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Chloride dysregulation, seizures, and cerebral edema: a relationship with therapeutic potential

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Abstract

Pharmaco-resistant seizures and cytotoxic cerebral edema are serious complications of ischemic and traumatic brain injury. Intraneuronal Cl^- concentration ($[\text{Cl}^-]_i$) regulation impacts both cell volume homeostasis and Cl^- permeable GABA_A receptor-dependent membrane excitability. Understanding the pleiotropic molecular determinants of neuronal $[\text{Cl}^-]_i$ —cytoplasmic impermeant anions, polyanionic extracellular matrix (ECM) glycoproteins, and plasmalemmal Cl^- transporters—could help identify novel anti-convulsive and neuroprotective targets. The cation- Cl^- cotransporters and ECM metalloproteinases may be particularly druggable targets for intervention. Here, we establish a paradigm that accounts for recent data regarding the complex regulatory mechanisms of neuronal $[\text{Cl}^-]_i$ and how these mechanisms impact neuronal volume and excitability. We propose approaches to modulate $[\text{Cl}^-]_i$ that are relevant for two common clinical sequela of brain injury: edema and seizures.

Keywords

GABA; brain development; chloride; seizures; cerebral edema; inhibition; NKCC1; KCC2; traumatic brain injury; extracellular matrix

The role of Cl^- in neuronal excitability

GABA and glycine are the main inhibitory neurotransmitters in the mature central nervous system (CNS). Ligand-gated activation of GABA_A receptors (GABA_AR , see Glossary)

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opens a membrane anion conductance. The current flowing through these channels is carried mainly by Cl^- and to a lesser extent by bicarbonate (HCO_3^-) ions [1].

Both inward and outward neuronal Cl^- currents are frequently observed because the driving force for Cl^- movement across the membrane is small and can be readily reversed. In the adult brain, $[\text{Cl}^-]_i$ is 10 – 20 times lower than the extracellular Cl^- concentration, leading to a large driving force for inward Cl^- diffusion. However, this diffusive driving force is almost perfectly matched by the electrical force that arises from the negative resting membrane potential of neurons, which drives negatively charged Cl^- ions out of the cytoplasm. Consequently, whether Cl^- flows into or out of GABA or glycine-gated channel depends on the precise relationship between the reversal potential of Cl^- (E_{Cl^-} , see Glossary) and the neuron's membrane potential. E_{Cl^-} is the membrane voltage at which no Cl^- current flows. If the membrane potential is more negative than E_{Cl^-} , GABA_AR activation will result in outward flow of Cl^- from the neuron and membrane depolarization. If the membrane potential is positive to E_{Cl^-} , then GABA_AR activation results in inward Cl^- flow to the neuron and membrane hyperpolarization (Fig. 1). E_{Cl^-} is very sensitive to millimolar changes in $[\text{Cl}^-]_i$. A 1–2 mM change in $[\text{Cl}^-]_i$ can be significant in terms of the effect of GABA_AR activation on a neuron's membrane potential (Fig. 1). Importantly, E_{Cl^-} may be positive relative to the neuron's membrane potential but still not positive enough to activate excitatory voltage-dependent Na^+ and/or Ca^{2+} currents. In this case of “shunting inhibition”, GABA might be depolarizing but still inhibitory, because it clamps the membrane potential below the activation point of the excitatory conductances [2–5].

The mature brain has a low neuronal $[\text{Cl}^-]_i$ which favors inward Cl^- flow through GABA_ARs and leads to membrane hyperpolarization. However, during early development, $[\text{Cl}^-]_i$ may be significantly higher so that GABA_AR activation promotes outward Cl^- flow and membrane depolarization [6] (Fig. 1). In addition to immature neurons, higher neuronal $[\text{Cl}^-]_i$ and consequent GABA-mediated depolarization are also observed in mature dorsal root ganglia sensory neurons [7] and in many brain regions that accumulate Cl^- after acute injury. These injuries include hypoxic-ischemic encephalopathy (HIE, see Glossary) [8,9], traumatic brain injury [10–12] and prolonged seizures [13,14]. There are two immediate consequences of injury-related Cl^- accumulation. First, it may enhance excitatory GABA signaling by increasing $[\text{Cl}^-]_i$, which may lower seizure threshold and alter anticonvulsant efficacy. Second, Cl^- entry into cells through cation- Cl^- cotransporters (CCCs, see Glossary) is associated with water and cation movement (e.g. KCC2, KCC3, NKCC1) [15,16]. This results in an increase in both cellular volume and $[\text{Cl}^-]_i$ unless compensatory mechanisms are activated. Clinically, these two pathological consequences of ion and water fluxes may contribute to early seizures and cytotoxic edema after acute brain injury.

The aims of this review are to explore the mechanistic interplay among neuronal $[\text{Cl}^-]_i$ regulation, cytotoxic edema, as well as seizures, and to propose a framework where we can consider why drugs that modulate Cl^- cotransport and neuronal volume may have beneficial effects for seizure treatment. The neuronal $[\text{Cl}^-]_i$ regulatory mechanisms are central to these phenomena and our understanding is changing. Thus, we will first briefly review the critical determinants of Cl^- homeostasis.

[Cl⁻]_i during development: a tale of two transporters

Neuronal [Cl⁻]_i measurement is studied using a variety of techniques that have included gramicidin perforated cell recordings [17,18], single-channel E_{NMDA} / E_{GABA} measurements [19] and single wavelength Cl⁻ imaging [20,21]. These techniques minimize [Cl⁻]_i perturbation caused by invasive electrophysiological techniques such as sharp and whole cell recordings. Using these techniques, the [Cl⁻]_i of CNS neurons was observed to progressively decrease during early development in most species [6].

The neuronal [Cl⁻]_i change during early development was originally correlated with the differential protein expression patterns of two related electroneutral *SLC12A* family CCCs [22]: NKCC1 (canonically importing 2 Cl⁻ with 1 Na⁺ and 1 K⁺ ion per transport cycle) and KCC2 (canonically exporting 1 K⁺ and 1 Cl⁻ ion) both of which *can reverse their transport directionality* [23–28]. In addition to CCCs, other transporters and channels have been implicated in [Cl⁻]_i regulation including, Na⁺-driven Cl⁻ - bicarbonate exchanger (NDCBE) [29], chloride channels (CLC-2) [30–32] and the Cl⁻/HCO₃⁻ exchanger AE3 [33]. Here we focus on the NKCC1 and KCC2 cotransporters. The electroneutral nature and the canonical stoichiometries of their transported ions leads to predicted [Cl⁻]_i equilibria of 60 mM for NKCC1 [24,34] and about 3 mM for KCC2 [23,28] at commonly observed Na⁺ and K⁺ concentrations. Developmentally, the NKCC1 transporter was thought to be expressed earlier than KCC2 which explained the high [Cl⁻]_i observed at this age [35–37], while KCC2 was considered to be expressed later during development [38,39,37,40]. However, recent studies suggest that both NKCC1 and KCC2 are expressed outside these developmental windows [41]. Thus, these new results argue against the notion that the expressed transporter species determine the [Cl⁻]_i. Moreover, it is becoming increasingly clear that the transcript and protein expression level of the CCCs do not always accord with the predicted effects on [Cl⁻]_i and GABA_AR function due to post-translational covalent modifications such as protein phosphorylation [42,43].

