oncotic cell death.

A recent study demonstrated that SUR1-TRPM4 can physically assemble with astrocytic AQP4 to form a novel heteromultimeric water/ion channel complex [95]. Primary astrocytes in vitro showed that TRPM4 and AQP4 were closely co-localized and synergistically mediated a fast, high-capacity TM water influx that drove astrocytic swelling [95]. The same study also validated their finding in vivo in a mouse model of brain edema, and speculated that the co-assembly of AQP4 and SUR1-TRPM4 may further amplify the role of TRPM4 as a negative regulator of Ca^{2+} influx [95]. Nonetheless, future studies are required to demonstrate the functional implications of SUR1-TRPM4-AQP4 complex in the context of acute brain injuries.

Sulfonyleureas, such as glibenclamide and tolbutamide, are potent inhibitors of SUR-regulated channels, including K_{ATP} and NC_{ca,ATP}. SUR1-TRPM4 can be inhibited by glibenclamide (EC_{50} = 48 nM) with high affinity and specificity. Glibenclamide administration in vitro effectively prevented SUR1-TRPM4-mediated Na^{+} influx, cytotoxic edema, and oncotic cell death [86]. Furthermore, in vivo effects of glibenclamide on brain edema have been widely studied in different rat models of ischemic stroke, including thromboembolic stroke, pMCAO, and tMCAO models [96]. In all of these studies, glibenclamide blocked the ischemia-induced upregulation of NC_{ca,ATP} Channels and improved stroke outcomes. In regard to TBI, rats treated with glibenclamide showed attenuated BBB disruption, brain water content, and contusion size [97].

Clinically, the FDA-approved, antidiabetic medication glibenclamide (aka, glyburide) appeared promising in both Phase-I and Phase-II trials (Remedy Pharmaceuticals) [98, 99]. Intravenous glibenclamide administration in vitro showed that TRPM4 and AQP4 were closely co-localized and synergistically mediated a fast, high-capacity TM water influx that drove astrocytic swelling [95]. The same study also validated their finding in vivo in a mouse model of brain edema, and speculated that the co-assembly of AQP4 and SUR1-TRPM4 may further amplify the role of TRPM4 as a negative regulator of Ca^{2+} influx [95]. Nonetheless, future studies are required to demonstrate the functional implications of SUR1-TRPM4-AQP4 complex in the context of acute brain injuries.

Chloride Channels

Cl^{-} is the most abundant anion in humans under physiological conditions. The intracellular chloride level, [Cl^{-}]_{i} in neurons is tightly regulated to ensure appropriate electrical responses to the inhibitory neurotransmitters γ-aminobutyric acid (GABA) and glycine. Specifically, low [Cl^{-}]_{i} stimulates Cl^{-} influx through GABA_{A} and glycine receptors, resulting in hyperpolarization. High [Cl^{-}]_{i} in contrast, stimulates Cl^{-} efflux, resulting in depolarization [103]. Regulation of [Cl^{-}]_{i} is also essential to maintain normal cell volume. This is achieved either through regulatory volume increase (RVI) or regulatory volume decrease (RVD), the processes by which osmotically shrunken or swollen cells are restored to their original volumes [103]. The putative candidates for [Cl^{-}]_{i} regulation in the context of cerebral edema are the NKCC, KCC, VRAC, TMEM16A, cystic fibrosis (CF) transmembrane conductance regulator (CFTR), and voltage-gated chloride channels (CICs).

NKCC

First described by Geck et al., NKCCs are membrane proteins that maintain cell volume by RVI [104]. Two isoforms of NKCCs are currently characterized; NKCC1 is found in most mammalian cells whereas NKCC2 is exclusively found in the kidney [105]. In the CNS, the NKCC1 isoform (132 kDa) is expressed in neurons, astrocytes, endothelial cells, and epithelial cells of the choroidplexus [105]. Under physiological conditions, cell shrinkage triggers NKCC1 activation and the electroneutral influx of 1 Na^{+}, 1 K^{+}, and 2 Cl^{-}, coupled with an isosmotic water flow [104]. NKCC1 is a secondary-active transporter, and its activity is driven by TM Na^{+} and K^{+} gradients as established by Na^{+}/K^{+} ATPase [105].

The overall structure of NKCC1 is a dimer, consisting of 12 TM domains with intracellular N- and C-termini [106]. NKCC1 activation is associated with phosphorylation at its N-terminal domain and its activity can be stimulated by various factors that are well-implicated in acute brain injuries [107]. These factors include high extracellular K^{+}, excitatory amino acids (EAAs), hypoxia, aglycemia, and arginine vasopressin [108, 109]. As previously mentioned, extracellular K^{+} is rapidly elevated in the early stages of acute ischemic stroke, reaching 20 times its original concentration [30]. The elevated [K^{+}]_{o} creates a large driving force, and stimulates NKCC1 activity in neurons and the surrounding astrocytes to facilitate rapid K^{+} uptake [105]. Since K^{+} returns to the cells with Na^{+}, Cl^{-}, and water, the net effect is an increase in

Our lab has shown that K_{ATP} (Kir6.2/SUR1) channels are neuroprotective through potentially inducing cellular hyperpolarization during ischemia and hypoxic preconditioning (HPC) [35, 101]. Previously, we demonstrated that pretreatment with tolbutamide in neonatal mice abolished the effects of HPC-induced neuroprotection and worsened neurodegeneration following hypoxic-ischemic insults. Contrary, pretreating with a K_{ATP} activator, diazoxide, enhanced neuroprotection [101]. Our lab also showed that administering tolbutamide to mice subjected to pMCAO resulted in exacerbated brain damage and poorer behavioral outcomes. Opposing effects including reduced brain damage and improved behavioral outcomes were obtained using the K_{ATP} activator diazoxide on the pMCAO model [102]. Furthermore, a subsequent meta-analysis illustrated that patients with type-2 diabetes mellitus (T2DM) had a greater odds ratio for stroke morbidity when treated with sulfonyleureas [102]. A possible explanation is that anti-type-2 diabetes medication sulfonyleureas may have interfered with the neuroprotective role of the K_{ATP} channels in mediating membrane hyperpolarization during ischemia as well as HPC. Taken together, sulfonyleureas may have nonspecific effects on K_{ATP} (Kir6.2/SUR1), as well as NC_{ca,ATP} channels; further investigations are needed to elucidate their individual effects when specific blockers for each channel have been identified.

