European Journal of Medical Genetics xxx (xxxx) xxx–xxx

Contents lists available at ScienceDirect

European Journal of Medical Genetics

journal homepage: www.elsevier.com/locate/ejmg

Megalencephalic leukoencephalopathy with subcortical cysts: A personal biochemical retrospective

Raúl Estévez[a,c,*], Xabier Elorza-Vidal[a,c], Héctor Gaitán-Peñas[a,c], Carla Pérez-Rius[a], Mercedes Armand-Ugón[a], Marta Alonso-Gardón[a], Efren Xicoy-Espaulella[a], Sònia Sirisi[a,b,c], Tanit Arnedo[a,c], Xavier Capdevila-Nortes[a], Tania López-Hernández[a], Marisol Montolio[a], Anna Duarri[a,b], Oscar Teijido[a], Alejandro Barrallo-Gimeno[a,c], Manuel Palacín[c,d], Virginia Nunes[b,c]

[a] Unitat de Fisiologia, Departament de Ciències Fisiològiques, IDIBELL-Institute of Neurosciences, Universitat de Barcelona, L'Hospitalet de Llobregat, Spain
[b] Unitat de Genètica, Departament de Ciències Fisiològiques, Laboratori de Genètica Molecular, IDIBELL-Universitat de Barcelona, L'Hospitalet de Llobregat, Spain
[c] Centro de Investigación en Red de Enfermedades Raras (CIBERER), ISCIII, Spain
[d] Institute for Research in Biomedicine, University of Barcelona, Barcelona, Spain

ARTICLE INFO

Keywords:
Leukodystrophy
MLC1
GlialCAM
ClC-2
Astrocyte

ABSTRACT

Megalencephalic leukoencephalopathy with subcortical cysts (MLC) is a rare type of leukodystrophy characterized by dysfunction of the role of glial cells in controlling brain fluid and ion homeostasis. Patients affected by MLC present macrocephaly, cysts and white matter vacuolation, which lead to motor and cognitive impairments. To date, there is no treatment for MLC, only supportive care. MLC is caused by mutations in the *MLC1* and *GLIALCAM* genes. MLC1 is a membrane protein with low identity to the Kv1.1 potassium channel and GlialCAM belongs to an adhesion molecule family. Both proteins form a complex with an as-yet-unknown function that is expressed mainly in the astrocytes surrounding the blood–brain barrier and in Bergmann glia. GlialCAM also acts as an auxiliary subunit of the chloride channel ClC-2, thus regulating its localization at cell–cell junctions and modifying its functional properties by affecting the common gate of ClC-2. Recent studies in *Mlc1*-, *GlialCAM*- and *Clcn2*-knockout mice or *Mlc1*-knockout zebrafish have provided fresh insight into the pathophysiology of MLC and further details about the molecular interactions between these three proteins. Additional studies have shown that GlialCAM/MLC1 also regulates other ion channels (TRPV4, VRAC) or transporters (Na^+/K^+-ATPase) in a not-understood manner. Furthermore, it has been shown that GlialCAM/MLC1 may influence signal transduction mechanisms, thereby affecting other proteins not related with transport such as the EGF receptor. Here, we offer a personal biochemical retrospective of the work that has been performed to gain knowledge of the pathophysiology of MLC, and we discuss future strategies that may be used to identify therapeutic solutions for MLC patients.

1. Introduction

Megalencephalic leukoencephalopathy with subcortical cysts (MLC; MIM 604004) is a white matter disorder associated primarily with myelin and astrocyte vacuolation (van der Knaap et al., 2012). It is an infantile-onset hereditary disease characterized by early-onset macrocephaly (van der Knaap et al., 1995a). Magnetic resonance imaging (MRI) is used to diagnose the disease in childhood (van der Knaap et al., 1995b). Brain MRI shows diffuse signal abnormality and swelling of the cerebral white matter and the presence of subcortical cysts, mainly in the anterior temporal regions (Singhal et al., 1996). Clinically, MLC patients present deterioration of motor functions with ataxia and spasticity, epileptic seizures and mental decline. In contrast to other leukodystrophies, MLC progresses very slowly, but minor head trauma and common infections exacerbate clinical conditions (van der Knaap et al., 2012). Many factors seem to affect the severity of the disease; even siblings with the same mutation may present differences in its phenotypic expression of the disease (Pascual-Castroviejo et al., 2005). Other patients have been shown to present a similar clinical picture at first, but subsequent MRIs have shown an improvement or even

* Corresponding author. Facultat de Medicina, Departament de Ciències Fisiològiques, Universitat de Barcelona-IDIBELL, C/Feixa Llarga, s/n 08907 L'Hospitalet de Llobregat, Barcelona, Spain.
E-mail address: restevez@ub.edu (R. Estévez).

http://dx.doi.org/10.1016/j.ejmg.2017.10.013
Received 21 July 2017; Received in revised form 14 September 2017; Accepted 22 October 2017

normalization (van der Knaap et al., 2010). This phenotype has been called MLC2B (MIM 613926), and these patients may present different phenotypes that range from a transient benign form of MLC to macrocephaly and mental retardation with or without autism (Lopez-Hernandez et al., 2011a).

In the 1990s, it was hypothesized that the MRI pattern recognition of white matter disorders could be used to classify leukodystrophies and also to identify the genes involved (van der Knaap et al., 1991). Although this method is now recognized as standard (van der Knaap and Bugiani, 2017), it revolutionized the field of white matter disorders. This technique was used in genetic linkage studies to identify the first gene locus for MLC associated to an autosomal recessive trait (Topcu et al., 2000) and, subsequently, the disease gene, which was named *MLC1* (Leegwater et al., 2001). *MLC1* mutations were detected in most MLC patients (about 80% of patients) (Ilja Boor et al., 2006; Leegwater et al., 2002; Riel-Romero et al., 2005; Yuzbasioglu et al., 2011; Kariminejad et al., 2015; Xie et al., 2012; Montagna et al., 2006; Wang et al., 2011), but some families did not present linkage to the *MLC1* locus (about 20% of patients), thus indicating genetic heterogeneity (Patrono et al., 2003; Blattner et al., 2003). Since *Clcn2* knockout mice show myelin vacuolation similar to that of MLC patients (Blanz et al., 2007), *CLCN2* mutations were screened in those patients without linkage to *MLC1*, but no pathogenic mutations were identified (Scheper et al., 2010). While looking for MLC1-interacting proteins, our group identified GlialCAM as the most abundant protein to interact with MLC1 (Lopez-Hernandez et al., 2011a). Subsequently, the analysis of the *GLIALCAM* gene (also called *HEPACAM*) in MLC patients without MLC1 mutations identified pathogenic mutations with recessive inheritance in those MLC2A patients (Lopez-Hernandez et al., 2011a). Furthermore, mutations with a dominant inheritance in *GLIALCAM* were also identified in MLC2B patients (Lopez-Hernandez et al., 2011a).

In this review, we offer a biochemical retrospective of the molecular pathogenesis of MLC. Readers are asked to consult other recent reviews for the clinical aspects of the disease (van der Knaap and Bugiani, 2017; Schiffmann and van der Knaap, 2004; Kaye, 2001; Brignone et al., 2015).

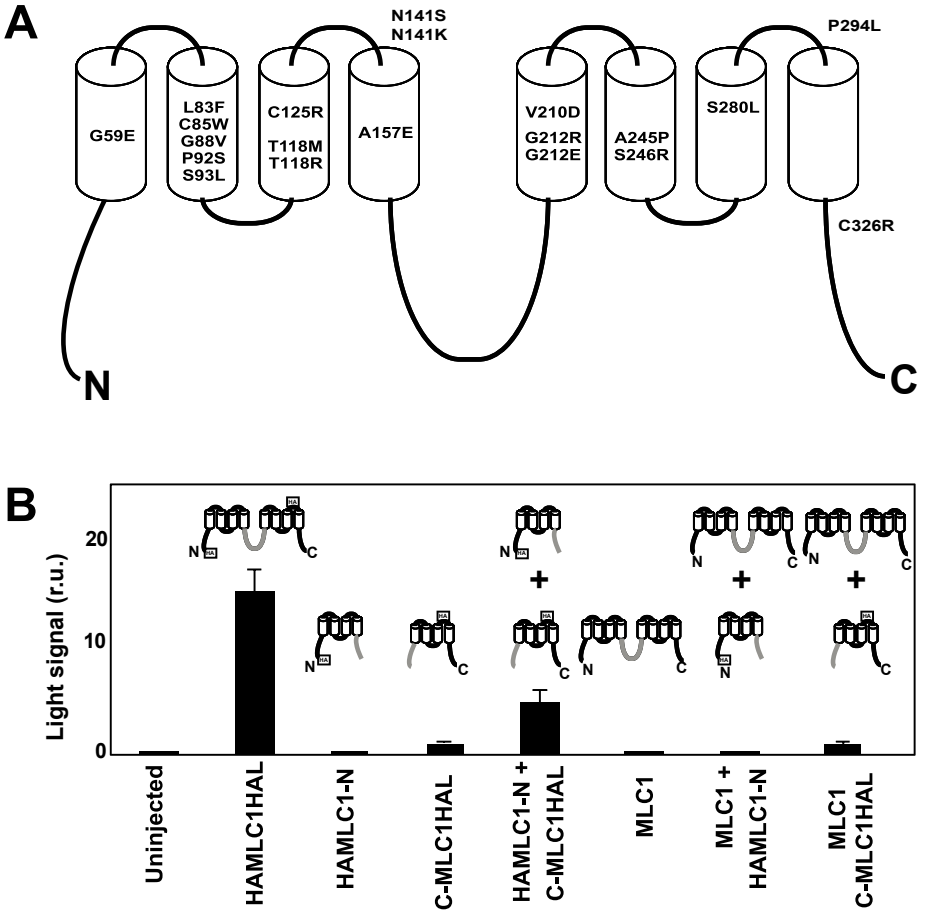
2. Biochemical studies of the MLC1 protein

