Supplementary material

Aquaporin-4 and GPRC5B: old and new players in controlling brain oedema

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Supplementary methods

STUDY OVERSIGHT | With approval of the Institutional Review Board, we performed genetic studies in patients with MRI-based MLC diagnosis and no pathogenic variants in MLC1 and GLIALCAM. The diagnosis of MLC is based on MRI criteria: 1) diffuse cerebral white matter signal abnormalities with swelling of the abnormal white matter, 2) cysts or near-cystic abnormalities in the anterior temporal subcortical white matter, 3) relatively preserved central white matter structures. Blood is received from patients, parents and sometimes unaffected siblings for DNA diagnostic purposes. Lymphocytes are isolated from blood of patients and lymphoblasts are created using Epstein-Barr virus to allow RNA analysis. Sanger sequencing of the *MLC1* and *GLIALCAM* genes is performed as previously described; if unrevealing RNA and Multiplex Ligation-dependent Probe Amplification analysis of *MLC1* is executed.[1-3]

GENETIC ANALYSIS | Whole exome sequencing (WES) was performed on genomic DNA from three unrelated patients (patients 1-3). For one patient parental DNA was included to form a family trio. We analysed the data as previously described.[4] Variant filtering was executed under the hypothesis of autosomal recessive or autosomal dominant *de novo* inheritance. We focused on rare variants, exonic as well as intronic, filtering for a minor allele frequency (MAF) of less than 1% for recessive inheritance and less than 0.1% for dominant inheritance in public databases (dbSNP, 1000 Genomes Project, Exome Variant Server (EVS) and NHLBI Exome Sequencing Project).

Two siblings, patients 4 and 5, without possible pathogenic variants in the gene identified in patients 1-3, had consanguineous parents. We executed single nucleotide polymorphism (SNP) array analysis (CytoScan HD array, Affymetrix, USA) according to the manufacturer's protocol to identify runs of homozygosity larger than 1 Mb and overlapping regions (Nexus version 7, BioDiscovery, USA). SNP array-based genotypes were created using Chromosome Analysis Suite 2.1.0.16 (Affymetrix, USA).

Validation and segregation of the identified *GPRC5B* (MIM 605948, NM_016235.2) and *AQP4* (MIM 600308, NM_001650.5) variants was performed by Sanger sequencing.

PLASMIDS | Human AQP4 fused with C-terminal GFP in the pDEST47 expression vector was a kind gift of Dr. Alex Conner (University of Birmingham, UK).[5] The Ala215Thr variant was introduced using the Seamless Ligation Cloning Extract (SLiCE) cloning method.[6] GPRC5B plasmids (containing wild-type GPRC5B or either of the two patient variants) were generated in house, starting from commercially obtained GPRC5B plasmids (Eurofins, the Netherlands). Isoforms 1 (NP_057319.1) and 2 (NP_001291700.1) of human GPRC5B were cloned in the pcDEF3 mammalian expression vector.[7] An N-terminal HA-tag (which followed the predicted[8] signal peptide Met1-Ala27 in isoform 1), and a C-terminal fluorescent protein (mVenus) was added. By adding silent restriction sites (NdeI and NheI) surrounding the area of the patient variant, Ile176dup and Ala177dup variants of GPRC5B were subcloned into the wild-type plasmid.

CELL LINES | HEK293T cells were cultured in Dulbecco's Modified Eagle Medium (DMEM), high glucose, pyruvate, supplemented with 10% fetal bovine serum in a humidified CO_2 incubator (37°C / 5% CO_2). Medium of cells used for immunofluorescence and calcein quenching experiments was in addition, supplemented with 1% pen/strep (Gibco, 15140122) HEK293T cells were plated 24 hours before transfection and assayed 24 hours post transfection. Cells were transfected with wild-type or Ala215Thr AQP4 plasmid using Neuromag (OZ Biosciences, France) according to manufacturer's instructions. For mock conditions the same transfection protocol was used, but without adding DNA.

MDCK cells were cultured in Dulbecco's Modified Eagle Medium, high glucose, pyruvate, supplemented with 10% foetal bovine serum in a humidified CO_2 incubator (37°C / 5% CO_2). To establish stable cell lines, cells were transfected and kept as described.[9] Briefly, cells were plated in 6 well plates and transfected with wild-type or Ala215Thr AQP4 plasmid using branched PEI according to manufacturer's instructions. Stably transfected cells were selected using 700ug/mL G418 for two weeks, and single colonies were picked to establish stably expressing cell lines. Colonies were grown to confluence under G418 selection and AQP4 expression was then checked using cell surface biotinylation and western blot.

