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Brain white matter oedema due to ClC-2 chloride channel deficiency: an observational analytical study

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Summary

Background **Mutant mouse models suggest that the chloride channel ClC-2 has functions in ion and water homoeostasis, but this has not been confirmed in human beings. We aimed to define novel disorders characterised by distinct patterns of MRI abnormalities in patients with leukoencephalopathies of unknown origin, and to identify the genes mutated in these disorders. We were specifically interested in leukoencephalopathies characterised by white matter oedema, suggesting a defect in ion and water homoeostasis.**

Methods **In this observational analytical study, we recruited patients with leukoencephalopathies characterised by MRI signal abnormalities in the posterior limbs of the internal capsules, midbrain cerebral peduncles, and middle cerebellar peduncles from our databases of patients with leukoencephalopathies of unknown origin. We used exome sequencing to identify the gene involved. We screened the candidate gene in additional patients by Sanger sequencing and mRNA analysis, and investigated the functional effects of the mutations. We assessed the localisation of ClC-2 with immunohistochemistry and electron microscopy in post-mortem human brains of individuals without neurological disorders.**

Findings **Seven patients met our inclusion criteria, three with adult-onset disease and four with childhood-onset disease. We identified homozygous or compound-heterozygous mutations in *CLCN2* in three adult and three paediatric patients. We found evidence that the *CLCN2* mutations result in loss of function of ClC-2. The remaining paediatric patient had an X-linked family history and a mutation in *GJB1*, encoding connexin 32. Clinical features were variable and included cerebellar ataxia, spasticity, chorioretinopathy with visual field defects, optic neuropathy, cognitive defects, and headaches. MRI showed restricted diffusion suggesting myelin vacuolation that was confined to the specified white matter structures in adult patients, and more diffusely involved the brain white matter in paediatric patients. We detected ClC-2 in all components of the panglial syncytium, enriched in astrocytic endfeet at the perivascular basal lamina, in the glia limitans, and in ependymal cells.**

Interpretation **Our observations substantiate the concept that ClC-2 is involved in brain ion and water homoeostasis. Autosomal-recessive *CLCN2* mutations cause a leukoencephalopathy that belongs to an emerging group of disorders affecting brain ion and water homoeostasis and characterised by intramyelinic oedema.**

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Introduction

ClC-2 is a chloride channel that is almost ubiquitously expressed in the human body.[1] It is present in plasma membranes and is activated by hyperpolarisation, acidic extracellular pH, and osmotic cell swelling.[1–4] Its functions in human physiology are a matter of debate. Roles in gastric acid secretion, lung development and function, and nephronogenesis have been suggested, but not confirmed.[1,5] Information about the localisation of ClC-2 within the brain comes from rodent studies, which have detected ClC-2 in oligodendrocytes, astrocytes (especially in endfeet), and pyramidal and non-pyramidal neurons in the hippocampus.[6,7] Evidence suggests that ClC-2 modulates postsynaptic responses to GABA by affecting intracellular chloride concentration in neurons[8] and that ClC-2 regulates neuronal excitability,[9,10] but the physiological relevance of these findings has not been confirmed.

Physiological roles of proteins can be gleaned from diseases caused by mutations in their genes. No human disease has been unequivocally related to mutations in *CLCN2*, the gene encoding ClC-2. Heterozygous mutations in *CLCN2* were described as a cause of idiopathic generalised epilepsies,[11–17] but this finding was later refuted[1,18] and the original paper was retracted.[17] Because mutant mice without functional ClC-2 have a leukoencephalopathy with intramyelinic oedema,[5,6,19] ClC-2 was proposed to have a role in brain ion and water

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homoeostasis;[5,6] however, this hypothesis has not been confirmed in human beings. A known human disorder characterised by intramyelinic oedema is megalencephalic leukoencephalopathy with subcortical cysts.[20–22] Mutations in *MLC1* have been identified in 75% of patients with this disorder.[23,24] No mutations in *CLCN2* were reported in the patients without *MLC1* mutations.[25] Recently, mutations in *HEPACAM* (also called *GLIALCAM*), which encodes the glial cell adhesion molecule (GlialCAM)—a putative auxiliary subunit of both ClC-2 and MLC1[26,27]—were identified in patients without *MLC1* mutations.[26]

We aimed to study patients with leukoencephalopathies of unknown origin to define novel disorders characterised by distinct patterns of MRI abnormalities[28] and identify the mutated genes. We were specifically interested in leukoencephalopathies with MRI evidence of intramyelinic oedema. When we identified *CLCN2* mutations in a group of such patients, we sought further evidence for ClC-2's function in human physiology.

Methods

Study design and patients

In this observational analytical study, we used MRI criteria to recruit adult and paediatric patients from the Amsterdam and Paris leukoencephalopathy databases containing MRI images of patients with leukoencephalopathies of unknown origin submitted from all over the world. Patients with prominent signal abnormalities in the middle cerebellar peduncles, midbrain cerebral peduncles, and posterior limbs of the internal capsules were included in the study.

We studied the MRI results of each patient and scored the appearance and distribution of signal abnormalities. We assessed apparent diffusion coefficient (ADC) values in areas of abnormal signal. Signal intensity on T_2-weighted and T_1-weighted images and ADC values provide information on tissue microstructure. High ADC values indicate large water spaces; low ADC values indicate small water spaces. Because the study was multi-institutional, MRIs were done with different pulse sequences on machines from different vendors. Therefore, we used a robust cutoff ADC value of 60×10^{-5} mm²/s, below which the diffusion was considered to be restricted. Subtle diffusion restriction could, therefore, have been missed.

We obtained approval from the institutional review boards of the participating centres in Paris and Amsterdam (Hôpital Pitié-Salpêtrière and VU University Medical Centre) and informed consent from patients and their families.

Procedures

Exome sequencing was undertaken in the three patients with adult-onset disease under the assumption that these patients had one disease with autosomal recessive inheritance. In patient 3, who has consanguineous parents, we identified homozygous regions (appendix), in which we searched for homozygous mutations. One paediatric patient came from a family with X-linked disease inheritance, and this family participated in another exome sequencing study focused on X-linked disorders. We then used Sanger sequencing to sequence the *CLCN2* gene in both the adult and paediatric patients; *GJB1,* which encodes the gap junction beta-1 protein (also known as connexin 32), was sequenced in the male patient without *CLCN2* mutations (appendix).