Systematic measurements of [Cl⁻]_i in hundreds of neurons [44,45] with genetically-encoded ratiometric fluorophores such as Clomeleon [46], that also do not perturb the intracellular milieu, has provided additional insight into the developmental regulation of [Cl⁻]_i. These studies have revealed that neuronal [Cl⁻]_i does not decrease in all neurons simultaneously during development. Instead, [Cl⁻]_i varies widely among neurons at similar developmental time points. Even within a single neuron, [Cl⁻]_i varies considerably among different subcellular compartments [25,47]. While early gramicidin recordings first suggested inter-neuronal [Cl⁻]_i variance [17], this has become more evident using fluorometric techniques due to the sheer number of neurons whose [Cl⁻]_i can be measured simultaneously. The wide variability in inter-neuronal [Cl⁻]_i suggests that the effects of GABA_A signaling in individual neurons will differ depending on the local [Cl⁻]_i and the neuron's membrane potential fluctuations [5,48]. The [Cl⁻]_i variance among subcellular compartments may also explain why GABA_AR currents have different reversal potentials within the same neuron depending on subcellular location [49].

How do neurons maintain this variety of [Cl⁻]_i with only two cotransporters, each of which has a single value of intracellular Cl⁻ equilibrium? One possibility is a “push-pull system”

where transporters work in opposite directions and contribute to Cl^- influx and efflux. This will result in a weighted average of $[\text{Cl}^-]_i$ depending on the inward and outward transport rates, phosphorylation states and protein expression. However, the pull-push mechanism has been challenged [25,48]. First, a great deal of energy is wasted when two oppositely-directed transporters work against each other [5]. Both KCC2 and NKCC1 can change the local concentration of Cl^- by several mM per second [24,50]. If the two transporters were working against each other, ATP would be drained at a similar rate as Na^+/K^+ -ATPase worked to maintain the cationic gradients that provide the energy for Cl^- transport. Second, some neurons can maintain high $[\text{Cl}^-]_i$ without the expression of NKCC1 [51–53], and some neurons in dorsal root ganglia can have a low $[\text{Cl}^-]_i$ even in the absence of KCC2 activity [20]. Third, both KCC2 and NKCC1 transporters can operate in “reverse” – i.e., NKCC1 can mediate Cl^- export, and KCC2 can mediate Cl^- import [23–25,27,28]. This reverse operation can occur because the transporters are ATP-independent and the direction of the ions’ movement is dictated only by the extra- and intracellular free energies of the transported species. This reverse-mode transport can be assessed by measuring $[\text{Cl}^-]_i$ before and after transport block. If $[\text{Cl}^-]_i$ increases after inhibition, this indicates an outward transport. Instead, if $[\text{Cl}^-]_i$ decreases after transport inhibition, it indicates a net inward transport. In a series of experiments using hundreds of neocortical and hippocampal neurons expressing Clomeleon, we demonstrated that in general, neurons with high $[\text{Cl}^-]_i$ tend to lose Cl^- after transport block (indicating that they were actively transporting Cl^- inward), and the reverse is true for neurons with low $[\text{Cl}^-]_i$ [25]. These observations were equally true in immature and mature neurons and whether NKCC1 or KCC2 was blocked. The results support the idea that there is no canonical direction for either NKCC1 or KCC2 in *all* conditions. Consequently, the hypothesis of a simple decline in the “NKCC1:KCC2 protein expression ratio” accounts for the GABA excitatory to inhibitory developmental sequence is almost certainly too simplistic a notion.

Cl^- : the neuronal “packing peanut”

If CCCs are equally likely to be running forwards as backwards, and their range of expression is wide during development, what other factors might contribute to the setting of neuronal $[\text{Cl}^-]_i$? We recently proposed that immobile intra- and extracellular anions displace Cl^- , and thereby help establish the baseline $[\text{Cl}^-]_i$ [25,48]. Gibbs-Donnan effects (see Glossary) are generated by immobile intra- and extracellular anions and the free K^+ and Cl^- membrane permeability engendered by equilibrative CCCs. These effects are congruent with the concentration of impermeant anions present in the cytoplasm and the density of variably-sulfated glycosaminoglycans (see Glossary) that comprise the extracellular matrix (ECM) [54]. The negative charge of intracellular impermeant anions and extracellular sulfates displace local Cl^- creating regions of low $[\text{Cl}^-]$ both in the intracellular and extracellular compartments (Fig. 2). The neuronal distribution of Cl^- by charge displacement might be analogous to the distribution of foam packing peanuts in a shipping box, where the shipped contents are the anionic macromolecules, the box is the neuronal membrane and the packing peanuts are the Cl^- ions. This is not meant to imply that the neuronal membrane is as rigid as a shipping container. However, the cytoplasmic space is as effectively constrained by regulatory mechanisms [55], as the space within the shipping container.

Variations in the local concentration and the charge of large immobile anionic species, including intracellular polyglutamylated neuronal microtubules that are negatively charged at intracellular pH [56,57], as well as the dense and entirely post-translationally sulfated glycosaminoglycans of the ECM [54,58,59] will create opposing Gibbs-Donnan effects [60] that stabilize the intra- and extracellular Cl^- microdomains independently of secondarily active transport [25] (Fig. 2). Therefore, the polyanionic macromolecular charges will displace Cl^- and determine the local $[\text{Cl}^-]$ at equilibrium, i.e. *in the absence of ongoing energy expenditure at rest*. The intact olfactory epithelium and the isolated olfactory receptor neurons are examples of the importance of the ECM impermeant anions in transmembrane Cl^- homeostasis. NKCC1 is essential for the high $[\text{Cl}^-]_i$ in the isolated olfactory receptor neurons in which the ECM and its Gibbs-Donnan effects have been lost. However, NKCC1 is not required for a depolarizing Cl^- efflux in the intact preparation in which the ECM and the corresponding Gibbs-Donnan effect are preserved [61,62].

