Biochemistry to the Rescue: A ClC-2 Auxiliary Subunit Provides a Tangible Link to Leukodystrophy

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ClC-2 is a broadly distributed chloride channel with an enigmatic neurophysiological function. In this issue of *Neuron*, Jeworutzki et al. (2012) use a biochemical approach to identify GlialCAM, a protein with a defined link to leukodystrophy, as a ClC-2 auxiliary subunit.

Auxiliary subunits of ion channels are central players in the exquisite electrical tuning of the central nervous system. While they do not directly form ion-channel pores, auxiliary subunits can substantially alter channel properties through interaction with the pore-forming subunits. The effects of these interactions include modulation of sensitivity to ions and signaling molecules, alteration of voltage dependence and activation/inactivation kinetics, and changes in localization and trafficking. The combination of these effects amplifies the functional diversity of ion channels. Discovery of auxiliary subunits has occurred through diverse avenues, from early biochemical approaches to more recent genetic screening and genetic linkage analyses, and now—as exemplified here—back to biochemical approaches tied to modern mass spectrometry.

ClC-2 is a chloride-selective channel broadly expressed in every type of tissue (Jentsch, 2008). In the brain, ClC-2 is found in neurons, astrocytes, and oligodendrocytes (Blanz et al., 2007). In neurons, it is agreed that ClC-2 contributes to input resistance, though it is currently debated whether it serves principally as an influx or efflux pathway for chloride ions (Ratté and Prescott, 2011; Rinke et al., 2010). In glia, ClC-2 is essential for myelin integrity, as evidenced by progressive myelin vacuolation in the ClC-2 knockout mouse (Blanz et al., 2007). The similarity of this phenotype to that observed with disruption of glial Kir4.1 potassium channels (Neusch et al., 2001) or glial connexins Cx32 and Cx47 (Menichella et al., 2003) together with the similarity in expression patterns of the three types of ion channels strongly suggests a role of ClC-2 in ion homeostasis by the glial syncytium. The glial syncytium is a connexin channel-mediated coupling between astrocytes and oligodendrocytes, which plays a crucial role in buffering ions. In conjunction with Kir4.1, the glial syncytium is essential for regulating K^+ concentrations in narrow extracellular spaces between neurons and glia. ClC-2 may contribute to this process by facilitating parallel movement of Cl^- to maintain electroneutrality and may also contribute to $[Cl^-]$ and $[H^+]$ regulation (Blanz et al., 2007). Defects in ion homeostasis upon disruption of ClC-2, Kir4.1, or Cx32/47 probably lead to osmotic imbalances that drive the observed myelin vacuolation (Brignone et al., 2011).

The myelin vacuolation in the ClC-2 knockout mouse mimics the pathology observed in human cystic leukoencephalopathies, suggesting ClC-2 mutations as potential culprits in disease. However, extensive searches failed to reveal any ClC-2 mutations linked to these disorders (Blanz et al., 2007; Scheper et al., 2010). Among the human cystic leukoencephalopathies is megalencephalic leukoencephalopathy with subcortical cysts (MLC). This disorder is characterized by increased head circumference and abnormal myelin with cystic lesions. Mutations associated with the disease were identified in a previously uncharacterized gene designated *MLC1* (Leegwater et al., 2001). Mutations in the *MLC1* gene account for about three-quarters of the MLC cases. The protein encoded by *MLC1* is an integral membrane protein with multiple transmembrane segments expressed in astrocyte endfeet in the perivascular, subependymal, and subpial regions. Its function remains unknown. Surprisingly, MLC1 is not expressed in oligodendrocytes, the site of the primary pathology in MLC.

In order to identify other genes that might be involved in MLC, van der Knaap and colleagues searched for proteins that biochemically interact with MLC1. GlialCAM, an IgG-like cell adhesion molecule, was identified using mass spectrometric analysis of affinity-purified MLC1. GlialCAM is expressed predominantly in astrocytes, oligodendrocytes, and a subset of pyramidal neurons in the brain and, as hoped, genetic analysis of MLC patients revealed mutations in the gene encoding GlialCAM. Experiments with heterologous expression demonstrated that GlialCAM is required for localization of MLC1 to cell-cell contacts in astrocytes. In the absence of GlialCAM or with expression of disease-associated GlialCAM mutants, MLC1 is targeted to the plasma membrane but not specifically to cell-cell contacts. These results suggest a trafficking defect of MLC1 as a potential pathophysiolgical mechanism in MLC.