CLCN2 mRNA was quantified in fibroblasts of adult patients with *CLCN2* mutations. Transient transfections of wild-type or mutant ClC-2-V5-His6 expression plasmids in COS7 cells were used to study the functional effects of *CLCN2* mutations (appendix).

To define which cell types express ClC-2 in healthy mature human brain, we examined the posterior limb of the internal capsule, frontal white matter, frontal cortex, and ependyma with fluorescence immunohistochemistry of tissue obtained at autopsy from nine patients aged 1–75 years (appendix) who succumbed to heart disease or extracerebral neoplasm. None of the patients had neurological symptoms and neuropathological examination showed no abnormalities.

Immunohistochemistry (appendix) was done on frozen tissue using antibodies against ClC-2, GlialCAM, MLC1, glial fibrillary acidic protein (GFAP; an astrocyte marker), oligodendrocyte transcription factor-2 (OLIG2; an oligodendrocyte marker), neuronal nuclear antigen (NeuN; a neuronal marker, also known as RBFOX3), platelet endothelial cell adhesion molecule-1 (PECAM1; an endothelial marker, also known as CD31), and phosphorylated and non-phosphorylated neurofilament H (SMI31 and SMI32, respectively; axonal markers). The anti-ClC-2 antibodies were validated for specificity (appendix). All sections were counterstained with DAPI (4□,6-diamidino-2-phenylindole; nuclear staining).

Electron microscopy was done on tissue from the posterior limb of the internal capsule (appendix). Sections were incubated with ClC-2, GlialCAM, and MLC1 antibodies.

Role of the funding source

The sponsors of the study had no role in study design, data collection, data analysis, data interpretation, or writing of the report. The corresponding authors had full access to all the data in the study and had final responsibility for the decision to submit for publication.

Results

Seven patients met our inclusion criteria, three with adult-onset disease and four with childhood-onset disease. The three patients with adult-onset disease were unrelated (patients 1–3; table 1). They had mild cerebellar ataxia and a variable combination of chorioretinopathy with visual field defects, optic neuropathy, and headaches. One patient had a schizophrenia-like disorder. The three patients with childhood-onset disease and *CLCN2*

See Online for appendix

mutations (patients 4–6) had mild cerebellar ataxia and a variable combination of mild spasticity, visual field defects, learning disabilities, and headaches (table 1).

The patient with X-linked disease (patient 7; table 1) presented with nystagmus at 12 months. He developed incapacitating cerebellar ataxia and spasticity. Clinical evidence of peripheral nerve involvement was absent, although recent neurophysiological investigations revealed mild motor neuropathy. Affected male family members had the same clinical and MRI findings. Carrier females had no abnormalities.

All patients had prominent signal abnormalities and decreased ADC values in the posterior limbs of the internal capsules, cerebral peduncles in the midbrain, pyramidal tracts in the pons, and middle cerebellar peduncles (figure 1; appendix). All patients with adult-onset disease had additional signal abnormalities in specific brainstem tracts and the cerebellar white matter (appendix). Cerebral hemispheric white matter abnormalities were mild and non-specific. In one patient (patient 1), low to borderline-low ADC values were also found in cerebral white matter areas (appendix).

In the paediatric patients, the same additional signal abnormalities in specific brainstem tracts and cerebellum were noted. Additionally, they had diffuse mild signal abnormality of the brain white matter, which was hyperintense relative to grey matter on T_2-weighted and T_1-weighted images (figure 1). Low to borderline-low ADC values were present in the cerebral and cerebellar hemispheric white matter and corpus callosum. The signal behaviour of the white matter on T_1-weighted and T_2-weighted images would typically suggest hypomyelination,[29] but hypomyelination is associated with increased size of water spaces and therefore increased ADC values.[30] The low white matter ADC suggests myelin microvacuolation instead of hypomyelination.[31] Increased intramyelinic water accounts for the mild T_2-signal changes.

As a result of exome sequencing and homozygosity mapping in patient 3, we identified ten homozygous single nucleotide substitutions and two homozygous insertion–deletions with a possible effect on gene or protein function that were absent from control databases (appendix). One was a homozygous in-frame deletion (p.Leu144_Ile145del) in *CLCN2*. Analysis of exome data

	Patient 1	Patient 2	Patient 3	Patient 4	Patient 5	Patient 6	Patient 7
Year of birth	1948	1952	1961	1996	1998	2001	1993
Sex/ancestry	f/north Africa	f/north Africa	f/north Africa	f/Europe	m/Europe	f/Europe	m/Europe
Consanguineous parents	No	No	Yes	Not known	No	Yes	No
Affected family members	No	No	No	No	No	No	Yes
Early psychomotor development	Normal	Normal	Normal	Normal	Normal	Normal	Normal, but has never walked without support
Age at first sign	44 years	57 years	30 years	12 years	6 years	3 years	12 months
Presenting signs	Action tremor, mild gait ataxia	Tinnitus, vertigo	Chorioretinopathy, psychosis	Learning disability, headache	Headache	Action tremor, mild gait ataxia	Nystagmus
Disease course	Stable	Progressive	Stable	Stable	Stable	Stable	Slowly progressive
Signs of the deterioration	None	Deafness	None	None	None	None	Motor deterioration
Headache	No	No	Yes	Yes, severe	Yes, severe	No	No
Cognitive level	Normal	Normal	Severe learning disability	Mild learning disability	Normal	Mild learning disability	Mild learning disability
Head circumference	Normal	Normal	Normal	Normal	Normal	Borderline macrocephaly	Borderline macrocephaly
Vision	Normal	20/80 right eye; 20/400 left eye	1/10 right eye; 1/50 left eye	Normal with glasses	Normal with glasses, strabismus	Normal	Normal
Visual field defects	No	Yes	Yes	Yes	No	Yes	No
Retina and optic nerve	Normal	Retinoschisis, bilateral optic neuropathy	Retinal atrophy, choroidal neovascularisation, bilateral optic neuropathy	Normal	Normal	Normal	Normal
Nystagmus	No	No	No	No	Yes	No	Yes
Hearing	Normal	Perceptive hearing loss	Normal	Normal	Normal	Normal	Normal
Spasticity	No	No	No	Yes, mild	No	Yes, mild	Yes, prominent
Ataxia	Yes, mild	Yes, mild	Yes, mild	Yes, mild	Yes, mild	Yes, mild	Yes, severe
Peripheral polyneuropathy	No	No	No	No	No	No	No clinical signs; mildly decreased motor NCV
Gait	Unstable	Unstable	Unstable	Unstable	Normal	Unstable	Wheelchair dependent

f=female. m=male. NCV=nerve conduction velocity.