The neuronal membrane is a selectively permeable diffusion barrier with a changeable electric field that compartmentalizes Cl^- and creates intracytoplasmic and transmembrane Cl^- gradients. In the absence of transmembrane Cl^- flux, the *intracytoplasmic* Cl^- microdomains must be at equilibrium, else large cytoplasmic ion fluxes would occur between adjacent microdomains. No energy is required to maintain local differences in $[\text{Cl}^-]_i$ that arise due to local differences in immobile anions concentrations. The intra- and extracellular Cl^- microdomains along a dendrite are not evidence of “an eternal motion machine” [63,64]. For example in Figure 2, diffusion of cytoplasmic Cl^- from the area of high concentration to the area of low concentration is held in check by the charge separation that would be induced by Cl^- diffusion, rather than the activity of the local CCCs [48]. In this case, the lack of cytoplasmic mobility of anionic macromolecules rather than a semi-permeable membrane is the basis of the Gibbs-Donnan effect [65,66]. The *transmembrane* Cl^- gradients are also at equilibrium in a system that includes operational membrane CCCs when there is no GABA_AR conductance. If the transmembrane conditions are changed by the opening of GABA_ARs, then the system will no longer be at equilibrium and Cl^- ions will flow across the membrane, moving charge until the potential across the membrane inhibits further Cl^- flux ($V_m = E_{\text{Cl}^-}$). When the GABA_AR conductance closes, the cytoplasmic and extracellular $[\text{Cl}^-]$ will have been slightly altered by the transmembrane Cl^- flux. The only way to restore the prior equilibrium state is via secondary active membrane transport mediated by CCCs. The essential action of CCCs is to return $[\text{Cl}^-]_i$ to its set point after normally occurring Cl^- perturbations. Therefore, the CCCs are a component of the Gibbs-Donnan system. Their role is to facilitate the movement of Cl^- , co-transported cations and water during rapid synaptic activity and therefore, to maintain the equilibrium conditions established by the local immobile anion concentrations [25,48,67,68]. While the $[\text{Cl}^-]_i$ set point is determined by the immobile anions on either side of the membrane, the importance of the CCCs in establishing the equilibrium is evidenced by the functional alterations in $[\text{Cl}^-]_i$ seen in both NKCC1 and KCC2 knockout animals [69–73].

The “packing peanut” hypothesis explains the developmental decrease in $[\text{Cl}^-]_i$. As the concentration of macromolecular anions in the cytoplasm rise during development [56,74], this will lead to a progressively larger displacement of cytoplasmic Cl^- , thus reducing $[\text{Cl}^-]_i$ independently of the expressed cotransporters. The common cytoplasmic immobile anions

are composed of carboxyl and other anionic groups (i.e. glutamic acid) on large proteins as well as phosphates on nucleic acid polymers, both of which vary in different subcellular compartments [75]. The uneven distribution of immobile anions between the soma and the processes could also explain the diversity of $[\text{Cl}^-]_i$ micro-domains in a single neuron [25,49,67]. The sum of all local macromolecular anionic species determines the local Cl^- concentration. For example, the concentration of nucleic acids may be locally increased in the nucleus and soma, [76–78], while the concentration of negatively charged actin and tubulin polymers are greatest in the cell processes [79,80]. The Gibbs-Donnan effect between these different compartments would lead to Cl^- micro-domains that would not require energy to be maintained (in the absence of transmembrane Cl^- signaling). In contrast, *energy would be required* to keep $[\text{Cl}^-]_i$ at steady state if micro-domains were maintained by oppositely-directed transporters [81].

While the intracellular impermeant anions are present in much higher concentration than Cl^- , the Cl^- to impermeant anions ratio in the extracellular space is unknown. The negative value of E_{Cl^-} indicates that on average, there is more Cl^- in the extra vs. intracellular space, but we have learned little about how the other anions are distributed in the extracellular space since Golgi first used cationic dyes to visualize the sulfates in the ECM [82]. The extracellular macromolecules are of interest because they are created without templates, their degree of sulfation may reflect neuronal activity [83,84] and they turn over very slowly [85]. Moreover, the ECM is considered the fourth essential element of a chemical synapse [86,87] and it is implicated in multiple processes including synaptic plasticity through modulation of GABAergic inhibition, intrinsic neuronal excitability, integrin signaling and activities of L-type Ca^{2+} channels, NMDA receptors and Rho-associated kinases [88]. However, a new function for the ECM sulfation pattern may be as a permanent and translatable record of neural activity by GABA_A currents reading out the distribution of $[\text{Cl}^-]_o$.

Neuronal volume and Cl^- , a close relationship

Neurons lack rigid walls and thus are unable to tolerate major differences in intra- versus extracellular pressure without dramatic volume alterations. To regulate water concentration and shape, neurons use different channels and transporters, including the CCCs, [15,55,89,90] as they lack aquaporin channels which would have allowed the flow of pure water [91]. Neurons have conserved mechanisms of cell volume regulation so that changes in neuronal volume (even if non-osmotic) are regulated by the same transport mechanisms as other cells. In neurons, water may move through solute transporters in stoichiometric linkage to solute transport, or it may permeate through the transporter in a “passive” or non-stoichiometric manner, driven by the osmotic gradient created by the solute transport [92]. Both mechanisms result in isosmotic transport of solute and water. Stoichiometric linkage is emphasized here because of the evidence that the osmotic gradient contributes to the free energy of solute transport [93], the difficulty to explain observed neuronal transmembrane Cl^- gradients without this free energy contribution [48], the modest neuronal expression of transporters that have “passive” water permeability such as SGLT1 and GAT-1, and the modest water permeability of such transporters compared to aquaporins [15,92,94,95] but see [96,97].

When the intracellular or extracellular osmolarity is altered due to different pathological conditions (ischemia, stroke, trauma, acute hyponatremia, etc.), neurons activate homeostatic counter-responses to offset the induced changes in cytoplasmic volume [98–100] (yet see [101]). These processes result in Regulatory Volume Increase (RVI) in response to cell shrinkage or Regulatory Volume Decrease (RVD) in response to cell swelling (Fig. 3) [55,102]. Acutely, RVI mechanisms are activated when acute cell shrinkage occurs or when there is water and solute loss from the neuron. RVI promotes Na^+ , K^+ , Cl^- and water accumulation via NKCC1 (as well as Na^+/H^+ and $\text{Cl}^-/\text{HCO}_3^-$ transporters), and thereafter leads to the production of idiogenic osmoles (e.g. inositol, betaine, etc.) if the osmotic perturbation persists [103]. In cell swelling, RVD triggers the acute loss of cytoplasmic K^+ , Cl^- , and water by the K^+-Cl^- cotransporters, the TASK-2 K^+ channel [104,105], and the volume-regulated anion channel, composed of LRRC8 heteromers [106–111]. In addition to the loss of inorganic ions, RVD also triggers the loss of non-essential organic osmolytes, which might also be mediated by LRRC8 channels [112].