Lymphocytes were isolated from blood of all three patients with a *GPRC5B* variant and five controls. Lymphocytes were activated by the Epstein-Barr virus to create lymphoblast cell lines, which were stored at -80°C. Batches of patient and control lymphoblasts were thawed and cultured in Rowell Park Memorial Institute (RPMI)-1640 medium supplemented with GlutaMAX and 10% fetal calf serum, 1% sodium pyruvate and 0.1% gentamicin in a humidified CO_2 incubator (37°C / 5% CO_2). For RVD experiments and immunohistochemistry, cells were plated on poly-L-lysine (PLL, P2636, Sigma-Aldrich, USA) coated glass coverslips in a 24-wells plate at a density of 50.000 cells/ml three to four days before use.

Generation of U251 cells overexpressing human MLC1 is described elsewhere.[10] Cells were cultured in DMEM with 10% fetal calf serum and 600 ug/ml G418. Cells were passaged in 1:3 dilution two times per week, and care was taken to not maintain cells for more than 20 passages. Two to three days before whole cell patch-clamp recordings cells were plated on 13 mm glass coverslips in a 24-wells plate with a density of 10.000 cells/ml. Transfection with wild-type, Ile176dup or Ala177dup GPRC5B plasmids (isoform 1 or 2) was performed 24 hours after plating using FUgene (Fugent LLC, USA) according to manufacturer's instructions (DNA:FUgene ration 1:3). For transfection, G418 was removed from the medium and 1% pen/strep (Gibco, 15140122) was added. Cells were recorded 24-48 hours after transfection.

IMMUNOHISTOCHEMISTRY AND ELECTRON MICROSCOPY | To study expression of GPRC5B in relation to other proteins in the human brain, tissue samples from the frontal lobe were obtained at autopsy from 2 unrelated control subjects aged 1 day (female, ependymoblastoma of the posterior fossa) and 54 years (male, myocarditis). Two patients with metachromatic leukodystrophy were also included; they were aged 4 (male) and 9 years (female), respectively, and died from disease progression. None of the control subjects had neurological symptoms. Routine neuropathological examination (gross examination of brain anatomy as well as review of Haematoxylin and Eosin-stained tissue sections from multiple brain areas) was normal.

For immunofluorescence staining, formalin-fixed paraffin-embedded (FFPE) tissue was cut in 5 µm-thick sections. Staining of FFPE tissue was carried out by rehydrating sections in xylene and alcohol. Slides were blocked in glycine (0.1 g / 100 ml) for 10 minutes at room temperature followed by heat induced antigen retrieval in citrate buffer (pH=6) or TRIS/EDTA buffer (pH=9). Primary antibodies against GPRC5B (1:100, sc-135260, Santa Cruz Biotechnology, USA), GFAP (1:500, AB5541, Sigma-Aldrich, USA), CD31 (1:50, M0823, DAKO, Agilent Technologies, USA), GlialCAM (1:100, MAB4108, R&D Systems, USA) and NEUN (1:100, MAB377, Merck Millipore, USA) were incubated overnight at room temperature. The next day slides were rinsed and incubated with fluorescent labelled antibodies (Alexa Fluor-488 & Alexa Fluor-594, 1:250). To reduce autofluorescence slides were quenched in Sudan Black (0.1% in 70% alcohol) for 10 minutes. After rinsing slides were mounted with Fluoromount-G® Mounting Medium (0100-01, SouthernBiotech, USA) containing DAPI (d9542, Sigma-Aldrich, USA). Pictures were taken using a Leica DM6000B microscope (Leica Microsystems, Germany).

