Megalencephalic leucoencephalopathy with cysts: defect in chloride currents and cell volume regulation

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Megalencephalic leucoencephalopathy with subcortical cysts is a genetic brain disorder with onset in early childhood. Affected infants develop macrocephaly within the first year of life, after several years followed by slowly progressive, incapacitating cerebellar ataxia and spasticity. From early on, magnetic resonance imaging shows diffuse signal abnormality and swelling of the cerebral white matter, with evidence of highly increased white matter water content. In most patients, the disease is caused by mutations in the gene MLC1, which encodes a plasma membrane protein almost exclusively expressed in brain and at lower levels in leucocytes. Within the brain, MLC1 is mainly located in astrocyte–astrocyte junctions adjacent to the blood–brain and cerebrospinal fluid–brain barriers. Thus far, the function of MLC1 has remained unknown. We tested the hypothesis that MLC1 mutations cause a defect in ion currents involved in water and ion homeostasis, resulting in cerebral white matter oedema. Using whole-cell patch clamp studies we demonstrated an association between MLC1 expression and anion channel activity in different cell types, most importantly astrocytes. The currents were absent in chloride-free medium and in cells with disease-causing MLC1 mutations. MLC1-dependent currents were greatly enhanced by hypotonic pretreatment causing cell swelling, while ion channel blockers, including Tamoxifen, abolished the currents. Down regulation of endogenous MLC1 expression in astrocytes by small interfering RNA greatly reduced the activity of this channel, which was rescued by overexpression of normal MLC1. The current–voltage relationship and the pharmacological profiles of the currents indicated that the channel activated by MLC1 expression is a volume-regulated anion channel. Such channels are involved in regulatory volume decrease. We showed that regulatory volume decrease was hampered in lymphoblasts from patients with megalencephalic leucoencephalopathy. A similar trend was observed in astrocytes with decreased MLC1 expression; this effect was rescued by overexpression of normal MLC1. In the present study, we show that absence or mutations of the MLC1 protein negatively
Impact both volume-regulated anion channel activity and regulatory volume decrease, indicating that megalencephalic leukoencephalopathy is caused by a disturbance of cell volume regulation mediated by chloride transport.

Keywords: leucodystrophy; MLC1; astrocytes; chloride currents; volume-regulated anion channel

Abbreviations: HEK293 = human embryonic kidney cells; I–V = current–voltage; MLC = megalencephalic leukoencephalopathy with subcortical cysts; Sf9 = Spodoptera frugiperda 9; VRAC = volume-regulated anion channel

Introduction

Megalencephalic leukoencephalopathy with subcortical cysts (MLC; MIM 604004) is an autosomal recessive brain disorder with infantile onset, first described in 1995 (van der Knaap et al., 1995). Patients with MLC develop macrocephaly during the first year of life. After several years, slowly progressive cerebellar ataxia and spasticity occur. Most patients become wheelchair-dependent by the age of 1–3 years. Brain MRI reveals diffuse signal abnormalities and increasing periventricular white matter signal changes. Stroke-like episodes are a frequent manifestation. After several years, slowly progressive cerebellar ataxia and spasticity occur. Most patients become wheelchair-dependent by the age of 1–3 years. Brain MRI reveals diffuse signal abnormalities and increasing periventricular white matter signal changes. Stroke-like episodes are a frequent manifestation.

In 2001, a gene for MLC, MLC1, was identified (Leegwater et al., 2001). This gene encodes a 377 amino acid plasma membrane protein with eight transmembrane domains, almost exclusively expressed in the brain and leucocytes (Boor et al., 2005). Within the brain the MLC1 protein is mainly expressed in astrocyte–astrocyte junctions adjacent to the blood–brain and CSF–brain barriers (Teijido et al., 2004; Boor et al., 2005; Duarri et al., 2011). MLC1 mutations all disrupt the membrane localization of the MLC1 protein (Duarri et al., 2008). We have recently shown that mutations in GLIALCAM, coding for hepatic and glial cell adhesion molecule GlialCAM, also cause MLC. GLIALCAM mutations also disrupt the localization of MLC1 (Lopez-Hernandez et al., 2011), indicating that MLC1 is central in the pathophysiology of MLC.

Thus far, the function of MLC1 has remained unknown. Amino acid sequence analysis reveals a weak similarity with potassium channel Kv1.1, ABC-2 type transporters and sodium:galactoside symporters (Leegwater et al., 2001; Teijido et al., 2004; Boor et al., 2005). Further, MLC1 contains an internal repeat that is found in several ion channel proteins (Teijido et al., 2004). These observations and the highly increased white matter water content prompted us to test the hypothesis that MLC1 plays a role in ion transport involved in cell volume regulation.

Materials and methods

The study was performed with approval of the Institutional Review Board. Written informed consent was obtained for the use of patient lymphoblasts. The MLC1 mutations of the patients, of whom lymphoblasts or MRIs were used, are given in Table 1.