These processes of RVI and RVD are examples that demonstrate how neuronal volume and Cl^- movement are linked through CCCs [113,114]. In turn, CCCs are regulated by $[\text{Cl}^-]_i$ and cell volume [16,89]. Consequently, altering these transporters will not only modulate $[\text{Cl}^-]_i$ but will also affect the neuronal volume. Rephrasing this, cotransporters are not well-suited *to set* the $[\text{Cl}^-]_i$ because of their large effects on cell volume. Rather, the cotransporters are well-suited to stabilize or maintain *both* neuronal volume and the particular local $[\text{Cl}^-]_i$ around pre-determined set points. As we have discussed, the $[\text{Cl}^-]_i$ set point is determined by the local concentrations of intra and extracellular immobile anions. The roles of the resulting Gibbs-Donnan effects and the CCCs are not affected by the level of synaptic activity. However, the CCCs become more active as GABAergic synaptic activity increases. CCCs are particularly active when there is high Cl^- flux through GABA_ARs, restoring baseline $[\text{Cl}^-]_i$ and preventing shifts in E_{Cl^-} that may reverse Cl^- flux through the GABA_ARs leading to inhibition failure.

The close relationship between cell volume and Cl^- movement indicates that the popular figures that depict neurons as rigid vessels with varying amounts of Cl^- “fluid” need to be updated (Fig. 3). The canonical figures depict $[\text{Cl}^-]_i$ varying as a function of transport activity, with no corresponding change in cell volume and suggesting a compressible space above the fluid level [115]. However, in real cells, this space is filled with hydrated macromolecules that make up the vast majority of intracellular anions. This gel is not compressible, so when the neuron’s saline volume changes, there is a correlated increase in total neuronal volume (Fig. 3).

The Gibbs-Donnan effect can induce significant osmotic gradients across the membrane, which is why textbook examples generally posit a rigid cell wall. However, Gibbs-Donnan effects do not require a semi-permeable membrane. The equilibrium can also be established between different cellular sub-domains with varying concentrations of structural macromolecular anions if the macromolecules are immobile (Fig. 2) [65,66]. Balanced Gibbs-Donnan effects are critical to avoid severe water shifts. These shifts would be induced by the large fraction of impermeant intracellular anions if there were not a corresponding concentration of osmotically-active impermeant molecules in the extracellular space.

Although the extracellular balancing role was initially ascribed to Na^+ [116], the effects of $[\text{Na}^+]_o$ are essentially balanced by $[\text{K}^+]_i$, and thus cannot serve to offset the effects of the intracellular anions. Modern experiments suggest a more complex system instead of $[\text{Na}^+]_o$ [25]. Glycoproteins (see Glossary) that comprise the ECM could offset much of the Gibbs-Donnan effects of the intracellular macromolecular anions and balance the Gibbs-Donnan forces. The ECM glycoproteins contain long, starch-like strings of glycosaminoglycans (GAG, see Glossary) [54,58,59]. Each disaccharide moiety of the GAG chains can contain 0 to 4 sulfate groups providing the capacity for very high negative charge density. GAGs near neuronal membranes are readily stained with cationic dyes, creating the perineural nets first observed by Golgi using ferric chloride and silver nitrate stains [117,118], and also observed by our group using the cationic stain Alcian Blue [25]. Subsets of these glycoproteins can also be recognized by antibodies and agglutinins [119,120]. The cationic staining suggests a high local negative charge density that would generate an extracellular Donnan effect, balancing the intracellular Donnan effect of anionic macromolecules. This delicate balance is upset in the setting of acute brain injury that can lead to neuronal cytotoxic edema.

Cytotoxic edema occurs in the setting of acute brain injury and is associated with neuronal and glial volume increases. Although the mechanisms of cytotoxic edema are complex and remain poorly understood, the net effects are clear: a cytoplasmic volume increase arising from the influx of extracellular Na^+ together with Cl^- and water [121–124]. Brain injuries that lead to this type of edema include infarction, trauma, and toxic/metabolic disturbances. Initially, cytotoxic edema was thought to occur primarily due to ATP depletion and consequent failure of the Na^+/K^+ -ATPase pump, resulting in the accumulation of cytoplasmic Na^+ . One difficulty with this model is K^+ . When the influx of Na^+ exceeds the capacity of the Na^+/K^+ -ATPase pump, neurons depolarize [125–127]. This activates Ca^{2+} , Na^+ and voltage-sensitive K^+ channels, causing K^+ efflux that is no longer balanced by the Na^+/K^+ -ATPase-mediated K^+ re-uptake. Thus, in the setting of pure Na^+/K^+ -ATPase dysfunction, neurons should gain Na^+ and lose K^+ , depleting the transmembrane gradients of both these ions as the entropy of the system increases. If Na^+ and K^+ fluxes are balanced, both electroneutrality and isotonicity is maintained. Cl^- influx via voltage-gated channels will occur to the extent that Na^+ influx exceeds K^+ efflux [128]. The excess NaCl would create an osmotic gradient for water to enter the cell in this circumstance, as discussed in the next paragraph. Consequently, when only Na^+/K^+ -ATPase is blocked, no neuronal swelling is expected, and none is observed [25,129]. However, cytotoxic edema is observed in conditions of energy failure and direct injury, conditions under which many homeostatic derangements are likely to occur in addition to reduced Na^+/K^+ -ATPase activity [121–124]. Therefore, reduced Na^+/K^+ -ATPase function does not seem to be the primary cause of cell swelling during cytotoxic edema. We must then ask: *what mechanisms result in net cytoplasmic entry of cations, Cl^- , and water after brain injury?*

During cytotoxic edema, net cytoplasmic Na^+ influx will depolarize the membrane and trigger Cl^- influx through voltage gated Cl^- channels or transporters such as SLC26A11 to maintain electroneutrality [130,131]. Cell swelling under these circumstances indicates a corresponding water influx, although the pathway for water entry is currently unclear. Water can enter neurons stoichiometrically with Na^+ and Cl^- through CCCs (including NKCC1), and a similar mechanism may exist for SLC26A11. However, direct osmotically-driven

water influx is not likely to be a major pathway, because central neurons lack aquaporins [15,90,102,121,122]. While some aquaporin genes are up-regulated in neurons after brain edema, their functional role in the development of cytotoxic edema is much less clear [132]. Movement of water via CCCs is observed in conditions that induce cell/dendritic edema including spreading depression [133,134]. Furthermore, cytotoxic edema can be lessened when CCCs are blocked with loop diuretics, pointing to the importance of CCCs in water movement during pathological conditions [10,25,135,136]. *Now, what is driving the activity of the CCCs?*

