Megalencephalic leukoencephalopathy with subcortical cysts protein 1 regulates glial surface localization of GLIALCAM from fish to humans

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Megalencephalic leukoencephalopathy with subcortical cysts (MLC) is a leukodystrophy characterized by myelin vacuolization and caused by mutations in MLC1 or GLIALCAM. Patients with recessive mutations in either MLC1 or GLIALCAM show the same clinical phenotype. It has been shown that GLIALCAM is necessary for the correct targeting of MLC1 to the membrane at cell junctions, but its own localization was independent of MLC1 in vitro. However, recent studies in Mlc1−/− mice have shown that GlialCAM is mislocalized in glial cells. In order to investigate whether the relationship between Mlc1 and GlialCAM is species-specific, we first identified MLC-related genes in zebrafish and generated an mlc1−/− zebrafish. We have characterized mlc1−/− zebrafish both functionally and histologically and compared the phenotype with that of the Mlc1−/− mice. In mlc1−/− zebrafish, as in Mlc1−/− mice, Glialcam is mislocalized. Re-examination of a brain biopsy from an MLC patient indicates that GLIALCAM is also mislocalized in Bergmann glia in the cerebellum. In vitro, impaired localization of GlialCAM was observed in astrocyte cultures from Mlc1−/− mouse only in the presence of elevated potassium levels, which mimics neuronal activity. In summary, here we demonstrate an evolutionary conserved role for MLC1 in regulating glial surface levels of GLIALCAM, and this interrelationship explains why patients with mutations in either gene (MLC1 or GLIALCAM) share the same clinical phenotype.

INTRODUCTION

Megalencephalic leukoencephalopathy with subcortical cysts (MLC) is a rare type of congenital leukodystrophy characterized mainly by myelin vacuolization and early onset of macrocephaly (1). Magnetic resonance imaging (MRI) of patient’s brains shows diffuse signal abnormality, and this method is used for diagnosis during childhood (2). MLC is caused by mutations...
in either MLC1 (MIM #605908) or GLIALCAM (MIM #611642) genes (3,4).

MLC1 encodes for a membrane protein, which is expressed in several brain barriers, mainly in ependymal cells, astrocytes surrounding blood vessels and Bergmann radial glia in the cerebellum. In all cells, MLC1 is localized at cell junctions (5–7). Surprisingly, MLC1 is not expressed in oligodendrocytes, the site of the primary pathology of MLC (8). Mutations have been found across the whole coding sequence (3,9–11), which cause protein folding defects that lead to degradation of MLC1 (12,13). The function of MLC1 is still unknown, although it has been related to the activation of volume-regulated anion currents (VRAC) (12,15), involved in cellular osmotic response. It has been indicated that a reduced VRAC activity may lead to intracellular water accumulation and to the vacuolization observed in patients (12,15). In addition, other studies have suggested that MLC1 cooperates with the Na+/K+-ATPase and the TRPV4 channel (17,18), although the exact mechanism is still unknown.

GlialCAM (also called HEPACAM due to its original discovery in hepatic cancer cells) is a cell adhesion molecule of the immunoglobulin family (19) that is mostly expressed in glial cells (20) and co-localizes with MLC1 at astrocyte–astrocyte junctions (4). MLC1 patients with recessive mutations in GLIALCAM (known as MLC2A type of MLC, MIM #613925) present the same phenotype as patients carrying recessive mutations in MLC1 (MLC1 type of MLC, MIM #604004) (4,21). Some MLC patients have dominant mutations in the GLIALCAM gene (MLC2B type of MLC, MIM #613926), and these are associated to various degrees of phenotypic expression, ranging from a transient benign form of MLC, to macrocephaly and mental retardation with or without autism (4,21).

Both data obtained from in vitro and from GlialCAM−/− mice have demonstrated that GlialCAM is necessary for MLC1 to exit the endoplasmatic reticulum and target cell junctions (13,15,22). Furthermore, a mistargeting of both GlialCAM and MLC1 to cell junctions has been shown by in vitro studies with most missense GLIALCAM mutations (either recessive or dominant) and with GlialCAM−/− knock-in mice harbouring a dominant mutation (4,12,13,22,23). In contrast, GlialCAM seems to be able to traffic to cell junctions by itself (4,19). Thus, the overlapping phenotype of MLC1 and MLC2A patients can be understood because of the cardinal role of MLC1 in the pathogenesis of MLC.

However, GlialCAM, but not MLC1, is detected in oligodendrocytes (4,20); it was also found that GlialCAM functions as an auxiliary glial-specific subunit of the broadly expressed CIC-2 chloride channel (24). Furthermore, MLC-related GLIALCAM mutations impair the trafficking of CIC-2 to cell junctions (23,24). Analyses of GlialCAM−/− and GlialCAM−/− mice have shown that localization of CIC-2 in glial junctions is dependent on GlialCAM (22). Thus, based on these in vitro and in vivo data, patients carrying recessive GLIALCAM mutations should be more severely affected than patients carrying recessive MLC1 mutations, as CIC-2 channel may also be affected in those carrying GLIALCAM mutations. However, there are no clinical differences between these two types of patients.

Recent studies with Mlc1−/− mice have shown that, contrary to what was expected based on the lack of direct interaction between MLC1 and CIC-2, GlialCAM and CIC-2 are mislocalized in astrocytes and oligodendrocytes when MLC1 is absent (22). Based on cell culture data, it was indicated that MLC1 and CIC-2 could indirectly interact through homotypic extracellular GlialCAM-mediated interactions (22), as previously suggested (25). These results indicate that lack of function of CIC-2 may be involved in the pathogenesis of MLC (22). Lack of CIC-2 function has also been associated with a distinct form of leukoencephalopathy (26).

The mislocalization of GlialCAM found in Mlc1−/− mice contradicts previous results obtained using RNA interference of Mlc1 in rat astrocytes and localization studies of GLIALCAM around blood vessels in a sample from an MLC1 patient (13). Although RNA interference may not completely reproduce the gene knockout phenotype, we have previously shown that Mlc1 RNAi induces a significant decrease in Mlc1 protein levels (12,13). Thus, it is unclear if GlialCAM is unstable without MLC1 only in mice, or whether this is a general mechanism.

To solve this controversy, we decided to examine the relationship between Mlc1 and GlialCAM in other animal species. We generated a zebrafish model for MLC based on the lack of mlc1. As in mice, characterization of the zebrafish model revealed that loss of mlc1 impairs the correct localization of glialcam. Re-examination of the brain biopsy from a MLC1 patient that had been previously studied revealed that GlialCAM was mislocalized in Bergmann glia. These results confirm that the MLC1-dependent localization of GlialCAM is a general mechanism, as data from Mlc1−/− mouse had shown. As in the RNA interference experiments, cultured astrocytes from Mlc1−/− mice show normal GlialCAM localization, but slightly reduced VRAC activity. However, we observed that mimicking neuronal activity in Mlc1−/− astrocyte cultures causes GlialCAM mislocalization. Hence, data presented here explain previous discrepancies and reveal a conserved activity-regulated role of MLC1 in determining the localization of GLIALCAM.

