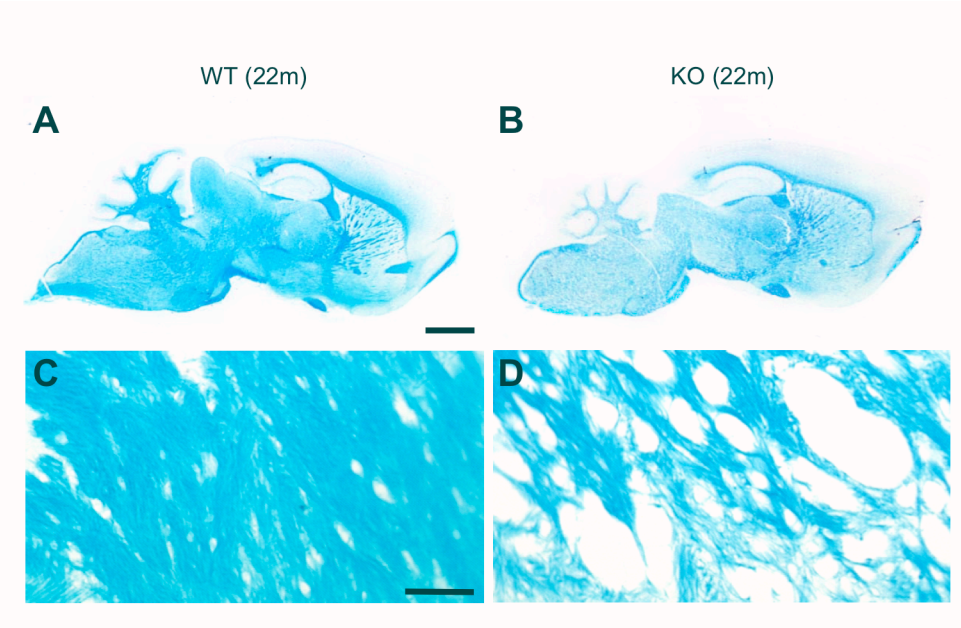
Supplementary Information

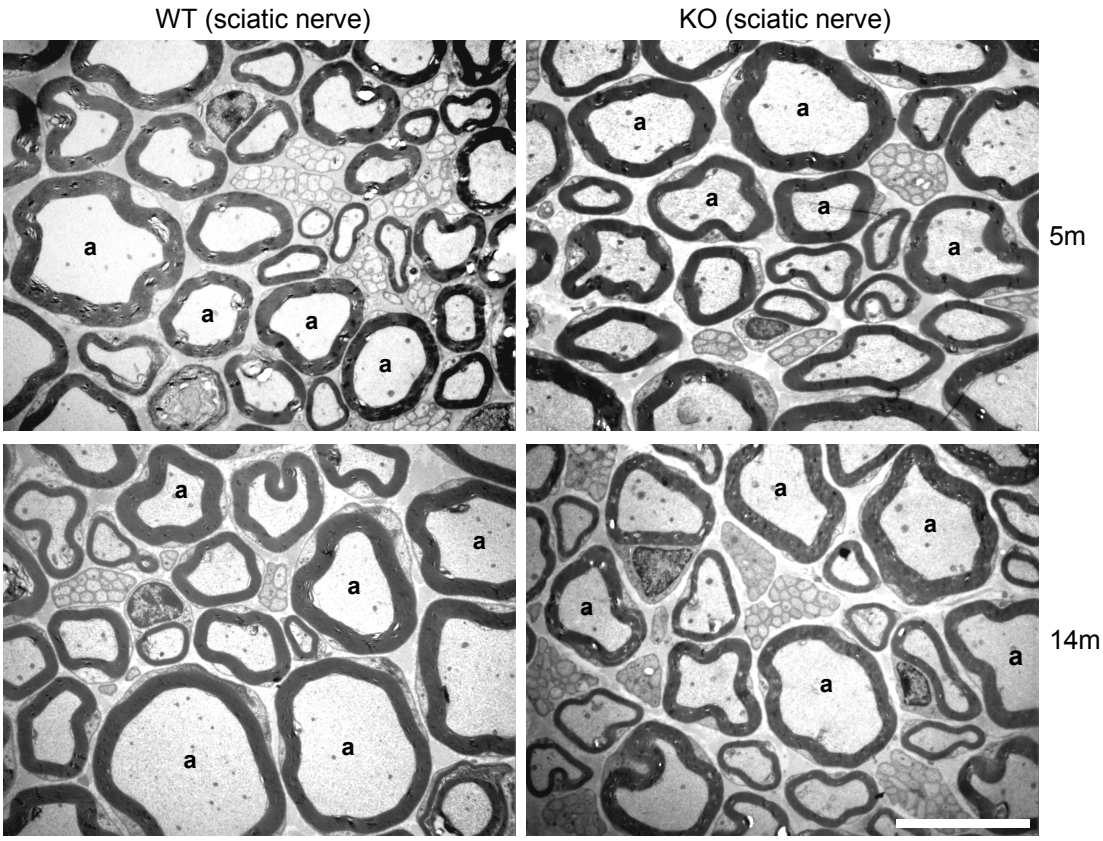
for '*Leukoencephalopathy upon disruption of the chloride channel ClC-2*' by Blanz et al.

Supplementary Figures



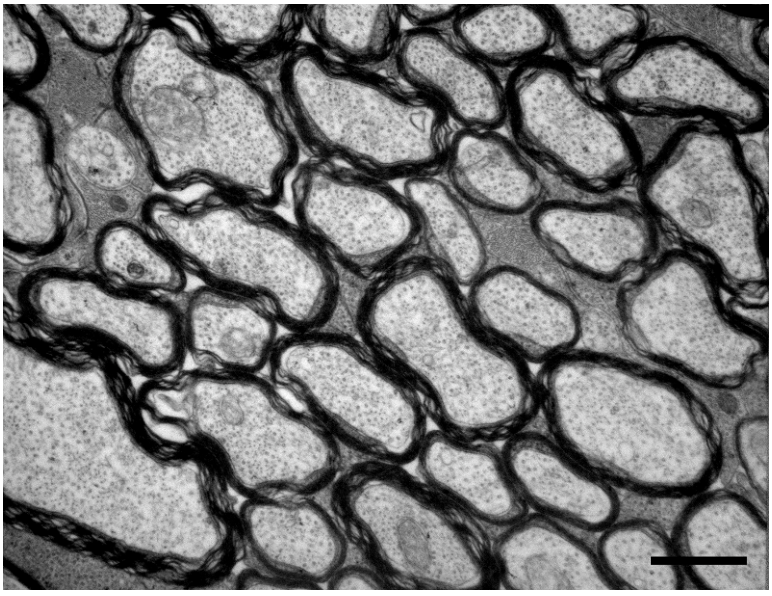
Suppl. Figure S1

Luxol blue staining for myelin of paraffin-embedded brain sections of 22-months (m) old WT (**A, C**) and ClC-2 KO (**B, D**) mice. Whereas the fiber tracts of the internal capsule are compact in WT brain (C), they are vacuolated and dispersed in the KO (D). (Scale bars: (A, B) 1 mm; (C, D) 0.1 mm).