One possible driver for NKCC1 is an increase in $[K^+]_o$, augmenting the driving force for inward influx of Na^+ , K^+ , $2Cl^-$ and water [135,137]. In addition, NKCC1 can also be up-regulated by phosphorylation via SPAK/OSR1 (behaving as “stress kinases”) and WNK3 (which behaves as a cellular “ Cl^- sensor”) which was evidenced in a model of ischemic stroke [138]. In depolarized neurons, the $[K^+]_o$ increases as a consequence of K^+ efflux from the cytoplasm. The loss of $[K^+]_i$ would shrink the cell, but because K^+ returns with Na^+ and Cl^- via NKCC1, the net effect is an increase in cell osmolarity and volume. Next, if Na^+ - K^+ -ATPase activity is reduced sufficiently to increase $[K^+]_o$, then $[Na^+]_i$ will also increase. This $[Na^+]_i$ increase reduces the driving force for inward ion transport via NKCC1, offsetting the effects of increased $[K^+]_o$. *Thus, transmembrane changes in $[K^+]$ and $[Na^+]$ may not result in a net change in driving force or a net influx of ions and water via NKCC1.*

KCC2-mediated transport of K^+ and Cl^- will reverse in high $[K^+]_o$ [23,26–28]. However, as for NKCC1, the “new” extracellular K^+ comes from the cytoplasm which reduces $[K^+]_i$. The reduction in $[K^+]_i$ and increase in $[K^+]_o$ strongly reduces the driving force for K^+ efflux. Although the effects of efflux of K_i on K_o might be mitigated by an increase in the extracellular space, this does not occur in these circumstances [139]. If the Cl^- gradient is unchanged, the reduction in K^+ gradient will strongly favor KCl influx via KCC2. Thus, some of the cytotoxic edema could be due to “reversed” KCC2 or KCC3 cotransport of K^+ , Cl^- and water in the setting of an unbuffered increase in $[K^+]_o$. This $[K^+]_o$ may return either to neurons or astrocytes. However, because this is a “U turn” for $[K^+]_i$, the overall effects on cell volume are likely to be modest and thus *changes in $[K^+]$ may not be the driving force for KCC2.*

Proposed hypothesis: Freeing trapped water

We propose a new contributing mechanism of cytotoxic cerebral edema: the mobilization of structural anions of the ECM as a consequence of brain injury. Up to four anionic sulfate groups can be attached to each disaccharide moiety of the glycosaminoglycan chains that comprise the ECM [54,140] displacing and substantially reducing local $[Cl^-]_o$. These sulfates can be released by activation of matrix metalloproteinases (MMPs, see Glossary) from injured neurons and glia, hydrolyzing the GAGs [141]. In addition, MMP activation leads to increase permeability of the blood-brain-barrier, leukocyte infiltration and progressive inflammatory actions that can allow the clearance of GAGs and may contribute to the later onset of vascular edema [142–144]. The released sulfates would then be replaced by Cl^- from the vascular space [139] increasing local $[Cl^-]_o$. This in turn would shift CCC-

mediated equilibrative transport of salt and water toward the cytoplasm, resulting in cytotoxic edema (Fig. 4).

Although there are many experiments that need to be performed to test this hypothesis, we recently demonstrated that the release of sulfated GAGs by Chondroitinase ABC, a bacterial analog of MMPs, resulted in the predicted large increase in $[Cl^-]_i$ [25]. An intriguing element of this experiment was that neuronal volume did not change, as would be expected by the cotransport of cations and water with Cl^- . This lack of volume change may have occurred because the neurons in this experiment were otherwise healthy and were able to employ RVD mechanisms [98] to compensate for the increased salt influx over the 2 hour course of the Chondroitinase ABC application.

There are two translational predictions of this model: 1) there should be a detectable increase in the concentration of released GAGs in the cerebrospinal fluid after brain injury, and perhaps in the blood and urine, that might serve as biomarkers correlating to the extent of ECM damage. 2) Cytotoxic edema may be ameliorated by MMP inhibitors. This last prediction is an important one because current therapies for cytotoxic edema are woefully inadequate [113,145] and improved outcomes after experimental brain injury have been observed with MMP inhibition, as described below.

Edema, MRI diffusion changes, seizures, and anticonvulsant resistance after brain injury

Severe cerebral edema increases total brain volume mainly by 2 mechanisms: 1) cytotoxic edema of neurons and astrocytes and 2) trans-capillary flux of Na^+ , other ions, water, and serum macromolecules due to increased blood brain barrier permeability (trans-vascular edema) [121,123]. Cytotoxic edema is associated with an increase in cellular volume and a marked decrease in extracellular volume [139]. This cellular edema results in a proportional decrease in the local apparent diffusion coefficient (ADC, see Glossary) that can be measured by brain magnetic resonance imaging (MRI, see Glossary). Thus, a reduction in ADC is a measure of local cytotoxic cerebral edema [146,147]. The intracellular space increase cannot occur without a reduction in the space occupied by the ECM, as gels are not compressible. Therefore, part of the extracellular volume reduction most likely reflects a decrease in the ECM that could arise from the release of activated MMPs from injured cells in addition to the movement of water from the extracellular to the intracellular space [141,148] (Fig. 4).

In vitro, a reduction in sulfated GAGs from the ECM concentration leads to an increase in $[Cl^-]_i$ [25]. We postulate that a reduction of ECM anionic macromolecules increases local $[Cl^-]_o$ and leads to an increase in $[Cl^-]_i$ through equilibrative CCC transport. Measuring the local $[Cl^-]_o$ next to a neuron as well as E_{Cl^-} in these circumstances would further test this hypothesis. If $[Cl^-]_o$ and $[Cl^-]_i$ were proportionately increased through the action of CCCs, this would increase the anionic flux through GABA_ARs [149] and increase the consequent membrane potential response, but would not change E_{Cl^-} (Fig. 5). However, in areas where E_{Cl^-} is depolarizing, the effect of GABA_ARs activation on membrane potential will be increased, enhancing the excitatory actions of GABA. Therefore, ECM disruption may

contribute to the correlation between cytotoxic edema and seizures in pathological conditions observed in animal models and humans. A few examples can bring light to this correlation:

Ischemic stroke: in this condition, neurons are injured due to a decrease in oxygen and oxidizable substrates as a consequence of blood flow interruption. Brain MRI of acute stroke shows restricted water diffusion [150] which maps to cell swelling [151]. In experimental hypoxic-ischemic injury, neuronal $[Cl^-]_i$ and volume are increased [9]. MMP expression is also increased in ischemic and hemorrhagic stroke in humans and animal models [152,153]. Furthermore, convulsions complicate around 5% of acute strokes [154] and a recent study demonstrated that seizure activity was more common after treatment with tissue plasminogen activator (t-PA) [155], which also activates MMPs. Although there are several potential mechanisms explaining the link between t-PA and seizure risk [156], it is of interest that one explanation is that t-PA might exacerbate ECM damage and lead to the consequent shift in Cl^- that alter GABA_ARs signaling. Finally, MMP inhibitors decrease cerebral edema [157] and improve early outcome in experimental stroke models [157,158] yet the optimal timing to use MMP inhibitors is unclear [157].