RESULTS

GlialCAM is also mislocalized in Mlc1−/− astrocytes close to ependymal cells

In situ hybridization and immunohistochemistry studies have demonstrated that MLC1 and GLIALCAM co-localize in astrocytes close to brain barriers, Bergmann glia and ependymal cells, and that GLIALCAM is additionally expressed in oligodendrocytes (4,6,7,20). In Mlc1−/− mice, GlialCAM is mislocalized in astrocytes, Bergmann glia and oligodendrocytes (22). We examined whether GlialCAM was also mislocalized in ependymal cells by means of electron microscopy. To our surprise, gold particle-labelled antibodies showed that Mlc1 is not present in ependymal cells, but rather in astrocyte–astrocyte contacts below ependymal cells (Fig. 1A and C). Control experiments in Mlc1−/− mice confirmed the specificity of the signal (Fig. 1B and D). GlialCAM co-localized with Mlc1 in subependymal astrocyte–astrocyte contacts (Fig. 1E and G), and, as observed previously in astrocytes and oligodendrocytes, GlialCAM was also mislocalized in astrocytes near ependymal cells in Mlc1−/− mice (Fig. 1F and H). Thus, we conclude that the absence of Mlc1 in mice causes mistargeting of GlialCAM in all types of glial cells.
Identification of MLC-related genes in zebrafish

To address whether this Mlc1-dependent mislocalization of GlialCAM was a conserved feature, we decided to generate an Mlc1-deficient animal model in another species. By means of sequence database inspection, we found that genes encoding the orthologue of human MLC1 are present in all vertebrates, including zebrafish (Danio rerio), that has a single orthologue (Supplementary Material, Fig. S1A). In contrast, metazoan model organisms, such as Drosophila melanogaster and Caenorhabditis elegans, which lack myelin, do not possess MLC1 orthologues (3). For GLIALCAM, we observed that the teleost-specific genome duplication yielded two glialcam paralogues: glialcama and glialcamb (Supplementary Material, Fig. S2A).

Sequence comparison between zebrafish mlc1 and its human ortholog showed an overall 53% identity that was higher (63%) at the putative transmembrane domains and the terminal part of the C-terminus (Supplementary Material, Fig. S1B). Both glialcam paralogues showed a 76% identity with human

**Figure 1.** GlialCAM does not localize to astrocyte junctions in subependymal astrocytes in Mlc1<sup>−/−</sup> mice. (A–D) EM immunolabelling of ependymal cells shows localization of Mlc1 in astrocyte–astrocyte junctions (arrows) near ependymal cells (A and C). Dotted lines highlight boundaries between ependymal cells (ec) and inner astrocytic processes (a). The signal was specific as it was absent in Mlc1<sup>−/−</sup> mice (B and D). The insets in (A) and (B) are shown amplified in (C) and (D). Scale bars: (A, B): 2 μm; (C, D): 500 nm. (E–H) EM immunolabelling images of GlialCAM in astrocyte junctions below ependymal cells (arrows) (E, G). GlialCAM protein could not be observed in astrocyte–astrocyte junctions in Mlc1<sup>−/−</sup> mice (F, H). The insets in (E) and (F) are shown amplified in (G) and (H). Scale bars: (E and F): 2 μm; (G and H): 500 nm.
GLIALCAM across the two extracellular Ig-domains and the putative transmembrane domain, but the conservation of the C-terminal intracellular domain was very low, saved for a stretch of residues in the central part (Supplementary Material, Fig. S2B).

We cloned zebrafish mlc1 and both glialcama paralogues, fused to an intracellular FLAG tag, and expressed them transiently in HeLa cells. Importantly, glialcama could be predominantly detected in cell junctions (Fig. 2A), while glialcamb was found in a diffuse manner (Fig. 2B). Like its human counterpart (13), glialcama was able to target zebrafish mlc1 (Fig. 2C–E) and rat chloride channel CIC-2 (24) (Fig. 2F–H) to cell junctions. Furthermore, glialcama and not glialcamb was able to modify the functional properties of rat CIC-2-mediated currents in *Xenopus* oocytes. Glialcama, as with the human ortholog (24), not only increased CIC-2-mediated currents, but also changed rectification and activation properties (Fig. 2I–L). To additionally verify that glialcama behaves as human GLIALCAM, we analysed glialcama variants containing MLC-related mutations in conserved residues (Supplementary Material, Fig. S2B) that have previously shown trafficking defects (13). As happened in human GLIALCAM (4), we observed that the introduction of MLC-related mutations in glialcama negatively affected its junctional localization without substantially interfering with its expression levels (Supplementary Material, Fig. S3A–C).

These results suggest that zebrafish glialcama, and not glialcamb, could perform the same physiological functions as human GLIALCAM. Therefore, we decided to focus our studies on this zebrafish parologue.

**Generation of a mutant mlc1 zebrafish line**

We generated a zebrafish mlc1 knockout line by sequencing the first exons of the *mlc1* gene on the ENU-based TILLING library at the laboratory of Dr C. Moens at the FHCRC (Seattle, USA). Unfortunately, we did not detect any stop, frameshift or splice site mutations that would potentially abolish normal protein expression. However, we detected a missense mutation (c.241T>G) in one allele (mlc1*fh328*), resulting in the substitution of a conserved hydrophobic residue (isoleucine) for a charged one (arginine; p.811>R) on the predicted first transmembrane domain of the protein. We hypothesized that the introduction of a positive charge in the transmembrane domain could be deleterious for its protein expression. In order to test this, we introduced the equivalent mutation (V64R, Supplementary Material) in human MLC1 carrying HA epitope tags (one at the N-terminus and the other extracellular) and assayed surface expression by an ELISA-based luminescence assay in *Xenopus* oocytes (Fig. 3A). We used oocytes because their incubation temperature (18°C) would reveal whether the mutation causes folding defects (27). No surface expression could be detected, confirming our hypothesis that the mutation was deleterious.

Therefore, the heterozygous founder zebrafish were pair-wise mated and out-crossed for several generations to eliminate any other background mutations from the original ENU mutagenesis (see Materials and Methods). As the mutation results in a novel *Dde1* restriction site, it was possible to perform RFLP genotyping, and gene sequencing confirmed the presence of the mutation (Fig. 3B and C). As our previous antibodies against human or mouse Mlc1 proteins did not recognize the zebrafish orthologue (data not shown), we developed a new rabbit polyclonal antibody that was able to detect zebrafish mlc1 (Fig. 3D and E). We assayed mlc1 expression in brain extracts from wild-type, heterozygous and homozygous mlc1*fh328* adult fish siblings. No mlc1 protein expression could be detected in homozygotes, validating the allele mlc1*fh328* as an mlc1 knockout line, which we will refer to as mlc1<sup>−/−</sup> zebrafish from now on (Fig. 3F). As with the *Mlc1<sup>−/−</sup>* mouse (22), the homozygous mlc1<sup>−/−</sup> zebrafish turned out to be viable and fertile, with the expected mendelian ratio among adult descendants.