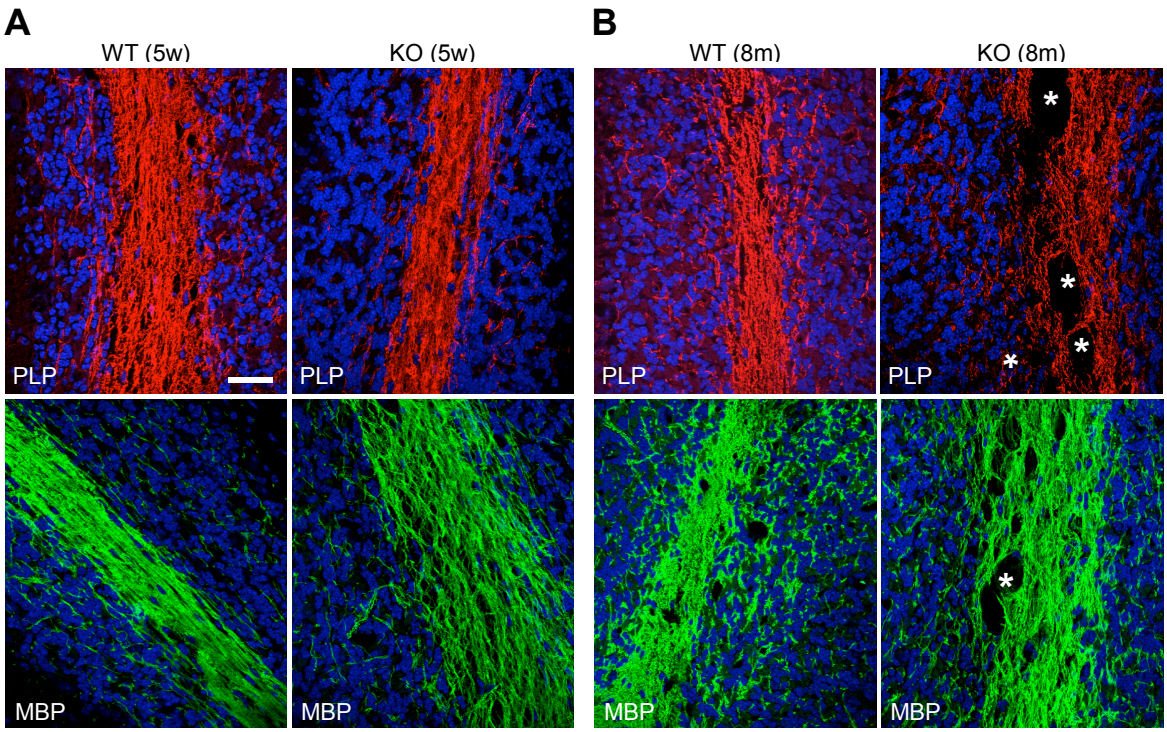
Suppl. Figure S2

Absence of pathological changes in the PNS of ClC-2 KO mice. Electron micrographs of sciatic nerve sections of 5 and 14 month (m) -old WT and *Clcn2*$^{-/-}$ mice are shown. Myelinated axons (a) of ClC-2 KO mice did not display the vacuolation of their myelin sheaths that was observed in the CNS. (Scale bars: 10µm)



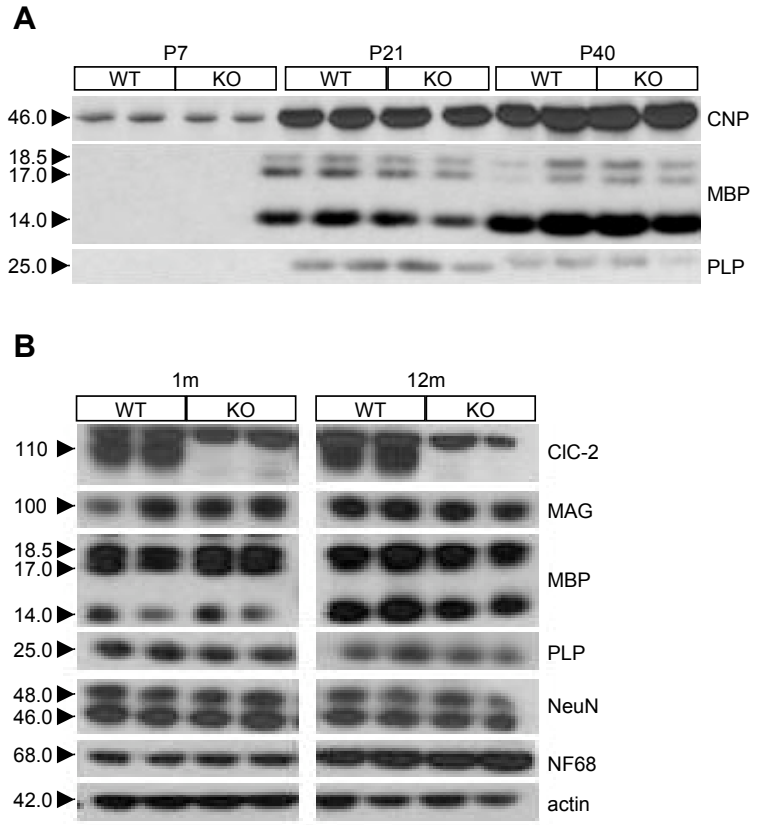
Suppl. Figure S3

Electron microscopy of an optic nerve of ClC-2 KO mice (7-week-old) demonstrates that this part of the CNS is spared from vacuolation. The bar indicates 1 µm.



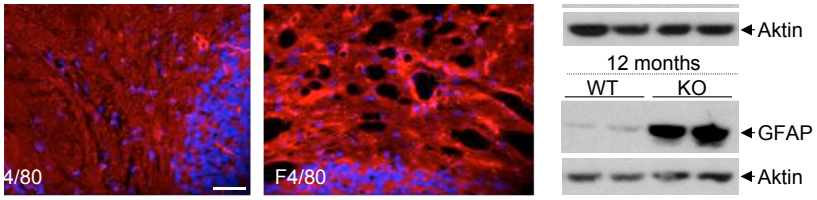
Suppl. Figure S4

Expression of myelin specific proteins in CNS fiber tracts. Cerebellar sections of 5 week (w) (**A**) and 8 month (m) old (**B**) ClC-2 KO and WT mice were immunostained for two CNS myelin proteins, the proteolipid protein (PLP) (red) and the myelin basic protein (MBP) (green). Nuclei were stained with TOTO (blue). While this analysis confirms the vacuolation of myelin (∗), the overall abundance of myelin does not seem to be changed (Scale bar: 0.1mm).