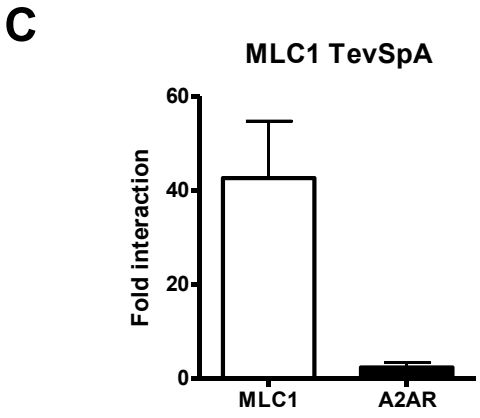
MLC1 encodes a membrane protein of still unknown function also known as MLC1. DNA databases provide clues that shed light on the function of novel genes (Nomura et al., 1994). MLC1 orthologues are found only in vertebrates, including the zebrafish, which has a single orthologue (Sirisi et al., 2014). Sequence comparison between all orthologues revealed that amino acids are more conserved in the putative eight transmembrane (TM) domains, including residues that are mutated in patients (Figs. S1 and 1A), and in the terminal part of the C-terminus that contains a stretch of three bulky hydrophobic valines (Fig. S1). Immunofluorescence experiments using antibodies directed against the N- or C-terminus indicated that both segments are intracellular (Boor et al., 2005; Teijido et al., 2004). Amino acid sequence analysis suggested that the *MLC1* gene might have evolved from a gene duplication of a minigene containing the first four TM domains. This can clearly be seen, as the predicted fourth and eighth TM segments contain a stretch of poly-leucine residues and the intracellular loop between TM segments 4 and 5 is not conserved among the different orthologues (Fig. S1). If we co-express in *Xenopus* oocytes the N- and C-terminal parts of MLC1 that has been split between TM segments 4 and 5, both parts are able to arrive to the plasma membrane (Fig. 1B), thus indicating that these two halves may interact with each other, probably through these poly-leucine stretches, as occurs with other membrane proteins (Gurezka et al., 1999). These experiments also indicated that the loops before TM4 and TM8 are extracellular. In addition to this internal oligomerization, we have conducted many different biochemical experiments, such as co-immunoprecipitation of differently tagged MLC1 subunits, enhanced trafficking assays (Teijido et al., 2004) and split-TEV (tobacco etch protease) (Capdevila-Nortes et al., 2012), which show that MLC1 may homo-oligomerize (Fig. 1C). This type of biochemical behaviour is found in many ion channel proteins (Fig. 1D) (Park et al., 2016). In line with this finding, MLC1 presents low identity (< 20% amino acid identity) with the potassium channel Kv1.1, which is involved in ataxia (Fig. S2). Furthermore, MLC patients may present epilepsy, which is typical in diseases caused by ion channel protein mutations (Haj-Yasein et al., 2011), but not in leukodystrophies. MLC1 also has the signature of ABC-2 type transporters and sodium-galactose transporters, thus suggesting it could be a transport protein. On the other hand, a PROSITE search also indicates that MLC1 contains a signature motif of the ribosomal protein S14 subunit that starts in the middle of transmembrane 2 and the beginning of the first intracellular loop (Fig. S1). However, it has been suggested that this motif is a false positive (Leegwater et al., 2001). Whether MLC1 plays a role in protein translation has not yet been investigated.

In light of all this evidence, the first theory regarding the possible function of MLC1 is that it could act as an ion channel. To test this hypothesis, we expressed MLC1 in *Xenopus* oocytes and HEK293 cells and performed voltage-clamp or patch-clamp measurements using different voltage protocols under normal conditions or after adding activating compounds, but no currents that could be assigned to MLC1 were detected (Teijido et al., 2004). It is important to note that we verified that the protein was expressed at the plasma membrane alone (Teijido et al., 2004). We also co-expressed MLC1 with the potassium channel Kv1.1 (Teijido et al., 2004) or with the potassium channel Kir4.1 (since both are expressed in the same cells) (Teijido et al., 2007), but no changes were observed after co-expression with MLC1. As is always the case in science, a negative result does not mean that this hypothesis is wrong. Since many ion channels need accessory subunits in order to be functional (Estevez et al., 2001), our interpretation at the time was that an MLC1-accessory subunit could be required to express ion channel activity, which was consistent with the fact that some MLC patients do not have mutations in *MLC1* (van der Knaap et al., 2010).

3. Identification of *GLIALCAM* as a second MLC gene

Six years ago, before NGS (Next generation sequencing) experiments represented standard methods (Fernández-Marmiesse et al., 2017), the search for new MLC genes was performed through genetic-linkage studies. However, this approach proved unsuccessful. Viewed retrospectively, this problem could have been solved actually very easily by sequencing patients' genome. At that time, our strategy was to identify MLC1-interacting proteins as candidate genes for MLC. Two types of experiments were performed: a split-ubiquitin yeast two-hybrid assay to test for membrane proteins (results are ongoing) and identification by mass-spectrometry of MLC1-associated proteins by affinity purification using antibodies directed against MLC1 (Lopez-Hernandez et al., 2011a). Our first, somewhat naive idea was that proteins that appeared in both methods were probably true MLC1-interacting proteins. Our initial results were very discouraging, as no protein appeared in both methods. Two experimental changes were key to the successful identification of GlialCAM as an MLC1-interacting protein. First, we decided to repeat affinity purification experiments using another detergent, since we suspected that the interaction could be sensitive to detergent, as is the case with, for example, cholesterol metabolism-related proteins (Yang et al., 2002). In fact, only a few detergents such as digitonin maintained this interaction (Fig. 2A). The sensitivity of the interaction of both proteins to detergents may explain why other groups did not identify GlialCAM as an MLC1-interacting protein (Sugio et al., 2017). The second key modification was to apply new protein quantification methods based on the peptide signal intensities that were extracted (Zolles et al., 2009; Muller et al., 2010; Bildl et al., 2012). This allowed us to conclude that GlialCAM had the second highest yield (after MLC1) in all affinity purifications (Lopez-Hernandez et al.,





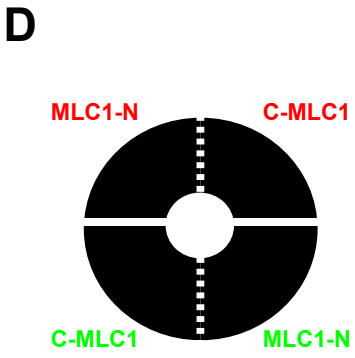


Fig. 1. Biochemical features of the MLC1 protein. A) A scheme of the MLC1 molecule, showing the predicted 8 transmembrane (TM) segments. The N- and C-terminal are intracellular. Some of the mutations identified in MLC patients and characterized (Duarri et al., 2008) are shown. The position of the N and the C termini are indicated. **B)** MLC1 surface expression can be reconstituted by expressing separately two halves of the MLC1 protein (HAMLC1-N + C-MLC1HAL). MLC1 has been split at the intracellular loop between TM segments 4 and 5 (in grey). Surface expression was determined by a luminescence-based assay, detecting the HA epitope tag (HAL), which is inserted in the extracellular loop between TM segments 7 and 8. In contrast, expression of full-length MLC1 (without HA tags) does not improve surface expression of the C-terminal half of MLC1, suggesting that these halves in the full-length MLC1 are not free to interact with split MLC1. For clarity, a scheme of each of the constructs expressed in *Xenopus* oocytes is provided and we indicated the N and C termini of full-length MLC1. The experiments were repeated at least twice. **C)** Example of typical split-TEV experiments used to detect interactions between MLC1 molecules. The A2A receptor is used as a negative control. **D)** A schematic model of MLC1 putative quaternary structure that integrates the results obtained in B (split MLC1, dotted lined) and in C (homo-oligomerization). We considered an antiparallel structure, as this is usually found in ion channels (Park et al., 2016). We imagined that a central pore could be formed by elements of each group of four transmembrane segments.

2011a). Importantly, an estimate of the abundance of the interacting protein suggested that both proteins directly interact (Lopez-Hernandez et al., 2011a). Marjo van der Knaap's research group identified two mutations in *GLIALCAM* (inherited in a recessive form) in MLC2A patients and one *GLIALCAM* mutation (inherited in a dominant form) in MLC2B patients (Lopez-Hernandez et al., 2011a) (Fig. 2B). Thus, this friendly collaboration between a biochemical group and a genetic group yielded exciting results.

GlialCAM (Glial cell adhesion molecule) is a type-I transmembrane protein (N-terminus extracellular, due to the presence of a signal peptide) that contains two immunoglobulin (Ig)-like domains (Fig. 2B). The domain proximal to the transmembrane domain is C2 type and the more distant domain is V-type. The C-terminus contains many phosphorylation sites and is also very rich in proline residues (Fig. S3). GlialCAM was originally identified as a silenced gene in human hepatocellular carcinoma (hence the reason for the name HepaCAM), suggesting that it could act as a tumour suppressor (Chung Moh et al., 2005). A very low expression of GlialCAM was found in the liver, and GlialCAM knockout mice do not show any evidence of having an increased incidence of tumours (Hoegg-Beiler et al., 2014).

The fact that GlialCAM and MLC1 directly interact was demonstrated following the *in vitro* expression of both cDNAs in a heterologous system such as HeLa or HEK cells (Lopez-Hernandez et al., 2011b). Thus, different methods such as co-immunoprecipitation between differentially tagged GlialCAM or MLC1 proteins, FRET (fluorescence resonance energy transfer), BRET (bioluminescence resonance energy transfer) and split-TEV indicated that the interaction between both proteins is direct (Lopez-Hernandez et al., 2011b) (Fig. 2C).

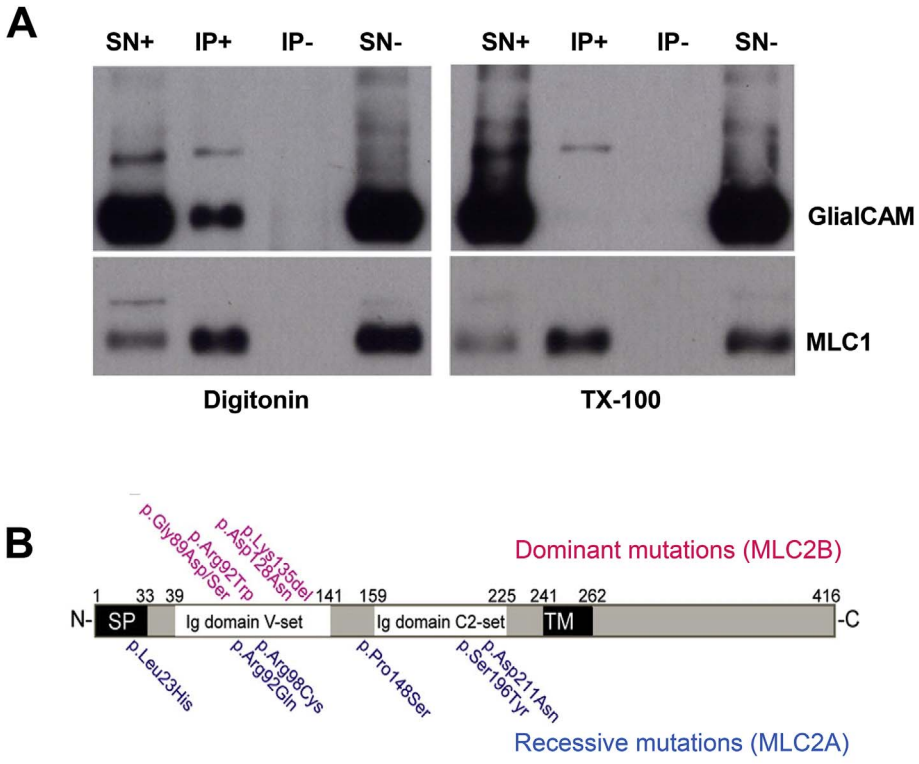


Fig. 2. Identification of GlialCAM as an MLC1-interacting protein and as a second MLC gene. A) Co-immunoprecipitation of mouse brain membrane proteins using a monoclonal antibody directed against MLC1. Proteins were solubilized in PBS1x, 150 mM NaCl and 1% detergent (digitonin or Triton X-100) at a 1:10 protein/detergent ratio. A Western blot was performed with rabbit polyclonal antibodies detecting GlialCAM or MLC1. **B)** Schematic structure of GlialCAM indicating the positions of the signal peptide (SP), IgV, IgC2 and transmembrane segment (TM). Some of the mutations found in MLC2B and MLC2A patients are also indicated. **C)** Interaction of MLC1 with GlialCAM detected by BRET (bioluminescence resonance energy transfer). Cells expressing a constant amount of GlialCAM-*R*luc (donor) with increasing amounts of MLC1-YFP (acceptor) are incubated with the luciferase substrate and the BRET signal is detected using a luminometer. The levels of MLC1-YFP are determined by measuring the fluorescence levels of YFP. The levels of GlialCAM-Rluc are determined by measuring the luminescence signal 10 min after adding the substrate. The BRET ratio is the acceptor emission at 527 nm relative to the donor emission at 475 nm 1 min after adding the substrate. Due to the overlap of the emission spectra, it is necessary to subtract the light detected at 527 nm that is contributed by the donor emission, which is calculated in cells expressing only GlialCAM-Rluc. The saturation of the curve indicates that the interaction is not due to random collisions. Similar results have been found by FRET (Förster resonance energy transfer). **D)** Typical example of split-TEV experiments used to detect interactions between GlialCAM molecules. The 4F2 molecule is used as a negative control.