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intracellular osmolarity and cytotoxic edema [103, 110]. In fact, both neurons and astrocytes of NKCC1\(^{-/-}\) mice exhibited reduced intracellular Na\(^+\) accumulation, and cytotoxic edema after oxygen-glucose deprivation (OGD) [110]. In astrocytes, K\(^+\)-induced NKCC1 activation also leads to the release of EAAs such as glutamate, which in turn stimulates NKCC1 activity [111]. NKCC1\(^{-/-}\) astrocytes showed decreased EAAs release and astrocytic swelling compared to wild-type astrocytes in response to either elevated [K\(^+\)\(_{in}\)], or OGD [110, 112]. This K\(^+\)-induced NKCC1 activation in astrocytes can be completely abolished by either removal of extracellular Ca\(^{2+}\) or blocking the L-type voltage-dependent Ca\(^{2+}\) channels, suggesting that the activation of NKCC1 under conditions of high [K\(^+\)\(_{in}\)], is mediated through Ca\(^{2+}\)-dependent pathways [108].

Unlike astrocytes and neurons, where NKCC1 is primarily responsible for cytotoxic edema, NKCC1 activation in BBB endothelial cells mediates ionic and vasogenic edema following injuries [113]. Specifically, the luminal-facing NKCC1 facilitates the influx of Na\(^+\), which is then pumped out through the abluminal-facing Na\(^+\)/K\(^+\) ATPase [113]. Consistent with in vitro studies, the expression of NKCC1 is upregulated and correlated with cell swelling and brain edema in both ischemic stroke and TBI models in rats [80, 114–116]. In addition, both NKCC1\(^{-/-}\) and NKCC1\(^{+/-}\) mice showed less brain damage following tMCAO, with a ~45% and ~30% reduction, respectively, in infarction volume [110]. Brain edema was also significantly attenuated in both NKCC1\(^{-/-}\) and NKCC1\(^{+/-}\) mice [110]. Together, these studies suggest that targeting NKCC1 may be beneficial in reducing cerebral edema following TBI and acute ischemic stroke.

The FDA-approved loop diuretic bumetanide is a potent inhibitor of NKCC1 and NKCC2. Low doses of bumetanide can specifically inhibit NKCC1, without producing the diuretic effects associated with NKCC2 inhibition. In animal studies, intravascular administration of bumetanide significantly reduced infarction volume and brain edema in rats after pMCAO [80]. Bumetanide is also protective against TBI in terms of reducing brain concussion and brain edema [115]. These effects of bumetanide are potentially mediated though anti-neuroinflammatory pathways, including the inhibition of TRPV4 and phosphorylation of MEK, ERK, and Akt [115].

Since bumetanide contributes to Cl\(^-\) and GABAergic regulation, it has been shown to have promising therapeutic effects in neurological disorders related to impaired GABAergic signaling in clinical trials. These neurological disorders include temporal lobe epilepsy, autism spectrum disorder, and Parkinson’s disease. Clinical trials using bumetanide to treat ischemic and TBI-related edema have not yet been performed.

It is worth mentioning that bumetanide does not readily cross the BBB under physiological conditions. This is due to its high ionization (>99%) and plasma protein binding (>95%) properties [117, 118]. The activation of NKCC1 in endothelial cells, in fact, promotes the uptake of systemically administered bumetanide [80, 105]. To improve the brain bioavailability of bumetanide, a series of prodrugs with higher lipophilicity have been recently identified. Compared with other prodrugs, administration of the ester prodrug BUM5 obtained a higher brain concentration of bumetanide [119]. BUM5 was also thought to be more efficacious than bumetanide in rodent models of epilepsy [119, 120]. A novel NKCC1 inhibitor STS66, with high BBB penetration, has been proposed very recently to have a higher efficacy than BUM5 and bumetanide in reducing infarction and cerebral edema after ischemic stroke in mice [121]. Together, these findings suggest that compounds that specifically target NKCC1 with a better BBB penetration property will hold more therapeutic potential in treating brain diseases related to altered Cl\(^-\) level.

Note that the function of NKCC1 requires ATP indirectly, and thus is mostly involved in early stages of edema development, during which the activity of the Na\(^+\)/K\(^+\) ATPase is still adequate, and the ion gradients are not completely collapsed. In this context, it might be valuable to couple an inhibitor of NKCC1 with a drug that targets the later stages, when ATP is depleted and NKCC1 activity is compromised. The SUR1-TRPM4 channels, as mentioned above, are only transcribed and expressed under injury conditions, suggesting that they may play a more predominant role in the later stages of cerebral edema. Thus, using a combined regimen of bumetanide plus glibenclamide may therefore offer greater clinical outcomes.