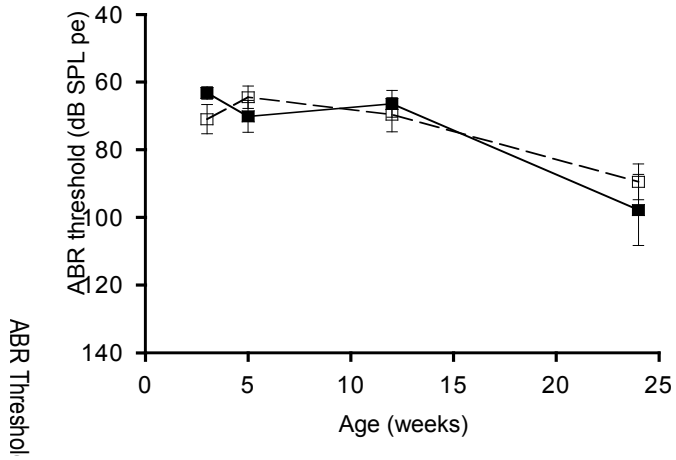
Suppl. Figure S5

Glial and neuronal markers in *ClCn2*$^{-/-}$ and WT mice. (**A**) Upregulation of oligodendrocyte markers during myelinogenesis in WT and ClC-2 KO mouse brain. Crude brain lysates were prepared from WT and *Clcn2*$^{-/-}$ mice (KO) at postnatal (P) days 7, 21 and 40 and analyzed by Western blotting for the expression of oligodendrocyte-specific proteins cyclic-2'-3'-nucleotide phosphodiesterase (CNP), MBP and PLP. Two animals per genotype were used for each time point and 20µg of proteins loaded per lane. In *Clcn2*$^{-/-}$ and WT mice, MBP, PLP and CNP levels increased to a similar extent, indicating normal myelinogenesis. (**B**) Expression of neuronal and myelin proteins in brain. Western blot analysis of brain lysates obtained from 1 and 12 month-old WT and *Clcn2*$^{-/-}$ (KO) mice showed comparable expression of the myelin-specific proteins MAG (myelin associated glycoprotein), MBP and PLP. The expression of the neuronal marker proteins NF68 and NeuN were not changed in ClC-2 KO brain lysates either. Lysates from two animals per genotype and time point were loaded (20µg protein per lane). Staining for actin served as a loading control.



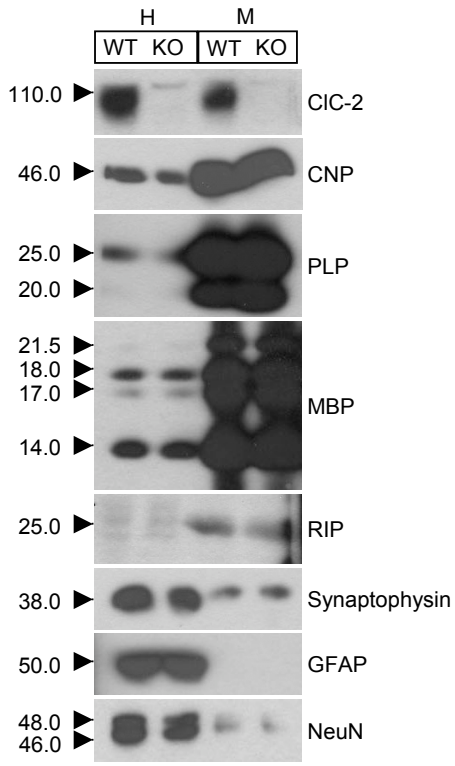
Suppl. Figure S6

Activation of microglia and reactive astrogliosis in *Clcn2*$^{-/-}$ brain. (***A***) immunohistochemistry (red) for F4/80, a surface marker of activated microglia, reveals an inflammatory response in a section of cerebella from 8-month-old WT (left panel) and *Clcn2*$^{-/-}$ mice (right panel). Nuclei are counterstained with TOTO (blue). The bar in (*A*) indicates 40µm. (***B***) Western blot analysis of cerebellar lysates from mice of both genotypes reveals a strongly increased expression of the astrocyte marker GFAP (glial fibrillary acidic protein) in *Clcn2*$^{-/-}$ cerebellum from 1 to 12 months of age, indicative of reactive astrogliosis. Actin served as loading control.



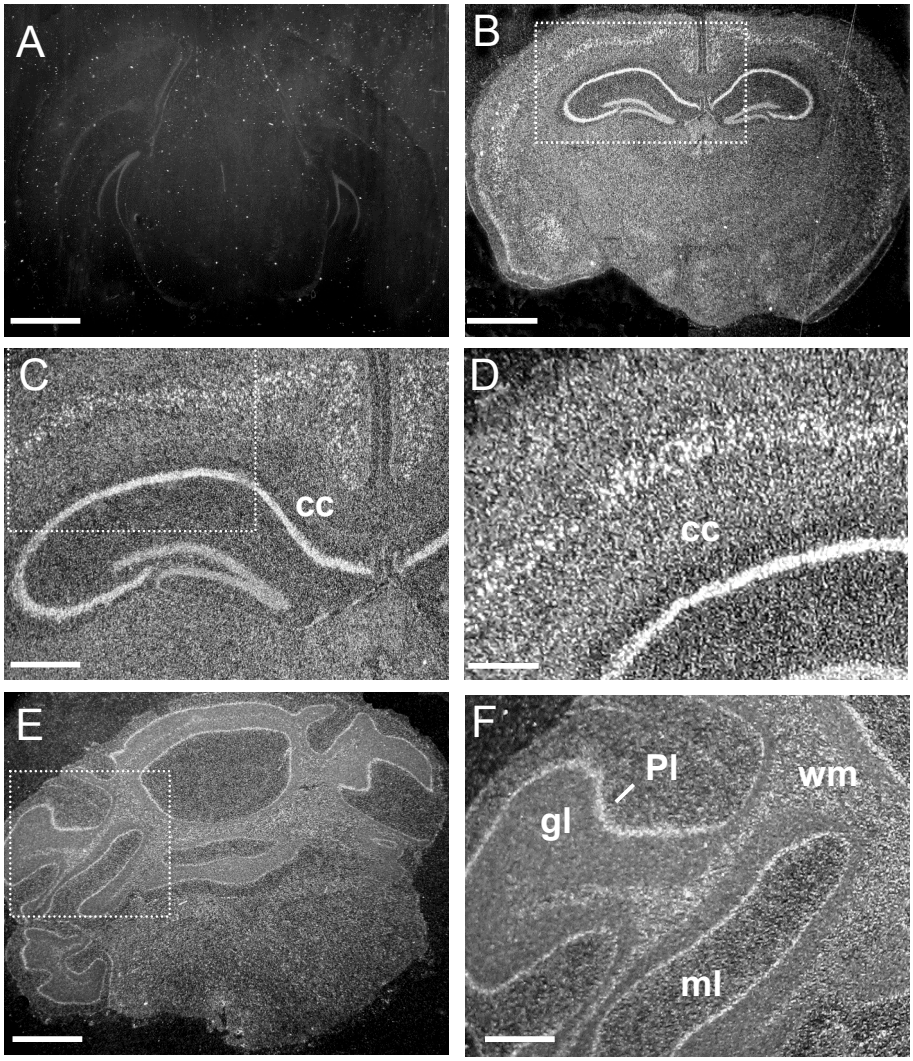
Suppl. Figure S7

Auditory brainstem responses to clicks in *Clcn2*$^{-/-}$ (□) and WT (■) mice after the onset of hearing at P14 until an age of 24 weeks. Hearing thresholds at 3 (WT n=8; KO n=5), 5 (WT n=14; KO n=18), 12 (WT n=16; KO n=15) and 24 (WT n=4; KO n=10) weeks were statistically indistinguishable. The age-related hearing loss (presbyacusis) was equally pronounced in both genotypes. Standard errors are indicated by bars.