Furthermore, GlialCAM co-expression changed the subcellular localization of MLC1 from the endoplasmic reticulum/plasma membrane and concentrated it in cell–cell junctions in heterologous cells (Lopez-Hernandez et al., 2011a, 2011b). Analysis of deletions and chimeric GlialCAM proteins suggested that the extracellular domain of GlialCAM is necessary for both cell junction targeting and mediating interactions with MLC1 (Capdevila-Nortes et al., 2015). The confinement of MLC1 in cell–cell junctions by GlialCAM *in vitro* recapitulates the MLC1 expression observed in astrocyte–astrocyte junctions in primary astrocytes or in tissue by EM (electron microscopy) Immunogold (Teijido et al., 2007; Duarri et al., 2011) (Fig. 3A). It should be noted that EM Immunogold is the only method that is resolutive enough to address the localization of MLC1 in tissue, since other methods such as confocal microscopy and post-embedding EM may yield ambiguous results in the tiny membranes of the astrocytic endfeet, where MLC1 is mostly located (Boor et al., 2005; Teijido et al., 2004; Bugiani et al., 2017; Dubey et al., 2015; Ambrosini et al., 2008) (Fig. 3A). Studies by confocal microscopy in Bergmann glia represent an alternative approach, as they possess long, straight processes (Teijido et al., 2004).

In summary, we obtained bona-fide genetic and biochemical evidence that indicated that GlialCAM is a subunit of MLC1 required for its confinement at cell junctions. We then tried to measure currents in *Xenopus* oocytes and HEK cells, but no currents were observed (unpublished results). Thus, our hypothesis that a missing subunit may be required for the functional expression of MLC1 was therefore disproved. We then decided to perform some biochemical experiments to address the role of GlialCAM in MLC1 cell biology. Unexpectedly, a lack of GlialCAM caused by RNA interference in primary astrocytes or knockout mice not only abolished MLC1 localization at junctions, but also dramatically reduced the total expression of MLC1 and its localization at the plasma membrane (Hoegg-Beiler et al., 2014; Bugiani et al., 2017; Capdevila-Nortes et al., 2013). These results allowed us to conclude that GlialCAM is an essential MLC1 subunit that acts as a chaperone to ensure that MLC1 reaches the plasma membrane and concentrates at cell–cell junctions. Why, then, does overexpressed MLC1 reach the plasma membrane in heterologous cell lines? Our explanation was that low GlialCAM expression levels (even those not detected by western blot) are enough to rescue MLC1 retention in the endoplasmic reticulum (ER) (Capdevila-Nortes et al., 2013). On the other hand, the overexpression of MLC1 may saturate a possible ER

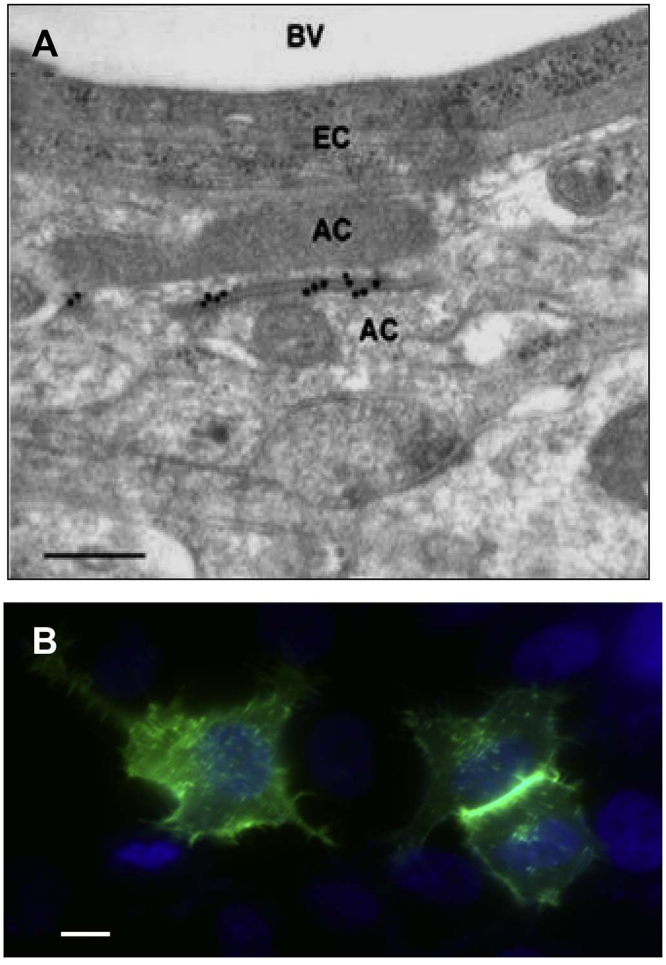


Fig. 3. Localization of MLC1 at astrocyte–astrocyte junctions is determined by GlialCAM-mediated transinteractions. A) Example of immunolabelling showing the localization of MLC1 (18 nm gold particles) at astrocyte–astrocyte junctions in mouse tissue. No gold particles were observed in endothelial-astrocyte junctions. Scale bar: 500 nm. **B)** Localization of GlialCAM in cell junctions depends on GlialCAM transinteractions. Thus, single-transfected cells are not located in junctions whereas junctional localization is observed in double-transfected cells. Scale bar: 20 μm. MLC1 requires co-expression with GlialCAM in order to reach cell–cell junctions.

retention mechanism that may operate on MLC1 and is not associated with GlialCAM. *In vitro*, the confinement of MLC1 to cell–cell junctions depends on the expression levels of GlialCAM, which is observed only at high expression levels (Capdevila-Nortes et al., 2013).

Several GlialCAM protein properties are crucial to its capacity to concentrate at cell–cell junctions. First, GlialCAM homo-oligomerizes with itself through the extracellular domain, as revealed by split-TEV (Lopez-Hernandez et al., 2011b) (Fig. 2D). This homo-oligomerization seems to be important for the role of the C-terminus in localization at cell–cell junctions (Capdevila-Nortes et al., 2015). The C-terminus probably mediates interactions with the cytoskeletal actin network (Moh et al., 2009a). On the other hand, the extracellular domain of GlialCAM also interacts homophilically in trans through the extracellular domain (Hoegg-Beiler et al., 2014; Capdevila-Nortes et al., 2015), as can be seen in single- or double-transfected pairs with GlialCAM: GlialCAM was located at cell–cell junctions in only the double-transfected cells (Fig. 3B). Cis-homo-oligomerization seems to be required for the establishment of sufficient trans-homophilic interactions, as observed in mutants found in MLC (see below) (Lopez-Hernandez et al., 2011b). GlialCAM do not interact in trans with MLC1, as demonstrated by experiments in which cells independently transfected with GlialCAM or MLC1 were mixed (Hoegg-Beiler et al., 2014).

Although GlialCAM concentrates at cell–cell junctions when expressed alone in cell lines, MLC1 also increased the proportion of GlialCAM present at junctions, thus suggesting that MLC1 may favour the formation of GlialCAM and thereby increase its propensity to localize at cell–cell junctions (Lopez-Hernandez et al., 2011b). One of the many unexpected findings associated with MLC is that a lack of MLC1 in knockout zebrafish (Sirisi et al., 2014), knockout mice (Hoegg-Beiler et al., 2014; Dubey et al., 2015) and in a MLC patient (Sirisi et al., 2014) leads to GlialCAM mislocalization. Surprisingly, GlialCAM mislocalization was not observed in primary astrocytes from *Mlc1* knockout mice (Sirisi et al., 2014). However, the application of a depolarizing solution containing extracellular potassium causes GlialCAM mislocalization in astrocytes from *Mlc1* knockout mice, but not in wild-type astrocytes (Sirisi et al., 2014), recapitulating the biochemical defect observed *in vivo*. This suggests that this GlialCAM trafficking process may depend on MLC1 function and perhaps extracellular potassium levels, which may be linked to increased neuronal activity. It could also be that in primary astrocytes in basal conditions MLC1 is not working properly, although functional defects have also been observed in primary astrocytes in basal or in hypotonic conditions, suggesting that this is not the case. Clarification regarding the mechanistic details underlying the internalization of GlialCAM in *Mlc1* knockout mice is still required.

4. GlialCAM/MLC1 directly regulates the function of chloride channel ClC-2 in glial cells

For the interaction of GlialCAM and MLC1 to be physiologically relevant, both proteins must co-localize in native tissue. This was in fact the case in the astrocytic endfeet or Bergmann glia (Lopez-Hernandez et al., 2011a), but GlialCAM was also detected in oligodendrocytes (Favre-Kontula et al., 2008), where MLC1 is not present. This suggested that GlialCAM might interact with other proteins in this cell type. Quantitative mass spectroscopy of GlialCAM-interacting proteins identified mostly peptides from GlialCAM and MLC1, but also from the chloride channel ClC-2 (Barrallo-Gimeno et al., 2015a; Jeworutzki et al., 2012). ClC-2 is expressed in neurons, astrocytes and oligodendrocytes (Blanz et al., 2007; Jentsch et al., 2002; Ratte and Prescott, 2011; Sik et al., 2000; Nobile et al., 2000). The interaction between GlialCAM and ClC-2 was also direct, as demonstrated after both proteins were expressed in cell lines by co-immunoprecipitation and split-TEV (Jeworutzki et al., 2012). As occurs with MLC1, GlialCAM directs ClC-2 to cell–cell junctions and, more importantly, modifies the functional properties of the ClC-2-mediated current (see below).