KCCs

KCCs are another group of cotransporters that are critical for Cl\(^-\) homeostasis. KCCs were first discovered in red blood cells, where they are entirely responsible for RVD [122]. Four different subtypes of KCCs (KCC1–4) have been identified. Early work described the association between human malignancy and upregulated KCC expression and activity; namely, augmented transport rates were found to accompany increased KCC1, -3, and -4 mRNA levels in cervical cancer cells [123]. This enhanced osmotic sensitivity, supported by KCC-mediated Cl\(^-\) transport, is consistent with the ability of glioma cells to thrive in the edematous environment they often present with. In the DS5-MG cell line as well as in glioma cells from acute patient biopsies, 30%-40% of Cl\(^-\) efflux during RVD occurs through KCC1 and -3a, primarily within the initial stages of volume regulation. KCC-specific inhibitor, [(dihydroindenyl)oxy] alkanoic acid (DIOA), was observed to inhibit the hypotonic-activated Cl\(^-\) current and resultantly slow the RVD [124].

Although the KCC family have been detected in glioma, cervical cancer cells and recently, lens epithelial cells, the KCC2 isofrom (123.6 kDa) is generally thought to be neuronal-specific [125, 126]. Unlike other subtypes, which are activated only after osmotic stress, KCC2 is distinct in its activation under isotonic conditions. Structurally, KCC2 consists of 12 TM domains with intracellular N- and C-termini [127]. The C-terminus contains multiple phosphorylation sites, which are critical for KCC2 regulation [127]. Under physiological conditions, KCC2 maintains Cl\(^-\) homeostasis and cell volume by extruding Cl\(^-\) along with K\(^+\) in the stoichiometry of 1:1 [127, 128]. The direction of ion transport via KCC2 is reversible and is largely driven by the electrochemical gradient of K\(^+\) [127, 128]. By maintaining low [Cl\(^-\)], KCC2 ensures the hyperpolarizing inhibitory effects of GABA and glycine by keeping the reversal potential of Cl\(^-\) below the membrane potential (E\(_{Cl}\) < V\(_{m}\)) in mature neurons [129].

Impaired KCC2 activity and Cl\(^-\) imbalance have been associated with several neurological conditions including traumatic and ischemic injuries, epilepsy, neuropathic pain, and schizophrenia [130-132]. In excitotoxic injuries, the large increase in extracellular K\(^+\) causes a significant reduction in KCC2-mediated Cl\(^-\) extrusion. In extremely high [K\(^+\)]\(_{in}\) conditions, KCC2-mediated transport of K\(^+\) and Cl\(^-\) may reverse and promote edema formation by facilitating Cl\(^-\), K\(^+\), and water influx [103].

More importantly, impaired Cl\(^-\) extrusion could lead to a positive shift in E\(_{GABA}\). The resultant reduction in GABAergic and glycineergic inhibition may initially promote mechanisms of plasticity and recovery, resembling developmental features in the immature brain [128]. However, [Cl\(^-\)], overload, as generated in severe injuries, can lead to a depolarizing shift in E\(_{GABA}\). Subsequent Cl\(^-\) efflux through GABA\(_A\)R could instead, generate a large depolarizing GABA current [103, 128]. This switch of GABA from inhibitory to excitatory contributes to hyperexcitability [128]. Thus, it is not surprising that the two common clinical sequelae of ischemic stroke, namely edema and seizures, are related to impaired KCC2 activity and expression as well as the dysregulation of Cl\(^-\) [103].

Altered KCC2 expression and Cl\(^-\) homeostasis have been reported in experimental models of ischemic stroke and TBI. Specifically, hippocampal slices subjected to OGD showed a
KCC2 in injured neurons seems to be dependent on GABA A-mediated signaling in mature neurons, and improved the maintenance of hyperpolarizing GABAergic inhibition reduced the glutamate-induced downregulation of KCC2 [135]. A recent study using the controlled cortical impact model of TBI in rats also showed decreased mRNA and protein expression of KCC2 in the injured cortex and these changes were associated with cerebral edema and functional deficits [136]. Although not yet well understood, BDNF/TrkB and glutamate-dependent pathways have been proposed to contribute to the downregulation and/or inactivation of KCC2 in acute brain injuries. A recent study using dissociated rat neurons demonstrated that the glutamate-mediated activation of NMDARs and Ca2+ influx led to dephosphorylation of KCC2 at Ser940, causing a significant loss of KCC2 function [137]. NMDAR activation also leads to KCC2 cleavage by the calcium-activated protease calpain [138]. Conversely, inhibiting dephosphorylation of Ser940 or using a calpain inhibitor reduced the glutamate-induced downregulation of KCC2 and improved the maintenance of hyperpolarizing GABAergic inhibition [137]. Multiple studies have also proposed that BDNF is one of the upstream regulators of KCC2; BDNF downregulates KCC2 expression through TrkB-mediated signaling in mature neurons while upregulating KCC2 in immature neurons or injured neurons [129, 139–141]. This up-regulatory effect of BDNF on KCC2 in injured neurons seems to be dependent on GABA A-mediated depolarization, and is also speculated to promote mechanisms of repair and recovery [141]. Melatonin has been implicated in restoring KCC2 expression, attenuating brain edema, cell death, and neurological deficits after TBI in rats [136]. These neuroprotective effects of melatonin were mediated partially through the BDNF/ERK pathway, although the detailed mechanisms remain undefined [136].

The contribution of the KCC2 to cell swelling and the mechanisms of KCC2 regulation in acute brain injuries have not yet been elucidated as understanding these mechanisms has proven challenging. KCC2+/− mice die shortly after birth due to respiratory failure or spontaneous generalized seizures [142, 143]. Hypomorphic KCC2-deficient mice also exhibit increased seizure susceptibility and anxiety-like behaviors [144]. Furthermore, compounds like furosemide and bumetanide have been shown to inhibit KCC, however, their inhibitory effects are more potent towards NKCC1 than KCC, especially at low doses [117].