Why are seizures infrequent in stroke victims? The shifts in Cl^- induced by ECM injury are complex. If the dominant effect is an increase in $[Cl^-]_o$, then CCCs will mediate equilibrative increase in $[Cl^-]_i$ that will not alter E_{Cl^-} . In addition, many neurons that could be excited by GABA may be in the ischemic core, and have other ionic derangements that preclude action potential firing (e.g. persistent membrane potential depolarization with consequent inactivation of sodium channels). More functional neurons farther out in the penumbra may have deranged E_{Cl^-} but their inputs and/or outputs could be reduced if the relevant axons traverse the ischemic core. Indeed, EEG studies of acute stroke support this notion as they demonstrate both more frequent epileptiform activity for cortical strokes [155], and more plentiful focal epileptiform activity than is manifested as convulsions [159]. When damage is more global, as in hypoxic ischemic encephalopathy (HIE), it is common to find electrographic evidence of cortical seizures without clinically apparent convulsive activity, a phenomenon termed electro-clinical dissociation, or uncoupling [160]. One mechanism for uncoupling may be a more profound derangements of GABA signaling in the cortex vs. subcortical structures [44]. A prediction of this uncoupling mechanism is that patients exhibiting uncoupling may have more cortical than thalamic diffusion changes seen in brain MRI.

Traumatic brain injury (TBI)

Acute convulsive seizures complicate severe TBI [161,162]. The advent of long term EEG monitoring has demonstrated a higher proportion of sub-clinical electrographic seizures in patients suffering from TBI, especially children [163,164]. In this context, brain MRIs evidence restricted water diffusion in injured areas in a manner similar to stroke. TBI areas are also associated with diffusion weighted imaging changes (DWI, see Glossary) in animal models [165]. *In vitro*, hippocampal brain slicing (a severe example of TBI) produces an increase in $[Cl^-]_i$ and volume in the most superficial, and hence injured, neurons. Furthermore, the neurons closest to the cut edge with the highest $[Cl^-]_i$ are often silent and

do not participate in network activity [10]. This $[Cl^-]_i$ increase in neurons closest to the edge of the brain slice is also observed in neocortical and thalamic brain slices at all ages through early development suggesting that this may be a global phenomenon after severe trauma [12]. Interestingly, there is also an up-regulation of MMP in mice after TBI [141,166]. Therefore, TBI is another example of changes in neuronal volume, $[Cl^-]_i$ increase and seizures.

Neonatal seizures: the neonatal brain is prone to seizures after brain injury [167]. One of the most common insults to the neonatal brain resulting in seizures is HIE (See Glossary) [168]. Brain MRIs of neonates imaged in the first 5 days after HIE demonstrates elevated DWI signals, indicating cerebral edema and neuronal injury [169,170]. A change in mental status and seizures are among the most common presentations of HIE in neonates [171]. Importantly, the most common anti-convulsive medications used at this age (phenobarbital, phenytoin, and benzodiazepines) have poor effectiveness [37,172,173] which is correlated with high $[Cl^-]_i$ in the neocortex during early development [44,45]. This high $[Cl^-]_i$ can be further exacerbated by Cl^- accumulation during ensuing seizure episodes [13,174]. Why are seizures more common after brain injury in neonates compared to the mature brain? One possibility, relevant to this review is the developmental increase of both the sulfation pattern of the ECM [175] and the concentration of intra-cytoplasmatic immobile anions [74,176]. Consequently, the neonatal brain would be expected to have relatively low $[Cl^-]_o$ and corresponding high $[Cl^-]_i$; the latter has been demonstrated repeatedly [177].

The relation between $[Cl^-]_i$ and the effectiveness of a GABA_ARs allosteric modulator like phenobarbital is clear in the thalamo-cortical slice [44]. In this preparation, thalamus and neocortex are connected and are able to produce synchronous seizures. However, there is a differential effect of phenobarbital in these two connected structures. Thalamic seizures decrease while this drug is ineffective in the neocortex. This is in turn correlated with the higher $[Cl^-]_i$ and excitatory actions of GABA in the neocortex than the thalamus during early development. When bumetanide is applied at a dose that blocks NKCC1 and lowers $[Cl^-]_i$ in neurons with high $[Cl^-]_i$ [25], phenobarbital becomes effective in the neocortex yet does not alter phenobarbital's effect in the thalamus that had initial lower $[Cl^-]_i$ [44]. The preferential effect of phenobarbital in different brain regions can explain the electro-clinical dissociation seen in so many seizing infants [44,160]. The high $[Cl^-]_i$ during early development can also explain the occasional paradoxical (pro-convulsive) effect of benzodiazepines in human neonatal neocortex [45] and the ineffectiveness of phenobarbital in the neonatal seizing hippocampus [37,172].

Epilepsy

Neurons and glia in seizure foci undergo significant cellular swelling before and during ictal activity as a result of ionic influx stimulated by severe persistent excitation [178,179]. Compared to cells that rapidly return to their baseline volume after removal of the excitatory stimulus, human epileptic hippocampal neurons recover more slowly and exhibit cell swelling that lasts up to an hour after hyper-excitation [180]. Seizure animal models also demonstrate neuronal Cl^- accumulation [13,174] that may contribute to the reduced efficacy of GABAergic allosteric modulators such as benzodiazepines during prolonged seizure

activity. Similarly to the other injuries discussed above, prolonged seizures are associated with increased DWI signals seen in brain MRIs even after a single seizure [181,182]. Although the DWI changes could occur due to hypoxic injury associated with ictal hypoventilation, the DWI changes are also evident in prolonged focal seizures that cause less respiratory compromise [183].

New treatment venues: exploiting the linked triad of cellular volume, ECM and $[Cl^-]_i$

Recent research findings have given us a better picture of the interplay among $[Cl^-]_i$, neuronal volume, and seizures. This improved understanding has opened venues for novel approaches to treat seizures by modifying the ECM, $[Cl^-]_i$ and cell volume.

Reducing $[Cl^-]_i$

When the ECM is damaged, a compensatory increase in $[Cl^-]_o$ induces an increase in $[Cl^-]_i$ via equilibrative CCC-dependent transport (Fig. 4). This increase in $[Cl^-]_i$ should be reduced by CCC inhibitors and there is evidence supporting this idea. For example, the addition of bumetanide (an NKCC1 blocker and FDA-approved diuretic) to phenobarbital reduces seizure activity in the hippocampus and the neocortex *in vivo* and *in vitro* [184,44,185,186]. Importantly, inhibiting the NKCC1 transporter decreases the neuron's $[Cl^-]_i$ if they have a high initial $[Cl^-]_i$ [25], thus making GABA_ARs activation more inhibitory in this group of neurons. However, excitatory actions of GABA during early development are essential for maturation of cortical neurons *in vivo* [187], for proper excitatory synapse formation and for dendritic development of newborn cortical neurons [188]. Consequently, caution should be exercised with prolonged drug treatment during early development that prevent the normally elevated $[Cl^-]_i$ in the developing brain as it could lead to altered brain maturation.