By contrast, the co-expression of MLC1 with ClC-2 does not modify the functional properties of ClC-2. Moreover, when the same anti-MLC1 polyclonal antibodies used to co-immunoprecipitate GlialCAM were used, no ClC-2 was purified, which suggested that GlialCAM/ClC-2 and GlialCAM/MLC1 were independent complexes (Lopez-Hernandez et al., 2011a). However, this view has recently changed. A newly developed mouse monoclonal anti-MLC1 antibody was used to co-precipitate ClC-2 and GlialCAM (Sirisi et al., 2017a). Furthermore, the immunoprecipitation of ClC-2 using anti-GlialCAM antibodies on brain membranes from *Mlc1*-KO was dramatically reduced (Sirisi et al., 2017a). These new results together with proximity ligation experiments in primary astrocytes (Sirisi et al., 2017a) suggest that, in astrocytes, GlialCAM, MLC1 and ClC-2 may form a ternary complex in which GlialCAM acts as a bridge between ClC-2 and MLC1 (Fig. 4).

Importantly, in *Xenopus* oocytes, transfected HEK293 cells (Jeworutzki et al., 2012) and oligodendrocytes *in vivo* (Hoegg-Beiler et al., 2014), GlialCAM modifies the functional properties of the ClC-2-mediated Cl^- current, thus increasing current amplitudes and changing activation and rectification properties by affecting the common gate that operates in both protomers of the CLC channels (Jeworutzki et al., 2014). It seems that the first three amino acids (Ser-Leu-Tyr) of the transmembrane segment of GlialCAM are essential for the activation of ClC-2 currents, but not for targeting or biochemical interactions

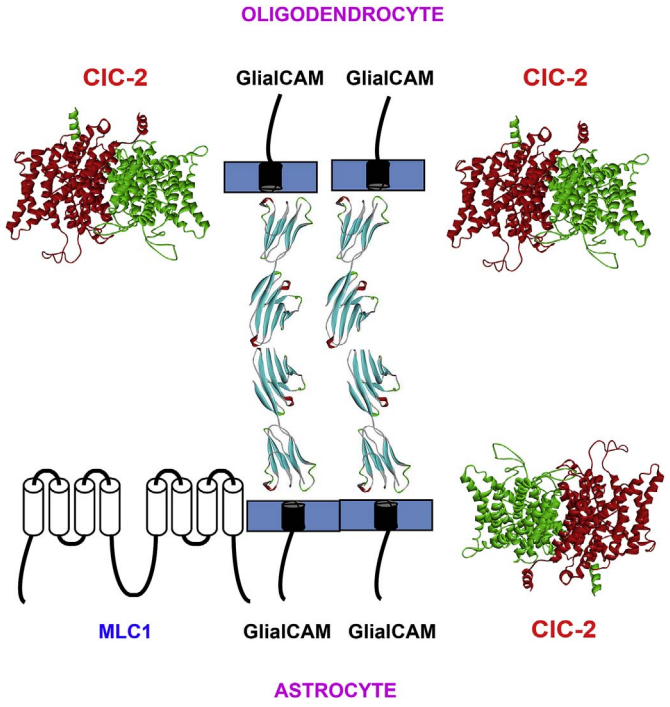


Fig. 4. Model of the complex formed between GlialCAM, MLC1 and ClC-2 in junctions between astrocytes and oligodendrocytes. GlialCAM, MLC1 and ClC-2 form homo-oligomers, in the case of ClC-2 dimers, as shown in the crystal structure of a mammalian ClC-K channel. MLC1 is an astrocytic protein, whereas GlialCAM and ClC-2 are found in astrocytes and oligodendrocytes. GlialCAM is capable of interacting in trans with GlialCAM molecules from other cells (in this case oligodendrocytes, but the model can also be extrapolated to astrocyte–astrocyte junctions). In addition, GlialCAM is capable of hetero-oligomerizing with MLC1 and ClC-2 in a different manner: it is an obligatory subunit for MLC1, as it is needed for its endoplasmatic reticulum exit and its plasma membrane/junctional localization. In the case of ClC-2, it works as an auxiliary subunit, as expression at the plasma membrane does not depend on GlialCAM but its expression at cell junctions depends on GlialCAM. Thus, a lack of GlialCAM causes MLC1 and ClC-2 mislocalization. A lack of MLC1 also causes GlialCAM mislocalization in astrocytes by unknown mechanisms, leading to GlialCAM and ClC-2 mislocalization in oligodendrocytes due to the lack of trans-interactions.

(Capdevila-Nortes et al., 2015). It is not yet clear what parts of ClC-2 interact with GlialCAM; such information may help to shed light on the mechanistic details of the common gating process (Zuniga et al., 2004). However, recordings of ClC-2 currents in Bergmann glia in slices from wild type, *GlialCAM* and *Mlc1* KO mice (Hoegg-Beiler et al., 2014) or primary rat or mouse wild-type astrocytes in culture (Sirisi et al., 2017a) (which express GlialCAM and MLC1) do not show any modifications to currents. In astrocytes in culture, it was shown that GlialCAM did not interact with ClC-2 (Sirisi et al., 2017a). Here, the situation changed following incubation with a depolarizing solution for a short time. In this condition, ClC-2 forms a ternary complex with GlialCAM and MLC1, as revealed by immunofluorescence, co-immunoprecipitation, proximity ligation assays and patch-clamp measurements (Sirisi et al., 2017a). Furthermore, the localization of ClC-2 at junctions in primary astrocytes following incubation with a depolarizing solution was blocked when Mlc1 was absent or GlialCAM was depleted by RNAi (Sirisi et al., 2017a). We have recently conducted similar experiments in primary astrocytes derived from *GlialCAM* KO mice and have obtained similar results (data not shown). Patch clamp experiments in depolarizing conditions have not been performed in Bergmann glia. However, since these cells show long, straight processes, recording the ClC-2 activity during these processes could be hindered by an access resistance problem, and the recording of the ClC-2 activity of these cells in slices may reflect only the ClC-2 present in the soma (Hoegg-Beiler et al., 2014). Nevertheless, based on results from recordings in primary astrocytes and oligodendrocytes in slices, we suggest that the chloride influx at positive voltages mediated by GlialCAM/MLC1/ClC-2 in astrocytes and GlialCAM/ClC-2 in oligodendrocytes may be needed to compensate for an excess of potassium (Kofuji and Newman, 2004). In addition, with all these evidences, we propose that GlialCAM/MLC1/ClC-2 in astrocytes may also interact in trans with GlialCAM/ClC-2 in oligodendrocytes through GlialCAM, which would explain why an astrocytic MLC1 knockout causes GlialCAM and ClC-2 mislocalization in oligodendrocytes (Hoegg-Beiler et al., 2014) (Fig. 4).

5. Effect of MLC mutations on GlialCAM and MLC1

Several mutations have been identified in *MLC1* (Fig. 1A; for a detailed table, see the recent review (van der Knaap et al., 2012)). These mutations have been studied after the expression of MLC1 alone in different systems such as *Xenopus* oocytes (Montagna et al., 2006; Teijido et al., 2004; Lopez-Hernandez et al., 2011b; Duarri et al., 2008), HeLa cells (Teijido et al., 2004; Lopez-Hernandez et al., 2011b; Duarri et al., 2008), human astrocytoma cells (Xie et al., 2012; Lanciotti et al., 2012, 2016) and primary astrocyte cultures (Lopez-Hernandez et al., 2011b; Capdevila-Nortes et al., 2013; Duarri et al., 2008), and endogenously in monocytes from MLC1 patients (Duarri et al., 2008; Petrini et al., 2013) and in a brain biopsy from an MLC patient containing an *MLC1* mutation (Lopez-Hernandez et al., 2011b). In general, most of the mutations studied attenuated the cell surface expression of MLC1. As occurs with many other diseases caused by mutations that affect membrane proteins, such as cystic fibrosis, the experiments involving expression in heterologous systems revealed differences that depended on the cell system and possibly on the expression levels of each mutant and the performance of the quality control machinery (in the endoplasmic reticulum and the plasma membrane) (Drumm et al., 1991; Sharma et al., 2004). These differences included mutation P92S, which showed normal levels at the plasma membrane in *Xenopus* oocytes, but a dramatic reduction in transfected HeLa cells (Duarri et al., 2008). These experiments suggested that this mutation is temperature sensitive, as oocytes are incubated at lower temperatures than Hela cells. Another example is mutation S246R, which presents a lower surface expression in primary astrocytes than HeLa cells and *Xenopus* oocytes. By contrast, the few studies that address the endogenous MLC1 protein (as monocytes from MLC patients containing the S246R mutation) showed a complete absence of MLC1 protein (Duarri et al., 2008). We would therefore suggest that most mutations in *MLC1* may cause MLC1 misfolding, which affects protein degradation and trafficking. No correlation can be established between the phenotype of patients and their genotype based on biochemical studies of these mutations, as we speculate that MLC1 protein levels would be reduced in all cases. Co-expression with GlialCAM stabilized the MLC1 mutants, and even corrected its localization at cell–cell junctions, thus demonstrating that GlialCAM is an obligatory subunit of MLC1 that protects it from ER-associated degradation (Capdevila-Nortes et al., 2013).

In contrast to *MLC1* mutations, most *GLIALCAM* missense mutations do not affect GlialCAM protein expression, with the exception of mutation L23H, which affects the signal peptide of GlialCAM (Fig. 2B) (Lopez-Hernandez et al., 2011a, 2011b). Most of the mutations, which are found in MLC2A or MLC2B patients, affect the localization of GlialCAM at cell–cell junctions and, as a consequence, also affect the trafficking of MLC1 and ClC-2 to cell–cell junctions (Lopez-Hernandez et al., 2011a, 2011b; Arnedo et al., 2013; Arnedo et al., 2014). None of the mutants studied presented a reduction in the interaction with MLC1 or ClC-2 or a lack of ClC-2 functional activation (Arnedo et al., 2014). Mutants that affect trafficking can be classified into two groups: some show defective homo-oligomerization and others show normal homo-oligomerization (Arnedo et al., 2014). We assume that this means that only those with normal homo-oligomerization have defective trans interaction, although there is a lack of evidence for this. Mutations found in MLC2B patients also act as dominant mutations in trafficking assays

(Lopez-Hernandez et al., 2011a). However, it remains unclear why *GLIALCAM* mutations are dominant or recessive. Two striking examples are mutation R92Q (recessive) and mutation R92W (dominant) (Fig. 2B). A knockin mouse with a dominant mutation found in MLC2B patients (G10S) has been generated and studied (Hoegg-Beiler et al., 2014). It shows partially defective trafficking of GlialCAM, MLC1 and ClC-2 to cell–cell junctions (Hoegg-Beiler et al., 2014). In view of the fact that MLC1 and GlialCAM expression in humans is higher in the early years of life (Bugiani et al., 2017; Dubey et al., 2015), the phenotypic reversibility of MLC2B in patients (van der Knaap et al., 2010) could be explained by the fact that they still have enough GlialCAM/MLC1 proteins to perform their functions in the latter stages of life. In contrast, the protein levels in the early years of life are not high enough for this active myelination process. According to this hypothesis, it should be possible to improve MLC patients' phenotype by introducing some GlialCAM/MLC1, even in adults. However, recent results have shown that overexpression of MLC1 in mice could have severe consequences and lead to cavitation (Sugio et al., 2017). Thus, expression levels of MLC1 should be controlled to avoid toxic effects.