Recent inhibitors of KCC include DIOA, ML077, and VU0463271 [145–147]. Among these, VU0463271 has been identified to block KCC2 with the highest potency (IC50 = 61 nM) and display >100-fold selectivity towards KCC versus NKCC1 [147]. Administration of VU0463271 led to a depolarizing shift in E_cl, suggesting that this silencing KCC2 using shRNA increased [Cl−], and neuronal susceptibility to excitotoxicity [145]. The effects of KCC2 knockdown or VU0463271 in ischemic stroke and TBI have not yet been addressed. Nevertheless, considering the dual function of KCC2 in both cell volume and GABAergic signaling regulation, pharmacological agents that rescue or activate, rather than inhibit KCC2 function may be a potential therapeutic strategy for attenuating brain edema and hyperexcitability in various neurological disorders.

**VRAC**

VRACs or volume-sensitive outwardly rectifying anion channels (VSORs) are widely expressed, Cl−-permeable anion channels. VRACs maintain cell volume through RVD, and mediate the swelling-induced Cl− current I_clswell [148, 149]. Under physiological conditions, RVD via VRACs is accomplished by the efflux of Cl− coupled with K+ and subsequent water efflux, leading to cell shrinkage [149]. Other than cell volume regulation, I_clswell has been implicated in various cellular functions such as synaptic transmission, cell proliferation, migration, and apoptosis [149].

VRAC is ubiquitously expressed in mammalian tissues. VRAC currents were first reported in 1988 by Cahalan and Lewis in human T lymphocytes and Hazama and Okada in human intestine epithelial cells [150, 151]. Both groups showed that application of a hypotonic solution activated the outward rectifying I_clswell while the inhibition of I_clswell coincided with a reduced RVD [150, 151]. In the CNS, VRAC or VRAC-like currents have been reported in neurons, astrocytes, and microglia [149]. Despite decades of extensive research, the molecular nature of VRAC remains ill-defined. Recent studies identified using genome-wide RNAi screening have identified the leucine-rich repeat-containing 8 (LRRC8) proteins as molecular components of VRAC. VRAC was proposed to be a hetero-hexameric complex (~800 kDa) composed of LRRCA8 (also known as SWELL1) with at least one other LRRC8 parologue (LRRC8B-E) [152, 153]. These LRRC8 paralogues form assemblies with distinct biophysical properties. More recent studies suggest that LRRC8A can also assemble into homohexameric channels [154, 155]. In fact, knockdown of LRRC8A using siRNA abolished I_clswell suggesting that the LRRC8A is indispensable for VRAC-mediated conductance [153]. Point mutations in LRRC8A also caused a significant change in its anion selectivity [152]. In addition to Cl−, VRACs are permeable to other anions such as I− and amino acids, including glutamate, aspartate, and taurine, and other small osmolytes. Besides cell swelling, VRAC activation can be induced isovolumetrically by various cellular factors. Low intracellular ionic strength, for example, directly activates VRAC [156]. Under isosonic conditions, VRAC activation can also be initiated by Na+ overload via glutamate receptors and ATP [157, 158].

In acute ischemic stroke and TBI, overactivation of VRACs due to persistent depolarization results in Cl− influx, rather than efflux as in RVD [148]. Using the OGD model, Zhang et al. showed a progressive increase in I_clswell amplitude over the period of post-OGD reperfusion due to VRAC activation [157]. The same study also revealed that OGD-induced I_clswell was significantly attenuated by inhibitors of both AMPAR (NBQX) and NMDAR (AP-5), suggesting that the activation of VRAC and the induction of I_clswell require excessive Na+ loading via these glutamate receptors [157].

Upon activation, VRACs can mediate the release of EAs such as glutamate and aspartate, and can modulate neuronal excitability by changing Cl− permeability independent of the GABA A receptors [149, 150]. An important pathophysiological implication in ischemic or traumatic injuries is that astrocytes at the site of injury rapidly swell, activate VRACs and release EAs [148, 160]. Although the permeability to glutamate is not as high as to Cl−, VRAC-mediated EAA release is sufficient to evoke depolarization and increase [Ca2+]i in adjacent neurons, exacerbating excitotoxic cell death [160]. Consistently, a recent in vivo study demonstrated that astrocyte-specific LRRCA8 knockout mice were protected after tMCAO, with reduced glutamate-dependent neuronal excitability [158].

Studies showed that ATP released from damaged or swollen neurons and astrocytes significantly increased VRAC-mediated EAA release from astrocytes [161]. Since the activation of VRACs in astrocytes is dependent on exogenous ATP, the VRAC-mediated EAA release was more profound in the penumbra, where neuronal intracellular ATP level remains high [159].

VRAC inhibitors, including tamoxifen and the more selective ethacrynic acid derivative, DCPIB, have shown neuroprotection against ischemic stroke [162, 163]. DCPIB in vitro decreased neuronal intracellular Cl− concentration induced by low ionic strength and abolished OGD-induced cell death [38, 157]. DCPIB also significantly reduced infarction volume and improved neurobehavioral deficits in vivo in a rat tMCAO model [162]. These neuroprotective effects were elicited through the inhibition...
of VRAC-mediated EAA release and Cl\textsuperscript{−} influx \cite{159, 162}. DCPIB has been previously defined as a potent and selective VRAC inhibitor and does not inhibit other Cl\textsuperscript{−} channels (CFTR, CIC, CaCC) at concentration ranges used for VRAC. A more recent study, however, suggested that DCPIB may also target other glutamate transporters pathways, such as GLT-1 and connexin hemichannel in glial cells \cite{163}. Therefore, the in vivo effect of DCPIB on reducing EAA release may not be solely attributed to its action on VRACs. Further research on characterizing the specific mechanisms of DCPIB action is required. Nevertheless, these studies concluded that targeting VRAC may provide a new therapeutic approach to cerebral edema following acute brain injuries.