Cell culture

Culture media for human embryonic kidney (HEK293) and HeLa cells consisted of DMEM/HAM-F10 (1:1) (Invitrogen) supplemented with 10% foetal calf serum (Invitrogen). Spodoptera frugiperda (Sf9) insect cells were cultured in suspension in serum-free HyQ SFX-Insect medium (Perbio) at 27°C. Isolation and immortalization of human lymphoblasts were performed as previously described (van Kollenburg et al., 2006). Lymphoblasts were cultured in RPMI-1640 medium (Invitrogen), supplemented with 10% foetal calf serum. Primary rat astrocytes were isolated and cultured as described (van Kollenburg et al., 2006), with the exception that we used trypsin precipitation and lipofectamine (Invitrogen), respectively, with a construct expressing wild-type or mutant MLC1 (MLC1_Ser93Leu or MLC1_Cys326Arg).

HEK293 and HeLa cells were used for patch clamp experiments 24–48 h after transfection. Constructs for expression in Sf9 insect cells were made by cloning the sequences for green fluorescent protein (to mark transfection) or adenovirus (wild-type or mutant) into pIEX5 (Novagen). Sf9 cells were transfected with Insect GeneJuice (VWR). Sf9 cells were used 48–72 h after transfection.

Table 1 MLC1 mutations

<table>
<thead>
<tr>
<th>Patient</th>
<th>Mutations</th>
<th>Patient material used</th>
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<tbody>
<tr>
<td>Patient 1</td>
<td>c.135_136insC, p.Cys46LeufsX34 (homozygous)</td>
<td>Lymphoblasts, MRI</td>
</tr>
<tr>
<td>Patient 2</td>
<td>c.736A&gt;C, p.Ser246Arg (homozygous)</td>
<td>Lymphoblasts</td>
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Transfection and infection

HEK293 and HeLa cells were transfected using calcium phosphate precipitation and lipofectamine (Invitrogen), respectively, with a construct expressing green fluorescent protein (pEGFPN1, Clontech) to mark transfection either alone or in combination with constructs expressing wild-type or mutant MLC1 (MLC1_Ser93Leu or MLC1_Cys326Arg). HEK293 and HeLa cells were used for patch clamp experiments 24–48 h after transfection. Constructs for expression in Sf9 insect cells were made by cloning the sequences for green fluorescent protein (to mark transfection) or adenovirus (wild-type or mutant) into pIEX5 (Novagen). Sf9 cells were transfected with Insect GeneJuice (VWR). Sf9 cells were used 48–72 h after transfection.

Astrocytes were infected with adenoviruses (multiplicity of infection [= ratio of infectious virus particles to cells] of 4–6) and used after 6–8 days for patch clamp experiments. Generation of adenoviruses for expression of wild-type or mutant (Ser246Arg) haemagglutinin-tagged MLC1 has been described (Duarri et al., 2008).

Small interfering RNAs

The generation of adenoviruses for expression of small interfering RNA against nucleotides 405–425 of the coding region of rat MLC1 or a sequence without messenger RNA target as control has been described (Duarri et al., 2011). Sequences are given in Supplementary Table 1.
MLC1 expression

Total RNA was isolated using RNA-Bee (BioConnect). Complementary DNA was made with 5 μg total RNA with SuperScript™III (Invitrogen) according to the vendor’s instruction. Expression of MLC1 messenger RNA was studied by reverse transcription PCR as described (Boor et al., 2006), or quantitative PCR using SYBR® Green according to the vendor’s protocol (Applied Biosystems). For primers see Supplementary Table 1. The values obtained were corrected for the amount of glyceraldehyde 3-phosphate dehydrogenase messenger RNA in each sample (ΔΔCt). In addition, the relative expression of MLC1 messenger RNA in the separate cell types as compared with its expression in the brain sample was calculated.

Immunocytochemistry

For immunofluorescence staining, cells were fixed with phosphate-buffered saline containing 4% paraformaldehyde for 5 min, permeabilized with 1% goat serum, 0.9% NaCl and 0.1% saponin in phosphate-buffered saline for 10 min at room temperature and blocked with 5% goat serum, 0.9% NaCl and 0.05% saponin in phosphate-buffered saline for 30–60 min. Primary antibodies were diluted in 1% goat serum, 0.9% NaCl and 0.01% saponin in phosphate-buffered saline and cells were incubated overnight at 4 °C. Rabbit anti-mouse MLC1 (1:100) (Teijido et al., 2004), rabbit anti-human MLC1 (1:100) (Teijido et al., 2004; Dauri et al., 2008), chicken anti-ribosomal CREST antibody were used. Cells were washed and incubated for 1 h at room temperature with secondary antibodies: Alexa-488 goat anti-chicken, Alexa-568 goat anti-rabbit, Alexa-594 goat anti-mouse. Coverslips were mounted in Vectashield® mount medium (Vector Laboratories) with 1.5 μg/ml 4',6-diamidino-2-phenylindole (DAPI; Sigma) and visualized using a Leica DM6000B microscope.