A few GlialCAM mutations, however, do not present trafficking defects. One mutation (P148S) is defective only when expressed in *Mlc1* knockout astrocytes (Arnedo et al., 2014). We suggest that, in much the same way as GlialCAM stabilizes MLC1, MLC1 may also stabilize this mutant. Some other GlialCAM mutants, such as D211N, show a very interesting phenotype. They do not show any trafficking defects in any cell types, including *Mlc1*-deficient astrocytes (Arnedo et al., 2014). As discussed above, GlialCAM is internalized in the presence of a depolarizing solution when MLC1 is absent (Lopez-Hernandez et al., 2011b). In contrast, these mutants are never internalized, so they behave as gain-of-function mutations (Arnedo et al., 2014). However, in primary astrocytes, ClC-2 is translocated to cell–cell junctions in the presence of a depolarizing solution (Sirisi et al., 2017a), and the overexpression of these mutants blocks this translocation (Sirisi et al., 2017a). We speculate that this process of ClC-2 translocation after depolarization depends on the internalization of GlialCAM and subsequent recycling to the plasma membrane, which may be stabilized at the surface by MLC1. However, the mechanistic details of this process have yet to be studied in depth.

6. Other proteins regulated by GlialCAM/MLC1

Before the chloride channel ClC-2 was identified as a GlialCAM-interacting protein (Jeworutzki et al., 2012; Barrallo-Gimeno and Estévez, 2014), it was hypothesized that other chloride channels may be involved in MLC. In astrocytes, the activity of the volume-regulated anion channel (VRAC) is crucial for restoring astrocyte cell volume after a hypotonic shock in a process called regulatory volume decrease (RVD) (Hoffmann et al., 2009; Ernest et al., 2005). The channel releases chloride and other organic anions such as taurine, glutamate and even ATP (Lutter et al., 2017; Gaitán-Peñas et al., 2016; Voss et al., 2014; Qiu et al., 2014). Thus, it has been shown that, even in lymphoblast from patients that expresses undetectable levels of MLC1 protein by western blot, monocytes from patients, primary rat astrocytes with reduced expression of MLC1 and GlialCAM by RNAi and mouse astrocytes from Mlc1 knockout (Dubey et al., 2015; Capdevila-Nortes et al., 2013; Petrini et al., 2013), that VRAC activity is reduced, but not completely abolished. Conversely, the overexpression of MLC1 enhances VRAC activity in primary astrocytes. At that time, we hypothesized that MLC1 may directly or indirectly influence the VRAC channel, but it could not be the channel itself, as the channel is expressed in all the cells in our body. The recent identification of LRRC8 heteromers (LRRC8A as the main subunit, plus different combinations of LRRC8B, C, D and E) as the molecular correlates of VRAC represents a new tool to shed light on how GlialCAM/MLC1 may influence this channel (Voss et al., 2014; Syeda et al., 2016; Hyzinski-García et al., 2014). Recent results in GlialCAM knockout mice (Bugiani et al., 2017) and unpublished immunoaffinity purification results from our group suggest that these proteins do not interact directly (*manuscript in progress*). Thus, the regulation may be indirect.

Other proteins related to ionic homeostasis have been identified as putative MLC1-interacting proteins, including the Na,K-ATPase (Sugio et al., 2017; Brignone et al., 2011), the potassium channel Kir4.1 (Lanciotti et al., 2012), the calcium-permeable channel TRPV4 (Lanciotti et al., 2012), the water channel AQP4 (Lanciotti et al., 2012), connexin 43 (Wu et al., 2016) and the vacuolar ATPase (Brignone et al., 2014), the proton pump that regulates acidity. These proteins were identified by yeast two-hybrid (Y2H) or affinity purification methods such as pull-downs, co-fractionation and affinity purification using antibodies. However, there is no experimental evidence that these proteins interact directly with GlialCAM or MLC1 after they have been transfected in heterologous systems using standard biochemical methods. For the Na,K-ATPase there are contradictory results, since one group identified by Y2H MLC1 binding to the beta subunit (Brignone et al., 2011), whereas the other group identified binding to only the alpha subunit and excluded by immunoprecipitation that MLC1 interacted with the beta subunit (Sugio et al., 2017). Following this contradiction, the first group suggested that hypo-osmotic conditions increased MLC1 membrane expression and favoured MLC1/beta association (Brignone et al., 2011). In contrast, the other group indicated that MLC1 overexpression decreases the formation of heterodimers between the alpha and beta subunits compromising the activity of the ATPase (Sugio et al., 2017). In contrast, the activity and membrane expression was not altered in *Mlc1* KO astrocytes (Sugio et al., 2017). Furthermore, in all the cases studied its localization is not altered (as happens for instance with ClC-2) in *GlialCAM* and *Mlc1* knockout mice (Bugiani et al., 2017; Dubey et al., 2015).

In our opinion, it should first be verified that GlialCAM/MLC1 interacts directly with these proteins *in vitro* after expressing in cell lines and second, demonstrate that this interaction is lost in knockout or RNAi-depleted models to be completely sure that it is not a non-physiological indirect consequence of the over-expression. Only then would it be possible to interpret these changes as a direct consequence of GlialCAM/MLC1 function. On the other hand, due to compensatory mechanisms or biological differences (as it is with the timing of myelination between mice and humans (Dubey et al., 2015)), not always transgenic mouse models fully recapitulate pathological mechanisms. One then should validate this interaction in human-derived samples, as it has been done for instance in the interaction between GlialCAM and MLC1 (Lopez-Hernandez et al., 2011b). One clear example is the VRAC channel: VRAC has been shown to be regulated by multiple mechanisms (Mongin, 2015; Stauber, 2015; Jentsch et al., 2015; Pedersen et al., 2016) such as adhesion, GPCRs, phosphorylation and oxidation, processes that may also be regulated/influenced by MLC1 (Lanciotti et al., 2016). Even knockdown of AQP4 affects the VRAC channel (Benfenati et al., 2007). Possibly, MLC1 and also AQP4 do not interact directly with the VRAC channel but its deletion/over-expression may influence it by unknown mechanisms.

Other proteins that have been shown to be interactors of the GlialCAM/MLC1 complex are members of the dystrophin-glycoprotein complex (Ambrosini et al., 2008; Boor et al., 2007) like syntrophin, dystrobrevin and others such as caveolin (Lanciotti et al., 2010) and ZO-1 (Duarri et al., 2011). Their interactions have been demonstrated by means of affinity purification experiments using antibodies or pull-down experiments using intracellular regions of the MLC1 protein. We could consider these proteins as scaffolding proteins, which could regulate the targeting of GlialCAM/MLC1 (Bragg et al., 2006; Fanning and Anderson, 2009). In this case, one would not expect the localization of these proteins to change in *GlialCAM* or *Mlc1* knockout mice unless they interact directly. One example is the direct interaction between syntrophin and AQP4 (through a PDZ domain of syntrophin). In this case, as expected, syntrophin knockout affects the localization of AQP4 at the perivascular endfeet (Amiry-Moghaddam et al., 2003) but the

opposite is also true (Amiry-Moghaddam et al., 2003). In contrast, in AQP4 knockout there was a modest reduction in the localization of dystrophin and the localization of β-dystroglycan was unaltered (Amiry-Moghaddam et al., 2003). In MLC-related proteins, localization of MLC1 was unaltered in dystrophin KO, α-dystrobrevin KO and utrophin KO (Duarri et al., 2011). Importantly, Immunogold-EM showed that these proteins do not co-localize in human and rat tissue, since they are present in endothelial-astrocyte surfaces at the endfeet, whereas GlialCAM and MLC1 are present in astrocyte-astrocyte contacts (Duarri et al., 2011). Furthermore, the localization of syntrophin, α- and β-dystroglican was not altered in *Mlc1* and *GlialCAM* knockout mice (Bugiani et al., 2017; Dubey et al., 2015). In contrast, the protein ZO-1 co-localize with MLC1 at astrocyte–astrocyte junctions by immunogold cytochemistry and also in cultured astrocytes (Duarri et al., 2011). The discovery that MLC1 co-purify with caveolin is interesting (Lanciotti et al., 2010; Moh et al., 2009b), since MLC1 contains a caveolin-binding-like motif (Fig. S1) and MLC1 expression have been detected in caveolar membrane rafts in astrocytes and rat/mouse brain (Lanciotti et al., 2010). However, in both cases, its localization was also unaltered in *GlialCAM* and *Mlc1* knockout mice (Bugiani et al., 2017; Dubey et al., 2015). More experiments are needed to unravel whether there is a direct interaction between these proteins or whether they are associated through an unknown bridge protein.

Recently, it has been shown that overexpression of MLC1, but not mutated MLC1, favours epidermal growth factor receptor (EGFR) degradation and inhibits EGF-induced Ca^{2+} entry, ERK1/2 and PLCγ1 activation and calcium-activated KCa3.1 potassium channel function: all molecular pathways that are involved in the stimulation of astrocyte proliferation (Lanciotti et al., 2016). In fact, previous experiments by our group indicate that the expression of MLC1 is much higher in AraC-arrested astrocytes (Duarri et al., 2011). If these results are validated in knockout animals, they could be highly significant, since they suggest that MLC1 may also influence signal transduction processes, which could explain the heterogeneity of the effects observed after the overexpression or depletion of MLC1. Understanding the mechanisms of these processes is key to identifying therapeutic solutions for MLC patients.

7. A model of MLC pathogenesis

I began working on MLC in 2003, when I created my own group after working on a postdoctoral research project as part of a group led by Professor Thomas Jentsch, a very bright scientist who focuses primarily on chloride channels (Jentsch et al., 2002). In 2001, Marjo van der Knaap's group identified the first MLC gene (Leegwater et al., 2001). I had read that MLC was characterized by myelin vacuolization. Since chloride fluxes are usually linked to water movements, I thought that MLC could be due to a defect in new chloride channels, which was my recent area of expertise, so it seemed an interesting project to work on. Looking back, I can see that this naive hypothesis was correct. Furthermore, many surprises have emerged in research into the molecular mechanisms of the pathogenesis of this disease by different groups. The first surprise was that, although brain biopsies had indicated that vacuoles are mostly present in the outer layer of myelin (van der Knaap et al., 1996), MLC1 is expressed only at the cell contacts between astrocytes around the endfeet and in Bergmann glia (Hoegg-Beiler et al., 2014). It is not present in neurons, as initially indicated but later corrected by our group due to the lack of a control *Mlc1* knockout when the first experiments were performed (Teijido et al., 2004, 2007; Hoegg-Beiler et al., 2014). Moreover, initial confocal experiments suggested that it is expressed in ependymal cells, but this was revealed to be untrue by electron microscopy experiments with knockout animals as a control (Sirisi et al., 2014). How, then, do defects in an astrocytic protein cause vacuoles in myelin? The first studies to use RNAi in astrocytes and re-examination of a brain biopsy in MLC patients indicated that vacuoles are also present in astrocytes and show swelling (Duarri et al., 2011). Studies with *Mlc1* and *GlialCAM* knockout mice showed that astrocytic swelling precedes myelin vacuolization and the degree of white matter vacuolization parallels the increased thickness of perivascular astrocyte processes, thus suggesting that astrocytic swelling leads to water retention in myelin (Bugiani et al., 2017; Dubey et al., 2015). On the other hand, as GlialCAM is mislocalized in oligodendrocytes in *Mlc1* knockout mice, ClC-2 activity is altered in oligodendrocytes, as shown in recordings from slices (Hoegg-Beiler et al., 2014). Therefore, both processes may occur simultaneously and oligodendrocytes are perhaps more sensitive to osmotic alterations. Future research on GlialCAM and/or ClC-2 cell-specific knockout mice is perhaps required to resolve this issue.

