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Response to Comments on "Local impermeant anions establish the neuronal chloride concentration"

J. Glykys, **V. Dzhala**, **K. Egawa**, **T. Balena**, **Y. Saponjian**, **K. V. Kuchibhotla**, **B. J. Bacskai**, **K. T. Kahle**, **T. Zeuthen**, and **K. J. Staley**

We address the concerns of Voipio et al. regarding Donnan equilibria and then the concerns of Luhmann et al. regarding technical aspects of our study. We appreciate their interest in our paper and the opportunity to clarify theoretical and technical aspects describing the influence of Donnan equilibria on neuronal chloride ion (Cl_i) distributions.

Regarding Donnan energetics, Voipio et al. state: "If Cl− were initially in equilibrium across a membrane, then the mere introduction of immobile negative charges (a passive element) at one side of the membrane would, according to their line of thinking, cause a permanent change in the local electrochemical potential of Cl−, *thereby leading to a persistent driving force for Cl- fluxes"*. (italics added). The non-italicized section of the quote is a concise description of Donnan effects on the Cl^- distribution, and the resultant electrochemical potential is the Donnan potential [1]. The italicized section of the quote is the point of confusion.

The Donnan potential is the membrane voltage at which the system is at equilibrium, i.e. at which there is no free energy available to do work such as moving Cl^- across the membrane. The free energy to drive membrane Cl^- currents is supplied by the process that shifts the membrane potential away from the Donnan potential, and thereby shifts the system away from equilibrium. The driving force for electrogenic Cl^- flux across the membrane depends on the difference between the shifted membrane potential and the Donnan potential, not the Donnan potential itself. Without the addition of energy to the system to shift the membrane potential, there will be no net ion flux across the membrane in a system at equilibrium.

Regarding the distribution of impermeant charges, Voipio et al. express two concerns. First, they cite charge screening studies to support their argument that impermeant anion distributions do not alter either the bulk [Cl^-] or the free energy of Cl^- flux across the membrane. For sufficiently thin anion layers, we agree. Charge screening studies concern the effects of the charged polar heads of phospholipids comprising lipid bilayers. These are not the distributions that we describe:

Intracellularly, most intracellular anions cannot permeate $GABA_A$ receptor-operated channels, and these impermeant anions are not confined to a thin layer along the membrane. Nevertheless, Voipio et al. are concerned that "A consequence of the logic of Glykys et al. is that local charges could even reverse 'the polarity of local $GABA_A$R signaling' ". Since the classic studies of Coombs, Eccles and Fatt [2], it has been routine to manipulate the Cl^- equilibrium potential in intracellular recordings by replacing various amounts of chloride in the recording electrode solution with impermeant anions. Thus, there is overwhelming

electrophysiological evidence that (exogenous) intracellular impermeant local charges can alter intracellular [Cl] and thereby change the polarity of $GABA_AR$ signaling.

Extracellularly, bulk cerebrospinal fluid has a high Cl^- concentration and few impermeant charges. However, neurons are not apples bobbing in a sea of cerebrospinal fluid. Rather they are embedded in a gelatinous extracellular matrix comprised of polyanionic biopolymers that are sufficiently dense to impart the matrix with a tortuosity that far exceeds that of cerebrospinal fluid [3]. Although the exceptionally high charge density of these anionic biopolymers is well-established [4], the actual spatial distribution of fixed anionic charges in the brain's extracellular matrix has only rarely been considered [5] and merit more study.

Voipio et al. express a second concern regarding the distribution of intracellular impermeant anions: " a gradient in cytosolic impermeant charge density would create opposing $[Cl^-]_i$ and electrical potential gradients within the cell. However, under these conditions, the electrochemical potential of Cl^- would be uniform within the cell." This was our point also. If the electrochemical potential for $[Cl^-]_i$ is uniform within the neuron, then oppositely-directed Cl^- cotransport is not required to maintain differences in subcellular $[Cl^-]_i$[6]. While the electrochemical potential of Cl^- would be uniform within the cell, the electrochemical potential of Cl^- *across the cell membrane* would not be uniform at subcellular locations containing differing concentrations of impermeant anions, as has been repeatedly observed[7, 8].