**Expression pattern of mlc1 in the adult zebrafish brain and retina**

We determined the expression pattern of *mlc1* in adult tissues by RT-qPCR (Fig. 4A) and western blot (Fig. 4B). This showed that *mlc1* is mainly expressed in the brain and at reduced levels in the eye. We performed immunofluorescence experiments to determine which cell types express mlc1 in the adult brain, using the mlc1<sup>−/−</sup> zebrafish as a control. Prominent immunoreactivity was observed at the ventricular and pial surface of the brain, and around some blood vessels (Fig. 4C, D, F). Additionally, mlc1 immunoreactivity was observed in radial processes within the brain parenchyma (Fig. 4D). Lack of staining in the mlc1<sup>−/−</sup> zebrafish confirmed the specificity of our results (Fig. 4E). Staining of mlc1 in the transgenic line *flila:GFPl*, which expresses GFP in the vascular endothelium (28), confirmed that the mlc1 protein is present at astrocytic perivascular processes (Fig. 4F). mlc1 staining around brain ventricles and radial-like structures was also positive for glial fibrillary acid protein (gfap) antibody (Fig. 4G and H), indicating that these are astroglial-like cells (29). Thus, expression of mlc1 in the zebrafish brain is very similar to that found in humans.

We also analysed the localization of mlc1 in the retina. High expression of mlc1 was detected at the inner limiting membrane of the retina. Specificity of the antibody was confirmed by lack of staining in *mlc1<sup>−/−</sup>* zebrafish retina (Fig. 4I and J). Double immunostaining with gfap demonstrated that mlc1 was strongly expressed in Müller glia end-feet in the inner limiting membrane (Fig. 4K). Furthermore, we also observed weak mlc1 and gfap co-expression at fine radial glial processes away from the vitreal surface of the retina (Fig. 4K).

**Glialcama localization in the adult brain and retina is affected in mlc1<sup>−/−</sup> zebrafish**

As with *mlc1*, we determined the pattern of expression of glialcama using RT-qPCR in adult tissues. mRNA for glialcama was more broadly expressed compared with mlc1, but showed the highest expression levels in the brain. Detection of glialcama protein in tissue extracts by western blot using a newly developed glialcama antibody (Supplementary Material, Fig. S3D) confirmed its higher expression in the brain (Fig. 5B), and its presence in the eye.

We next addressed the localization of glialcama in brain and retina from wild-type and mlc1<sup>−/−</sup> zebrafish (Fig. 5C and D). Similar to mlc1, we detected strong immunoreactivity to glialcama in radial glial cell bodies and their processes (Fig. 5C and D), which were also positive for gfap (Fig. 5E). In contrast,
Figure 2. Characterization of the zebrafish orthologues of Mlc1 and Glialcam. (A and B) glialcama and glialcamb were transfected separately in HeLa cells and detected through the FLAG epitope with immunofluorescence. While glialcama is specifically located in cell–cell junctions, glialcamb protein presented a diffuse location. Scale bar: 10 μm. (C–E) Co-transfection of glialcama and mlc1 in HeLa cells reveals their co-localization at cell junctions. Scale bar: 10 μm. (F–H) Co-transfection of glialcama and rat CIC-2 in HeLa cells reveals their co-localization at cell junctions. Scale bar: 10 μm. (I–L) A typical current trace mediated by rat CIC2 (I) expressed in oocytes and after co-expression with glialcama (J) or glialcamb (K). Only glialcama is able to modify the functional properties of CIC-2. The apparent inactivation observed sometimes at very negative voltages is an artefact caused by chloride depletion inside the oocytes (J). (L) Average steady-state current–voltage relationship of CIC-2 (black circles) and CIC-2 co-expressed with glialcama (white circles) or with glialcamb (triangles). Glialcama increased CIC-2-mediated conductance and changed its rectification properties.
a more diffuse glialcama staining was observed in the same brain regions in mlc1 \(^{-/-}\) zebrafish (Fig. 5F). We addressed if lack of detection was due to reduced protein expression by western blot using brain extracts, but no changes in protein expression levels were observed (Fig.5G). We also studied glialcama localization in the retina, as its laminar arrangement could allow easier visualization of subcellular mislocalization. As observed for mlc1, glialcama was highly expressed at Müller glia end-feet at the inner limiting membrane (Fig. 5H and I). This expression was absent in mlc1 \(^{-/-}\) zebrafish. Thus, as we observed in the brain, glialcama was mislocalized in the retina of mlc1 \(^{-/-}\) zebrafish (Fig. 5J).

We can conclude that lack of mlc1 in zebrafish causes glialcama mislocalization in glial cells in the brain and retina, in a similar manner to that found in Mlc1 \(^{-/-}\) mice (22).

Comparison of mouse and zebrafish Mlc1 knockout phenotypes

The phenotypes of Mlc1 mouse and zebrafish knockouts were compared. Histopathology of brain sections from Mlc1 \(^{-/-}\) mice revealed largely restricted myelin vacuolization in fibre tracts of the cerebellum (22). In agreement with these histology results, \(T_2\)-weighted MRI of the brain of Mlc1 \(^{-/-}\) mice showed lesions in white matter tracts of the cerebellum from about 6 months of age that increased with age (Fig. 6A and B). Measurement of brain volume revealed that the whole brain is bigger in Mlc1 \(^{-/-}\) than in wild-type mice at the same age (Fig. 6G).

In zebrafish, we analysed the brain of four adult (14 months old) mlc1 \(^{-/-}\) zebrafish by MRI. We only observed very minor lesions in the telencephalon and mesencephalon (Fig. 6C–F) which showed similar relaxation times as cerebrospinal fluid, thus suggesting that they were due to increased fluid. As in mice, measurements of different brain areas revealed that the telencephalon seems larger in comparison with the whole brain in mutant than in wild-type zebrafish (Fig. 6H).

Thus, both animal models showed two typical MLC features: megalencephaly and increased fluid accumulation. However, we failed to observe the presence of vacuoles in any region of the zebrafish brain by general histological procedures (eight different animals; Supplementary Material, Fig. S4A and B) or electron transmission microscopy (four different animals; Supplementary Material, Fig. S4E and F). We conclude that the mlc1 \(^{-/-}\) zebrafish develops less severe lesions than the Mlc1 \(^{-/-}\) mice in the brain, which in turn also develops a less severe phenotype than humans.