Another exciting finding was the discovery that the ClC-2 chloride channel is associated with GlialCAM (Jeworutzki et al., 2012), since this harked back to my past work in finding subunits of CLC channels (Estevez et al., 2001; Barrallo-Gimeno et al., 2015a). This was followed by our initial hypothesis that ClC-2 dysfunction could fully explain the pathogenesis of MLC (Maduke and Reimer, 2012). However, this rather naive idea was incorrect. Several findings contradict it: i) MLC1 has been shown to influence many other proteins and activities, such as the volume-regulated anion channels (Capdevila-Nortes et al., 2013). ii) Double knockout of GlialCAM and ClC-2 has a stronger vacuolation phenotype that the knockout of ClC-2 alone, thus suggesting that GlialCAM may affect other processes (Hoegg-Beiler et al., 2014). iii) Mutations in *CLCN2* have been identified in a different type of leukodystrophy (CC2L-related leukodystrophy) that manifests a phenotype that is different to the phenotype of MLC patients (Depienne et al., 2013; van der Knaap et al., 1993). It can therefore be suggested that ClC-2 dysfunction may contribute to MLC pathogenesis, but this is not the whole story.

The third exciting finding was recently reported by the group led by Elena Ambrosini, which indicated that GlialCAM/MLC1 might influence signal transduction cascades (Lanciotti et al., 2016). In light of the results of the changes observed in GlialCAM and ClC-2 after incubation with a depolarizing solution, a completely unproven model of MLC pathogenesis has now emerged. We speculated that MLC1 may be a potassium sensor (Sirisi et al., 2017b), or in another words, a neuronal activity sensor in astrocytes. If the homology of the potassium channel Kv1.1 is studied in depth (Fig. S2), the selectivity filter or residues involved in the pore are not conserved, unlike some features of the voltage sensor of the channel. We speculate that the localization of GlialCAM/MLC1 at astrocyte–astrocyte junctions or astrocyte–oligodendrocyte junctions could play a functional role in the process of sensing ions. It allows changes in the concentration of potassium, for instance, to be detected in advance, since small increases in the content of these ions in the tiny volume occupied by junctions increase their concentration dramatically. In view of Elena's results that MLC1 may affect different signal transduction processes, it may affect different proteins, such as astrocytic ion channels and transporters, via unknown mechanisms after detection of potassium concentration changes, which may help re-establish the homeostasis of ions and water (Fig. 5). As our colleague Professor Enrico Bertini suggested (Enrico's personal communication), MLC may be considered a metabolic disease that affects multiple processes, each of which helps regulate homeostasis. The fact that many processes are affected could explain the heterogeneity of phenotypes observed in MLC patients, even in siblings with the same mutation (Riel-Romero et al., 2005). On the other hand, GlialCAM and MLC1 may also play different roles such as acting as scaffolding for other proteins, as is the case with ClC-2. As shown for other membrane proteins, such as the chloride/proton antiporter ClC-7 (Barrallo-Gimeno et al., 2015b), several functions may co-exist in GlialCAM/MLC1, each with different levels of importance for the phenotype of MLC patients.

Thus, in terms of finding a treatment for MLC patients, we envisaged several strategies that can be established. A gene therapy approach using adeno-associated viruses may obviously be beneficial, as shown in

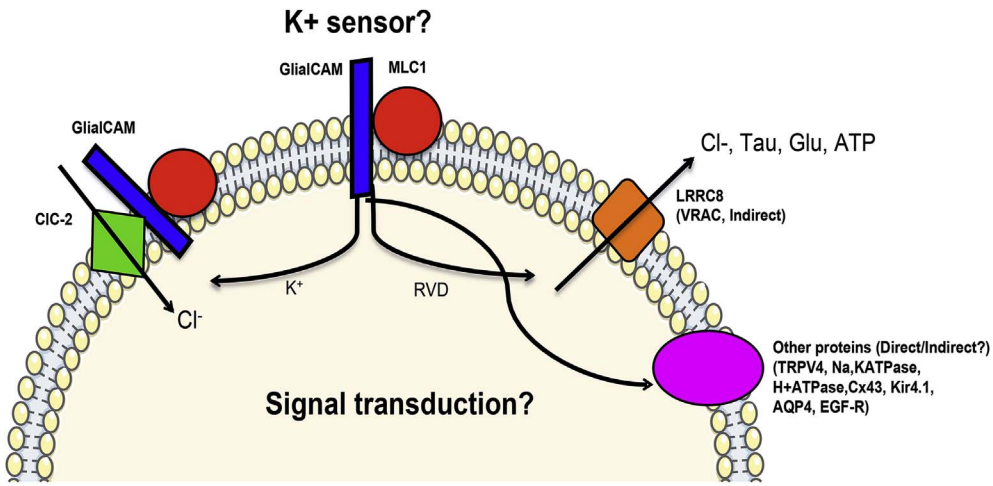


Fig. 5. Hypothetical model of the functional role of GlialCAM/MLC1 and ClC-2 in the molecular pathogenesis of MLC. We hypothesized that GlialCAM/MLC1 forms a potassium sensor, based on the homology of MLC1 to the Kv1.1 potassium channel and biochemical experiments performed by our group in primary astrocytes. The detection of changes in extracellular potassium concentration that may increase during periods of high neuronal activity will be easily sensed in the tiny astrocyte–astrocyte or astrocyte–oligodendrocyte junctions, as small changes in potassium content will cause considerable increase in potassium concentration. We have observed that in cultured primary astrocytes, increases in potassium concentration led to the formation of a complex between GlialCAM, ClC-2 and MLC1. Thus, ClC-2 changes its activity from that of an inward rectifier chloride current, only opened at negative voltages, to that of an ohmic chloride current. We speculated that chloride influx at positive voltages may be important to compensate positive potassium charges, which could be important in the process of potassium siphoning. In an indirect manner, GlialCAM/MLC1 may regulate the volume-regulated anion channel (VRAC), which is formed by LRRC8 proteins and that mediates efflux of chloride, osmolytes such as taurine and other molecules that act as neurotransmitters like glutamate or ATP. The activation of this channel is important in the process of regulatory volume decrease (RVD) that happens in astrocytes during hypotonic shock. Possibly, the activation of VRAC and other channels, transporters or receptors is mediated in a direct or indirect manner through signal transduction events that still have to be defined.

other brain disorders (Georgiou et al., 2017). Special care should be taken to control expression levels, since too much MLC1 could also be deleterious. Pharmacological chaperones that could restore MLC1 expression at the plasma membrane could also be used, in the same way as they have been tested in other diseases such as cystic fibrosis (Verkman and Galietta, 2009; Brown et al., 1996; Thomas et al., 1995). Finally, understanding the way in which GlialCAM/MLC1 influences signal transduction processes and identifying pharmacological methods to manipulate them could also represent a potential strategy for ameliorating the symptoms of MLC patients.

Acknowledgements

This work was supported in part by grants from the European Leukodystrophies Association (ELA) Research Foundation (ELA2012-014C2B) to RE and VN, Ministerio de Ciencia e Innovación (SAF SAF2012-31486 to RE and SAF2015-70377 to RE and ABG), Generalitat de Catalunya ((SGR2014-1178) to RE), (SGR2014-541) to VN)), Instituto de Salud Carlos III (bodies linked to MICINN and FEDER): (ERARE) to RE, FIS PI13/00121 to VN. RE is a recipient of an ICREA Academia prize. ABG is a Serra-Hunter fellow. We thank to all collaborators on the MLC studies.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ejmg.2017.10.013.

References

Ambrosini, E., Serafini, B., Lanciotti, A., et al., 2008. Biochemical characterization of MLC1 protein in astrocytes and its association with the dystrophin-glycoprotein complex. Mol. Cell Neurosci. 37, 480–493.

Amiry-Moghaddam, M., Williamson, A., Palomba, M., et al., 2003. Delayed K+ clearance associated with aquaporin-4 mislocalization: phenotypic defects in brains of alpha-syntrophin-null mice. Proc. Natl. Acad. Sci. U. S. A. 100, 13615–13620.

Arnedo, T., Aiello, C., Jeworutzki, E., et al., 2013. Expanding the spectrum of megalencephalic leukoencephalopathy with subcortical cysts in two patients with GLIALCAM mutations. Neurogenetics. http://dx.doi.org/10.1007/s10048-013-0381-x.

Arnedo, T., López-Hernández, T., Jeworutzki, E., et al., 2014. Functional analyses of mutations in HEPACAM causing megalencephalic leukoencephalopathy. Hum. Mutat. 35, 1175–1178.

Barrallo-Gimeno, A., Estévez, R., 2014. GlialCAM, a glial cell adhesion molecule implicated in neurological disease. Adv. Neurobiol. 8, 47–59.

Barrallo-Gimeno, A., Gradogna, A., Zanardi, I., Pusch, M., Estévez, R., 2015a. Regulatory-auxiliary subunits of CLC chloride channel-transport proteins. J. Physiol. 593, 4111–4127.

Barrallo-Gimeno, A., Gradogna, A., Zanardi, I., Pusch, M., Estévez, R., 2015b. Regulatory/Auxiliary subunits of CLC chloride channel/transport proteins. J. Physiol. http://dx.doi.org/10.1113/JP270057.

Benfenati, V., Nicchia, G.P., Svelto, M., Rapisarda, C., Frigeri, A., Ferroni, S., 2007. Functional down-regulation of volume-regulated anion channels in AQP4 knockdown cultured rat cortical astrocytes. J. Neurochem. 100, 87–104.

Bildl, W., Haupt, A., Müller, C.S., et al., 2012. Extending the dynamic range of label-free mass spectrometric quantification of affinity purifications. Mol. Cell Proteomics 11 M111.007955.

Blanz, J., Schweizer, M., Auberson, M., et al., 2007. Leukoencephalopathy upon disruption of the chloride channel ClC-2. J. Neurosci. 27, 6581–6589.