Table 1: **Clinical findings**

in the other adult patients revealed the same homozygous non-sense mutation (p.Trp570X) in *CLCN2* (table 2; appendix). *CLCN2* was the only gene that contained at least one homozygous or at least two heterozygous, possibly deleterious variants in all three patients that were absent from exome data from individuals with other diseases analysed at the same time (appendix).

Analysis of the coding sequence of *CLCN2* by Sanger sequencing in the paediatric patients identified homozygous or compound-heterozygous *CLCN2* mutations in three patients (table 2; appendix). All mutations were predicted to be pathogenic and none was present in healthy controls of the same origin or in the International Hapmap Project and the 1000 Genomes Project databases (appendix).

Sequencing of the exome of the paediatric patient without *CLCN2* mutations who had an X-linked family history revealed a novel missense mutation (p.Pro174Ser) in *GJB1* (table 2; appendix). This mutation was confirmed in his mother and in one living similarly affected male family member.

Quantification of the *CLCN2* mRNA in fibroblasts from patients and controls by real-time quantitative PCR showed that the mRNA with the p.Trp570X mutation was substantially downregulated compared with that of controls, by contrast with the mRNA with p.Leu144_Ile145del (appendix). Treatment with emetine, an inhibitor of non-sense mediated decay, rescued the mRNA expression, suggesting that p.Trp570X leads to degradation of the mutant mRNA, although a substantial fraction of the mRNA subsists. We could not confirm the presence of the truncated protein because expression of ClC-2 was undetectable in fibroblasts. Transient expression of the protein with the p.Trp570X mutation in COS7 cells showed that the truncated protein, if expressed, has an aberrant localisation restricted to the Golgi apparatus (appendix).