Nevertheless, bumetanide (or analogs with improved blood-brain-barrier penetration [189]) could be employed during *acute* seizures to decrease $[Cl^-]_i$ so that the anti-convulsive medications that work by allosterically modulating GABA_ARs, such as phenobarbital, become more effective. While a recent pharmacokinetic clinical trial with no control group showed that bumetanide, as an add on medication, did not improve seizure outcome in a population of human neonates with seizures associated with HIE [190], it is difficult to make conclusions regarding efficacy from a study designed to address bumetanide pharmacokinetics, in part because 5 of the 14 neonates included in the analysis had no seizures during the baseline period [191]. The publication of the actual seizure raster diagrams strongly suggest an effect of bumetanide [192], although without a control group this can only be considered suggestive, because most neonatal seizures gradually decline over the first week of life [193]. Another clinical trial is currently studying pharmacokinetic and safety data of bumetanide in a different population of human neonates with seizures (NCT00830531) so this issue may be resolved in the near future. Drugs that modulate KCC2 or KCC3 (increasing their activity) to pump Cl^- out of neurons with high $[Cl^-]_i$ could be another approach to reduce transiently the elevated $[Cl^-]_i$ [194,195].

Reversing membrane NaCl influx

Neurons do not have aquaporins and thus have limited permeability to free water. Rather, water moves with ions through the CCCs and other transporters in response to ionic and osmotic gradients, as discussed above. Mannitol, an osmotic agent that is commonly used to treat increased intracranial pressure, decreases neuronal volume as well as neuronal $[Cl^-]_i$ in the hippocampal organotypic preparation [25]. This is congruent with the idea that water moves out of the neuron with cations and Cl^- , through CCCs [25] in response to the increase in extracellular osmolarity produced by mannitol. In some animal models and even human trials, mannitol has been shown to decrease epileptiform activity [196–198]. Therefore, agents that increase extracellular osmolarity may aid in decreasing $[Cl^-]_i$.

Reducing the driving force for NaCl influx

Our recent experiments have demonstrated that $[Cl^-]_i$ is balanced by intracellular and extracellular immobile anions [25]. When immobile anions in the ECM are released, Cl^- ions take their place, triggering a corresponding increase in $[Cl^-]_i$ (Fig. 4). Neuronal damage and death may lead to the release and activation of MMPs that further disrupt the local ECM, which may initiate an avalanche of further cell death and ECM degradation. Yet, many questions are still unanswered. Could the “stabilization” of the ECM with MMP inhibitors prevent an increase in $[Cl^-]_i$ as well as cell edema? Would there be no net change in brain volume, only a stabilization of the ratio of intracellular to extracellular space? These are interesting questions with potential clinical significance. We have not yet considered the balance between the ECM and the plasma, nor the cell volume signaling distortion by ECM damage. This is an innovative avenue for research into the pathophysiology and treatment of cerebral edema and the anticonvulsant-resistant seizures associated with acute brain injury.

All of these treatments lines have the common goal of controlling cell edema and reducing $[Cl^-]_i$. At least for bumetanide, this approach has shown to decrease seizure burden *in vitro* and *in vivo* [37,44,186,199]. We look with attentiveness to find if other drugs that decrease $[Cl^-]_i$ and cell edema also lead to seizure reduction.

Concluding Remarks and Future Perspectives

New insights into neuronal $[Cl^-]_i$ regulation, the pathogenesis of seizures, and its link with cell volume homeostasis and ECM has provided new potential strategies for seizure management. Advances in clinical management will require further *in vitro* experimentation to better understand the link between $[Cl^-]_i$, cellular volume and the ECM (see Outstanding Questions). An increased knowledge of the interplay among neuronal volume, $[Cl^-]_i$ and the ECM may yield pharmacological tools that could treat both seizures and cerebral edema associated with brain injury. Further understanding of the ECM and its degradation during pathological conditions may also give clues into novel neuroprotective strategies.

OUTSTANDING QUESTIONS BOX

- What are the local concentrations of large immobile anionic species on either side of the neurons' membrane (intra- and extra-cellularly) and how do they change during development?

- What are the local Cl^- concentrations around the GABA_A receptors, and do they undergo lasting changes in response to neuronal activity?
- Does the sulfation of the extracellular matrix change over time, and what are the determinants of the local sulfate concentration?
- Would prevention of extracellular matrix breakdown after brain injury ameliorate detrimental $[\text{Cl}^-]_i$ and neuronal volume changes?

What are the main mechanisms of neuronal volume regulation at play when the extracellular matrix is distorted?

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GLOSSARY

Apparent diffusion coefficient (ADC)

MRI sequence based on measuring the magnitude of diffusion of water molecules within a tissue. It is clinically calculated from diffusion weighted imaging and it is inversely related to the DWI value. Molecular diffusion of water in tissues reflects its interactions with macromolecules and membranes. Therefore, the relationship between tissue and diffusion is complex. In general, dense cellular tissues or those with cellular swelling exhibit lower ADC values.

Cation-Chloride Cotransporters (CCCs)

Plasma membrane symporters that belong to the *SLC12A* family of solute carriers. They include, among others, the neuronal K-Cl cotransporter, KCC2, and the Na-K-2Cl cotransporter, NKCC1.

Diffusion weighted imaging (DWI)

MRI sequence based upon measuring the random Brownian motion of water molecules within a voxel of tissue.

Gibbs-Donnan effect

It is also called the Donnan effect, Donnan law, and Gibbs-Donnan equilibrium. It explains the impact of a non-permeable ion on the behavior of permeable ions on either side of a semi-permeable membrane: the concentrations of the permeable ions cannot equalize on either side of the membrane, despite their free diffusion. As a consequence, osmotic and electrical gradients develop across the membrane.

GABA_A Receptors (GABA_AR)

These are hetero-pentameric ligand-gated ion channels permeable to Cl^- and to a lesser extend bicarbonate. Their physiological and pharmacological properties depend on their

subunit composition. These receptors are modulated by anesthetics, ethanol, neurosteroids, and anticonvulsive drugs including barbiturates and benzodiazepines.

Glycoproteins (proteoglycans)

Diverse family of proteins consisting of one or more glycosaminoglycans chains covalently linked to a core protein. Glycoproteins are a major component of the extracellular matrix.

Glycosaminoglycans (GAG)

Heterogeneous family of linear, starch-like polysaccharides composed of a repeating disaccharide unit. The identity of the disaccharide unit results in a classification of five major groups: chondroitin sulfate, heparin, heparan sulfate, dermatan sulfate, keratan sulfate and hyaluronic acid. The disaccharide moieties of the GAG chains can contain between 0 and 4 sulfate groups providing the capacity for very high negative charge density.