**TMEM16A**

TMEM16A, the first of ten members of the Anoctamin or TM protein 16 family, was first reported in 2008 by three independent groups to confer Ca\textsuperscript{2+}-activated Cl\textsuperscript{−} currents \cite{164, 166}. TMEM16A and close paralog TMEM16B, have since been identified to function as calcium-activated chloride channels (CaCCs) with differing activation and deactivation kinetics and [Ca\textsuperscript{2+}]\textsuperscript{−} activation thresholds that suggest a prominent role for the latter in native olfactory sensory neuron CaCC activity \cite{167}. Meanwhile, TMEM16A expression coincides with that of endogenous CaCCs including secretory epithelial cells, airway and vascular smooth muscle cells, sensory neurons, and various endothelial cell types \cite{164}. Accordingly, under physiologic conditions, anion permeation via TMEM16A regulates mucus secretion, smooth muscle contraction, gut mobility, and RVD \cite{164, 168}.

Homology modeling of fungal homolog, nhTMEM16, and recent single-particle cryoEM of TMEM16A revealed the homodimeric structure consisting of 10 TM domains, two independently activated ion conduction pores as well as highly conserved Ca\textsuperscript{2+} binding sites and pore-lining residues critical for Cl\textsuperscript{−} selectivity and channel gating \cite{169-171}. As a CaCC, TMEM16A exhibits both \(V_m\) and [Ca\textsuperscript{2+}]\textsuperscript{−} gating properties; at low [Ca\textsuperscript{2+}], the current is largely voltage-dependent and outwardly rectifying, however as [Ca\textsuperscript{2+}]\textsuperscript{−} increases, Ca\textsuperscript{2+}− alone may induce a strong Cl\textsuperscript{−} current characterized by a linear I−V relationship \cite{168, 172}. Extracellular Cl\textsuperscript{−} has also been suggested to stabilize the open conformation, and allosterically modulate Ca\textsuperscript{2+} sensitivity of TMEM16A \cite{172}.

In endothelial cells, pathological or agonist-induced high cytosolic Ca\textsuperscript{2+} activates TMEM16A, leading to Cl\textsuperscript{−} efflux and cell volume modulation \cite{173}. However, isovolumetric conditions may also activate CaCCs, implicating TMEM16A in the cell shrinkage that initiates apoptotic volume decrease \cite{174}. Interestingly, Almaça et al. showed that through an ATP-purinergic P2Y receptor binding mechanism and ERK1/2 activation, TMEM16A contributes to swelling-activated Cl\textsuperscript{−} conductance. siRNA-TMEM16A was found to suppress \(I_{Cl\ellowell}\) and RVD in three separate cell lines under increased extracellular hypotonicity, while TMEM16A overexpression augmented swelling-activated Cl\textsuperscript{−} currents. Furthermore, TMEM16A inhibitor, CACChin-A01, was observed to significantly reduce \(I_{Cl\ellowell}\) to an extent comparable to both tamoxifen and nonspecific Cl\textsuperscript{−} channel blocker, DIDS. \(I_{Cl\ellowell}\) was also nearly abolished in epithelial cells of TMEM16A\textsuperscript{−/−} mice \cite{175}.

Although significant progress in understanding the molecular and structural underpinnings of TMEM16A function has been made, the precise interaction mechanisms of modulators have yet to be clearly addressed \cite{168}. Much of recent literature has explored the therapeutic effects of TMEM16A inhibitors on cancer cell lines given the role of TMEM16A-activated signal transduction on cancer cell proliferation and migration – TMEM16A overexpression is commonly accounted for by gene amplification in malignant tumors, and cell-specific signaling pathways in non-tumor cells under pathological conditions \cite{174, 176}. In addition, TMEM16A dysfunction has been associated with CF, hypertension, and more recently, stroke \cite{176}.

TMEM16A was recently proposed to contribute to ischemic damage after being identified in cardiac vascular endothelial cells of mice; TMEM16A-mediated \(I_{ClCa}\) was found to be potentiated under hypoxic conditions via increased protein expression levels, and enhanced channel sensitivity to [Ca\textsuperscript{2+}], and membrane depolarization \cite{173}. Moreover, TMEM16A was shown to play a role in regulating BBB permeability following ischemic stroke. TMCAO of adult mice resulted in a marked upregulation of TMEM16A, with particular abundance in brain epithelial cells. CaCCinh-A01, TMEM16A antagonist, attenuated brain infarction and functional deficits, and preserved BBB integrity by preventing the loss of tight junction proteins, and downregulating ICAM-1 as well as neutrophil adhesion and infiltration \cite{177}. In vitro, silencing of TMEM16A was also found to rescue OGD-induced transendothelial permeability by suppressing the NF-κB-ICAM-1 signaling pathway \cite{177}. It is important to note that T16ainh-A01, another well-documented TMEM16A inhibitor, did not yield the same protective effects; CaCCinh-A01 is distinct in its ability to both inhibit channel activity as well as reduce TMEM16A protosomal turnover, while T16ainh-A01 has no effect on channel expression levels \cite{178}. Moreover, MONNA is another compound that exerts inhibitory effects on TMEM16A-mediated \(I_{ClCa}\). While ample evidence supports the antagonizing effects of these small molecule inhibitors, recent literature has demonstrated that T16ainh-A01, CaCCinh-A01, and MONNA were still able to induce vasorelaxation in rodent resistance vessels under Cl\textsuperscript{−}−free conditions, thereby challenging their selectivity for TMEM16A Cl\textsuperscript{−} conductance \cite{179}.

While the role of TMEM16A in cerebral edema following acute brain injury remains elusive, its contribution to both \(I_{Cl\ellowell}\) and \(I_{ClCa}\) combined with the effects of TMEM16A inhibition on preventing BBB leakage has profound implications for acute stroke patients and warrant further research \cite{180}.