Luhman et al. raise several technical questions for which we provide the following clarifications. Regarding the sensitivity of Clomeleon, For a ratiometric fluorophore with a Kd of ~ 100 mM, the change in the fluorescence ratio is 1% Δ ratio per 1–2 mM change in Cl_i for Cl_i between 1 and 20 mM[8, 9, 10]. This sensitivity is sufficient to test our hypotheses.

Regarding changes in Cl_i due to slicing-induced injury, we made extensive use of hippocampal organotypic slice cultures, in which injured neurons have been cleared. Stacks of images along the Z axis were analyzed, where the initial image was acquired on average 6 μm below the surface for organotypic hippocampal slices and 56 μm from the surface for acute neocortical slices. The variance in Cl_i in acute slices was increased compared to organotypic preparations, which we attribute to the effects of trauma. However, the variance in Cl_i in the organotypic hippocampi strongly support the central hypotheses of the paper. We look forward to the results of in vivo experiments as they become feasible.

Regarding the variance in Cl_i, including immature preparations: A key finding driving the current study is the substantial variance in neuronal Cl_i, which has also been reported by other groups using Clomeleon[8, 11] as well as perforated patch [12] and dual cell-attached recordings [13]. Intra-neuronal Cl_i is also variable[7, 8]. Rather than being an experimental deficiency, we propose that the variability of the Cl transmembrane gradient is a fundamental a feature of the brain's composition.

Regarding the effects of NKCC1 inhibition, the data we present are consistent with the cited studies. Data in Fig 3H, I are from two different populations of neurons, and well within the range of values shown in Figure 1B,C. NKCC1 inhibition reduces Cli in neurons with high initial Cli, and increases Cli in neurons with low initial Cli (supplemental figures S1B, S2B,

S3)[14, 15]. Fluorometric techniques sample dozens to hundreds of neurons. Electrophysiological studies, including our earlier studies, report a handful of recorded cells selected based on the experimenter's preferences for cell turgor; in light of our findings regarding the relation between neuronal volume and Cli, such selection could readily bias small samples of neurons.

Regarding knockout studies of transporters, as stated in the concluding sentence of the summary of our study, cation-chloride transporters are critically important for restoring Cli and volume after signaling transients. The sequelae of chronic cation-chloride cotransport inhibition[16] do not invalidate our hypotheses.

Regarding NaKATPase, the suggested experiment was not included because we had previously reported that perforated patch assays of Cli during NaKATPase inhibition showed only very modest changes in Cli that were well within the range we would predict [17]. Anoxia and consequent energy failure have many more effects than NaKATPase inhibition, and we would not equate these two manipulations.

Regarding the permeability of gluconate, this anion permeates a variety of chloride channels with permeabilities ranging from 10 to 40% of chloride[18, 19, 20], which is ample for the experiment we performed. The interesting hypotheses put forward as to why the experiment utilizing weak organic acids might not work would only be valid if proton buffering was purely passive, i.e. only in the absence of proton pumps and exchangers. We and others, including Luhmann's group [21], have also altered the cytoplasmic concentration of relatively impermeant anions by introducing gluconate directly from the recording pipette solution. This approach of altering A_i eliminates the dependence on membrane transport or permeation. Much larger reductions in Cl_i can be demonstrated with this technique, and the data robustly support the idea that A_i and Cl_i are inversely related

Regarding the seizure experiments, our confirmation of the predicted correlation between neuronal volume and Cli changes during seizures has not been previously reported.

We accept that this is a complex topic to introduce in a short communication, and we appreciate the opportunity to provide clarifications based on the theoretical and technical questions raised here. These questions do not affect the validity of our conclusions. Cl homeostasis and GABA signaling are more complex than we initially envisioned. This provides additional capacity for information storage and transmission, and broad opportunities for further research.

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Luhmann references

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