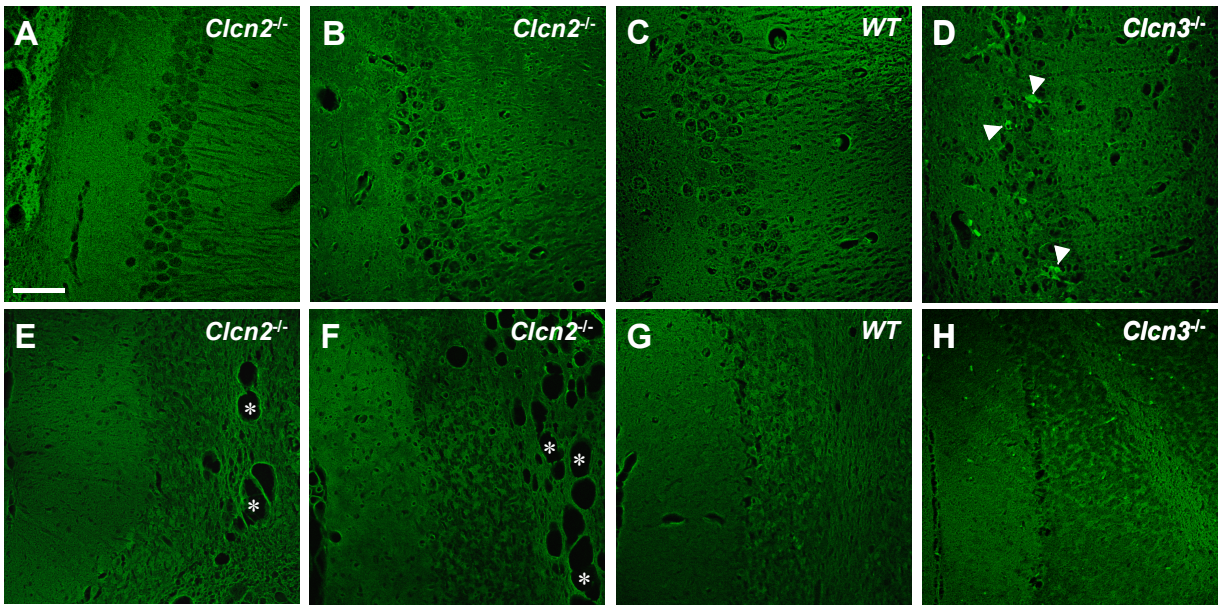
Suppl. Figure S8

Myelin composition in WT and *Clcn2*$^{-/-}$ mice (KO). Myelin was purified from brains of 6 to 8 month-old WT and ClC-2 KO mice by sucrose gradient centrifugation as described (Norton and Podulso, *J Neurochem* 21, 749-57 (1973)). 20µg of the initial crude lysate (H) and of myelin (M) were analyzed by SDS-PAGE and Western blotting. Membranes were probed with antibodies against MBP, PLP, RIP, CNP, glial acidic fibrillary protein (GFAP), synaptophysin, NeuN and ClC-2. In ClC-2 KO mice, the abundance of myelin specific proteins and their enrichment in the myelin fraction was comparable to WT. Different isoforms are indicated for MBP (14/17/18.5/21.5kDa), PLP (20/25kDa) and NeuN (46/48kDa). The contamination of myelin fractions with non-myelin proteins was low because their contents of NeuN, synaptophysin and GFAP was low compared to the homogenate. Compared to these proteins, ClC-2 was much less decreased in the myelin fraction, suggesting that its presence in this preparation is not caused by a contamination with neurons or astrocytes. Hence, ClC-2 is present in myelin.



Suppl. Figure S9

In situ hybridization of ClC-2 of an adult mouse brain. Darkfield photomicrographs of coronal sections are shown. (***A***) The sense control probe gave only very low background signals. (***B***) The ClC-2 message was detected throughout the brain including the corpus callosum (cc) and the cortex (***C***,***D***). In the cerebellum (***E,F***) the Purkinje cell layer (Pl) and the white matter tracts (wm) were intensely labeled, whereas the granular cell layer (gl) and the molecular layer (ml) had a lower signal intensity. Scale bar in (*A*) and (*B*) corresponds to 1 mm, in (*C*) 0.1 mm, in (*D*) 0.05 mm, in (*E*) 0.02 mm and (*F*) 0.01mm. **Methods:** In situ hybridization was done as described in Bösl et al., *EMBO J* 2001. Specificity was confirmed by using sense controls, and by comparison of two independent antisense probes that gave consistent results.



Suppl. Figure S10

Fluro-Jade C staining of brain sections of mice of different genotypes (*Clcn2*$^{-/-}$, ***A, E*** (3 months); ***B, F*** (21 months); WT, ***C, G*** (21 months) and *Clcn3*$^{-/-}$ (Stobrawa et al., 2001), ***D, H*** (1 month)). (***A***-***D***), sections of the CA1 region of the hippocampus, (***E***-***H***), sections of the cerebellum with stratum moleculare, stratum granulosum and white matter. Many neuronal cell bodies are stained in the degenerating hippocampus of *Clcn3*$^{-/-}$ mice (some are indicated by arrows), but no staining above background could be detected in brain sections from 3 and 21 months old *Clcn2*$^{-/-}$ mice, including the cerebellum which is prominently affected by vacuolation (∗). Fluoro-Jade dyes have been shown to label neurons that degenerate under various circumstances (Schmued et al., 2005), but can stain also other cell types (e.g. Damjanec et al., Brain Res. 1128, 40-49 (2007)). Scale bar 50µm for A-D and 75µm for E-H. **Methods:** 10µm thick sections from paraffin embedded brains were deparaffinized by two 10 min changes of xylene and rehydrated through a graded alcohol series. After a short rinse in distilled water the slides were incubated in 0.06% potassium permanganate for 10min and in 0.0001% Fluoro-Jade C solution (Chemicon) for another 10 minutes. The slides were then rinsed, air dried and coverslipped in DPX (Fluka). Sections were analized with a Leica SP2 confocal microscope.