Electrophysiology

Voltage clamp experiments were performed at 20–22 °C using the tight-seal, whole-cell variant of the patch clamp technique as described previously (Hamill et al., 1981) and currents were amplified using an AXOPATCH-200A amplifier (Axon Instruments). In all experiments, unless mentioned specifically, we used iso-osmotic chloride-selective bath and pipette solutions.

In experiments with HeLa cells, HEK293 cells, lymphoblasts and astrocytes, the iso-osmotic bath solution contained in mM: 140 N-methyl-D-glucamine-Cl, 2 CaCl2, 2 MgCl2, 10 HEPES [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid], 0.1 LaCl3 (average osmolarity 295 mOsm/l, pH 7.4), and the pipette (resistance of 3–7 MΩ) solution contained in mM: 40 N-methyl-D-glucamine-Cl, 100-N-methyl-D-glucamine-gluconate, 2 MgCl2, 10 HEPES and Ca2+-ethylene glycol tetraacetic acid (EGTA) buffer (buffered to pH 7.2, average osmolarity 290 mOsm/l), unless mentioned otherwise.

In SF9 cells, the iso-osmotic bath solution contained in mM: 180 N-methyl-D-glucamine-Cl, 2 CaCl2, 2 MgCl2, 10 MES [2-N-(morpholino)ethanesulfonic acid] (average osmolarity 365 mOsm/l, pH 6.2) and the pipette (resistance of 3–6 MΩ) solution contained in mM: 50 N-methyl-D-glucamine-Cl, 120-N-methyl-D-glucamine-gluconate, 2 MgCl2, 10 N-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid (TES). The extracellular pH and osmolarity mimics that of the HyQ SFX-Insect medium (Larsen et al., 1996; Xiong et al., 1999).

Hypo-osmotic pretreatments were executed as previously described (Lascola and Kraig, 1996). The hypo-osmotic solution had a 40% reduced osmolarity as compared with the iso-osmotic solution.

Adenovirus-infected astrocytes were treated with 0.025% trypsin for 5 min before recording.

Where indicated, 10 μM Tamoxifen (Tocris Bioscience), 1 mM zinc (Sigma), 100 μM NPPB (5-nitro-2-(3-phenylpropylamino)benzoic acid; Sigma) or 200 μM DIDS (4,4-Dioctadecyl-4′,4′-disulphonic acid disodium salt hydrate; Sigma) was added to the bath solution. For the inhibition of chloride channels in astrocytes, Tamoxifen had to be used in reduced concentration (1 μM instead of 10 μM) and had to be present during both trypsin pretreatment and electrophysiological recording, as described before (Zhang et al., 1994). The other blockers were added to the bath solution after the trypsin treatment. In all lymphoblast experiments, 2 mM ATP (as Mg2+ salt) was present in the pipette-filling solution.

The electrophysiology protocols are summarized in Fig. 1. The holding potential was −60 mV. Currents were measured with voltage ramps of −120 to +120 mV, or −100 to +100 mV for SF9 cells and astrocytes in steps of 0.14 mV/ms. Sample interval was 250 μs. Data were filtered at 2 kHz. Each current measurement was an average obtained from five ramps, with an interval of 1 s between the ramps. Voltage ramps were repeated for all current–voltage curves and bar figures (Figs 2–5). In addition to the voltage ramps, we also recorded current traces obtained from a step voltage protocol (holding potential −60 mV, 250 ms steps ranging from −120 to +60 mV, spaced 10 mV). For SF9 cells and astrocytes we used a step voltage protocol ranging from −100 to +100 mV. Supplementary Figs 4 and 5B are the only figures incorporating currents that showed a stable holding current and a low series resistance (≤6 MΩ) throughout the experiment were analysed. Mean capacitance of the different cell types are given in Table 2. Voltage clamp protocol, data acquisition, storage and analysis were carried out using commercial software (pClamp 9.1, Axon Instruments).

Potassium currents were measured, as previously described (Benfenati et al., 2007), using bath solution containing (in mM) 140 NaCl, 4 KCl, 2 MgCl2, 2 CaCl2, 10 HEPES and 5 glucose; pH was adjusted to 7.4 with NaOH and osmolarity was adjusted to ~315 mOsmol/l with mannitol. The intracellular (pipette) solution contained (in mM) 144 KCl, 2 MgCl2, 5 EGTA and 10 HEPES; pH was adjusted to 7.2 with KOH and osmolarity was adjusted to ~300 mOsmol/l with mannitol (Benfenati et al., 2007).