As glialcama and mlc1 are co-expressed in Müller cells in the retina, we also studied and compared retina morphology between mice and zebrafish. As in Mlc1 \(^{-/-}\) mice (22), the retina of mlc1 \(^{-/-}\) zebrafish did not show lesions after histological staining (Supplementary Material, Fig. S4C and D). We also
evaluated the visual function by recording electro-retinograms (ERG) in Mlc1−/− mice and mlc1−/− zebrafish (Supplementary Material, Fig. S5). No significant differences of the means of b-wave amplitude between wild-type and mlc1−/− fish at any light condition were observed (Supplementary Material, Fig. S5A). The relation of normalized b-wave amplitude and the light intensity (inset in Supplementary Material, Fig. S5A) showed complete overlaps between the two groups, suggesting that the sensitivity in mlc1−/− fish does not differ from wild-types. Similar observations have been found in 2-month-old adults (Supplementary Material, Fig. S5B). Similar tests were performed on 1-year-old adult Mlc1−/− mice. Analysis of the

Figure 4. mlc1 expression and localization pattern in adult zebrafish. (A) qPCR with RNA from adult tissues shows the expression of mlc1 almost exclusively in the brain, and at a lesser level in the eye. (B) Western blot with the custom mlc1 antibody shows its expression on the brain and eye. As controls, we used extracts from mlc1-transfected and untransfected HeLa cells. (C–F) Immunofluorescence detection of mlc1 in the zebrafish adult brain. (C) mlc1 is expressed in radial glia processes (arrow) and pial end-feet (arrowheads). (D) mlc1 expression surrounds the ventricle of the brain (open arrow). (E) No immunoreactivity is observed in mlc1−/−. Arrowheads point to the pial surface of the brain. Scale bars: 20 μm. (F) Localization of mlc1 (red) in glial perivascular processes (arrowheads) around blood vessels (green) in the brain of fli1a::GFP transgenic zebrafish. Scale bar: 10 μm. (G and H) mlc1 co-localizes with the astrocytic protein gfap in the zebrafish adult brain at the pial (arrowhead in G) and ventricular surfaces of the brain (open arrow in H). Asterisk labels the ventricle. Scale bars: 15 μm. Inset: gfap and mlc1 co-expression at the cell body and radial processes of an astrocyte located in a subependymal zone. Scale bar: 15 μm. (I–K) Localization of mlc1 on the retina. (I) mlc1 is expressed in the inner limiting membrane (arrows) of the retina. (J) No immunoreactivity against mlc1 is observed in mlc1−/− zebrafish retina. (K) Double immunofluorescence with gfap (red) confirms mlc1 expression (green) in radial glia processes and Müllner glia end-feet (arrow) at inner limiting membrane. Scale bars: 100 μm.
amplitudes of the different components of the ERG showed no significant differences between wild-type and \( Mlc1^{-/-} \) mice (Supplementary Material, Fig. S5C).

**Human GLIALCAM is also mislocalized in the Bergmann glia of a MLC1 patient**

Our studies with \( Mlc1^{-/-} \) and \( mlc1^{-/-} \) zebrafish have revealed mislocalization of GLIALCAM in astrocytes and oligodendrocytes. However, previous studies from a biopsy of a MLC patient indicated that GLIALCAM was not mislocalized around blood vessels in the cortex (13).

We re-examined the localization of GLIALCAM in the cerebellum of this same human brain biopsy. We focused on the cerebellum, as it is more severely affected in \( Mlc1^{-/-} \) mice (22). As previously shown (13), we could not detect MLC1 in Bergmann glia from the MLC patient (Fig. 7A–F). In addition, staining with GLIALCAM antibodies revealed that it was in fact mislocalized in Bergmann glia in the MLC patient (Fig. 7G–L). Control staining with GFAP revealed that these changes were not due to defects in the integrity of the tissue. Thus, we can conclude that the mislocalization of GLIALCAM caused by the absence of MLC1 is a conserved feature from fish to humans, even though the phenotype is more severe as we climb the evolutionary ladder.

**VRAC activity in cultured astrocytes from \( Mlc1^{-/-} \) mice is still present**

We cultured astrocytes from \( Mlc1^{-/-} \) mice and analysed GLIALCAM localization. Unexpectedly, lack of Mlc1 did not change GLIALCAM localization (Fig. 8A–D) and expression (Fig. 8E) in \( Mlc1^{-/-} \) astrocyte cultures. This result agreed with previous experiments that showed that knocking down Mlc1 in rat astrocytes changed neither the expression nor the localization of GLIALCAM in cell junctions (13).

As MLC1 have been related with the activity of VRAC (14, 15), we analysed chloride currents in \( Mlc1^{-/-} \) astrocyte cultures. In wild-type and \( Mlc1^{-/-} \) astrocytes, replacing intra-and extracellular Na\(^+\) and K\(^+\) ions with Cs\(^+\) (to block sodium and potassium currents) and with symmetric Cl\(^-\) (Fig. 8F), we showed instantaneous activation and time-dependent inactivation at more positive potentials (Fig. 8F and Supplementary Material, Fig. S6A–D). These data indicated that hypotonicity-activated VRAC channels in wild-type and \( Mlc1^{-/-} \) mouse astrocytes which are functionally identical to those described in rat astrocytes (31, 32). The observation that the putative CIC-2 inhibitor cadmium (33) did not affect the hypotonicity-activated currents (Supplementary Material, Fig. S6E and F) strongly indicated that these Cl\(^-\) currents were not mediated by CIC-2, which is also present in mouse astrocytes (34). Comparison of VRAC amplitudes between wild-type and \( Mlc1^{-/-} \) astrocytes indicated that VRAC was slightly reduced in astrocytes obtained from \( Mlc1^{-/-} \) mouse (Fig. 8G). These results support the view that Mlc1 does not mediate directly...
Figure 6. MRI of $Mlc1^{+/+}$ mice and $mlc1^{+/+}$ zebrafish. (A and B) MRI of mice from different ages show progressive presence of white matter lesions (arrows) in the cerebellum of $Mlc1^{+/+}$ animals (B), but not in wild-type mice (A). (C–F) MRI of adult zebrafish reveals the presence of small lesions in the mesencephalon (thick arrow in D) and telencephalon (arrows in F) in $mlc1^{+/+}$ zebrafish. Hyperintense signals in (D) and (F) may correspond to fat containing tissue in or around the brain. (C) and (E) correspond to images from wild-type zebrafish. Scale bars: 1 mm in (C) and (D), 500 μm in (E) and (F). (G) Brain volume is higher in $Mlc1^{+/+}$ mice. Brain volume was estimated by quantifying the area of consecutive MRI images using ImageJ. Data correspond to seven wild-type and seven $Mlc1^{+/+}$ mice ($P < 0.0001$). (H) Telencephalon appears bigger relative to the whole brain in $mlc1^{+/+}$ zebrafish ($P < 0.05$).
VRAC current. To detect whether the non-statistically significant VRAC reduction may lead to astrocyte vacuolation, we stained wild-type (Fig. 8H) and $Mlc1^{2/2}$ astrocytes (Fig. 8I) with the freely diffusible fluorescent reagent calcein. Vacuoles were more frequently observed in $Mlc1^{2/2}$ astrocytes (Fig. 8J).