Suppl. Table 1. List of differentially regulated genes identified by microarray analysis

GeneBank Nr.	% of WT	Gene Description
NM_008161	840	glutathione peroxidase 3
NM_009263	251	secreted phosphoprotein 1 (spp1)
NM_010422	206	hexosaminidase b
NM_008710	183	nicotinamide nucleotide transhydrogenase
NM_021281	181	cathepsin s
NM_021355	180	Fibromodulin
NM_013590	178	Lysozyme
NM_008597	176	matrix gamma carboxyglutamate protein
NM_011289	164	ribosomal protein l27
NM_026055	164	ribosomal protein l39
NM_011106	163	protein kinase inhibitor
NM_007572	162	complement component 1
NM_015786	159	h1 histone family, member 2
NM_010701	157	leukocyte cell derived chemotaxin 1
NM_009121	156	Spermidinespermine n1-acetyl transferase
NM_008287	151	heat responsive protein 12
NM_026611	149	ribonuclease t2
NM_015786	146	h1 histone family, member 2
NM_009460	142	ubiquitin-like 1
NM_011992	141	reticulocalbin 2
NM_009255	139	serpine2 proteinase inhibitor
NM_145925	138	pituitary tumor-transforming 1 interacting protein
NM_009112	137	s100 calcium binding protein a10
NM_013737	137	phospholipase a2, platelet-activating factor
NM_019953	137	transmembrane protein 4
NM_080639	136	tissue inhibitor of metalloproteinase 4
NM_009818	135	catenin alpha 1
NM_009242	135	secreted acidic cysteine rich glycoprotein
NM_023243	135	cyclin h
NM_018730	135	ribosomal protein l36
NM_009093	135	ribosomal protein s29
NM_009761	134	adenovirus e1b protein
NM_016844	133	ribosomal protein s28
NM_011662	132	protein tyrosine kinase binding protein
NM_025523	131	nadh dehydrogenase (ubiquinone) 1
NM_013492	131	Clusterin
NM_016844	130	similar to ribosomal protein s28
NM_009227	130	small ribonucleinprotein e
NM_080644	129	calcium channel
NM_009242	128	secreted acidic cysteine rich glycoprotein
NM_009096	126	ribosomal protein s6
NM_022325	124	cathepsin z
NM_027015	122	ribosomal protein s27
NM_007788	121	casein kinase ii
NM_207523	119	ribosomal protein l23a
NM_028044	119	calponin 3
NM_009169	118	split handfoot deleted gene 1
NM_021345	79	butyrate-inducedtranscript 1
NM_022424	78	fibronectin type iii
NM_008747	75	neurotensin receptor 2
NM_008407	75	inter-alphatrypsin inhibitor
NM_011674	74	udp-glucuronosyltransferase 8

NM_145942	74	3-hydroxy-3-methylglutaryl-coa synthase 1
NM_146177	72	suv420h2 (histone methylation)
NM_146741	71	myelin proteolipid protein (plp)
NM_009325	71	thromboxane a2 receptor
NM_145942	71	3-hydroxy-3-methylglutaryl-coa synthase 1
NM_010758	70	myelin associated glycoprotein (mag)
NM_010354	70	gelsolin
NM_010762	68	myelin and lymphocyte protein (mal)
NM_009851	68	cd44 antigen
NM_019581	67	gtp-binding like protein 2
NM_178405	67	na/k_atpase
NM_008883	67	plexin a3
NM_009548	67	zinc finger protein 179
NM_007428	64	angiotensinogen2
NM_008614	62	myelin oligodendrocytic basic protein (mobp)
NM_007529	60	brevican
NM_010777	57	myelin basic protein (mbp)
NM_009900	17	chloride channel 2

Genes differentially expressed in ClC-2 KO mice. RNA from cerebella of 5 week-old male WT and ClC-2 KO littermates (n=4, each genotype) was used for microarray analysis on murine genome chips from Affymetrix (type MOE430A/B). This analysis revealed a differential regulation of several genes, which includes genes involved in inflammatory processes (upregulated, underlined in dark grey) as well as myelin specific genes (downregulated, underlined in light grey). Genes are ordered according to the magnitude in their change in transcription level in ClC-2 KO mice. This change is given as the mean value of the different chips in % of WT levels (3rd column). Those genes found to be differentially expressed (≤ 25%) in at least 55% of the pairwise comparisons as well as significantly changed in the statistical analysis judged by the t-test (5%) were chosen for further investigation. The complete Affymetrix data set has been submitted to the Arrayexpress database (www.ebi.ac.uk/arrayexpress) under accession number (E-MEXP-1028).

Suppl. Table 2. Validation of differentially regulated Genes by quantitative Real Time PCR

GeneBank Nr.	5w: Chip (% of WT)	5w: qRT (% of WT)	SEM	24w: qRT (% of WT)	SEM	Gene description
NM_008161	840	520	81	427	73	glutathione peroxidase 3
NM_009263	251	209	12	406	37	secreted phosphoprotein 1 (Spp1)
NM_010422	206	132	14	168	10	hexosaminidase b
NM_021281	181	212	13	281	65	cathepsin s
NM_013590	178	258	46	647	44	lysozyme
NM_007572	162	191	26	288	16	complement component 1
NM_010701	157	181	12	270	17	leukocyte cell derived chemotaxin 1
NM_146741	71	70	6	80	4	myelin proteolipid protein (PLP)
NM_010758	70	78	8	107	21	myelin associated glycoprotein (MAG)
NM_010762	68	66	6	80	6	myelin and lymphocyte protein (MAL)
NM_008614	62	60	2	64	3	myelin oligodendrocytic basic protein (Mobp)
NM_010777	57	69	3	74	3	myelin basic protein (MBP)
NM_010130	-	132	8	281	65	F4/80
NM_010277	-	124	4	219	7	GFAP
NM_009923	-	101	4	106	6	CNP

All listed genes were first identified to be differentially regulated in an Affymetrix microarray analysis, with the exception of F4/80, GFAP and CNP. To confirm the results from expression profiling, the RNA used for the microarray analysis, as well as RNA prepared from additional ClC-2 KO and WT littermates at the age of 5 weeks (n=2, each genotype) was analyzed. Genes highlighted in dark grey are involved in inflammatory processes, whereas genes displayed in light grey are myelin specific. GeneBank accession numbers are stated in column 1. The change in transcript level obtained from the microarray analysis is shown in column 2 (in % of WT), whereas column 3 indicates the qRT-PCR results (in % of WT) for these transcripts in ClC-2 KO mice. Column 4 gives the standard error of the mean (SEM) for the qRT-PCR data. The house-keeping gene hypoxanthine-guanine phosphoribosyl-transferase (HPRT) served as control. To correlate the time course of gene expression with that of myelin vacuolation, RNA prepared from cerebella of 24 weeks (24w) old animals (n=3, each genotype) was additionally analyzed. These results are given in columns 5 and 6.

Suppl. Table 3. DNA sequence variants identified in patients with leukodystrophy.

Exon	cDNA	protein	LD patients
17	c.1928_1929 insCCG	A643_R644insR	1
17	c.2003 G>T	S668T	34
18	c.2063 G>A	R688Q	5
19	c.2154 G>C	E718D	2

Sequence variants identified in DNA samples of a cohort of 150 Caucasian patients with leukodystrophy (LD). All variants were identified in a heterozygous state. One patient with leukodystrophy was compound heterozygous for the A643_R644insR (i.e. an insertion of an arginine between residues 643 and 644) variant on one allele and for both the R688Q and E718D variants on the other allele. The R688Q and E718D sequence variants were previously found in patients with epilepsy and suggested to possibly underlie the disease (d'Agostino et al., *Neurology* 63, 1500-1502 (2004)). However, we are not aware of an epilepsy phenotype in any of these patients. The S668T polymorphism (d'Agostino et al., *Neurology* 63, 1500-1502 (2004)) was found at a frequency comparable to that deposited in the NCBI dbSNP database (rs9820367). The A643_R644insR, S668T, R688Q and E718D polymorphisms were recently also identified by Stogmann et al. (*Neurogenetics* 7, 265-268 (2006)).