Imaging of regulatory volume decrease

Astrocytes and lymphoblasts were loaded with 5 μM Calcein-AM (Molecular Probes) for 20 min at 37 °C (Crowe et al., 1995). Changes in cell volume of single lymphoblasts, plated on poly-ornithine-coated glass cover slips, were monitored by measuring cell surface area. Changes in cell volume of single astrocytes, plated on glass cover slips, were analysed by measuring calcein fluorescence using the calcein-quenching method (Solov et al., 2004). Chloride selective iso- and hypo-osmotic (~40%) solutions were used, as described in the electrophysiology section. To ensure that differences between cells were dependent on anion permeability rather than cation permeability, lymphoblasts were pretreated with 40 μM/ml and astrocytes with 10 μg/ml gramicidin (Sigma) for 5 min prior to the start of the experiment. Cells were bathed in the iso-osmotic solution (20–22 °C) and transferred to a continuously perfused (5 ml/min) recording chamber, equipped with a microscope with a ×10 objective. An image was taken every 30 s. At the beginning of each experiment, images were obtained for...
5 min in the iso-osmotic solution to establish the baseline. Cells were perfused with the hypo-osmotic solution for 30 min, after which the iso-osmotic solution was reintroduced and images were taken for another 10 min. The cell surface or average fluorescence of each cell in the acquired images was calculated using ImageJ software (National Institutes of Health). Changes in cell surface and average fluorescence were expressed as \( S_t / S_0 \) and \( F_t / F_0 \) ratios, respectively, where \( S_0 \) and \( F_0 \) are the average cell surface area and fluorescence under iso-osmotic conditions at the beginning of the experiment. The curve from maximum swelling to the end of the hypo-osmotic treatment was fitted with an exponential curve using Microsoft Excel and the time constant of this exponential decay was determined for each cell.

Statistical analysis

Group measures were expressed as mean ± standard error of the mean; error bars also indicate standard error of the mean. We used the Student’s t-test to assess the statistical significance of differences between control and experimental conditions.

Results

MLC1 expression-related chloride currents in transfected cells

To address the question whether MLC1 could have a role in ion transport, we transfected different cell types with constructs expressing wild-type MLC1 and used the whole-cell patch clamp technique to test whether ionic currents were induced. Figures 2–5 illustrate current profiles in the cell types investigated, each figure representing a specific cell type. Quantitative results of all electrophysiological experiments are given in Table 2.

Initial patch clamp data in transfected HEK293 cells expressing either green fluorescent protein as a control or wild-type MLC1, and using different recording media indicated that MLC1 contributed to small ionic currents that become visible in particular at highly positive membrane potentials and are carried either by an influx of anions or an efflux of cations (data not shown). To test which ion carries the currents observed in MLC1-transfected HEK293 cells, we first eliminated the contribution of the monovalent cations sodium and potassium in the pipette and bath solutions by the impermeant cation \( N \)-methyl-D-glucamine. Under these conditions, a small inward current was observed at negative potentials in HEK293 cells transfected with the control green fluorescent protein construct that reversed at 0 mV (Fig. 2A, Table 2). In MLC1-transfected HEK293 cells, a stronger inward current was observed at negative potentials, which also reversed \( \approx \) 0 mV and showed outward rectification (Fig. 2B, Table 2). Anion channels have a very low permeability for gluconate (Nilius and Droogmans, 2003). Replacing chloride by gluconate in bath and pipette filling solutions almost completely abolished the inward and outward currents (Fig. 2B), demonstrating that the MLC1 expression-induced current is carried by chloride. Given the
nature of the voltage clamp protocol, the current measured in MLC1-overexpressing cells appeared to be a steady-state current.

HEK293 cells have a low endogenous MLC1 expression (Supplementary Table 2), which was undetectable in antibody staining (data not shown). To exclude influence of the low endogenous MLC1 expression in HEK293 cells, we also tested HeLa cells, which lack endogenous MLC1 expression (Supplementary Table 2). In HeLa cells, the current profiles were highly similar to those observed in HEK293 cells (Fig. 2A and C). For practical reasons we used HEK293 cells for further experiments.

We studied chloride currents in transfected Sf9 insect cells, which lack the MLC1 gene and therefore any possible endogenous MLC1 expression. Expression of MLC1 in Sf9 cells did not affect chloride currents under standard conditions (Fig. 3A). Because MLC1 is localized in astrocytes (Schmitt et al., 2003; Teijido et al., 2004; Boor et al., 2005) and many chloride currents in astrocytes are volume sensitive (Abdullaev et al., 2006), we applied pretreatment with hypo-osmotic solutions (Fig. 1C) in transfected Sf9 cells to induce cell swelling. Hypotonic pretreatment robustly increased the currents at negative and positive voltages in MLC1-expressing Sf9 cells (Fig. 3B, Table 2). As observed in MLC1-transfected HEK293 and HeLa cells (Fig. 2A and C) the current profiles of the MLC1-transfected Sf9 cells after hypo-osmotic pretreatment also showed outward rectification with a small negative reversal current.

### MLC1 expression-related chloride currents in primary astrocytes

In the brain MLC1 is predominantly expressed in astrocytes. We therefore studied chloride currents in cultured rat primary astrocytes. We chose rat astrocytes, because these are readily available
and have been used before for similar patch clamp experiments (Lascola and Kraig, 1996; Abdullaev et al., 2006). In these cells, MLC1 was demonstrated in the cell plasma membrane and the endoplasmic reticulum (Supplementary Fig. 3).