**CFTR**

CFTR is a cAMP-activated Cl\textsuperscript{−}−selective channel belonging to the superfamily of ABC transporters. It is unique amongst other ABCs in that it functions as an ion channel \cite{181}. CFTR is a large protein (180 kDa), consisting of 12 TM domains, 2 cytosolic nucleotide-binding domains (NBDs), and a regulatory (R) domain \cite{181}. The NBDs are responsible for ATP hydrolysis, and phosphorylation of the R domain is required for channel opening, which leads to bidirectional Cl\textsuperscript{−} transport down its electrochemical gradient \cite{181}. Besides Cl\textsuperscript{−}, CFTR is permeable to bicarbonate, thiocyanate, and other anions.

Although the brain is not traditionally viewed as an affected organ in CF, patients with CF who underwent lung transplants are associated with a high risk of developing neurologic complications, such as seizure and stroke, among other lung transplant recipients \cite{182, 183}. In the CNS, CFTR is mainly expressed in neurons, but not in glial cells \cite{181}. A study by Zhang et al. showed that CFTR acted to prevent neuronal apoptosis following cerebral ischemia-reperfusion in mice \cite{184}. Stimulating CFTR with its activator forskolin suppressed apoptosis whereas silencing CFTR by siRNA enhanced apoptosis \cite{184}. These effects of CFTR were thought to be mediated by regulating oxidative stress and glutathione in mitochondria-dependent pathways.

The role of CFTR in edema formation is primarily studied in the lung, where CFTR regulates the secretion and absorption of alveolar fluid. Specifically, CFTR expression was upregulated in pulmonary edema, whereas inhibition of CFTR decreased alveolar fluid secretion \cite{185}. Consistent with this, CFTR\textsuperscript{−/−} mice were protected from pulmonary edema \cite{185}. In the CNS, the upregulation of CFTR mRNA and protein has been associated with infection-induced cerebral edema through unknown mechanism \cite{186}. A very recent study has also established a strong relationship between cerebral artery CFTR expression and impaired cerebrovascular reactivity as well as
perfusion in mouse models of heart failure and subarachnoid hemorrhage [187]. High-affinity inhibitors as well as potentiators and correctors of CFTR have been largely identified via high-throughput screening in the last two decades. The CFTR potentiator VX-770/ivacaftor has been shown to be highly effective in restoring channel activity and has been approved for treating patients with a variety of CFTR mutations [188]. To rescue the specific F508del mutation, CFTR correctors, such as VX-809/lumacaftor, have been discovered to improve folding and stability of CFTR at the cell surface [188].

Regarding CFTR inhibitors, the thiazolidinidine CFTRinh-172 (IC$_{50}$ = 300 nM) and the glycine hydrazide GlyH101 (IC$_{50}$ = 5 μM), both identified by Verkman and colleagues in the early 2000s, are mostly studied [189, 190]. Both CFTRinh-172 and GlyH101 have been shown to significantly reduce alveolar Cl$^-$ and fluid secretion in different animal models of pulmonary edema [185, 191]. CFTRinh-172 has also been implicated in blocking toxin-induced intestinal Cl$^-$ and fluid secretion [190, 192]. A third class of CFTR inhibitors, the pyrimido-pyrrolo-quinaxolinediones, including PPQ-102 (IC$_{50}$ = 90 nM) and its related compound BPO-27 (IC$_{50}$ = 8 nM) were later discovered by the same group, with improved potency, solubility, and stability [193].

Recent comparative studies, however, suggested that GlyH101 and PPQ-102 are not CFTR-selective. At concentration ranges used to inhibit CFTR, the VRAC- and CaCC-mediated Cl$^-$ conductance can also be inhibited by these two compounds [194, 195]. The most-selective CFTR inhibitor to date is CFTRinh-172 as it does not affect CaCC and only exerts minor inhibition on VRAC conductance even with high concentrations [194].

While CFTR is capable of driving edema formation, further research is required to investigate its functional role in the context of cerebral edema following acute brain injuries. The effects of selective and potent CFTR inhibitors in managing cerebral edema also remain to be evaluated.

CICs
Nine members of the CIC family, facilitating voltage-dependent Cl$^-$ transport, have been identified. While the CIC family share a dimeric structure and Cl$^-$ gradient dependency, CIC-1, -2, -Ka, and -Kb function as plasma membrane Cl$^-$ channels, while CIC-3 to -7 operate as Cl$^-$/H$^+$ exchangers localized to intracellular compartments [196, 197]. Several members of the CIC family have been proposed as viable candidates for cell volume regulation and osmotic homeostasis, primarily CIC-2 and -3 [198]. CIC-2 are widely expressed in mammalian tissue and activated upon hyperpolarization and mild decreases in extracellular pH levels [196]. Although the inwardly rectifying Cl$^-$ conductance and anion selectivity characteristic of CIC-2 are distinct from typical I$_{CL,swell}$. CIC-2 was demonstrated to contribute to volume-sensitive changes of hepatoma tissue culture (HTC) cells. Microinjection of HTC cells with CIC-2 antibodies inhibited volume-activated currents and delayed hypotonic-induced cell volume recovery [198]. Furthermore, administration of DIDS, to which CIC-2 is largely resistant, was found to block ~40% of volume-activated currents, implicating the involvement of CIC-2 in the remaining 60% [198]. The presumptive role of CIC-2 in regulating cerebral ion homeostasis is furthermore illustrated by the proposition of CLCN2, encoding CIC-2, as a candidate gene for leukodystrophy. CIC-2$^{-/-}$ mice were found to display CNS myelin vacuolation accompanied by enhanced microglial activation and impaired BBB, hypothesized to result from disrupted ion homeostasis of the astrocytic/oligodendrocytic network [199]. The pathology of CIC-2$^{-/-}$ mice resembles human leukodystrophy and although controversial, evidence does suggest that CLCN2 mutations are associated with leukoencephalopathies characterized by intra- myelinic edema [200]. An observational analytical study found that CLCN2 mutations leading to CIC-2 loss-of-function occurred in 6/7 patients that presented with such disease criteria. Interestingly, healthy controls exhibited abundant CIC-2 membrane expression in the ganglial synctium, a network of glial cells specialized for osmotic homeostasis in myelinated axons [200].