Supplementary Material and Methods

Histology. Semi- and ultrathin sections were prepared from animals that were deeply anesthetized with ketanest and rompun and perfused transcardially with PBS, followed by a mixture of 2% paraformaldehyde and 3% glutaraldehyde in 0.1 M phosphate buffer. The brains, optic nerves and sciatic nerves were dissected and left in the fixative overnight. 50µm thick saggital vibratome sections were prepared from the brain. All tissue was postfixed with 1% OsO4 and further dehydrated in an ascending series of ethanol and embedded in Epon (Roth). Semi-thin sections were stained with toluidine blue. Ultrathin sections were examined with a Zeiss EM 902. For histological analysis, paraffin sections (5µM) from material immersed in Bouin's fixative were used.

Quantitative morphology. To quantify the vacuolated space, semi-thin sections of the cerebellum of 2, 5 and 14 month-old ClC-2 KO and WT mice (n=3 each) were prepared as described above. From each animal, 10 random, independent pictures of comparable regions of the cerebellar white matter of the middle peduncle were taken (63x lens) and the white area determined using the MetaMorph software (Molecular Devices) and expressed in % of the total area analyzed. Values are given as mean ± SE. Distances between major dense lines (MDLs) of myelin were measured using EM pictures (at a 50,000x magnification) of myelinated cerebellar axons from 2 month-old mice of each genotype (3 animals each). Sections were prepared as described above. In 20 randomly selected independent fields, distances between MDLs in the myelin sheaths of at least 30 axons per animal were determined and are given as mean ± SE.

Immunohistochemistry on brain sections. Brains of mice perfused with 4% paraformaldehyde (PFA) in 0.1M phosphate buffer (PB) were incubated in 30% sucrose in 0.1M PB for at least 24h before preparing free-floating cryo-sections (50 µm). For immunohistochemistry, sections were rinsed in 0.1M PB and blocked in 0.1M PB with 0.2% BSA, 4% NGS, 0.5% Triton-X-100 for 2h at RT. After incubation with the first antibody, sections were rinsed with 0.1M PB/ 0.5% Triton-X-100 and incubated with secondary fluorescent antibodies (Alexa fluor 488 and 546, Molecular Probes, 1:2000). Immunofluorescence was examined by confocal microscopy (Leica TCS SP2).

To test for the integrity of the blood brain barrier, horseradish peroxidase (HRP type II from Sigma) was dissolved in 100µl sterile PBS and injected in the vena cava of anesthetized mice (0.2 mg/g body weight). Brains were dissected 10min after injection and postfixed in 4 % PFA/PBS at 4°C overnight. 50µm free-floating sections were incubated in diaminobenzidine (DAB; 5%) for 20min and counterstained with methyl green.

Western blot analysis. Mouse brains were homogenized in 20mM Tris-HCl pH 7.4, 140mM NaCl, 5mM EDTA, 1% Triton-X-100 with protease inhibitors (Complete^{R}, Roche). Following sonification and incubation on ice for 30min, the homogenate was cleared by centrifugation at 14000xg for 15min at 4°C. Equal amounts of protein were separated on SDS polyacrylamide gradient gels (4-12.5%, Invitrogen), blotted onto nitrocellulose, and labeled with rabbit α-ClC-2 (Bösl et al., *EMBO J.* 20, 1289-99 (2001)), rabbit α-actin (Sigma), rat α-MBP (Chemicon), mouse α-CNP (Dianova), mouse α-PLP (Chemicon), mouse α-MAG (Chemicon), mouse α-RIP (Hybridoma Bank), mouse α-synaptophysin (Sigma), mouse α-GFAP (Sigma), mouse α-NeuN (Chemicon), mouse α-NF68 (Sigma). Secondary antibodies were conjugated to HRP (Chemicon) and detected by chemiluminescence (SuperSignalWest, Pierce).

Preparation of myelin. Myelin was prepared essentially as described. (Norton and Podulso, *J Neurochem* 21, 749-57 (1973)). To obtain crude myelin, brains were homogenized in 20vol of 0.32M sucrose in 10mM HEPES pH 7.4 (5 strokes with a loose pestle and 7 strokes with a tight pestle). The homogenate was layered over 0.85M sucrose, centrifuged at 25000rpm for 30min. The crude myelin was removed from the interphase, suspended in 10vol ice cold water, centrifuged at 25000 rpm for 15min. The pellet was resuspended in water and centrifuged at 25000rpm for 15min. Each myelin pellet was 2x resuspended in water, centrifuged at 10000 rpm for 15min. The myelin pellets were suspended in 0.32M sucrose, layered over 0.85M sucrose and centrifuged at 25000rpm for 30 min. The myelin layers were again suspended in water, shortly centrifuged and the pellet resuspended in 10mM HEPES buffer (pH7.4) with 1% Triton-X-100. 20µg purified myelin and the same amount of brain homogenate were used for SDS-PAGE and Western-Blot analysis.

Preparation of total RNA from WT and *Clcn2*$^{-/-}$ cerebellum for microarrray analysis. Cerebella of 2 weeks (n=3, each genotype) and 5 (n=4, each genotype)

weeks old male WT and *Clcn2*$^{-/-}$ littermates were harvested and immediately frozen in liquid nitrogen and stored at -80°C. The frozen tissue was homogenized in 1000µl TRIZOL (Gibco BRL) with 2ml syringes (first ∅ 10x 0.90 x 40mm, second ∅ 10x 0.45 x 25mm needle) and then incubated at RT for 5min before addition of 0.2x vol TRIZOL/chloroform. The mixture was shaken vigorously for 15 sec and incubated at RT for 3min before centrifugation for 10min at 4°C at 10000rpm. RNA was pelleted from the upper phase by adding 0.4ml isopropanol, vortexing, incubation for 10 min at RT and centrifugation at 14000rpm, 4°C, for 15 min. The RNA pellets were washed twice with 75% EtOH and dried at RT before resuspending in 100µl RNAse-free water. The concentration and purity of the RNA was determined by measuring the optical density at 260 nm and 280 nm. An aliquot of the RNA (1–2µg) was analyzed by TAE-agarose gel electrophoresis. The total RNA was then further purified using RNeasy columns according to the manufacturer's protocol (Qiagen).