In uninfected astrocytes, small-amplitude chloride currents were observed that were increased by hypo-osmotic pretreatment (data not shown). To alter expression of MLC1 we infected the astrocytes with adenoviruses expressing human haemagglutinin-tagged MLC1 to increase expression or with adenoviruses expressing small interfering RNA directed against MLC1 to reduce expression (Supplementary Fig. 3A and G; Duarri et al., 2011). Astrocytes infected with adenoviruses expressing, respectively, LacZ or small interfering RNA without messenger RNA target served as controls (Supplementary Fig. 3E). Adenovirus-infected

![Image of Figures 2](https://academic.oup.com/brain/article-abstract/134/11/3342/314911)

**Figure 2** MLC1-expression-related chloride currents in HEK293 and HeLa cells. (A) Current–voltage (I–V) relationships of control (black trace, n = 9) and MLC1-transfected HEK293 cells (red trace, n = 7). (B) I–V relationship of control HEK293 cells (black trace, n = 3) and MLC1-transfected HEK293 cells (red trace, n = 4) with replacement of chloride in both bath and pipette-filling solutions by equimolar amounts of gluconate. (C) I–V relationships of control (black trace, n = 4) and MLC1-transfected HeLa cells (red trace, n = 8). (D) I–V relationship of MLC1-transfected HEK293 cells (red trace, n = 7) after adding 10 μM Tamoxifen (light blue trace, n = 4) to the bath solution. (E) The average I–V relationship of whole-cell chloride current in control (black trace, n = 4), MLC1_Ser93Leu (dark green trace, n = 6) and MLC1_Cys326Arg (light green trace, n = 5) expressing HEK293 cells. (F) The amplitude of the chloride current at 120 mV in control HEK293 cells (black bar, n = 6), HEK293 cells overexpressing wild-type (wt) MLC1 (red bar, n = 6), MLC1_Ser93Leu (dark green bar, n = 6) and MLC1_Cys326Arg (light green bar, n = 5). Currents measured in HEK293 cells overexpressing wild-type MLC1 were significantly different from those measured in control cells and cells overexpressing mutant MLC1. The I–V profiles of the MLC1-transfected cells were outwardly rectifying. Statistically significant differences are indicated by asterisks (*P < 0.05; **P < 0.01). GFP = green fluorescent protein.
astrocytes were less viable after hypo-osmotic treatment than uninfected cells, showed greater difficulty to seal to the patch pipette and were generally very difficult to record from. Instead of hypo-osmotic pretreatment we, therefore, used a mild treatment with trypsin, which causes morphological changes in the astrocytes and induces the same chloride currents as hypo-osmotic pretreatment (Lascola and Kraig, 1996). In trypsin-treated astrocytes expressing LacZ or non-targeted small interfering RNA, the chloride current profiles were similar to those seen in MLC1-transfected Sf9 cells after hypo-osmotic pretreatment (Fig. 4A and B), although the peak current amplitudes of the astrocytes were higher. This difference can mainly be explained by the difference in size and, thus the membrane capacitance of the different cell types (Table 2). Note that within experiments with a certain cell type, the sizes of the cells were similar to each other (Table 2); differences in cell size therefore cannot underlie the observed changes in currents under different experimental conditions.

Overexpression of wild-type MLC1 by infection of astrocytes resulted in a large increase in the amplitude of the currents after trypsin pretreatment compared with cells overexpressing LacZ as control (Fig. 4A–C, Table 2).

The expression of the endogenous MLC1 protein was reduced by ~90% by small interfering RNA directed against MLC1 (Supplementary Fig. 3E–H, Supplementary Table 2). In these silenced astrocytes chloride currents recorded after trypsin pretreatment were significantly reduced (Fig. 4B and C, Table 2).

In cells with reduced MLC1 levels due to small interfering RNA, overexpression of human wild-type MLC1 restored the reduced chloride currents to high levels (Fig. 4C, Table 2), indicating that the effects were specific to the loss of MLC1.

In these experiments with primary astrocytes, the chloride current profiles showed heterogeneity (Supplementary Fig. 4) similar to previously reported findings in astrocytes (Lascola and Kraig, 1996). Heterogeneity was seen under all conditions (MLC1 overexpression or knock-down and in the respective controls). The different activation and inactivation characteristics are most likely explained by the presence of other chloride channels.

Potassium and chloride are the most important ions in the regulation of water homeostasis by astrocytes. We therefore tested whether potassium currents would be influenced by MLC1 in

![Figure 3](https://academic.oup.com/brain/article-abstract/134/11/3342/314911/0)