Blattner, R., Von Moers, A., Leegwater, P.A., Hanefeld, F.A., Van Der Knaap, M.S., Kohler, W., 2003. Clinical and genetic heterogeneity in megalencephalic leukoencephalopathy with subcortical cysts (MLC). Neuropediatrics 34, 215–218.

Boor, P.K., de Groot, K., Waisfisz, Q., et al., 2005. MLC1: a novel protein in distal astroglial processes. J. Neuropathol. Exp. Neurol. 64, 412–419.

Boor, I., Nagtegaal, M., Kamphorst, W., et al., 2007. MLC1 is associated with the dystrophin-glycoprotein complex at astrocytic endfeet. Acta Neuropathol. 114, 403–410.

Bragg, A.D., Amiry-Moghaddam, M., Ottersen, O.P., Adams, M.E., Froehner, S.C., 2006. Assembly of a perivascular astrocyte protein scaffold at the mammalian blood-brain barrier is dependent on alpha-syntrophin. Glia 53, 879–890.

Brignone, M.S., Lanciotti, A., Macioce, P., et al., 2011. The beta1 subunit of the Na,K-ATPase pump interacts with megalencephalic leucoencephalopathy with subcortical cysts protein 1 (MLC1) in brain astrocytes: new insights into MLC pathogenesis. Hum. Mol. Genet. 20, 90–103.

Brignone, M.S., Lanciotti, A., Visentin, S., et al., 2014. Megalencephalic leukoencephalopathy with subcortical cysts protein-1 modulates endosomal pH and protein trafficking in astrocytes: relevance to MLC disease pathogenesis. Neurobiol. Dis. 66, 1–18.

Brignone, M.S., Lanciotti, A., Camerini, S., et al., 2015. MLC1 protein: a likely link between leukodystrophies and brain channelopathies. Front. Cell Neurosci. 9, 66.

Brown, C.R., Hong-Brown, L.Q., Biwersi, J., Verkman, A.S., Welch, W.J., 1996. Chemical chaperones correct the mutant phenotype of the delta F508 cystic fibrosis transmembrane conductance regulator protein. Cell Stress Chaperones 1, 117–125.

Bugiani, M., Dubey, M., Breur, M., et al., 2017. Megalencephalic leukoencephalopathy with cysts: the *Glialcam* -null mouse model. Ann. Clin. Transl. Neurol. 4, 450–465.

Capdevila-Nortes, X., Lopez-Hernandez, T., Ciruela, F., Estevez, R., 2012. A modification of the split-tobacco etch virus method for monitoring interactions between membrane proteins in mammalian cells. Anal. Biochem. 423, 109–118.

Capdevila-Nortes, X., López-Hernández, T., Apaja, P.M., et al., 2013. Insights into MLC pathogenesis: GlialCAM is an MLC1 chaperone required for proper activation of volume-regulated anion currents. Hum. Mol. Genet. 22, 4405–4416.

Capdevila-Nortes, X., Jeworutzki, E., Elorza-Vidal, X., Barrallo-Gimeno, A., Pusch, M., Estévez, R., 2015. Structural determinants of interaction, trafficking and function in the ClC-2/MLC1 subunit GlialCAM involved in leukodystrophy. J. Physiol. 593, 4165–4180.

Chung Moh, M., Hoon Lee, L., Shen, S., 2005. Cloning and characterization of hepaCAM, a novel Ig-like cell adhesion molecule suppressed in human hepatocellular carcinoma. J. Hepatol. 42, 833–841.

Depienne, C., Bugiani, M., Dupuits, C., et al., 2013. Brain white matter oedema due to ClC-2 chloride channel deficiency: an observational analytical study. Lancet Neurol. 12, 659–668.

Drumm, M.L., Wilkinson, D.J., Smit, L.S., et al., 1991. Chloride conductance expressed by

delta F508 and other mutant CFTRs in Xenopus oocytes. Science 254, 1797–1799.
Duarri, A., Teijido, O., Lopez-Hernandez, T., et al., 2008. Molecular pathogenesis of megalencephalic leukoencephalopathy with subcortical cysts: mutations in MLC1 cause folding defects. Hum. Mol. Genet. 17, 3728–3739.
Duarri, A., Lopez de Heredia, M., Capdevila-Nortes, X., et al., 2011. Knockdown of MLC1 in primary astrocytes causes cell vacuolation: a MLC disease cell model. Neurobiol. Dis. 43, 228–238.
Dubey, M., Bugiani, M., Ridder, M.C., et al., 2015. Mice with megalencephalic leukoencephalopathy with cysts: a developmental angle. Ann. Neurol. 77, 114–131.
Ernest, N.J., Weaver, A.K., Van Duyn, L.B., Sontheimer, H.W., 2005. Relative contribution of chloride channels and transporters to regulatory volume decrease in human glioma cells. Am. J. Physiol. Physiol. 288, C1451–C1460.
Estevez, R., Boettger, T., Stein, V., et al., 2001. Barttin is a Cl- channel beta-subunit crucial for renal Cl- reabsorption and inner ear K+ secretion. Nature 414, 558–561.
Fanning, A.S., Anderson, J.M., 2009. Zonula occludens-1 and -2 are cytosolic scaffolds that regulate the assembly of cellular junctions. Ann. N. Y. Acad. Sci. 1165, 113–120.
Favre-Kontula, L., Rolland, A., Bernasconi, L., et al., 2008. GlialCAM, an immunoglobulin-like cell adhesion molecule is expressed in glial cells of the central nervous system. Glia 56, 633–645.
Fernández-Marmiesse, A., Gouveia, S., Couce, M.L., 2017. NGS technologies as a turning point in rare disease research, diagnosis, and treatment. Curr. Med. Chem. 24. http://dx.doi.org/10.2174/0929867324666170718101946.
Gaitán-Peñas, H., Gradogna, A., Laparra-Cuervo, L., et al., 2016. Investigation of LRRC8-mediated volume-regulated anion currents in Xenopus oocytes. Biophys. J. 111, 1429–1443.
Georgiou, E., Sidiropoulou, K., Richter, J., et al., 2017. Gene therapy targeting oligodendrocytes provides therapeutic benefit in a leukodystrophy model. Brain 140, aww351.
Gurezka, R., Laage, R., Brosig, B., Langosch, D., 1999. A heptad motif of leucine residues found in membrane proteins can drive self-assembly of artificial transmembrane segments. J. Biol. Chem. 274, 9265–9270.
Haj-Yasein, N.N., Jensen, V., Vindedal, G.F., et al., 2011. Evidence that compromised K(+) spatial buffering contributes to the epileptogenic effect of mutations in the human kir4.1 gene (KCNJ10). Glia 59, 1635–1642.
Hoegg-Beiler, M.B., Sirisi, S., Orozco, I.J., et al., 2014. Disrupting MLC1 and GlialCAM and ClC-2 interactions in leukodystrophy entails glial chloride channel dysfunction. Nat. Commun. 5, 3475.
Hoffmann, E.K., Lambert, I.H., Pedersen, S.F., 2009. Physiology of cell volume regulation in vertebrates. Physiol. Rev. 89, 193–277.
Hyzinski-García, M.C., Rudkouskaya, A., Mongin, A.A., 2014. LRRC8A protein is indispensable for swelling-activated and ATP-induced release of excitatory amino acids in rat astrocytes. J. Physiol. 592, 4855–4862.
Ilja Boor, P.K., de Groot, K., Mejaski-Bosnjak, V., et al., 2006. Megalencephalic leukoencephalopathy with subcortical cysts: an update and extended mutation analysis of MLC1. Hum. Mutat. 27, 505–512.
Jentsch, T.J., Stein, V., Weinreich, F., Zdebik, A.A., 2002. Molecular structure and physiological function of chloride channels. Physiol. Rev. 82, 503–568.
Jentsch, T.J., Lutter, D., Planells-Cases, R., Ullrich, F., Voss, F.K., 2015. VRAC: molecular identification as LRRC8 heteromers with differential functions. Pflugers Arch. http://dx.doi.org/10.1007/s00424-015-1766-5.
Jeworutzki, E., Lopez-Hernandez, T., Capdevila-Nortes, X., et al., 2012. GlialCAM, a protein defective in a leukodystrophy, serves as a ClC-2 Cl(-) channel auxiliary subunit. Neuron 73, 951–961.
Jeworutzki, E., Lagostena, L., Elorza-Vidal, X., López-Hernández, T., Estévez, R., Pusch, M., 2014. GlialCAM, a CLC-2 Cl(-) channel subunit, activates the slow gate of CLC chloride channels. Biophys. J. 107, 1105–1116.
Kariminejad, A., Rajaee, A., Ashrafi, M.R., et al., 2015. Eight novel mutations in MLC1 from 18 Iranian patients with megalencephalic leukoencephalopathy with subcortical cysts. Eur. J. Med. Genet. 58, 71–74.
Kaye, E.M., 2001. Update on genetic disorders affecting white matter. Pediatr. Neurol. 24, 11–24.
van der Knaap, M.S., Bugiani, M., 2017. Leukodystrophies: a proposed classification system based on pathological changes and pathogenetic mechanisms. Acta Neuropathol. http://dx.doi.org/10.1007/s00401-017-1739-1.
van der Knaap, M.S., Valk, J., de Neeling, N., Nauta, J.J., 1991. Pattern recognition in magnetic resonance imaging of white matter disorders in children and young adults. Neuroradiology 33, 478–493.
van der Knaap, M.S., Depienne, C., Sedel, F., Abbink, T.E., 1993. CLCN2-Related Leukoencephalopathy. University of Washington, Seattle. http://www.ncbi.nlm.nih.gov/pubmed/26539602, Accessed date: 29 April 2017.
van der Knaap, M.S., Barth, P.G., Stroink, H., et al., 1995a. Leukoencephalopathy with swelling and a discrepantly mild clinical course in eight children. Ann. Neurol. 37, 324–334.
van der Knaap, M.S., Valk, J., Barth, P.G., Smit, L.M., van Engelen, B.G., Tortori Donati, P., 1995b. Leukoencephalopathy with swelling in children and adolescents: MRI patterns and differential diagnosis. Neuroradiology 37, 679–686.
van der Knaap, M.S., Barth, P.G., Vrensen, G.F., Valk, J., 1996. Histopathology of an infantile-onset spongiform leukoencephalopathy with a discrepantly mild clinical course. Acta Neuropathol. 92, 206–212.
van der Knaap, M.S., Lai, V., Kohler, W., et al., 2010. Megalencephalic leukoencephalopathy with cysts without MLC1 defect. Ann. Neurol. 67, 834–837.
van der Knaap, M.S., Boor, I., Estevez, R., 2012. Megalencephalic leukoencephalopathy with subcortical cysts: chronic white matter oedema due to a defect in brain ion and water homoeostasis. Lancet Neurol. 11, 973–985.
Kofuji, P., Newman, E.A., 2004. Potassium buffering in the central nervous system. Neuroscience 129, 1045–1056.
Lanciotti, A., Brignone, M.S., Camerini, S., et al., 2010. MLC1 trafficking and membrane expression in astrocytes: role of caveolin-1 and phosphorylation. Neurobiol. Dis. 37, 581–595.
Lanciotti, A., Brignone, M.S., Molinari, P., et al., 2012. Megalencephalic leukoencephalopathy with subcortical cysts protein 1 functionally cooperates with the TRPV4 cation channel to activate the response of astrocytes to osmotic stress: dysregulation by pathological mutations. Hum. Mol. Genet. 21, 2166–2180.
Lanciotti, A., Brignone, M.S., Visentin, S., et al., 2016. Megalencephalic leukoencephalopathy with subcortical cysts protein-1 regulates epidermal growth factor receptor signaling in astrocytes. Hum. Mol. Genet. 25, 1543–1558.
Leegwater, P.A., Yuan, B.Q., van der Steen, J., et al., 2001. Mutations of MLC1 (KIAA0027), encoding a putative membrane protein, cause megalencephalic leukoencephalopathy with subcortical cysts. Am. J. Hum. Genet. 68, 831–838.
Leegwater, P.A., Boor, P.K., Yuan, B.Q., et al., 2002. Identification of novel mutations in MLC1 responsible for megalencephalic leukoencephalopathy with subcortical cysts. Hum. Genet. 110, 279–283.
Lopez-Hernandez, T., Ridder, M.C., Montolio, M., et al., 2011a. Mutant GlialCAM causes megalencephalic leukoencephalopathy with subcortical cysts, benign familial macrocephaly, and macrocephaly with retardation and autism. Am. J. Hum. Genet. 88, 422–432.
Lopez-Hernandez, T., Sirisi, S., Capdevila-Nortes, X., et al., 2011b. Molecular mechanisms of MLC1 and GLIALCAM mutations in megalencephalic leukoencephalopathy with subcortical cysts. Hum. Mol. Genet. 20, 3266–3277.
Lutter, D., Ullrich, F., Lueck, J.C., Kempa, S., Jentsch, T.J., 2017. Selective transport of neurotransmitters and –modulators by distinct volume-regulated LRRC8 anion channels. J. Cell Sci. 130 jcs.196253.
Maduke, M.C., Reimer, R.J., 2012. Biochemistry to the rescue: a ClC-2 auxiliary subunit provides a tangible link to leukodystrophy. Neuron 73, 855–857.
Moh, M.C., Tian, Q., Zhang, T., Lee, L.H., Shen, S., 2009a. The immunoglobulin-like cell adhesion molecule hepaCAM modulates cell adhesion and motility through direct interaction with the actin cytoskeleton. J. Cell Physiol. 219, 382–391.
Moh, M.C., Lee, L.H., Zhang, T., Shen, S., 2009b. Interaction of the immunoglobulin-like cell adhesion molecule hepaCAM with caveolin-1. Biochem. Biophys. Res. Commun. 378, 755–760.
Mongin, A.A., 2015. Volume-regulated anion channel-a frenemy within the brain. Pflugers Arch. http://dx.doi.org/10.1007/s00424-015-1765-6.
Montagna, G., Teijido, O., Eymard-Pierre, E., et al., 2006. Vacuolating megalencephalic leukoencephalopathy with subcortical cysts: functional studies of novel variants in MLC1. Hum. Mutat. 27, 292.
Muller, C.S., Haupt, A., Bildl, W., et al., 2010. Quantitative proteomics of the Cav2 channel nano-environments in the mammalian brain. Proc. Natl. Acad. Sci. U. S. A. 107, 14950–14957.
Nobile, M., Pusch, M., Rapisarda, C., Ferroni, S., 2000. Single-channel analysis of a ClC-2-like chloride conductance in cultured rat cortical astrocytes. FEBS Lett. 479, 10–14.
Nomura, N., Miyajima, N., Sazuka, T., et al., 1994. Prediction of the coding sequences of unidentified human genes. I. The coding sequences of 40 new genes (KIAA0001-KIAA0040) deduced by analysis of randomly sampled cDNA clones from human immature myeloid cell line KG-1. DNA Res. 1, 27–35.
Park, E., Campbell, E.B., MacKinnon, R., 2016. Structure of a CLC chloride ion channel by cryo-electron microscopy. Nature 541, 500–505.
Pascual-Castroviejo, I., van der Knaap, M.S., Pronk, J.C., Garcia-Segura, J.M., Gutierrez-Molina, M., Pascual-Pascual, S.I., 2005. Vacuolating megalencephalic leukoencephalopathy: 24 year follow-up of two siblings. Neurologia 20, 33–40.
Patrono, C., Di Giacinto, G., Eymard-Pierre, E., et al., 2003. Genetic heterogeneity of megalencephalic leukoencephalopathy and subcortical cysts. Neurology 61, 534–537.
Pedersen, S.F., Okada, Y., Nilius, B., 2016. Biophysics and physiology of the volume-regulated Anion Channel (VRAC)/Volume-Sensitive outwardly rectifying Anion Channel (VSOR). Pflugers Arch. http://dx.doi.org/10.1007/s00424-015-1781-6.
Petrini, S., Minnone, G., Coccetti, M., et al., 2013. Monocytes and macrophages as biomarkers for the diagnosis of megalencephalic leukoencephalopathy with subcortical cysts. Mol. Cell Neurosci. 56C, 307–321.
Qiu, Z., Dubin, A.E., Mathur, J., et al., 2014. SWELL1, a plasma membrane protein, is an essential component of volume-regulated anion channel. Cell 157, 447–458.
Ratte, S., Prescott, S.A., 2011. ClC-2 channels regulate neuronal excitability, not intracellular chloride levels. J. Neurosci. 31, 15838–15843.
Riel-Romero, R.M., Smith, C.D., Pettigrew, A.L., 2005. Megalencephalic leukoencephalopathy with subcortical cysts in two siblings owing to two novel mutations: case reports and review of the literature. J. Child. Neurol. 20, 230–234.
Scheper, G.C., van Berkel, C.G., Leisle, L., et al., 2010. Analysis of CLCN2 as candidate gene for megalencephalic leukoencephalopathy with subcortical cysts. Genet. Test. Mol. Biomarkers 14, 255–257.
Schiffmann, R., van der Knaap, M.S., 2004. The latest on leukodystrophies. Curr. Opin. Neurol. 17, 187–192.
Sharma, M., Pampinella, F., Nemes, C., et al., 2004. Misfolding diverts CFTR from recycling to degradation: quality control at early endosomes. J. Cell Biol. 164, 923–933.
Sik, A., Smith, R.L., Freund, T.F., 2000. Distribution of chloride channel-2-immunoreactive neuronal and astrocytic processes in the hippocampus. Neuroscience 101, 51–65.
Singhal, B.S., Gursahani, R.D., Udani, V.P., Biniwale, A.A., 1996. Megalencephalic leukodystrophy in an Asian Indian ethnic group. Pediatr. Neurol. 14, 291–296.
Sirisi, S., Folgueira, M., López-Hernández, T., et al., 2014. Megalencephalic leukoencephalopathy with subcortical cysts protein 1 regulates glial surface localization of GLIALCAM from fish to humans. Hum. Mol. Genet. 23, 5069–5086.
Sirisi, S., Elorza-Vidal, X., Arnedo, T., et al., 2017a. Depolarization causes the formation of a ternary complex between GlialCAM, MLC1 and ClC-2 in astrocytes: implications