Glialcam localization is dependent on Mlc1 with high extracellular potassium concentration

Why is GlialCAM mislocalized in $Mlc1^{2/2}$ astrocytes in tissue and not in culture? Mislocalization of GlialCAM in humans was better observed in Bergmann glia, which are bathed in a high extracellular potassium concentration due to cerebellar neuronal activity (35). Thus, we treated cultured astrocytes obtained from wild-type and $Mlc1^{−/−}$ mice with physiological medium or with an isosmotic solution with higher potassium concentration, mimicking neuronal activity. In wild-type astrocytes under high potassium concentration, there was no change on the localization of Mlc1 (Fig. 9A–D), but there was a slight increase of GlialCAM in cell junctions. When similar experiments were repeated in astrocytes from $Mlc1^{−/−}$, GlialCAM was detected intracellularly after high-potassium treatment (Fig. 9E–H). This effect was even more evident when astrocytes were infected with adenoviruses expressing human Flag-tagged GLIALCAM (Fig. 9I–L). Control staining with the junctional marker $β$-catenin revealed that these distribution changes caused by increasing extracellular potassium were specific to GlialCAM (Fig. 9M–P).
Figure 8. Cultured Mlc1−/− astrocytes do not show mislocalization of Glialcam and still present a VRAC response. (A and B) Immunofluorescence detection of Mlc1 in cultured astrocytes from newborn pups confirm the absence of the Mlc1 protein in Mlc1−/− mice. (C and D) Immunofluorescence detection of Glialcam in cultured astrocytes from newborn pups show that Glialcam protein is present in cell junctions in Mlc1−/− mice, unlike it is seen in vivo. Scale bar: 10 μm. (E) Western blot of brain extracts show no difference in Glialcam protein levels between wild-type and Mlc1−/− mice. (F and G) Depression of the hypotonicity-evoked chloride current with the Volume Regulated Anion Channel (VRAC) inhibitor DCPIB in wild-type (WT) and Mlc1−/− mouse cultured astrocytes. (F) Bar graph of membrane current densities (mean ± SEM) at +80 mV (upper bars) and −120 mV (lower bars) measured in isotonic (Iso CsCl) and hypotonic conditions (Hypo CsCl) and after addition of DCPIB (10 μM) to the hypotonic solution (Hypo CsCl + DCPIB) in wild-type (n = 9) and Mlc1−/− (n = 17) astrocytes. Statistical significance was calculated using paired two-tailed Student’s t-test; *P < 0.05, **P < 0.01, ***P < 0.001. (G) Bar graph of DCPIB-sensitive currents (mean ± SEM) induced by hypotonic challenge at −120 and +80 mV in wild-type (n = 7) and Mlc1−/− (n = 7) mouse astrocytes. Differences in current densities were not significant (ns). (H and I) Typical pictures of astrocytes obtained from wild-type (H) and Mlc1−/− (I) mice. Arrowheads point to vacuoles observed mostly in Mlc1−/− astrocytes. (J) Quantification of the number of cells containing vacuoles. A cell was positive for vacuolation if have at least three vacuoles of a size bigger than 0.5 μm. Scale bar: 10 μm.
DISCUSSION

We have analysed two animal models (zebrafish, mice) lacking MLC1 protein and compared them with a brain biopsy from an MLC patient. In all cases, localization of GlialCAM at cell junctions is abolished by lack of MLC1. In cultured mouse astrocytes from Mlc1−/−, this effect is only observed when mimicking neuronal activity.

Despite the fact that Mlc1 is one of the most astrocyte-specific genes (36,37) and its mutation leads to MLC (3), its precise function is still unknown. As MLC1 is a membrane protein with low homology to ion channels, a possible role as a channel or transporter has been suggested (6). However, no such function has been found so far. RNA interference experiments of Mlc1 in cultured astrocytes indicated that it may be a component or regulator of VRAC (14,15). Here, we demonstrate that VRAC activity is
still present in the absence of Mlc1. We conclude that MLC1 may affect VRAC activity in an indirect manner and is not a molecular component of VRAC. In agreement with this hypothesis, very recently it has been demonstrated that VRAC activity is mediated by LRRRC8 heteromers (38,39). Further work will be needed to define the biochemical relationship between MLC1/ GlialCAM and LRRRC8 proteins.

Further insight into MLC1 biology came from the finding that another MLC-causing gene, which encodes the cell adhesion molecule GLIALCAM (4), is necessary for stabilizing and targeting MLC1 to astrocyte junctions (4,15). In contrast, GLIALCAM targeting in vitro was independent of MLC1 (19,13). However, recent work performed with Mlc1−/− and GlialCAM−/− mice demonstrated that both mutants reproduce MLC symptoms and that Mlc1 is necessary for GlialCAM localization (22).

We decided to expand the repertoire of MLC animal models to solve this contradiction. We have characterized the orthologues of MLC1 and GLIALCAM in zebrafish. The zebrafish (and other teleost fishes) genome contains two glialcam paralogues of which only glialcama behaves as mammalian Glialcam in terms of subcellular localization and modulation of the chloride channel CIC-2 (24). Both mlc1 and glialcama show a similar expression pattern in the brain as murine Mlc1 and Glialcam (4). Mlc1 and glialcama are localized to glial cells especially around brain barriers, radial glia processes and end-feet, and also in Müller glia of the retina.

We have generated a zebrafish line carrying a mutation in mcl1 that abrogates its protein expression. However, the conservation of mcl1 in zebrafish does not translate into a severe MLC-like phenotype. We could only detect minor lesions and megalencephaly in the brain of mcl1−/− zebrafish by MRI, but not myelin vacuolization even in fish older than 1 year. Although in humans MLC is evident during the first year of life (1), mouse knockout models of both Glialcam and Mlc1 did not present vacuolization during their firsts months of life (22). This difference could reflect their heterochrony of myelination, and that MLC manifests when myelination is at its highest level (1). Zebrafish show regular compact myelin (40), but protein composition differs: P0 is the major component of CNS myelin, rather than PLP, like in mammals (41). In the transition from an aquatic to a terrestrial environment, there was a switch from P0 to PLP as the major CNS myelin protein, which provided more neuroprotection (42). Myelination in zebrafish larvae starts at 3 days post-fertilization (dpf), and myelin basic protein expression increases at least till 29 dpf (43), but new myelin deposition has been reported in trout even older than 1 year (44). Another significant difference with mammalian brains is the capacity of the adult zebrafish brain for neurogenesis (45) and even repair after injury (46). This is linked to the capacity for re-myelination (47,48). Therefore, it is possible to speculate that zebrafish are able to repair myelin sheath damage produced by the lack of mlc1 protein, so vacuolization never reaches a detectable stage by means of histological methods.