**Figure 3** MLC1-expression-related chloride currents in Sf9 cells. (A) Current–voltage (I–V) relationships of control (black trace, n = 8) and MLC1-transfected Sf9 cells (red trace, n = 9). (B) I–V relationship obtained after hypo-osmotic pretreatment of control (dark blue trace, n = 8) and MLC1-transfected Sf9 cells (purple trace, n = 12). (C) I–V relationship obtained after hypo-osmotic pretreatment of MLC1-transfected Sf9 cells (purple trace, n = 12) and after adding 2 μM Tamoxifen (light blue trace, n = 8) to the bath solution during the hypotonic pretreatment and to the isotonic medium during recording. (D) The amplitude of the chloride current obtained after hypo-osmotic pretreatment at 100 mV in Sf9 cells overexpressing wild-type (wt) MLC1 (purple bar, n = 12), in control Sf9 cells expressing green fluorescent protein (GFP) (blue bar, n = 8), and in Sf9 cells overexpressing mutant MLC1_Ser93Leu (dark green bar, n = 10) and mutant MLC1_Cys326Arg (light green bar, n = 10). Currents measured in Sf9 cells overexpressing wild-type MLC1 were significantly different from those measured in control cells and cells overexpressing mutant MLC1. The I–V profiles of the MLC1-transfected cells are outwardly rectifying. Statistically significant differences are indicated by asterisks (**P < 0.01).
astrocytes. Potassium currents were not affected by increased or decreased expression of MLC1 (Supplementary Fig. 5), showing that the currents observed are not caused by side effects of the altered MLC1 expression on, for example, voltage-gated potassium channels (Benfenati et al., 2007).

**MLC1 expression-related chloride currents in lymphoblasts**

MLC1 is expressed in the brain and in white blood cells. White blood cells are much easier to obtain from human patients and control subjects than astrocytes. We used human lymphoblasts endogenously expressing MLC1 for further studies. In control lymphoblasts, in which chloride currents were small under normal conditions, hypotonic pretreatment increased the currents at negative and positive voltages (Fig. 5A, Table 2) with current profiles similar to the profiles observed in MLC1-transfected Sf9 cells after hypotonic pretreatment (Fig. 3B).

**Effect of ion channel blockers**

Tamoxifen is known to block volume-regulated anion channels (VRACs; Zhang et al., 1994). Added to the bath solution it strongly reduced the chloride currents in MLC1-transfected HEK293 cells (Fig. 2D, Table 2). Tamoxifen also strongly inhibited the currents induced by hypotonic conditions in MLC1-transfected Sf9 cells (Fig. 3C), astrocytes overexpressing MLC1 (Fig. 4D) and control lymphoblasts (Fig. 5B). In all cases, currents were reduced ~80% at 100 mV (Table 2).

The rat astrocytes expressing human MLC1 were also used to test other ion channel blockers, i.e. Zinc, NPPB and DIDS (Lascola and Kraig, 1996; Benfenati et al., 2007). All blockers significantly reduced the chloride currents (Fig. 4D, Table 2).

**MLC1 mutations abolish chloride currents**

We introduced missense mutations, which have been observed in a homozygous state in patients, in MLC1 expression constructs.
Volume regulation is impaired in patient-derived lymphoblasts and MLC1-silenced astrocytes

We studied whether the failure to induce chloride currents in lymphoblasts of patients and the reduced chloride currents in MLC1-silenced astrocytes upon hypotonic shock was associated with an impaired regulatory volume decrease that follows hypotonia-induced cell swelling. Lymphoblasts and astrocytes were exposed to the chloride-selective hypotonic solution in the

Figure 5 Chloride currents in lymphoblasts. (A) Current-voltage (I–V) relationship of control lymphoblasts obtained after iso- (ISO, red trace, n = 13) and hypo-osmotic (HYPO, purple trace, n = 9) pretreatment. (B) I–V relationship of control lymphoblasts obtained after hypo-osmotic pretreatment (purple trace, n = 9) and after adding 10 μM Tamoxifen (light blue trace, n = 5) to the bath solution during the hypotonic pretreatment and to the isotonic medium during recording. (C) I–V relationship of lymphoblasts derived from Patient 1 obtained after iso- (black trace, n = 9) and hypo-osmotic (dark green trace, n = 7) pretreatment. (D) I–V relationship of lymphoblasts derived from Patient 2 obtained after iso- (black trace, n = 10) and hypo-osmotic (light green trace, n = 9) pretreatment. (E) The amplitude of the chloride currents obtained at 120 mV for control and patient lymphoblasts after iso- and hypotonic pretreatment and in the absence or presence of Tamoxifen, as indicated. Statistically significant differences with control cells and conditions (red) are indicated by asterisks (*P < 0.05; **P < 0.01).
presence of gramicidin. Gramicidin forms pores for monovalent cations, allowing free exchange mainly of potassium, thereby excluding this ion from playing a role in the regulatory volume decrease (Pasantes-Morales et al., 1994). Cell volume recovery was significantly slower ($P < 0.001$) in lymphoblasts from Patients 1 and 2 (Table 1) than in the three control lymphoblast lines (Fig. 6F). The same trend of decreased rate of cell volume recovery was observed ($P = 0.067$) in MLC1-silenced astrocytes (Fig. 6B–D and F). Although we observed a trend towards reduced maximum swelling after exposure to the hypotonic solution in MLC1-silenced astrocytes compared with control astrocytes ($P = 0.056$) (Fig. 6B), we did not observe a difference in maximum swelling between patient-derived and control lymphoblasts ($P < 0.001$; Fig. 6A). The normal cell volume recovery was restored in MLC1-silenced astrocytes by overexpression of human wild-type MLC1 (Fig. 6B, C, E and F). After reintroduction of chloride selective isotonic solution, regulatory volume increase was nearly absent in all lymphoblasts and astrocytes (Fig. 6A and B). Regulatory volume increase is highly dependent on influx of monovalent cations (Hoffmann et al., 2009), which were absent in our solutions.