The presence of GlialCAM in oligodendrocytes, which appear to lack MLC1, suggested that GlialCAM might bind to other proteins that also play a role in MLC. In this issue of *Neuron*, Estevez,

Pusch, and colleagues use a biochemical approach to identify ClC-2 as the crucial GlialCAM binding partner, thus reinvigorating the link between cystic leukoencephalopathies and ClC-2 (Jeworutzki et al., 2012). Their ensuing discovery that GlialCAM targets ClC-2 to cell contacts together with the phenotype of the ClC-2 knockout mouse strongly supports the hypothesis that altered ion flux across oligodendrocyte membranes leads to myelin vacuolization in MLC.

The expression of GlialCAM and ClC-2 in oligodendrocytes is consistent with the major pathology of MLC, but how could loss of MLC1, which is not expressed in oligodendrocytes, cause a similar phenotype? Genetic defects in MLC1, GlialCAM, and ClC-2 induce similar glial and myelin pathologies in both humans and mice, suggesting that all three proteins contribute to a common functional process. GlialCAM trafficks both ClC-2 and MLC1 to cell-cell junctions and has a robust effect on ClC-2 electrophysiological function; however, no biochemical or functional interaction between ClC-2 and MLC1 could be detected, and MLC1 expression and localization are not affected in the ClC-2 knockout mouse. Nevertheless, it remains possible that MLC1 and ClC-2 could interact indirectly. Indeed, an indirect interaction through GlialCAM could juxtapose MLC1 and ClC-2 across astrocyte-oligodendrocyte cell contacts (Figure 1), thus bringing MLC1 to the site of major pathology in the disease. But by what mechanism does the disease occur? It is known that ion movement through the glial syncytium is in delicate balance. Upsetting this balance by disruption of either gap junctions (which facilitate intraglial ion movement) or Kir4.1 potassium channels (which facilitate glial-extracellular ion movement) leads to myelin vacuolation. Thus, it is likely that ClC-2, in parallel to Kir4.1, contributes to ion homeostasis in the narrow extracellular spaces. While the precise mechanism of myelin vacuolation has not been defined, it probably arises from osmotic imbalances associated with the defect in ion homeostasis (Brignone et al., 2011). But what is the function of MLC1? Is it an ion channel as well? This remains a mystery and will require further study of MLC1 and investigations of how loss of MLC1 influences ion permeability across membranes of individual astrocytes and the glial syncytium.

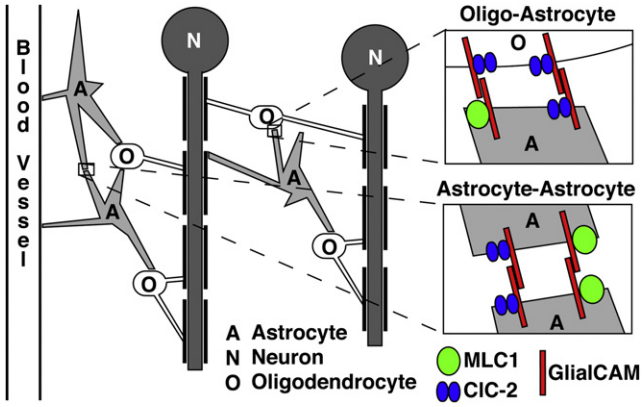

Figure 1. A Model for GlialCAM, MLC1, and ClC-2 in a Glial Ion Homeostasis Network

Astocytes and oligodendrocytes form a connexin-based glial syncytium that provides a network for ion homeostasis. GlialCAM, MLC1, and ClC-2 are localized at contacts within the glial syncytium. GlialCAM (red) is present at cell-cell contacts between endfeet of astrocytes and probably at contacts between oligodendrocytes and astrocytes. Through homotypic extracellular interactions and its interactions with ClC-2 (blue) and MLC1 (green), GlialCAM can target these proteins across from each other where they will share a local extracellular space. Note that GlialCAM and ClC-2 are present in both astrocytes and oligodendrocytes while MLC1 is expressed only in astrocytes.

In addition to changing ClC-2 localization, GlialCAM has an amazing effect on ClC-2 currents. In heterologous expression systems, coexpression of GlialCAM and ClC-2 results in large currents that retain ClC-2's characteristic anionic selectivity, but lack its signature rectification and slow activation by hyperpolarization. The increase in current is due solely to an effect on gating, as surface expression is unchanged (the cells used for recording lack cell-cell contacts), and no increase in current is observed in cells expressing only GlialCAM or GlialCAM plus ClC-5. While the effect of GlialCAM on ClC-2 currents in astrocytes is milder than in the heterologous expression systems (either because of lower relative GlialCAM expression or some other cellular difference), the observed increase in current and decrease in rectification could be physiologically important for bidirectional chloride transport. Regardless of whether the change in electrophysiological properties is important for glial physiology and myelin maintenance, GlialCAM is a fascinating new tool for investigating the biophysics of ClC-2 gating.