in megalencephalic leukoencephalopathy. Hum. Mol. Genet. 26, 2436–2450.
Sirisi, S.S., Elorza-Vidal, X., Arnedo, T., et al., 2017b. Depolarization causes the formation of a ternary complex between GlialCAM, MLC1 and ClC-2 in astrocytes: implications in megalencephalic leukoencephalopathy. Hum. Mol. Genet. 26, 2436–2450.
Stauber, T., 2015. The volume-regulated anion channel is formed by LRRC8 heteromers – molecular identification and roles in membrane transport and physiology. Biol. Chem. 396, 975–990.
Sugio, S., Tohyama, K., Oku, S., et al., 2017. Astrocyte-mediated infantile-onset leukoencephalopathy mouse model. Glia 65, 150–168.
Syeda, R., Qiu, Z., Dubin, A.E., et al., 2016. LRRC8 proteins form volume-regulated anion channels that sense ionic strength. Cell 164, 499–511.
Teijido, O., Martinez, A., Pusch, M., et al., 2004. Localization and functional analyses of the MLC1 protein involved in megalencephalic leukoencephalopathy with subcortical cysts. Hum. Mol. Genet. 13, 2581–2594.
Teijido, O., Casaroli-Marano, R., Kharkovets, T., et al., 2007. Expression patterns of MLC1 protein in the central and peripheral nervous systems. Neurobiol. Dis. 26, 532–545.
Thomas, P.J., Qu, B.H., Pedersen, P.L., 1995. Defective protein folding as a basis of human disease. Trends Biochem. Sci. 20, 456–459.
Topcu, M., Gartioux, C., Ribierre, F., et al., 2000. Vacuoliting megalencephalic leukoencephalopathy with subcortical cysts, mapped to chromosome 22qtel. Am. J. Hum. Genet. 66, 733–739.
Verkman, A.S., Galietta, L.J., 2009. Chloride channels as drug targets. Nat. Rev. Discov. 8, 153–171.
Voss, F.K., Ullrich, F., Münch, J., et al., 2014. Identification of LRRC8 heteromers as an essential component of the volume-regulated Anion Channel VRAC. Science. http://dx.doi.org/10.1126/science.1252826.
Wang, J., Shang, J., Wu, Y., et al., 2011. Identification of novel MLC1 mutations in Chinese patients with megalencephalic leukoencephalopathy with subcortical cysts (MLC). J. Hum. Genet. 56, 138–142.
Wu, M., Moh, M.C., Schwarz, H., 2016. HepaCAM associates with connexin 43 and enhances its localization in cellular junctions. Sci. Rep. 6, 36218.
Xie, H., Wang, J., Dhaunchak, A.S., et al., 2012. Functional studies of MLC1 mutations in Chinese patients with megalencephalic leukoencephalopathy with subcortical cysts. PLoS One 7, e33087.
Yang, T., Espenshade, P.J., Wright, M.E., et al., 2002. Crucial step in cholesterol homeostasis: sterols promote binding of SCAP to INSIG-1, a membrane protein that facilitates retention of SREBPs in ER. Cell 110, 489–500.
Yuzbasioglu, A., Topcu, M., Cetin Kocaefe, Y., Ozguc, M., 2011. Novel mutations of the MLC1 gene in Turkish patients. Eur. J. Med. Genet. 54, 281–283.
Zolles, G., Wenzel, D., Bildl, W., et al., 2009. Association with the auxiliary subunit PEX5R/Trip8b controls responsiveness of HCN channels to cAMP and adrenergic stimulation. Neuron 62, 814–825.
Zuniga, L., Niemeyer, M.I., Varela, D., Catalan, M., Cid, L.P., Sepulveda, F.V., 2004. The voltage-dependent ClC-2 chloride channel has a dual gating mechanism. J. Physiol. 555, 671–682.