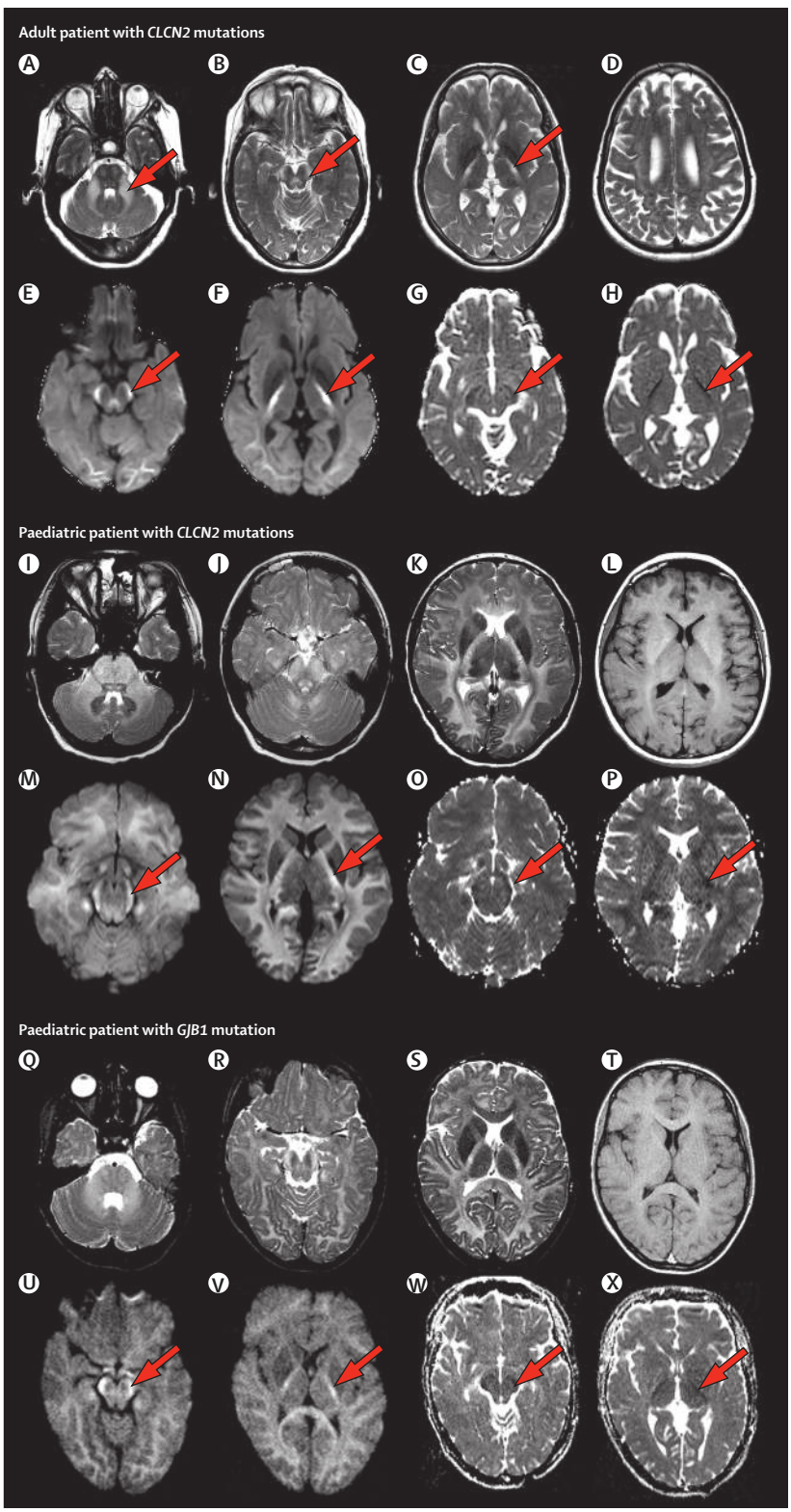

Figure 1: **MRI of brain in patients 1, 4, and 7**
MRI in patient 1 at the age of 63 years (A–H) shows signal abnormalities on T_2-weighted images (A–D) in the middle cerebellar peduncles (arrow in A), midbrain cerebral peduncles (arrow in B), and posterior limbs of the internal capsules (arrow in C). Slight signal abnormalities are present in the cerebral white matter (D). Diffusion-weighted images (E, F) show high signal in the midbrain cerebral peduncles (arrow in E), posterior limbs of the internal capsules (arrow in F), and to a lesser extent the posterior subcortical cerebral white matter (compare hyperintense posterior white matter to normal anterior white matter in E and F), suggesting restricted diffusion, confirmed by low ADC values on the ADC maps (low signal in G and H, arrows). MRI in patient 4 at the age of 14 years (I–P) shows diffuse mild T_2-hyperintensity (I–K) and T_1-hyperintensity (L) of all white matter structures. Prominent T_2-hyperintensity is seen in the basis pontis (I), middle cerebellar peduncles (I), midbrain cerebral peduncles (J), and posterior limbs of the internal capsules (K). Diffusion-weighted images (M, N) show increased signal in virtually all white matter, especially the midbrain cerebral peduncles (arrow in M) and posterior limbs of the internal capsules (arrow in N), suggesting restricted diffusion, confirmed by low ADC values on the ADC maps (arrows in O and P). MRI in patient 7 at the age of 14 years (Q–X) shows diffuse mild T_2-hyperintensity (Q–S) and T_1-hyperintensity (T) of all white matter structures. Prominent T_2-hyperintensity is seen in the middle cerebellar peduncles (Q), midbrain cerebral peduncles (R), and posterior limbs of the internal capsules (S). Diffusion-weighted images (U, V) show mildly increased signal in almost all white matter, especially the midbrain cerebral peduncles (arrow in U) and posterior limbs of the internal capsules (arrow in V), suggesting restricted diffusion, confirmed by low ADC values on the ADC maps (arrows in W and X).

	Exon	DNA	RNA	Protein	Genotype	Confirmation in parents*
***CLCN2* mutation**						
Patient 1	15	c.709G→A	..	p.Trp570X	Homozygous	..
Patient 2	15	c.1709G→A	..	p.Trp570X	Homozygous	..
Patient 3	4	c.430_435del	..	p.Leu144_Ile145del	Homozygous	..
Patient 4	11; 2 to part of 6	c.1143delT; c.64–1107_639del	r.1143delt; r.64_639delins82†	p.Gly382AlafsX34; p.Met22LeufsX5	Heterozygous; heterozygous	..
Patient 5	14	c.1499C→T	r.1499c→t	p.Ala500Val	Homozygous	Father and mother
Patient 6	8	c.828dupG	..	p.Arg277AlafsX23	Homozygous	Father and mother
***GJB1* mutation**						
Patient 7	2	c.520C→T	..	p.Pro174Ser	Hemizygous	Mother

*This column shows whether the mutation was confirmed in the father and mother of the affected patient. †Comprises a deletion of exons 2–5 and part of exon 6 and the insertion of intron 1 c.63+1097_64–1108.

***Table 2: CLCN2* and *GJB1* mutations**

The p.Leu144_Ile145del and p.Ala500Val mutations are thought to affect conserved hydrophobic aminoacids located in transmembrane domains of ClC-2, possibly leading to protein misfolding or misinsertion in the membrane. To test this hypothesis, we compared the expression and subcellular localisation of V5-His6-tagged ClC-2 with the p.Leu144_Ile145del or p.Ala500Val mutation to those of the wild-type protein in COS7 cells. Localisation of the p.Leu144_Ile145del ClC-2 and p.Ala500Val ClC-2 proteins was restricted to the endoplasmic reticulum, whereas wild-type ClC-2 was present in different subcellular compartments including the plasma membrane (figure 2A). Additionally, the amount of both mutated ClC-2 proteins transiently expressed in COS7 cells was lower than the amount of wild-type ClC-2 (figure 2B) and the mutated proteins hardly reached the plasma membrane (figure 2C and 2D). These findings suggest that p.Leu144_Ile145del ClC-2 and p.Ala500Val ClC-2 are trapped and degraded in the endoplasmic reticulum. Altogether, these results suggest that all mutations identified in the patients led to a complete or at least partial loss of function of the ClC-2 channel by different mechanisms.

Immunohistochemistry of the healthy brain samples revealed that virtually all GFAP-positive fibrous astrocytes in the posterior limb of the internal capsule and frontal white matter express ClC-2 on the surface of cell bodies and processes (figure 3A). ClC-2 immunopositivity had a fine punctate quality (figure 3A, inset), as expected for a membrane protein. Many ClC-2-positive astrocytic processes ran parallel and perpendicular to axonal bundles (figure 3A) and abutting axons (appendix). ClC-2 was enriched in perivascular astrocytes (figure 3B), where GlialCAM (figure 3C; appendix) and MLC1 (appendix) were also expressed. GlialCAM and MLC1 immunoreactivity overlapped consistently and extended further distally than ClC-2 into astrocytic endfeet approaching the basal lamina (figure 3C; appendix). A more discrete, punctate ClC-2 immunoreactivity was visible intermittently along axons (appendix). We detected many ClC-2-positive and GFAP-negative cells with the morphology of oligodendrocytes. In the frontal cortex, ClC-2 expression was detected only in scattered protoplasmic astrocytes around blood vessels and in astrocytes that are part of the glia limitans (figure 3D; appendix). In these locations, ClC-2 astrocytes coexpressed GlialCAM (figure 3E; appendix) and MLC1 (appendix). ClC-2 expression was also detected in the ependymal lining, again overlapping with GlialCAM (figure 3F; appendix) and MLC1 (appendix). No ClC-2-positive expression was noted in neuronal perikarya (figure 3G).