Preparation of cDNA from total RNA for microarray analysis. 21µg of purified total RNA were used to prepare cDNA using the Invitrogen double-stranded cDNA kit (#11917-010). Briefly, the RNA was annealed with 100pmol T7(dT)24 primer (GGCCAGTGAATTGTAATACGACTCACTATAGGGAGGCGG$(T)_{24}$) at 70°C for 10 min, quick-chilled on ice and centrifuged briefly. 4µl 5x reaction buffer, 2µl 0.1 M DTT and 1µl 10mM dNTPs were added and incubated at 42°C for 2 min. 2µl SuperScript™ II RT (200 U/µl) were added and incubated at 42°C for 1 h. The reaction was placed on ice, spun briefly and the second strand synthesis ingredients were added (91µl DEPC-H_2O, 30µl 5x Second Strand Reaction Buffer, 3µl 10mM dNTP mix, 1µl *E.coli* DNA Ligase (10U/µl), 4µl *E.coli* DNA Polymerase I (10U/µl), 1µl *E.coli* RNase H (2U/µl)) before incubation at 16°C for 2h. 2µl T4 DNA Polymerase (5U/µl) were added and the reaction was incubated for 5min at 16°C. The reaction was stopped by addition of 10µl 0.5M EDTA and phenol-chloroform extracted. The upper phase was transferred to a fresh tube, and the cDNA was precipitated with 0.5x vol 7.5M NH_4OAc and 2.5x vol. ice-cold absolute EtOH by vortexing and centrifugation for 20min at RT. The pellet was washed twice with ice-cold 70% EtOH and dried at 37°C for 10 min before resuspending in 12µl DEPC-treated water.

Preparation of biotin-labelled cRNA from cDNA and fragmentation of the cDNA for hybridization. Biotin-labelled cRNA was produced with the BioArray HighYield RNA Transcript Labelling Kit (ENZO) following the manufacturer's instructions. 10µl

of the cDNA was used as starting material, and the reaction was incubated in a water bath at 37°C for 5h. 100µg of the labelled cRNA were purified with RNeasy Mini colums (QIAGEN). 15µg of labelled cRNA per standard Affymetrix microarray were fragmented in a total volume of 30µl by incubating with 1x fragmentation buffer (provided as a 5x stock: 200mM Tris-acetate, pH 8.1, 500mM KOAc, 150mM MgOAc) at 94°C for 30 min.

Hybridization and scanning of Affymetrix microarrays. The hybridization of the labelled cRNA to Affymetrix Murine Genome Array MOE 430 A/B was performed according to the Affymetrix technical manual. The quality of the RNA was analysed by hybridization of Affymetrix Test 3 chips. The washed and stained microarrays were analysed with a GeneArray scanner (Agilent).

Evaluation of the microarrays. The evaluation of the microarrays was performed with the following programmes: Affymetrix Microarray Suite Version 5.0, Affymetrix MicroDB Version 3.0, Affymetrix Data Mining Tool Version 3.0. The evaluation was based on the statistical analysis using the Data Mining Tool programme and the pairwise comparison of all KO RNAs versus all WT RNAs (nine and sixteen comparisons) using the Microarray Suite program. The genes found to be differentially expressed (≤ 25%) in at least 55% of the pairwise comparisons as well as significantly changed in the statistical analysis judged by the t-test (5%) were chosen for further investigation and included in the respective tables of the *Supplementary Data*.

Validation of differentially expressed genes by quantitative RT-PCR. Total RNA was prepared from cerebella of WT and *Clcn2*$^{-/-}$ littermates as described above. The RNA was digested with 1µl RNAse-free DNAse (Ambion) for 20 min at 37°C in a total volume of 50µl. After purification with RNeasy columns (Qiagen), two aliquots of 5µg RNA were used for cDNA synthesis: The RNA was annealed with 1µl Oligo $p(dT)_{15}$ Primer (500µg/ml) for 10min at 65°C in a total volume of 10µl. The reaction was chilled on ice, spun briefly and supplemented with 4µl 5x First-Strand Reaction Buffer, 2µl 0.1M DTT, 2µl 10mM dNTP mix, 1µl RNase Inhibitor (RNaseOUT 40U/µl), vortexed gently and incubated at 42°C for 2 min. One of the aliquots was then supplemented with 1µl SuperScript™ II RT (200U/µl), the other aliquot with 1µl DEPC-treated water being the control, and the reactions were incubated at 42°C for

1 h. After heating to 70°C for 15min, the tubes were placed on ice, spun briefly and diluted for qRT-PCR. The qRT-PCR was performed in a ABI PRISM 7700 Sequence detection System (SDS 2.1 software) using the SYBR green PCR master mix (Applied Biosystems). The total volume of the reaction was 20µl, consisting of 10µl 2x SYBR green PCR master mix, 1µl diluted cDNA and 2.5pmol of each PCR primer. The reaction consisted of 2 min 50°C, 10 min 95°C and 45 cycles 15sec 95°C and 1 min 60°C. The reaction product was then slowly heated to 95°C to obtain the dissociation curve. All reactions were performed in triplicates with cDNAs from at least three different animal pairs. Fold changes were calculated and geometrically averaged. The primers were designed with the Primer Express Software (Applied Biosystems) and are listed in the following table.

5`-Sequence-3`	Primer
GTTCTTTGCTGACCTGCTGGA	HPRT-306F
TCCCCCGTTGACTGATCATT	HPRT-425R
AGCCTCGTCCCGTAGACAAAA	GAPDH-27F
TGGCAACAATCTCCACTTTGC	GAPDH-137R
AACCGTGGAGACCAAAGCACT	Lysozyme-190F
GCACTGCAATTGATCCCACAG	Lysozyme-302R
TGGCAATGGAGCAACTGCA	CathepsinS-68F
TTTCCCAGATGAGACGCCGTA	CathepsinS-190R
CAGTGACTGCAAAATCGACCC	ClC-2-2385F
CATAAGCATGGTCCACTCCCA	ClC-2-2488R
TGCTGAGAAGGCCAGTAAGGA	MBP-33F
TCCCCAAACACATCACTGTCC	MBP-143R
GGTGTGCTGCACTGTACAACCA	CNP-739F
GCCTTGCCATACGATCTCTTCA	CNP-839R
GTGAAAGGCAATCCAGGCAA	Complement Component-304F
TGGTTGGTGAGGACCTTGTCA	Complement Component-419R
TGTCCATCTGCAAAACAGCTG	PLP- 674F
CCGCCTGCATTCTATACAGCA	PLP-779R
ACGCAGAACCTTTCAACCCTG	F4/80-1062F
TCCGGACATCGTTTGGTGT	F4/80-1172R
TTCACTGGCACATAGTGGACG	GFAP-1105F
AATTAGGCACAAAGCCCCCAC	GFAP-1220R
GGCTTCCCTTCCAACCAATTT	Glutathion Peroxidase-305F
TTAATGACCTCACCATCCCGC	Glutathion Peroxidase-407R
TTCTCCAACCTCCAGATCCGA	Hexosaminidase-638F
TGCACTGCTTGGCATTGCT	Hexosaminidase-754R
TCCAGAAGGCTCCCAAGGATA	Leukocyte Chemotaxin-755F
GCAATCATGAAGGTGAGCAGG	Leukocyte Chemotaxin-864R

ClC-2 cDNA constructs. The (human) hClC-2 cDNA was subcloned into the expression vector pFrog. Point mutations were introduced by recombinant PCR. Constructs were verified by sequencing. After linearization with HpaI, capped RNA was transcribed using T7 RNA polymerase with the mMessage mMachine kit (Ambion, Austin, TX).