Absence of mlc1 in zebrafish brain leads to the misslocalization of glialcama, as we observed in Mlc1−/− mice. This prompted us to re-examine a human MLC biopsy. We found similar results in the cerebellum of the MLC patient. Thus, the reciprocal relationship between MLC1 and GlialCAM for their correct membrane localization has been conserved throughout evolution. This relationship also explains the fact that mutations in either MLC1 or GLIALCAM cause a similar phenotype in MLC1 and MLC2A patients (21).

It has been well described that MLC1 depends on GLIALCAM for exiting the endoplasmatic reticulum and reaching its junctional localization (15). However, a question remains: why does GLIALCAM mislocalize in the absence of MLC1 in vivo? We believe that MLC1 is not working as a chaperone of GLIALCAM, as GLIALCAM in vitro is able to traffic to cell junctions in the absence of MLC1. In agreement with this hypothesis, GlialCAM mislocalization in Mlc1−/− astrocytes in culture only appeared when mimicking neuronal activity by an increase in extracellular potassium. Thus, differences between in vivo and in vitro effects of MLC1 on GlialCAM localization may be due to the presence of interacting molecules in vivo that are not present in culture.

We also suggest that GlialCAM/MLC1 may have a role in the process of potassium siphoning by glial cells. As a result of neuronal electrical activity, potassium is released in order to repolarize the membrane potential, and this excess of potassium needs to be disposed of in a process known as ‘potassium siphoning’ (49). Glial cells surrounding neurons take up potassium by Kir4.1 channels and then transfer it through Connexin junctions to the astrocytic end-feet surrounding blood vessels, where it is eliminated. Ablation of Kir4.1 (50) or Connexins 32 and 47 (51) results in leukodystrophy characterized by water accumulation in myelin. Work with Mlc1−/−, GlialCAM−/− and Clcn2−/− mice models (22,60) and the identification of mutations in CLCN2 in human patients with leukodystrophy (26) have suggested that the chloride channel CIC-2 may also have a role in potassium-coupled chloride influx. In this sense, GlialCAM changes the rectification of the CIC-2 channel (24), allowing chloride influx at depolarized voltages. Interestingly, new studies with CIC-2 from C. elegans have also shown that the primary role of CIC-2 is to mediate chloride influx but not efflux (52). Unfortunately, the precise role of MLC1 in this process is still not known. However, based on these studies, we can speculate that it may have a regulatory role controlling the surface expression of GLIALCAM, which may be needed to regulate in turn the activity of CIC-2. Possibly, the severity of the phenotype caused by the depletion of Mlc1 from fish to mouse and human could be explained based on differences in neuronal activity between species, making Mlc1 or Glialcam more necessary to keep ionic homeostasis in the possibly more active human CNS.

In summary, our work show that the functional relationship between the two proteins causing MLC in vertebrates is evolutionary conserved. We have also increased the diversity of vertebrate animal models for this disease that can offer further insights into the molecular interactions between these two proteins. Our work also explains the similarity of phenotypes caused by mutations in either gene. More importantly, we uncovered a role for MLC1 in GlialCAM localization at astrocyte junctions in the presence of neuronal activity, offering new mechanistic insights into the pathophysiology of MLC.

MATERIALS AND METHODS

Zebrafish maintenance
AB or AB/TL strains were used in all the experiments. Adult fish were maintained in the animal facility at the University of
Barcelona, School of Medicine in Bellvitge Campus, under the standard culture conditions: 28°C, 14 h/10 h light/dark period (53). All experimental procedures conformed to the European Community Guidelines on Animal Care and Experimentation and were approved by animal care and use committees.

The mlc1fh328 allele was obtained from the laboratory of Dr Cecilia Moens, as a result of an ENU-based TILLING screening (62). The mutant line was generated in an islet1::GFP background, which was eliminated by out-crossing to AB wild-type fish. Similarly, two other embryonic recessive lethal phenotypes that were not linked to mlc1fh328 were eliminated by pair-wise mating and out-crossing. The mlc1fh328 mutation is a T/G transversion in mlc1 exon 3 that generates a novel Ddel restriction site (CTNAG), which was used for genotyping after amplifying the corresponding fragment of genomic DNA by PCR. The following oligonucleotides were used: TAAATGTGTCTATCTG TATCTGACC (underlined is a mutation introduced to eliminate an endogenous Ddel restriction site), 16 nucleotides upstream from the Ddel restriction site generated by the mutation) and TACTCTGCAAGCCAAAAGCA. Genomic DNA was obtained from tail clipping and extraction by Nucleo Spin Tissue kit (Macherey-Nagel).

Mlc1 knockout mice
Mlc1−/− mice were generated by ICS (Institut Clinique de la Souris, Strasbourg, France) as described previously (22).

Human samples
The MLC patient sample analysed here was already reported (13). Briefly, she carried a homozygous mutation in the MLC1 gene, resulting in an amino acid change (p.S69L) that leads to the reduced stability of the MLC1 protein and its practical absence.

Molecular biology
Plasmids used were constructed using standard molecular biology techniques employing recombinant PCR and the Multi-site Gateway system (Invitrogen). The integrity of all cloned constructs was confirmed by DNA sequencing. For localization studies in HeLa cells, proteins were C-terminally fused to the FLAG epitope or HA-tagged and cloned into the pcDNA3 vector (Invitrogen).

RT-PCR
Adult zebrafish were euthanized using an overdose of tricaine (MS222, Sigma), and tissues were quickly dissected and flash-frozen in liquid nitrogen. Total RNA was isolated with TRIZOL (Life Technologies) and retro-transcribed (RT-PCR) using random hexamers with SuperScript III system (Life Technologies). The following oligonucleotides pairs were used for qPCR: GCACGTTCAGTGGACAACGT and CACAACTATTGGCCTTCAG (mlc1), CCCACCACAAAGACTAAGC and CATCCTCAGTGGCTCTACTT (glialcama), and as internal controls (54): CTGGAGGCACGCCTAAACAT and ATCAAGAAGATGATCCGCTACCTATTAC (efla), TCT GGAGACGTGAAGAGTATGC and AGACGCACAATC TTGAGAGCAG (rpl13a). qPCR was performed with SYBR Select reagent (Life Technologies) in a StepOne apparatus (Life Technologies). The expression levels were calculated using the comparative C t method normalized to the internal control genes. The final results were expressed as the relative RNA levels as indicated in the corresponding figures, taking into account the efficiency of each primer pair with the Pfaffl method (55).

Generation of custom antibodies against zebrafish proteins
Immune sera against synthetic peptides from zebrafish mlc1 (QREEASQEVFSYAOQMST, corresponding to amino acids 2–19, in green in Supplementary Material, Fig. S1B) and zebrafish glialcama (EDPPTDGENTMHHSLPR, corresponding to amino acids 405–422, in green in Supplementary Material, Fig. S2B) were raised in rabbits using the services provided by Eurogentec. The peptide was coupled to keyhole limpet haemocyanin via a cysteine residue that has been added to the N-terminal end of the peptide for mlc1 and to the C-terminal of the peptide for glialcama. After four boosts of immunization, the antisera were affinity purified using the peptide covalently coupled to Sulpholink (Pierce). The polyclonal antibodies were tested by immunoblotting and immunofluorescence on HeLa cells expressing mlc1 or glialcama and on zebrafish brain tissue.