Electron microscopy confirmed that ClC-2 was abundantly present in white matter astrocytes and enriched in cell processes contacting abaxonal myelin (figure 4A) and contacting each other (figure 4B). At these sites, GlialCAM showed a similar distribution (figure 4C, and 4D). ClC-2 immunoreactivity was also visible inside axons and at the axonal surface contacting adaxonal myelin (appendix). By contrast, GlialCAM and MLC1 were not detected in axons or myelin (figure 4C). Around blood vessels, ClC-2 was found at astrocyte–astrocyte contacts in endfeet (figure 4B), a site where GlialCAM (figure 4D) and MLC1 (figure 4E) were present.

Discussion

We showed that autosomal recessive loss-of-function mutations in *CLCN2* cause a leukoencephalopathy with MRI evidence of myelin microvacuolation. These findings are similar to those in ClC-2-deficient mice and are in line with the notion that ClC-2 in the brain is involved in ion and water homoeostasis (panel).[5,6]

Our observations do not support the hypothesis that loss-of-function mutations in *CLCN2* confer an increased risk of epilepsy. None of the six patients with homozygous or compound heterozygous mutations in *CLCN2* had epilepsy or a family history of epilepsy, suggesting that partial or complete ClC-2 loss of function per se is insufficient to cause epilepsy.

The disease associated with *CLCN2* mutations is variable in age of onset and clinical presentation. Mild

cerebellar ataxia is the most consistent finding. Retinopathy, as reported in the ClC-2-deficient mice, was detected in only some of the patients, although the visual field defects in absence of other ophthalmological findings suggest that the other patients might have had a retinopathy that was too subtle to be picked up at funduscopy. ClC-2-deficient mice are sterile because of testicular degeneration; however, the only male patient with *CLCN2* mutations (aged 15 years) did not yet show signs of testicular degeneration. Although the clinical presentation of the patients is non-specific and does not allow a diagnosis, MRI findings in these patients are highly specific and do allow a diagnosis. We have also analysed the *CLCN2* gene in several other adult and paediatric patients who had similar MRI findings to those of study patients, but did not meet the MRI inclusion criteria, and we did not find *CLCN2* mutations in any of them (data not shown).

The phenotypic variability reported in the patients might at least in part be related to the extent of ClC-2 loss of function. Both the mutational variation and functional analyses suggest loss of function of ClC-2, but some