Discussion

Patients with MLC are normal at birth and develop macrocephaly during the first year of life (van der Knaap et al., 1995). MRI at that time reveals abnormal and swollen cerebral white matter with highly increased water content (van der Knaap et al., 1995; van der Voorn et al., 2006). Electron microscopy of brain tissue from a patient with MLC demonstrates that fluid-filled vacuoles within myelin sheaths (van der Knaap et al., 1996) and, to a lesser extent, astrocytic endfeet (Duarri et al., 2011) form the anatomic substrate of the white matter edema. Cerebral white matter contains little myelin at birth and acquires most of its myelin during the first year of life. Thus, the macrocephaly and neuroradiological characteristics of MLC develop during a period of rapid myelin deposition, suggesting that the vacuoles are formed during this process. We have shown previously that MLC1 mutations cause MLC (Leeuwen et al., 2001; Boor et al., 2006). The MLC1 protein is located mainly in the brain in astrocyte–astrocyte junctions adjacent to the blood–brain and CSF–brain barriers (Teijido et al., 2004; Boor et al., 2005; Duarri et al., 2011). Its function has remained unknown.

We demonstrate an association between MLC1 expression and chloride currents in different cell types, most importantly in patient-derived lymphoblasts and astrocytes, the cells that normally express the MLC1 protein. Previous negative results to find ion channel activity related to MLC1 may be due to the inability to identify the opening conditions of the channel or to the specific experimental conditions (Kaganovich et al., 2004; Teijido et al., 2004) (Supplementary Fig. 2). The ion substitution experiments, current–voltage profile, the activation by hypo-osmotic pretreatment causing cell swelling, and the sensitivity to inhibition by Tamoxifen and other ion channel blockers indicate that the increase in current amplitude induced by MLC1 expression is due to increased VRAC activity (Lascola and Kraig, 1996; Jentsch et al., 2002; Nilius and Droogmans, 2003; Abdullaev et al., 2006).

Water homeostasis and osmotic balance are vital in the brain and astrocytes are central in this process. They are highly sensitive to changes in extracellular osmolarity and can display prominent cell volume changes as part of the osmoregulatory process (Simard and Nedergaard, 2004; Nedergaard and Dirnagl, 2005; Benfenati et al., 2007; Benfenati and Ferroni, 2010). Any physiological or pathological osmotic perturbation induces a transmembrane flow of ions and water that rapidly restores the osmotic equilibrium and induces temporary swelling or shrinkage of cells, followed by regulatory volume decrease or regulatory volume increase, respectively, to normalize cell volume. The regulatory volume decrease is caused by activation of ion channels and transporters that allow effluxes of potassium, chloride, organic osmolytes and water (Pasantes-Morales et al., 2006). VRACs, which are activated by water fluxes, changes in cell shape and signal transduction events (Mulligan and MacVicar, 2006; Stutzin and Hoffmann, 2006), play an important role in the regulatory volume decrease. VRAC function is most likely dependent on multiple channels, which could be different for different cell types. The molecular identity of most of these channels is unknown (Jentsch et al., 2002; Nilius and Droogmans, 2003). Our study with patient-derived lymphoblasts shows that a defect in MLC1 not only decreases VRAC-related chloride currents but also the rate of the regulatory volume decrease. Similar findings were obtained from MLC1-silenced astrocytes, with the addition of a rescue experiment with human wild-type MLC1.

The variation in the rate of the regulatory volume decrease was larger in MLC1-silenced astrocytes than in patient lymphoblasts. Consequently, the $P$-value for the difference between MLC1-silenced and wild-type astrocytes was higher than for the difference between patient and control lymphoblasts. The larger variation in astrocytes is most likely due to variation in the degree of MLC1 knockdown in MLC1-silenced astrocytes, a problem not shared by patient lymphoblasts. As such, patient-derived lymphoblasts are a more consistent system to study effects of mutant or absent MLC1. The difference in degree of swelling of MLC1-silenced and wild-type astrocytes after hypotonic exposure was not observed in patient versus control lymphoblasts. Currently we have no explanation for this observation in astrocytes.

We performed our experiments in the presence of gramicidin and used chloride-selective medium, to enhance the dependency of the experiments on chloride. Yet, with this experimental set-up the regulatory volume decrease is not exclusively dependent on the MLC1-related chloride channel function; it depends in part on the efflux of organic osmolytes and on other chloride channels, not related to MLC1. This explains why the regulatory volume decrease is slower and not abolished in the absence of normal MLC1. MLC is not an immediately life-threatening disease. Complete abolition of the regulatory volume decrease is probably not compatible with life. At present it is still an open question whether MLC1 is a VRAC, a component of a VRAC or a protein that activates a VRAC, either directly or indirectly.