Cell culture and immunofluorescence microscopy
HeLa cells were grown on DMEM containing 10% (v/v) fetal bovine serum (Sigma) and 1% penicillin/streptomycin at 37°C in a humidity controlled incubator with 10% CO2. Cells were cultured and transfected with the Transfectin reagent (Biorad). Twenty-four hours after transfection, the cells were splitted and transferred into glass-covered petri dishes in which experiments were performed after further 24–48 h. To detect expressed proteins, immunofluorescence staining of cells was performed. They were fixed with phosphate-buffered saline (PBS) containing 3% paraformaldehyde for 15 min, blocked and permeabilized with 10% FBS and 0.1% Triton X-100 in PBS for 2 h at room temperature (RT). Primary antibodies were diluted in the same solution and incubated overnight at 4°C. Cells were washed and incubated for 2 h at RT with secondary antibodies. Cover slips were mounted in Vectashield medium (Vector Laboratories) with 1.5 μg/ml DAPI (Sigma) and visualized using an Olympus DSU spinning disk confocal.

Images were analysed using ImageJ. Pairs of cells were analysed manually and quantified as being in junctions or not being in junctions (around the plasma membrane or intracellular). Intensity profile analysis was used to discern the localization.

Histological staining methods
For immunofluorescence in zebrafish, individuals were deeply anaesthetized in 0.1% tricaine (MS222, Sigma) in fresh water, and then transcardially perfused with 4% paraformaldehyde. Fish heads were post-fixed for 24 h at RT. Then brains and eyes were dissected out, cryoprotected and frozen with liquid
sections were rinsed in PBS and pre-incubated with normal goat serum (Sigma, 1:100) for 1 h. Next, they were incubated with a primary antibody or a cocktail of primary antibodies overnight at room temperature. Antibody dilutions used were: rabbit anti-zebrafish mlc1, 1:100; rabbit anti-zebrafish glialcam, 1:100; mouse anti-gfap (ZIRC), 1:500; rat anti-GFP (Nacalai Tesque), 1:1000. After PBS washes, sections were incubated with secondary fluorescent antibodies (Alexa Fluor 488 or 568, 1:500, Invitrogen) for 1 h. After additional PBS washes, sections were mounted using the glycerol-based mounting medium.

For haematoxylin–eosin staining, we used paraffin sections from tissue fixed in Bouin’s fixative. Sections were de-waxed and stained using Meyer’s haematoxylin followed by eosin. Sections were dehydrated and mounted for imaging.

Zebrafish sections were observed and photographed either under a laser scanning confocal microscope (Nikon A1R) or a bright-field microscope (Nikon E100). Images were adjusted for brightness and contrast with Adobe Photoshop or Corel Photopaint, and they were assembled with Adobe Illustrator.

Human samples were processed and analysed as described previously (13).

Electron microscopy
For electron immunogold experiments, small samples of brain tissue were obtained and fixed in 4% paraformaldehyde and 0.1% glutaraldehyde in 0.12 M phosphate buffer, and processed. They were cryoprotected gradually in sucrose and cryosectioned. Mouse astrocyte cultures were performed as previously described from P0 to P2 mouse pups of the corresponding genotype (16). Calcein stainings were performed by treating the astrocytes with Calcein-AM 2 μM (Life Technologies) for 10 min and then with physiological solution (122 mM NaCl, 3.3 mM KCl, 0.4 mM MgSO4, 1.3 mM CaCl2, 1.2 mM KH2PO4, 25 mM Hepes, 10 mM glucose, pH 7.2, 300 mOsm/kg). In some experiments, astrocytes were treated for 6 h with physiological solution or high potassium medium (60 mM NaCl, 60 mM KCl, 0.4 mM MgSO4, 1.3 mM CaCl2, 1.2 mM KH2PO4, 25 mM Hepes, 10 mM glucose, pH 7.2, 300 mOsm/kg), to mimic neuronal activity. Then, they were fixed in 4% PFA and processed for immunofluorescence as described above.

Mouse magnetic resonance imaging
Mouse MRI experiments were conducted on a 7.0T BioSpec 70/30 horizontal animal scanner (Bruker BioSpin), equipped with a 12 cm inner diameter actively shielded gradient system (400 mT/m) in at least seven animals of the same age. Receiver coil was a phased array surface coil for mouse brain. Animals were placed in a supine position in a Plexiglas holder with a nose cone for administering anaesthetic gases (isoflurane in a mixture of 30% O2 and 70% CO2), fixed using a tooth bar, ear bars and adhesive tape. Tripilot scans were used for accurate positioning of the animal’s head in the isocentre of the magnet.

High-resolution T2-weighted images were acquired by using TurboRARE (rapid acquisition with rapid enhancement) sequence applying repetition time of 2969 ms, echo time of 36 ms, RARE factor = 8, 10 averages, slice thickness = 0.3 mm, number of slices = 25 for horizontal view, 25 coronal slices, field of view = 25 × 25 mm, matrix size = 240 × 240 pixels, resulting in a spatial resolution of 0.104 × 0.104 mm in 0.3 mm slice thickness.

T2 relaxometry maps were acquired with multislice-multiecho (MSME) sequence by applying 16 different TE5s, from 11 to 176 ms, TR = 4764 ms, slice thickness = 0.5 mm, number of coronal slices = 18, FOV = 20 × 20 mm and matrix size = 256 × 256 pixels, resulting in a spatial resolution of 0.078 × 0.078 mm in 0.50 mm slice thickness.

Zebrafish magnetic resonance imaging
Magnetic resonance microimaging (μMRI) of zebrafish was performed on a vertical wide-bore 9.4T Bruker Avance 400WB spectrometer, with a 1000 mT/m actively shielded imaging gradient insert (Bruker Biospin GmbH). A birdcage RF coil with an inner diameter 1 cm was used for excitation and detection. The system was interfaced to a Linux PC running Topspin 2.0 and Paravision 5.0 software (Bruker Biospin GmbH). For μMRI, adult zebrafish were euthanized and fixed in 4% buffered paraformaldehyde (Zinc Formal-Fixx, ThermoShandon) for 3 days and subsequently embedded in Fomblin. Before each μMRI measurement, the magnetic field homogeneity was optimized by shimming. Each session of measurements began with a multislice orthogonal gradient-echo sequence for position determination and selection of the desired region for subsequent experiments. High-resolution T2-weighted imaging was acquired by using TurboRARE (rapid acquisition with rapid enhancement) sequences with echo time (TE) = 10.5 ms; repetition time (TR) = 5000 ms; RARE factor = 4; slice thickness 0.2 mm; number of slices 15; field...
of view 12 × 12 mm; image matrix of 256 × 256 pixels, resulting in an in-plane resolution of 47 μm.