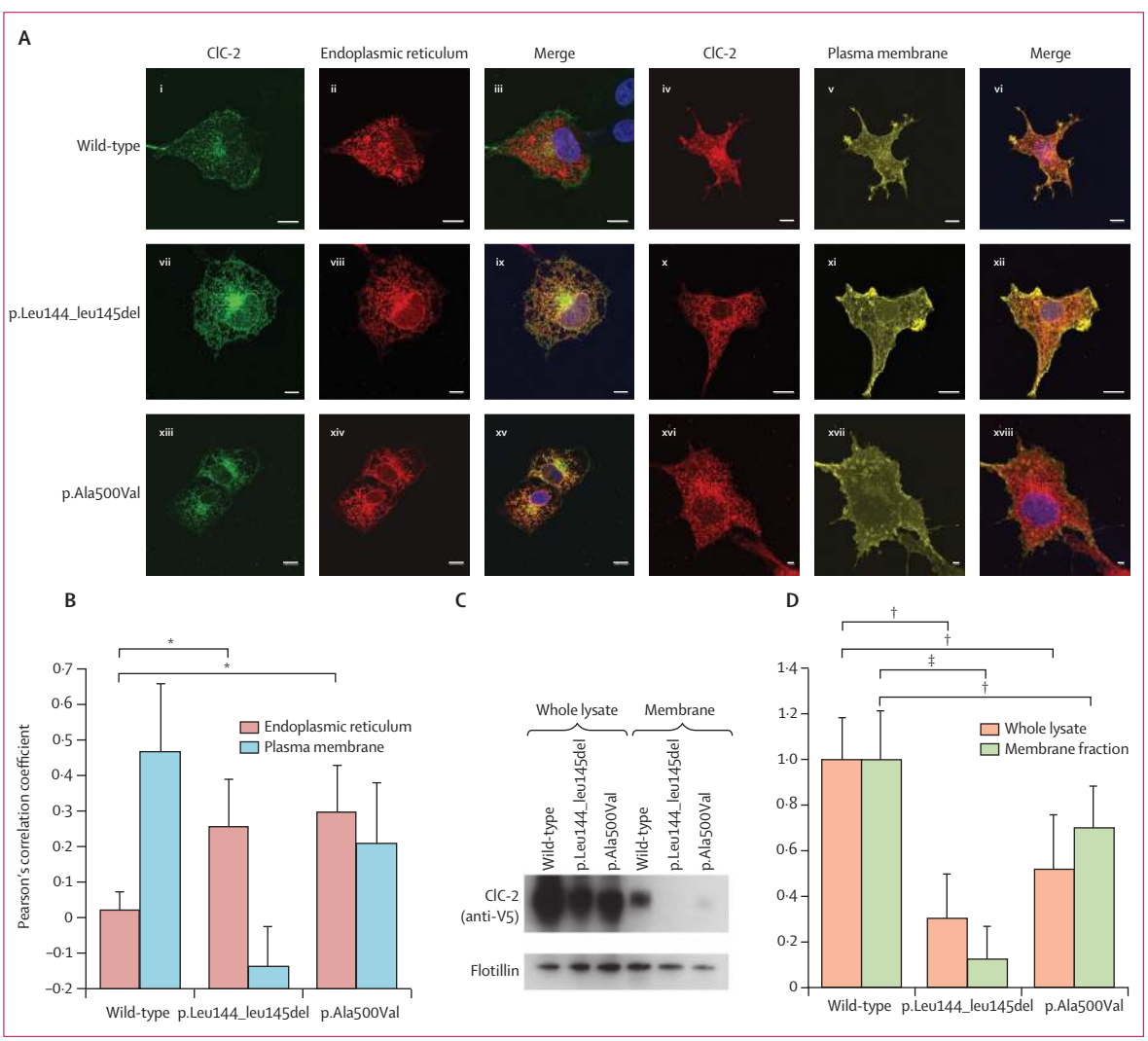

Figure 2: **Expression and subcellular localisation of wild-type and mutated ClC-2**
(A) Subcellular localisation of wild-type (i–vi) and mutated (p.Leu144_Ile145del [vii–xii]; p.Ala500Val [xiii–xviii]) ClC-2 proteins and colocalisation with markers of endoplasmic reticulum (anticalreticulin, red, [ii, viii, xiv]) or plasma membrane (pEYFP-Mem, yellow [v, xi, xvii]) by confocal microscopy. Note that wild-type ClC-2 is present at the plasma membrane and different subcellular compartments, whereas both mutant proteins are mainly seen in the endoplasmic reticulum. Scale bar=10 μm. (B) Quantification of colocalisation of wild-type or mutated ClC-2 with endoplasmic reticulum and plasma membrane using Pearson's correlation coefficient, including at least three cells per group. (C) Analysis of wild-type and mutated ClC-2 protein expression in whole lysates and plasma membranes by Western blot show that the expression of mutant ClC-2 protein transiently expressed in COS7 cells is lower than that of the wild-type protein, especially in the plasma membrane. The image shows the result of a representative experiment. Flotillin was used to control and normalise the protein load. (D) Quantification of wild-type and mutant ClC-2 proteins present in whole lysates and in plasma membranes shows that, overall, mutant ClC-2 proteins are not as highly expressed as wild-type ClC-2, suggesting that they are misfolded and unstable. By contrast with the wild-type form, both mutants are retained in the endoplasmic reticulum where they are probably degraded and hardly reach the plasma membrane. The values, obtained from at least three different experiments, were compared with the Mann-Whitney test. *p<0·05. †p<0·01. ‡p<0·001.

mutated proteins, in particular C-terminally truncated or missense mutants that reach the plasma membrane in substantial amounts, could possibly retain a partial function.

The present study adds to the growing insight into how the brain deals with the continuous shifts of ions and water related to action potentials. Brain white matter mainly consists of myelinated axons and its most important physiological function is impulse conduction. Action potentials are based on shifts in ions, which are obligatorily associated with osmotically driven shifts in water, needing rapid compensation. Any disturbance of the compensatory mechanisms might disrupt impulse transmission or lead to life-threatening changes in brain volume. Brain ion and water homoeostasis is complex and involves electrolytes and organic osmolytes, channels, and transporters, and coordinated processes such as regulatory volume decrease and increase to correct cell volume changes.[32,33] Astrocytes are central in this process. They are the most abundant and best connected cell type of the so-called panglial syncytium, a vast network of astrocytes, oligodendrocytes, and ependymal cells interconnected by gap junctions. The panglial syncytium is essential for long-distance disposal of ions and water.[32,33]

Action potentials are associated with disposal of submyelinic and intramyelinic potassium.[33] In myelinated axons, depolarisation is associated with influx of sodium at nodes of Ranvier; compensatory potassium efflux occurs in paranodal regions covered by myelin (appendix). Gap junctions help to transport potassium and osmotic water across myelin layers into astrocytes. Connexin 32 constitutes gap junctions in paranodal myelin; connexin 32 and connexin 47 constitute gap junctions between myelin and astrocytes. The astrocytic syncytium allows rapid dispersion of potassium and buffers the associated volume changes. This process of so-called potassium siphoning prevents action-potential-induced osmotic intramyelinic oedema.[33] Disruptions of molecular components of these ion and water homoeostatic pathways are associated with intramyelinic oedema in human diseases and mutant mouse models.[6,19,33–35] No myelin vacuolation occurs in mouse models in the absence of action potentials,[6,34] showing that hindrance of compensation of action-potential-induced ion and water shifts is the main factor leading to osmotic intramyelinic oedema.

Insight into the mechanisms and molecular components of brain ion and water homoeostatic pathways is still incomplete. Our study provides evidence that ClC-2 is one of these molecular components. The first observation that supports a role of ClC-2 in brain ion and water homoeostasis is the MRI evidence of myelin microvacuolation in patients with *CLCN2* mutations. The second observation is the presence of the same MRI abnormalities with evidence of myelin microvacuolation in a patient with a *GJB1* mutation. Mutations in *GJB1,* encoding connexin 32, lead to X-linked Charcot-Marie-Tooth disease. In addition to peripheral neuropathy, patients with X-linked Charcot-Marie-Tooth disease might have episodes of brain dysfunction, with MRI