Recessive MLC1 mutations are responsible for ~75% of the MLC patients (Leeuwen et al., 2001; Boor et al., 2006). We have recently shown that mutations in GLIALCAM, coding for
hepatic and glial cell adhesion molecule GlialCAM, also cause MLC (Lopez-Hernandez et al., 2011). Patients with recessive GLIALCAM mutations have classical MLC, while patients with dominant GLIALCAM mutations show transient clinical and MRI features of MLC (van der Knaap et al., 2010; Lopez-Hernandez et al., 2011). GlialCAM is an immunoglobulin-like cell adhesion molecule that is required for the proper localization of MLC1 in astrocytes–astrocyte junctions (Lopez-Hernandez et al., 2011). All types of MLC1 mutations cause decreased membrane expression of the MLC1 protein (Duari et al., 2008) and GLIALCAM mutations disrupt the localization of MLC1, which explains why MLC1 and GLIALCAM mutations cause the same disease. Why patients with dominant GLIALCAM mutations have transient features of MLC remains to be explained. In any case, this observation

Figure 6 MLC1-dependent regulatory volume decrease. Cells were bathed in a chloride-selective iso-osmotic solution that was switched to a hypo-osmotic chloride-selective solution for 30 min as indicated in (A–E) (black bar). Gramicidin was used in all experiments. (A) Average relative cell surface, obtained under iso- and hypo-osmotic conditions, from lymphoblasts of three controls (control 1, red trace, n = 34; control 2, dark grey, n = 14; and control 3, black trace n = 15) and two patients with MLC (Patient 1, dark green trace, n = 39; Patient 2, light green trace n = 15). (B) Average relative fluorescence, obtained under iso- and hypo-osmotic conditions, from astrocytes expressing non-targeted small interfering RNA (nt siRNA, dark blue, n = 11), small interfering RNA against rat MLC1 (purple, n = 9) or small interfering RNA against rat MLC1 in combination with expression of human wild-type (wt) MLC1 (orange, n = 7). Individual typical regulatory volume decrease fluorescence quenching traces fitted with an exponential curve are depicted for an astrocyte expressing non-targeted small interfering RNA (C), an astrocyte expressing small interfering RNA against rat MLC1 (D) and an astrocyte expressing small interfering RNA against rat MLC1 in combination with expression of human wild-type MLC1 (E). (F) Average regulatory volume decrease decay time constants of all lymphoblasts used for A (control 1, red; control 2, grey; control 3, black; Patient 1, dark green; Patient 2, light green) are indicated on the left. Average regulatory volume decrease decay time constants of all astrocytes used for B (non-targeted small interfering RNA, dark blue; small interfering RNA against rat MLC1, purple; small interfering RNA against rat MLC1 in combination with expression of human wild-type MLC1, orange) are indicated on the right. Statistically significant differences are indicated by asterisks (***P < 0.01).
suggests that MLC1 has its most important role during the process of myelin deposition. This conclusion is in line with another observation that all myelin-producing organisms have the MLC1 gene, whereas organisms that do not produce myelin do not (Boor et al., 2005).

Brain ion channel defects have been associated with epilepsy syndromes, migraine, dyskinesia and episodic ataxia (Cannon, 2006). There is no known human leucoencephalopathy related to ion channel dysfunction. There is also no known human disease caused by a defect in a chloride channel involved in cell volume regulation. The CLCN2-knockout mouse, however, has a leucoencephalopathy. CLCN2 encodes the chloride channel CIC-2, which, like MLC1, is localized in the brain in astrocytic endfeet lining blood vessels (Blanz et al., 2007). Remarkably, CLCN2-knockout mice display widespread cerebral white matter oedema and intramyelinic vacuole formation, similar to what is seen in the brain of human patients with MLC (van der Knaap et al., 1996; Blanz et al., 2007). Because of the striking similarities between patients with MLC and the CLCN2-knockout mice, CLCN2 was investigated as a possible candidate gene for MLC, but CLCN2 mutations were not found among patients with MLC without MLC1 mutations (Scheper et al., 2010). CIC-2 has a more widespread expression than MLC1 and CLCN2-knockout mice have involvement of other organs than the brain (Blanz et al., 2007). MLC1 is only present in leucocytes and the brain (Boor et al., 2005; Duarri et al., 2008), but patients only have a brain disease. This exclusive brain involvement is most likely explained by the much higher MLC1 expression in the brain than in leucocytes (Supplementary Table 2).

MLC is characterized by chronic cerebral white matter oedema and slow neurological deterioration. The fact that the CLCN2-knockout mice also display widespread cerebral white matter oedema and intramyelinic vacuole formation supports our conclusion that a disturbance of volume-regulated chloride channel activity underlies the white matter disease in MLC. At this point we do not know how a defect in cell volume regulation leads to water accumulation within astrocytic endfeet and myelin sheaths during myelination and to delayed-onset neurological deterioration. A better understanding of disease mechanisms is essential for the development of treatment. The transient MLC phenotype of the patients with a dominant GLIALCAM mutation suggests that there is a window of time, in which rescue of MLC1 function may prevent the disease or modify its course.

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Supplementary material

Supplementary material is available at Brain online.

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