T₂ relaxation time measurement in zebrafish brain was performed with MSME sequence by applying eight different TEs (8.5, 17.0, 25.5, 34.0, 42.5, 51.0, 59.5 and 68.0 ms); TR = 1.5 s; slice thickness 0.5 mm; number of slices 6, FOV 12 × 12 mm² and matrix size 256 × 256 pixels, resulting in an in-plane resolution of 47 μm. For calculation of T₂ relaxation time, regions of interest (ROIs) were drawn at various locations within the zebrafish brain. Another ROI in the muscle was used as an internal control. Means and standard deviation for T₂ relaxation times for each ROI were calculated.

Two-electrode voltage clamp, western and surface expression in Xenopus oocytes

*Xenopus* oocytes were injected and maintained as described (57). For rat CIC-2, 10 ng cRNA were injected. When co-expressing, 5 ng cRNA of *glialcama* or *glialcamb* were coinjected with rat CIC-2. Oocytes were perfused with ND96 (in mM): 96 NaCl, 2 KCl, 1.8 CaCl₂, 1.8 MgCl₂ and 5 HEPES/TRIS (pH 7.4). To estimate the specific ClC-2-mediated chloride currents, iodide (80 mM NaI replacing the NaCl), which blocks ClC-2-mediated outward currents (58), was applied in every experiment. Oocytes that did not exhibit a significant block were discarded. Currents were measured using a TEC-05X voltage amplifier and the CellWorks program (npi).

Western blot experiments and surface expression in oocytes were performed similarly as previously described (6).

Zebrafish electroretinogram recordings

ERGs were performed at 1-month and 2-month-old young adults (at least five animals). The young adults were anaesthetized by fish system water containing 0.75 mM 3-aminobenzoic acid methyl ester (MESAB, Sigma-Aldrich) and 4.6 mM NaHCO₃ before recording. All the experiments were performed at room temperature (20°C).

Fish were dark adapted for at least 30 min and then were placed on the filter paper in the middle of a plastic recording chamber which was filled by 1% agarose. The reference electrode was inserted into the agarose. After the fish was moistened by a drop of Ringer’s solution (111 mM NaCl, 2.5 mM KCl, 1 mM CaCl₂, 1.6 mM MgCl₂, 10 μM EDTA, 10 mM glucose and 3 mM HEPES buffer, adjusted to pH 7.7–7.8 with NaOH), the eye was removed by a loop made of tungsten wire. The loop was placed behind the eye and was pressed down to cut the optic nerve. Meanwhile forceps were used to pull the body and discard it. The isolated eye was then positioned to face the light source (ZEISS XBO 75 W). The recording electrode, filled with Ringer’s solution, was placed against the centre of the cornea. All these pre-recording steps were done under dim red illumination. Stimulation light intensity was 6000 lux and attenuated by neutral density filters. Stimulus duration was 100 ms and delivered 50 ms after the recording began. Electronic signals were amplified 1000 times by a pre-amplifier (P55 A.C. Preamplifier, Astro-Med Inc, Grass Technology) with a band pass between 0.1 and 100 Hz, digitized by DAQ Board NI PCI-6035E (National Instruments) via NI BNC-2090 accessories and displayed via self-developed NI Labview program (59). All the figures were prepared by Origin (Microcal Software).

Mouse electroretinogram recording

The mice were dark-adapted overnight before ERG recording. Under dim red light (λ > 600 nm), the mice were anaesthetized with an i.p. injection of a solution of ketamine (70 mg/kg, Ketalar®, Parke-Davies, S.L.) and xylazine (10 mg/kg, Rompun®) and kept on a heating pad to maintain normal body temperature at 37°C. A topical drop of 1% tropicamide (Coliciurs Tropicamida 1%, Alcon-Cusi, S.A.) were applied to the eyes of the experimental animals before ERG testing. Electrical responses were obtained with Burian-Allen electrodes (Hansen Labs) positioned on the cornea. A drop of methylcellulose (Methocel 2%, Novartis Laboratories CIBA Vision) was positioned on the cornea to improve electrical conductivity. The reference electrode was placed in the mouth, and the ground electrode at the base of the tail. After positioning the electrodes, the mice were placed in a Ganzfeld stimulator. Stimulation flashes were provided by a Ganzfeld dome light source, which ensured a uniform illumination of the retina and provided a wide range of light intensities. Electrical signals generated in the retina were amplified (×1000) and band filtered between 0.3 and 1000 Hz, using a Grass amplifier (CP511 AC amplifier, Grass Instruments). Electrical signals were digitized at 20 kHz, using a Powerlab data acquisition board (AD Instruments) and displayed on a PC computer monitor. The ERG responses were elicited by stimulation of the retina with light intensities ranging between 10⁻⁷ and 10² cd/m². A series of ERG responses were averaged (∼20 ERG traces) for each light intensity, after adjusting the time interval between flashes to ensure complete recovery of the response. Standard ERG waves were recorded according to the method recommended by the International Society for Clinical Electrophysiology of Vision (ISCEV).

Electrophysiological measurements on mouse astrocytes

Electrophysiological measurements were performed with the whole-cell configuration of the patch-clamp technique as previously described (60). Briefly, pipette had a resistance of 2–4 MΩ when filled with the intracellular solution. Membrane currents were amplified (amplifier List EPC-7) filtered at 2 kHz (-3 dB) and acquired at a sample rate of 5 kHz on a microcomputer for off-line analyses (pClamp 6, Axon Instruments and Origin 6.0, MicroCal). The access resistance (below 10 MΩ) was corrected for 60–80%. Current densities were calculated by dividing the current values measured at each membrane potential by the cell capacitance estimated from the capacitive transients of the recorded cells corrected by means of the analogical circuit of the patch-clamp amplifier. For the isolation of the Cl⁻ conductance, the external perfusing saline, called isotonic (Iso CsCl), was (mM): 122 CsCl, 2 MgCl₂, 2 CaCl₂, 10 TES, 5 glucose, pH 7.4 with CsOH and osmolality ~320 mOsm with mannitol. The intracellular (pipette) solution was composed of (mM): 126 CsCl, 2 MgCl₂, 1 EGTA, 10 TES, pH 7.2 and osmolarity ~300 mOsm with mannitol. Hypotonic extracellular solution (Hypo CsCl) ~260 mOsm was obtained by omitting mannitol. Osmolarity was measured with a vapour–pressure osmometer (Wescor 5500, Delcon). All salts and various concentrations of drugs were made in distilled water.
molecules were from Sigma. The DCPIB was prepared in DMSO at a concentration 1000-fold higher than that used in the perfusing saline.

SUPPLEMENTARY MATERIAL
Supplementary Material is available at HMG online.

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Conflict of Interest statement. None declared.

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