Several Cl$^-$ currents including I$_{CL,swell}$ have been attributed to CIC-3 [201, 202]. Early studies found that use of CIC-3 antisense oligonucleotide or CIC-3 antibody inhibited VRAC currents in multiple cell types, thereby identifying CIC-3 as a molecular component of volume-regulated cation channel (VRCC) regulation [201, 203]. As a VRCC, CIC-3 has been associated with proliferation and apoptosis in rodent vascular smooth muscle cells, both of which are associated with cerebrovascular remodeling in cardiovascular disease such as stroke [204]. However, the contribution of CIC-3 to I$_{CL,swell}$ has remained inconsistent partially due to the lack of a selective inhibitor [202]. Nevertheless, CIC-3 along with CIC-2, -5, -6, and -7 were recently proposed as the repertoire of Cl$^-$ channels contributing to RVD in human glioma cells; under changing osmolarities, glioma cells can notably regulate their volume, at times recovering to a volume lower than baseline levels [124]. Five members of the CIC family were identified as the candidates accounting for 60%-70% of the Cl$^-$ conductance during RVD. 5-nitro-2-(3-phenylpropylamino)benzoic acid was shown to act synergistically with Cd$^{2+}$ to inhibit CIC-mediated conductance, which corresponds with the respective sensitivities of CIC-3 and CIC-2 to these inhibitors [124]. The CIC family, particularly CIC-2 and -3, are notable candidate proteins for regulating ion homeostasis and swelling-induced RVD, and incite future study into their potential role in cerebral edema following acute injury such as stroke and TBI.

NCX
The sodium–calcium exchanger (NCX) is a bidirectional ion channel that maintains intracellular [Na$^+$] and [Ca$^{2+}$] homeostasis. All three mammalian isoforms of NCX (NCX1, NCX2, and NCX3) are expressed in neurons and glial cells, and are associated with various cellular functions such as neurotransmitter release and myelin formation [205]. Structurally, eukaryotic NCX consists of 10 TM domains with a large intracellular regulatory loop between TM5 and TM6 [206]. This loop includes two regulatory Ca$^{2+}$-binding domains, CBD1 and CBD2, which enable the dynamic regulation of [Ca$^{2+}$]i [206, 207].

The modes of ion transport via NCX depend on the electrochemical gradients of Na$^+$ and Ca$^{2+}$: Under physiological conditions, NCX operates in its forward mode. It maintains a low level of intracellular Ca$^{2+}$ by extruding one Ca$^{2+}$ in exchange with three Na$^+$, with an electronegative current [208]. Changes in [Na$^+$], are then balanced through Na$^+$/K$^+$ ATPase. In this forward mode, either an increase in [Na$^+$], or a decrease in [Ca$^{2+}$]i, leads to NCX inactivation and decreased NCX current [209]. The reverse mode of NCX, which mediates Na$^+$ efflux and Ca$^{2+}$ influx, has been associated with CNS injuries. In addition to the 3Na$:1Ca^{2+}$ transport ratio, two minor modes of NCX transport have also been proposed to operate at low rates [210].

In acute ischemic stroke, the mode of operation of NCX differs at the ischemic core and the penumbra. NCX operates in its forward mode to extrude Ca$^{2+}$ in the ischemic penumbra, where ATPase activity remains high and is still able to counterbalance the influx of Na$^+$ [211]. In contrast, ATPase activity is often compromised at the ischemic core. Na$^+$ overload, in addition to mediating cytotoxic edema, increases Em. When Em exceeds E$_{N_{K}}$, NCX will shift to its reverse mode [212]. This reverse mode of NCX operation has also been shown in vitro after severe, but not mild or moderate TBI [213]. Reversal of NCX can result in two opposing effects in the injury core; it increases Ca$^{2+}$-dependent cytotoxicity and decreases Na$^+$-dependent cell swelling [212, 214]. However, the net effect and the precise mechanism of NCX working in its reverse mode are still debatable. The expression and operation mode of the three NCX subtypes in permanent vs. transient
glomerular filtration and paracellular transport [191]. Collectively, these findings suggest a combined role for both neurotransmitters and extracellular ions in mediating brain edema.

**ION CHANNELS IN BRAIN EDEMA**

**Ischemic Brain Edema**

Ischemic brain edema occurs due to reduced cerebral perfusion, leading to decreased oxygen and glucose delivery to the brain. This results in increased cerebral metabolic demand, exacerbating the ischemic injury [230]. The presence of SGLT in BBB endothelial cells has been speculated in vitro, in vivo studies suggested an absence of SGLT-mediated glucose transport at the BBB under physiological conditions [230–232].

Enhancedgene or protein expression of both GLUT1 and SGLT1 at the BBB has been implicated in experimental models of ischemic stroke and TBI [231, 233, 234]. Consistent with these findings, brain imaging studies have suggested elevated glucose uptake and glycolysis hypermetabolism during ischemic stroke, especially in perinfarcted regions [234, 235]. Due to the high abundance of GLUTs at the BBB, they are thought to represent a major route for transendothelial water flux during ionic edema, as they have the ability to passively transport water, analogously to the AQP5 [13, 14]. In fact, mice pretreated with SGLT inhibitor, phlorizin, showed reduced infarction volume and brain edema after pMCAO [231]. Downregulating SGLT1 was also shown to be beneficial in TBI in terms of reducing cerebral edema and contusion volume [233]. These protective effects of SGLT inhibition are potentially due to reduced Na⁺ and water transport through the endothelial cell, as well as reduced acidosis and free radical production from the anaerobic metabolism of glucose [231].