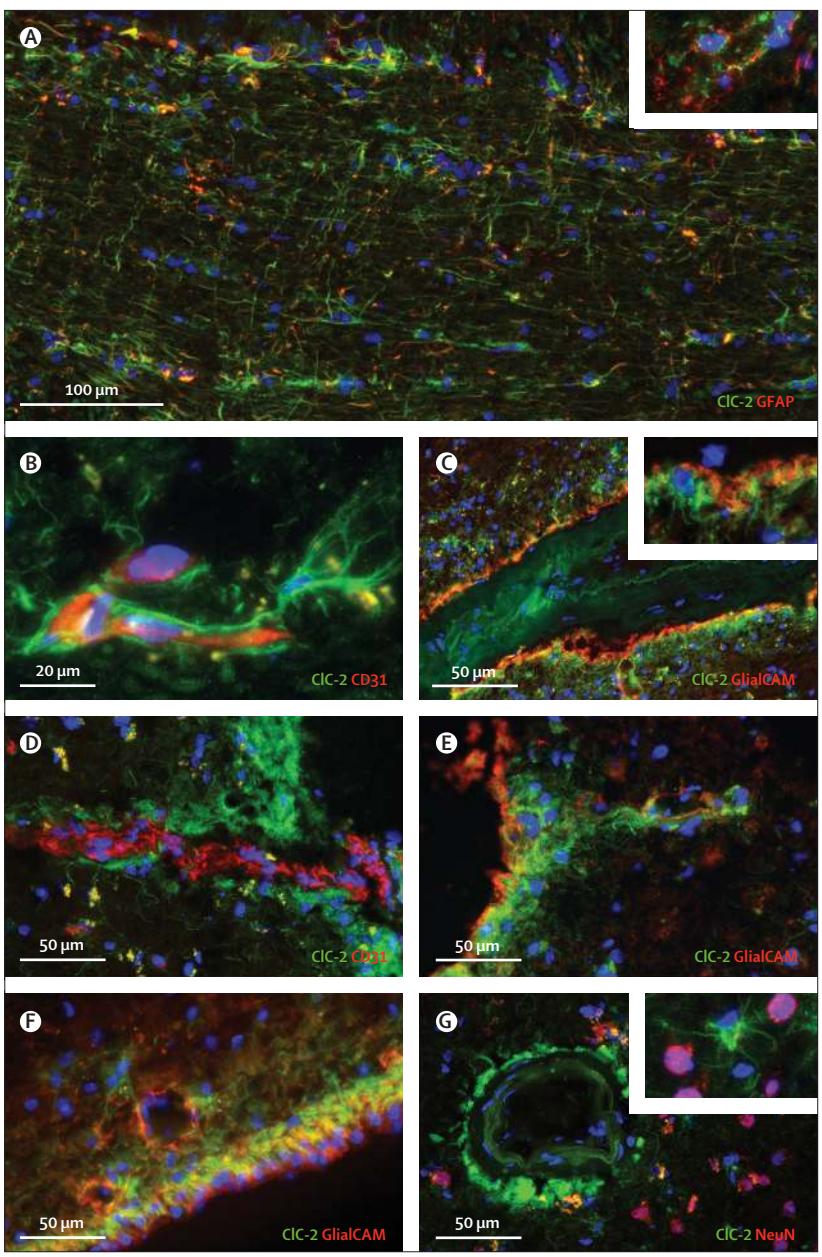

Figure 3: **Expression of ClC-2 in a healthy adult human brain**
Double immunohistochemical staining of the cerebral white matter for ClC-2 (green) and the astrocyte-specific marker GFAP (red) shows that all GFAP-positive astrocytes express ClC-2 (A). Note the long ClC-2-positive astrocytic processes extending parallel and perpendicular to the direction of the fibre bundle. ClC-2 shows a punctate immunoreactivity, as expected for a membrane protein (inset in A). Colabelling of the same tissue for ClC-2 (green) and the vascular endothelial cell marker CD31 (also known as PECAM1; red) shows that expression of ClC-2 is enhanced in the astrocytes surrounding the blood vessels deep in the parenchyma (B) and the penetrating vessels at the surface of the brain (D). Note the many fine astrocytic processes running along the white matter capillary (B). Perivascular astrocytes (C), subpial astrocytes (E), and ependymal cells (F) coexpress GlialCAM (red) with ClC-2 (green), but GlialCAM immunoreactivity extends further into the astrocytic processes and endfeet (inset in C). Double staining of the frontal cortex for ClC-2 (green) and the neuronal marker NeuN (red) shows that, in this location, ClC-2 immunoreactivity is restricted to perivascular astrocytes (G). No ClC-2 expression is seen in the NeuN-positive neuronal cells (inset in G). In all images the cell nuclei are stained with DAPI (4',6-diamidino-2-phenylindole; blue).

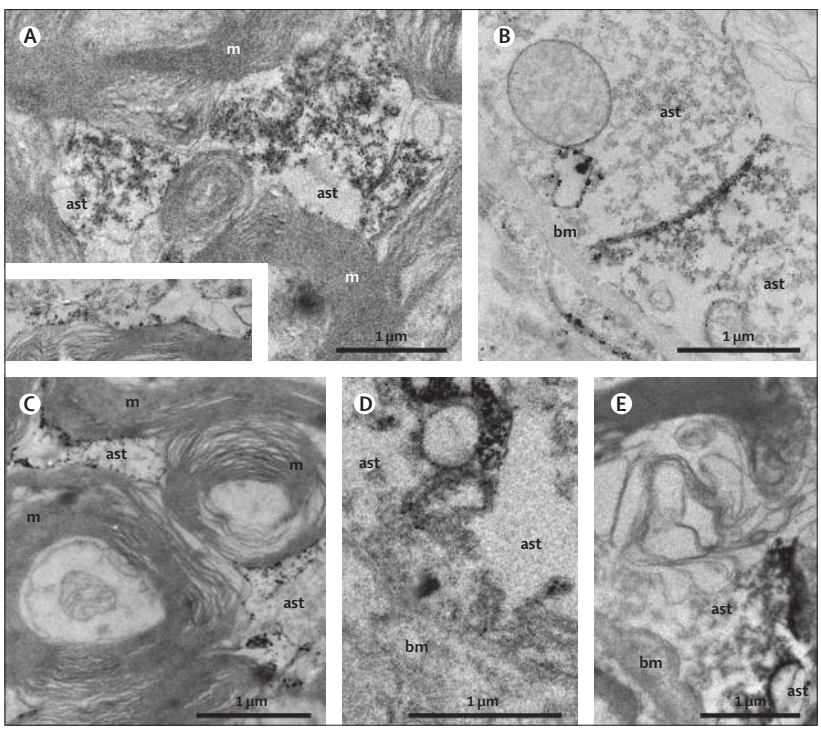

Figure 4: **ClC-2, GlialCAM, and MLC1 immunoreactivity in healthy adult white matter**
By use of electron microscopy, expression of ClC-2 is detected in astrocytic processes in between myelinated axons (A) and in contact with abaxonal myelin (inset in A). A similar localisation is observed for GlialCAM (C). Intense ClC-2 immunoreactivity is present in astrocytic endfeet surrounding blood vessels (B). Note the enhanced ClC-2 expression in the astrocyte–astrocyte contacts of the endfeet (B). At this site, GlialCAM (D) and MLC1 (E) are also expressed. ast=astrocyte. bm=basement membrane. m=myelin.