Hypoxic-ischemic encephalopathy (HIE)

Brain injury caused by oxygen deprivation to the brain due to perinatal asphyxia.

Magnetic Resonance Imaging (MRI)

Imaging technique used to visualize body structures based on nuclear magnetic resonance. Most MRI scans essentially map the location of water and fat in the body. Different contrasts can be generated between tissues by altering the pulse sequences that perturb hydrogen atoms and then measuring their subsequent relaxation.

Matrix metalloproteinases (MMPs)

These are zinc-containing endopeptidases. They are capable of degrading the extracellular matrix and cleaving other molecules (cell surface receptors, adhesion molecules, etc.). They are involved in fundamental processes such as cell proliferation, differentiation, adhesion, migration and apoptosis and in pathological conditions including epilepsy, neuro-inflammation, multiple sclerosis and autoimmune disorders.

Reversal (or equilibrium) potential (of a specific ion)

a neuron's membrane potential at which no current flows from one side of the membrane to the other. It is calculated by the Nernst equation using the specific ion intra- and extracellular concentration.

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TRENDS BOX

The neuronal intracellular chloride concentration ($[Cl^-]_i$) determines how the GABA neurotransmitter will alter a neuron's firing pattern as well as its cell volume. Thus, understanding the mechanisms that establish, maintain and regulate neuronal $[Cl^-]_i$ are essential if we are to achieve a better understanding of neuronal excitability and cell volume regulation.

Neuronal $[Cl^-]_i$ is high in certain pathological conditions including cell injury, seizures and epilepsy. This elevated $[Cl^-]_i$ contributes to a lower seizure threshold and altered anticonvulsant efficacy. The co-transport of cations, Cl^- and water may link cytotoxic edema to mechanisms of excitatory $GABA_A$ signaling, disinhibition and increased seizure risk after acute brain injury.

New data has emerged demonstrating that intracellular and extracellular immobile anions actually set the neuronal $[Cl^-]_i$ while the Cl^- co-transporters effectively facilitate the movement of this anion to reach this set-point. With the recognition of these new determinants of $[Cl^-]_i$, we can develop novel approaches to modulate the neuron's $[Cl^-]_i$ and its volume, and thus, treat two of the most common clinical sequela of brain injury: edema and seizures.

Figure 1. Cl^- flow and GABAergic transmission

A) Cl^- reversal potential calculated from the Nernst equation. No Cl^- flows through GABA_ARs when the $[\text{Cl}^-]_i$ (vertical dotted line) crosses the neuronal resting membrane potential (RMP, horizontal dotted line). Color strokes indicate $[\text{Cl}^-]_i$ values that lead to an inward flow of Cl^- (blue) and outward Cl^- flow (red). **B)** $[\text{Cl}^-]_i$ (green) depicted in neurons that lead to an outward (depolarizing, left) or inward (right, hyperpolarizing) Cl^- flow when GABA_ARs are open. E_{Cl^-} denoted by dashed line. Note that E_{Cl^-} can be variable (shaded violet) and while GABA can be depolarizing, it may not always reach action potential threshold (AP thr) and thus actually lead to shunting inhibition.

Figure 2. The Gibbs-Donnan effect and the setting of $[Cl^-]_i$

A) A neuron is represented with GABA_ARs (pentameric brown ovals) and Cl⁻ transporters (NKCC1, KCC2) yet with different actions when GABA_ARs open (*top*, depolarizing; *bottom*, hyperpolarizing). Immobile *extracellular* anions are represented by circles with sulfated branches (S⁻). Immobile *intracellular* anions are represented by circles with glutamic acid branches (E⁻). Green circles represent Cl⁻. The negative charge (with different Cl⁻ gradients) is balanced across the membrane in both sections of the cell. However, the top section has depolarizing Cl⁻ (outward flow of Cl⁻ and returning back to the neuron by NKCC1) while Cl⁻ is inhibitory in the lower section of the same neuron (inward flow of Cl⁻ with KCC2 pumping Cl⁻ outwards). There will be no ongoing cytoplasmic Cl⁻ diffusion from the high Cl⁻ area to the low Cl⁻ area because such diffusion would create charge separation.

Figure 3. CCCs are involved in neuronal volume regulation

Movement of Cl^- and cations in neurons by CCCs (NKCC1 and KCC2) also involves the movement of water through the transporters. The neuronal intracellular space is composed of macromolecules (bricks) not easily compressed. When Cl^- is co-transported with cations and water, it produces neuronal swelling (left) or shrinkage (right) unless other compensatory RVD or RVI mechanisms are activated. Middle neuron is baseline condition. CCCs: cation- Cl^- cotransporters.

Figure 4 (Key figure). Cytotoxic edema caused by degradation of the ECM

A) Effect of brain trauma on cytotoxic edema. Numbers correspond to the effects illustrated in B. **B)** Brain trauma directly produces a release of ECM surrounding the neuron (1). Matrix metalloproteinases (MMP) are released from neurons and glia (2) and further hydrolyze the ECM (3). The decrease in ECM (negative charges) leads to a local accumulation of $[\text{Cl}^-]_o$ next to the injured neuron. The $[\text{Cl}^-]_o$ increase leads to Cl^- , Na^+ , and water entry through NKCC1 (4). This increase in $[\text{Cl}^-]_i$ and higher local $[\text{Cl}^-]_o$ (see Fig. 5) will lead to larger depolarizing GABA currents in neurons with initial high $[\text{Cl}^-]_i$ (5) and cytotoxic edema (6). Immobile extracellular anions are represented by circles with sulfated

branches (S^-). Immobile intracellular anions are represented by circles with glutamic acid branches (E^-). Green circles represent Cl^- .

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Figure 5. Change in GABA_ARs mediated current by changes in [Cl⁻]_o

A) Goldman-Hodgkin-Katz current equation solution [149] keeping the resting membrane potential fixed at -60 mV with a Cl⁻ permeability of 4.05×10^{-8} m/sec [200] and varying [Cl⁻]_i and [Cl⁻]_o. The experimental local [Cl⁻]_o next to the GABA_ARs is unknown at this time. The dot-markers represent the inward current (outward Cl⁻ flow) leading to depolarization events depicted in **B**. **B)** Example of an increase in depolarizing GABA_ARs mediated Cl⁻ current leading to an action potential in ② when the local [Cl⁻]_o is increased (①, [Cl⁻]_o 30 mM; ②, [Cl⁻]_o 130 mM) with a proportional increase in [Cl⁻]_i (①, [Cl⁻]_i 6 mM; ②, [Cl⁻]_i 26 mM), resulting in no change in E_{Cl} (-43 mV).