In human studies, high blood glucose level has been described as an important predictor for early cerebral edema in patients with ischemic stroke, although the reasons for this phenomenon remain unknown [236, 237]. A recent postmortem study that examined 15 TBI cases found that the expression of SGLT1 and SGLT2 was significantly higher in contusional tissue, compared to that of the contralateral uninjured tissue or healthy brain tissue [238]. SGLT1 and SGLT2 in contusional regions also exhibit a biphasic pattern of increase that peaked at 72-h post injury, which may correlate with delayed secondary injuries after TBI [238].

Collectively, these findings suggest a combined role for both SGLT and GLUT in mediating glucose and water transport at the BBB under ischemic conditions [231]. To date, pharmacological inhibitors of GLUTs are extensively studied in T2DM, and multiple SGLT inhibitors have been approved by the FDA for treating diabetes. Our current understanding of brain glucose transport in acute brain injuries, however, is still limited, thereby warranting future investigations to elucidate the pathophysiological roles brain GLUTs play.

**PROTON-SENSITIVE CHANNELS/EXCHANGERS**

**ASIC**

ASICs are a class of ligand-gated, voltage-insensitive cation channels that belong to the epithelial sodium channel/degenerin family. ASICs are widely expressed in the CNS and activated by extracellular acidosis. Six subunits of ASIC (ASIC1a, ASIC1b, ASIC2a, ASIC2b, ASIC3, and ASIC4) have been identified, with ASIC1a having the highest proton sensitivity. Each subunit consists of 2 TM domains, a cysteine-rich extracellular loop, and intracellular N- and C-termini. The subunits may assemble into homomer or heteromeric ASIC2b/1a predominantly expressed [239, 240]. ASICs are generally more permeable to Na⁺, and to a lesser degree to Ca²⁺ and K⁺ [241]. Under physiological conditions, ASICs are noted for their roles in synaptic plasticity, nociception, and mechanosensation [242, 243]. ASICs have also been noted in ischemic stroke, multiple sclerosis, and epilepsy [13].

Extracellular acidosis in both ischemic stroke and TBI worsens secondary brain damage. With normal extracellular pH maintained around 7.3, ischemia and TBI typically induce a reduction of pH to 6.3 and 6.7, respectively, or even lower depending on the severity of the insult [243]. The build up of lactic acid rising from 1 to
NHEs are another major group of pH-regulated channels. Under physiological conditions, NHEs regulate pH, by the extrusion of 1 H⁺ in exchange of 1 Na⁺ [243]. In addition to pH regulation, NHEs also mediate RVI in osmotically shrunken cells by driving Na⁺ and water influx [243]. Structurally, NHEs consist of 12 TM segments, with intracellular N- and C-termini. Among the ten identified NHEs, NHE1 is the most abundantly expressed isoform in the CNS and is expressed in neurons, astrocytes, the luminal membrane of the BBB endothelial cells, and microglia [243].

Enhanced NHE1 activity has been associated with cerebral edema in ischemic stroke. In neurons and astrocytes, NHE1 activation contributes to Na⁺ overload and cytotoxic edema after OGD [250, 251]. This NHE1-mediated swelling is significantly reduced in NHE1−/− astrocytes [250]. A more recent study showed that selective knockout of astrocytic NHE1 reduced cerebral edema, infarct volume, and functional deficits after tMCAO [252]. NHE1−/− mice also exhibited reduced astroglisis and BBB breakdown, related to a reduced MMP-9 level [252]. NHE1 activity in BBB endothelial cells can also be stimulated by ischemic conditions to facilitate the influx of Na⁺ from the vasculature [253].

Two classes of pharmacological agents are known to inhibit NHE activity and have shown beneficial effects in experimental models of ischemic stroke [243]. The amiloride and its analogs dimethylamiloride and ethilsopropylamiloride inhibit NHEs activity in a nonselective manner [243]. However, as previously mentioned, amiloride is a nonspecific inhibitor as it also exerts inhibitory effects on ASICs and NCX [243]. Guanidine derivatives, including HOE-694, HOE-642 (cariporide), EMD-85131 (eniporide), and SM-20220, contribute to the second, and more selective class of NHE inhibitors [243]. Among all the guanidine derivatives, HOE-642 is the most selective and potent inhibitor of NHE1 and has demonstrated neuroprotection in reducing Na⁺ uptake and cerebral edema in both pMCAO and tMCAO models [243, 252, 253]. Furthermore, animals treated with a combination of HOE-642 and the NKCC1 inhibitor bumetanide, showed further reduction in cerebral edema and infarct volume in the initial hours following stroke onset compared to those treated with HOE-642 or bumetanide alone [253]. Based on current findings, NHE1 seems to be a promising target for attenuating cerebral edema in ischemic stroke. The involvement of NHE1 in TBI and the roles of other brain NHEs constitute an important direction for future studies.

SUMMARY
Brain edema is a complicated pathophysiological process that involves a variety of ion channels and transporters. Ion conduction of the same ion channel or transporter may also be altered at different stages of the edema. To date, the understanding of edema formation and propagation is limited. The discovery of effective anti-edematous drugs is challenging due to the dynamic nature of the process. As each ion channel and transporter has its distinct role in mediating brain edema, combinational therapeutic approaches targeting multiple ion channels or transporters may maximize the efficacy of anti-edematous therapy. The discovery of selective ion channel inhibitors thus constitutes a critical direction for future studies. Furthermore, continued investigations of the cellular and molecular mechanisms related to ion channel-mediated edema in acute brain injuries are necessary in order to yield novel and effective therapeutic strategies.

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ADDITIONAL INFORMATION

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