evidence of transient myelin vacuolation.[36] We have no explanation for why the mutation detected in the family that we studied has a persistent effect on brain myelin, without any fluctuation, both clinically and on serial MRI. The serious spasticity and cerebellar ataxia probably overshadow the neuropathy, which was only detected by neurophysiological studies after the *GJB1* mutation had been identified. Other cases of X-linked Charcot-Marie-Tooth disease with persistent clinical signs of brain dysfunction and white matter disease on MRI have been reported, but diffusion findings were not mentioned.[37] The third observation supporting the role of ClC-2 in brain ion and water homoeostasis is the ClC-2 membrane expression in the panglial syncytium, with enhanced expression around blood vessels, in the glia limitans, ependymal lining, and astrocyte–astrocyte contacts in the white matter. The fourth observation is the finding that ClC-2 colocalises with MLC1 and GlialCAM in astrocytic endfeet at the perivascular basal lamina and glia limitans.[26,27,38] As recently reviewed,[22] both *GLIALCAM* and *MLC1* mutations cause megalencephalic leukoencephalopathy with subcortical cysts,[23,26] a disease characterised by myelin vacuolation in the cerebral white matter, in which the corpus callosum, internal capsule, brainstem, and cerebellum are spared in comparison.[20,21] MLC1 is involved in chloride currents necessary for the astrocytic regulatory volume decrease after cell swelling.[39] GlialCAM is indispensable for correct localisation of MLC1 and ClC-2.[26,27] Results of our study suggest that ClC-2 is part of the same ion and water homoeostatic pathways as MLC1 and GlialCAM (appendix).

A limitation of our study is the rather small number of patients. The database on unclassified leukoencephalopathies in Amsterdam contains more than 3000 patients, but only a few patients fulfilled the MRI criteria. It could be that the ClC-2-related leukoencephalopathy is very rare, but we cannot exclude the possibility that the phenotypic variation is much wider and that we have not analysed a sufficient number of patients yet. Another issue is the specificity of the antibody against human ClC-2. To provide full proof, absence of all staining should be shown in brain tissue of a patient with two *CLCN2* null mutations. This tissue is not available. However, we did several experiments that support the specificity of the antibody and thus the validity of the immunohistochemical data (appendix).

Many questions remain. The exact role of ClC-2 in brain ion and water homoeostasis is unknown. Why defects in the same pathways cause different white matter disorders is unclear. ClC-2-related and connexin-32-related diseases preferentially affect white matter structures that are spared (relatively) by MLC1-related and GlialCAM-related disease. Although recessive mutations in *MLC1* and *GLIALCAM* lead to diffuse cerebral white matter oedema, sparing the corpus callosum, internal capsule, brainstem, and cerebellum, recessive mutations in *CLCN2* and *GJB1* preferentially cause white matter oedema of the internal capsule, brainstem structures, and cerebellum. Another intriguing question concerns the size of the myelin vacuoles. In MLC, ADC values in the affected white matter are highly increased,[40] suggesting large vacuoles and increased extracellular spaces, as confirmed by electron microscopy.[21] In ClC-2-related and connexin-32-related disease, ADC values in affected white matter are low, suggesting small intramyelinic vacuoles and extracellular spaces. Although GlialCAM is supposed to be a chaperone for both MLC1 and ClC-2,[26,27] recessive *GLIALCAM* mutations lead to a disease that is indistinguishable from the disease caused by *MLC1* mutations and does not share the MRI features of the disease caused by *CLCN2* mutations.[26] We noted that GlialCAM and MLC1 share restricted localisation in distal astrocytic processes, whereas ClC-2 has a more diffuse membrane localisation in astrocytes. These disease observations suggest that GlialCAM is a straightforward chaperone for MLC1, but that its relation with ClC-2 is more complex.

The growing group of known disorders affecting brain ion and water homoeostasis are typically relatively mild compared with other brain disorders, both in mice[5,6,19,35] and human beings.[20,24,26,36] The importance of the volume regulation process in the brain is emphasised by the

Panel: Research in context

Systematic review

We searched PubMed and Online Mendelian Inheritance in Man with the search terms "leukoencephalopathy", "MLC", "MLC1", "HepaCAM", "GlialCAM", "ClC-2", "CLCN2", "X-linked Charcot Marie Tooth", "connexin 32", and "GJB1", for articles in the English language published between 1969 and Dec 31, 2012. We also searched reference lists of identified papers. We studied reports on epilepsy and leukoencephalopathy related to mutations in the genes *CLCN2*, *MLC1*, *GLIALCAM*, and *GJB1*, both in mice and humans. Whole-exome sequencing is a well established and effective strategy to identify the gene mutated in several patients with the same disease with autosomal recessive and X-linked recessive inheritance. For interpretation of our molecular genetic findings we used Alamut2.1 (Interactive Biosoftware), 1000 Genomes, and HapMap databases.

Interpretation

The findings of our study, when added to those of previous work, suggest that there is no evidence for a role of *CLCN2* mutations in epilepsy, which should put a definitive end to the discussion. By contrast, following on from findings in rodents, our results show that ClC-2 is involved in brain ion and water homoeostasis in human beings. Defects in MLC1, GlialCAM, connexin 32, and ClC-2 all lead to leukoencephalopathies characterised by intramyelinic oedema. Observations in X-linked Charcot-Marie-Tooth disease caused by *GJB1* mutations, and megalencephalic leukoencephalopathy with subcortical cysts caused by dominant *GLIALCAM* mutations, show that intramyelinic oedema can be reversible. If means are found to target components of the ion and water homoeostatic pathways, this oedema could be successfully combated. Our study shows that ClC-2 is one of the molecular components, and might be a potential therapeutic target.

observation that it does not rely on just one protein or one pathway. Mutations in one protein only lead to partial and sometimes transient dysfunction. It is important to note that intramyelinic oedema can be reversible. Patients with megalencephalic leukoencephalopathy with subcortical cysts, caused by dominant *GLIALCAM* mutations, improve or recover spontaneously.[24,26] In most patients with a *GJB1* mutation, the white matter disease is transient.[36] If means are found to target components of the ion and water homoeostatic pathways, myelin oedema might be successfully combated.

Contributors

CDe, MB, CDu, NP, CvB, EP, and TEMA did the experiments or analysed the data (or both). CDe, FS, and MSvdK designed and supervised the study. CDe and MSvdK wrote the first draft of the manuscript. DG, VT, ET, FD, AB, CEdD-S, JSV, AV, GU, CY, SGF, VMK, JK, MK, and NIW contributed to patient data or contributed materials essential to the study (or both). All coauthors critically reviewed and approved the last version of the manuscript.

Conflicts of interest

We declare that we have no conflicts of interest.

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