GlialCAM is the third CLC auxiliary subunit to be discovered. The other two, Barttin (a ClC-K partner) and Ostm1 (a ClC-7 partner), were identified through their genetic links to disease. Though the genetics approach failed to identify ClC-2 binding partners, the Estevez lab's success using a biochemical approach here provides hope that additional CLC auxiliary subunits may soon be discovered. Such findings hold promise for clarifying our understanding of the diverse physiology displayed by CLC family members. For example, GlialCAM is expressed only in the brain, but ClC-2 is expressed ubiquitously. Though ClC-2 is functional in the absence of GlialCAM, evidence for the role of ClC-2 in cell junctions outside the CNS (Nighot et al., 2009) hints that new ClC-2 auxiliary proteins remain to be discovered. More intriguing and controversial is the possibility that ClC-3 auxiliary subunits might close the gap between seemingly irreconcilable reports on ClC-3 physiology. ClC-3 is in the branch of the CLC family that localizes to intracellular membranes and consists of chloride-proton antiporters (not channels). In accord with this classification, ClC-3 has been found to play physiological roles in endosomes and synaptic vesicles (Jentsch, 2008). However, ClC-3 has also been variously reported as a plasma-membrane channel that is regulated by cell volume (Xiong et al., 2010; Yang et al., 2011), CamKII (Cuddapah and Sontheimer, 2010; Wang et al., 2006), and acid (Matsuda et al., 2010), in a wide variety of cell types. While it has seemed doubtful that these findings could all be reconciled by auxiliary subunits (Clapham, 2001), the strong transformation of ClC-2's localization and electrophysiological properties by GlialCAM perhaps render this possibility

more likely. We hope that re-examination of these and other physiological puzzlers will be inspired by the success of Jeworutzki et al. (2012) in uncovering one of only a handful of known auxiliary subunits for the elusive CLC family.

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How Do Neurons Sense a Spike Burst?

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In this issue of *Neuron*, Xu et al. (2012) show that knock down of Syt1, a major Ca^{2+} sensor, impairs synaptic transmission similarly in different brain regions but with unexpected, region-specific behavioral outcomes.

Several decades ago, I used to listen to rock and roll by tuning in to Radio Free Europe with a small headphone, basically a magnetic coil and a metal diaphragm, so that the neighbors could not suspect my illegal activities. That of course was not the same thing as being in a concert hall, enjoying the entire frequency spectrum and perceiving the pitch, melody, harmony, and timbre content of the music but despite the high-pass filtering properties of the low quality earphone the rhythm and other remnant features of the broadcasted music made the experience still enjoyable. As engineers know, high-pass frequency filtering of signals makes communication poorer but not hopeless. Now suppose that we introduce high-pass filters in the communication lines between neurons in the brain. This is exactly what Xu et al., (2012) have accomplished, using molecular biological tools. They find that after such manipulation neuronal transmission becomes sluggish but is not completely abolished. For some structures and tasks, such as the hippocampus-dependent contextual fear learning task, high-pass filtering is tolerated, whereas for a prefrontal cortex-dependent remote memory recall, sluggishness of spike communication leads to a serious behavioral impairment.

Let's examine first how communication between neurons was achieved. Neurons communicate electrochemically. The upstream neuron generates a spike, which is broadcasted to all or most of its presynaptic terminals. Here, electricity is converted to chemically mediated synaptic transmission. This conversion process can be perturbed in multiple ways. For example, tetanus toxin (TetTox) can block transmitter release and thus completely eliminate synaptic communication. Other interventions can produce a more subtle interference. Synaptotagmin-1 (Syt1), together with other vesicle proteins, is essential for the docking and/or fusion of synaptic vesicles with the presynaptic plasma membrane following depolarization and Ca^{2+} influx in presynaptic bouton. Eliminating or interfering with Syt1 also impairs synaptic transmission to single, isolated spikes yet when high enough amount of Ca^{2+} enters the terminal in response to high-frequency spike activity chemical transmission is resumed, although it remains sluggish due to the asynchronous release of the transmitter (Maximov and Südhof, 2005). Put simply, interfering with Syt1 amounts to the introduction of a high-pass frequency filter: no or poor