***ClCN2* mutation analysis.** DNA samples of 150 patients diagnosed with leukodystrophy were analysed. The 24 coding exons of the *CLCN2* gene were PCR-amplified using intronic primers which are listed below. The PCR products were either directly sequenced or prescreened by SSCP-analysis. The accession number for the reference sequence for ClC-2 is NM_004366.

5`-Sequence-3`	Primer
GGCTGCCGGCGCGGACTTTGCG	Exon1-F
TAGGACAGGATTAGGGTAG	Exon1-R
CTCTCCCTGCGTTCCCACTCA	Exon2-F
CAGCAGCTCTAATGGCCTCTG	Exon2-R
GTGCCATCCTCCCCCTTCTTC	Exon3-F
AGTGTGGCATGGACGCAGGAG	Exon3-R
GTCGTATCAGCGTGGTCCACTG	Exon4/5-F
TTCATTCCCCAGCCTGCAGT	Exon4/5-R
CGACTCACCGCCTGCCCCACC	Exon6/7-F
CAGTCAGGTCGGGGTGTTGGGA	Exon6/7-R
TCAGTCCAGCCCCACAACCCTG	Exon8/9-F
AAGAGGCACCTGAGTGAAAAG-	Exon8/9-R
GCAGCCCGAGATGGTGCCAG	Exon9/10-F
TATACAACTCAAAGCAGCCTG	Exon9/10-R
CCACGACTGATGTGAAACCTAC	Exon11/12-F
GGTTAGCAGTGTACACGTGAG	Exon11/12-R
TGATCAGCGAGGTAGACTTCT	Exon13-F
GAGGCTGAGGAAGGTGCAGGC	Exon13-R
ACCCTCTCTCTTCCTAGTGCC	Exon14-F
CAGAGTCTCGGACCCTGTGGC	Exon14-R
CTGGTTGTGGAGGGTCTTCTG	Exon15-F
CTTGCTAGAGGTGGCTGTAGG	Exon15-R
CCATGCTGACCACAAACCAAG	Exon16-F
TGCATGCAGGCCACACAC	Exon16-R
CTCCCTCTCAGCTCCTGAGTC	Exon17/18-F
GCTGAGGCTCTGTGGACACAG	Exon17/18-R
CCTGTGTCTTCCTTACCTTTC	Exon19-F
GCAAGCTAGGAGGACAGGCTC	Exon19-R
ACTCCTCATCTTTCTGGTTTG	Exon20/21-F
CATGATGATTGAACACAACCAG	Exon20/21-R

GGAGTGAGGTCAGCTACAG	Exon22-F
GTCCTGAGAGCCTAGACCAGC	Exon22-R
GAGGAGTCTGACATCTGGGTC	Exon23-F
ATGCACATTCTGGGCTGACGG	Exon23-R
CCTCGTCCATCAAGTCCAATC	Exon24-F
GACCCAGATGTCAGACTCCTC	Exon24-R

Expression in *Xenopus laevis* oocytes

Individual stage V to VI oocytes were obtained from anaesthetized frogs and isolated by collagenase treatment. Synthesis of cRNA was carried out with the SP6 mMessage mMachine kit (Ambion, Austin, TX) from constructs cloned into the pTLN oocytes expression vector. 10ng of ClC-2 cRNA were injected into oocytes. The same total amount was used in co-injection experiments, which contained a 1:1 WT:mutant cRNA mixture. Following injection, oocytes were kept at 17°C in MBS solution (88mM NaCl, 2.4mM $NaHCO_3$, 1mM KCl, 0.41mM $CaCl_2$, 0.33mM $Ca(NO_3)_2$, 0.82mM $MgSO_4$, 10mM HEPES, pH 7.6).

Electrophysiology

3-5 days after injection, currents were measured in ND96 (96mM NaCl, 2mM KCl, 1.8mM $CaCl_2$, 1mM $MgCl_2$, 5mM HEPES, pH 7.4) at room temperature in two-electrode voltage clamp recordings using a Turbotec 10C amplifier (npi electronics, Tamm, Germany) and pClamp8 software (Axon Instruments, Union City, CA). The following voltage protocol was used: starting from a holding potential of -30mV, cells were clamped for 10s to test potentials between +20 and –155mV in steps of 25mV, followed by a 2s tail pulse to +40mV and a 2s pulse to –30mV.

Data were obtained from three different batches of oocytes with at least six oocytes measured per batch and cRNA injection scheme. The total numbers of oocytes for the respective constructs are given in the figure legend. Currents from individual oocytes were normalized to the average current from that batch (the particular current used for normalization is indicated in the figure legend). The normalized currents thus obtained were averaged with those obtained from the other oocytes batches. Data are given as arithmetic means ± S.E.M.

Auditory Brainstem Response. Auditory evoked brain stem responses (ABR) to clicks were recorded in anesthetized animals (16mg/kg xylazin hydrochloride (Rompun, Bayer) and 120mg/kg ketamin hydrochloride (Ketanest, Parker-Davis) that

were kept warm on a heating pad. Bioelectric potentials were recorded by subdermal silver wire electrodes at the vertex (reference), forehead (ground) and at both mastoids. Acoustic click stimuli were generated by a Beyer DT-48 earphone coupled closed-field into the external auditory meatus with a conical earpiece. An integrated probe microphone (Brüel & Kjær 4135) in the earpiece served to monitor the actual sound signal. Acoustic click stimuli (~200μs duration) had a flat frequency spectrum (± 5 dB) with an upper corner frequency of 5.5 kHz.

Alternating clicks were applied at a rate of 21/s and averaged 400 to 2000 times. Stimulus intensities were varied starting at 117dB peak equivalent SPL [dB SPL pe] in increments of 20dB except near threshold where 5dB steps were used. Latency analysis was performed visually at 117dB SPL pe blinded, without knowledge of the genotype.

Examination of the seizure threshold. To test the seizure susceptibility, seizures were induced by the proconvulsive substances pentylenetetrazol (PTZ) or fluorethyl. After an initial period of immobility or normal activity, generalized seizures became apparent in the form of forelimb and hindlimb clonus, occasionally accompanied by rearing and falling. Only the latency from drug application to generalized seizures was recorded.

Fluorethyl: Seizures were induced by fluorethyl, which was evaporated (10ml/min) in a closed chamber as described *(*Prichard et al., *J Pharmacol Exp Ther* 166, 170-8 (1969)). Always one pair of WT, *Clcn2*$^{-/-}$ or WT and *Clcn2*$^{+/-}$ mice of comparable weight and same sex were analyzed at the same time.

PTZ: Mice were injected intraperitoneally with the pro-convulsive substance pentylenetetrazol (PTZ) at a dose of 50mg/kg body weight. Mice were monitored for 10min. For those mice that did not develop seizures during that time, the latency was set to 10min.