For immunoelectron microscopy, human brain tissue was immersion fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4), cryoprotected in 10, 20 and 30% sucrose, quick-frozen on dry ice, and cryosectioned into 40 µm-thick sections for free floating immunolabelling. Sections were incubated with GPRC5B (1:100, sc-135260, Santa Cruz Biotechnology, USA) as a primary antibody for 24 hr, rinsed and incubated with BrightVision Poly-HRP-α-Rabbit IgG (DPVR-55HRP, ImmunoVision Technologies Co., Daly City, CA). To visualize the peroxidase, sections were incubated in a diaminobenzidine (DAB) solution containing 0.03% H_2O_2. The DAB reaction product was then intensified by a gold-substituted silver peroxidase method.[11] Sections were post-fixed for 20 minutes in 1% OsO4 supplemented with 1,5% potassium ferricyanide. The material was then dehydrated and embedded in epoxy resin. Ultrathin sections were examined and photographed using a FEI Tecnai 12 electron microscope (FEI company, US). Negative control sections in which primary antibody was omitted showed no labelling. Digital images were taken at ×9,300 to x13,000 magnification using a Veleta camera (Emsis GmbH, Germany) using Radius software (Olympus, Germany). 50 images were analysed to assess labelling patterns.

Staining of HEK293T cells was performed as follows: cells were plated on 13 mm PLL coated glass coverslips in a 24-wells plate. 24 hours after plating cells were transfected as described earlier. Membranes and Nuclei were stained before fixation using a Wheat Germ Agglutinin (WGA) Conjugate (WGA633, 29024-1, Biotium, USA) dissolved at 5 µg/mL in phenol red-free $HBSS^{-/-}$ (10 minutes at 37°C) and Hoechst (Sigma-Aldrich, USA) respectively. Next, cells were fixated in 2% paraformaldehyde (PFA) and stored in PBS. Coverslips were washed two times with PBS, followed by block of non-specific staining by 1 hour of incubation in blocking buffer (U3510, Sigma-Aldrich, USA). Coverslips were mounted on glass slides using Fluoromount-G® Mounting Medium (0100-01, SouthernBiotech, USA) and cured overnight. Z-stacks with a z step size of 0.2 µm were obtained at RT using an inverted Nikon Eclipse Ti A1 confocal microscope (Nikon, Japan) with an oil-immersion objective lens (Nikon Apochromat 60X/NA 1.4). Hoechst was excited at 407.7 nm (emission filter: 440-500 nm), AQP4-GFP was excited at 483.8 nm (emission filter: 500 nm – 550 nm) and WGA at 635.8 nm (emission filter: 639 nm – 720 nm).

Analysis of AQP4 expression on membrane vs cytosol was determined as follows: multiple high-resolution z-stacks were obtained from stained wild-type or Ala215Thr AQP4 expressing HEK293T cells. Quantification of the fluorescence was performed by manually drawing lines through 20-30 transfected cells per condition using ImageJ software. Care was taken to draw lines perpendicular to the membrane on both sides, without crossing the cell nucleus (for examples see Figure 3A). Fluorescence intensity for both the green (AQP4-GFP) and red (WGA633 membrane dye) channels was extracted. To determine membrane fluorescence, the two peaks in the red channel indicating the plasma membrane crossings were identified for each line, and mean green fluorescence was determined in a 500 nm area centred on each peak. For cytosolic fluorescence, mean green fluorescence in the central area 1 µm from each peak in the red channel was determined. To calculate membrane enrichment, mean membrane fluorescence was divided by mean cytosolic fluorescence.

CELL SURFACE BIOTINYLATION | For AQP4 cell surface biotinylation, cells were plated in 24 well plates 24 hours before transfection and biotinylated 24 hours post transfection. HEK293T cells were transfected with wild-type or Ala215Thr AQP4 plasmid using PEI according to manufacturer's instructions. For mock conditions the same transfection protocol was used, but without adding DNA. Biotinylation was performed as described.[12] In short, a cell-impermeable biotinylation reagent was used (EZ-Link Sulfo-NHS-SS-Biotin; ThermoFisher Scientific, USA). After lysis, 96-well Pierce™ NeutrAvidin™ coated plates (ThermoFisher

Scientific, USA) were used to pull down the biotinylated proteins which then were quantified using anti AQP4 antibody (ab128906, Abcam, UK).

CALCEIN-QUENCHING ASSAY | For experiments in HEK293T cells, 10.000 cells/well were plated in PLL coated half area clear bottom black 96-well plates (Greiner cellstar, Greiner Bio-One, Austria). 24 hours post plating, cells were transfected using 200 ng plasmid DNA and 0.5 µl NeuroMag per well. 24 hours post transfection, transfection efficiency was checked using a fluorescent microscope, following which cells were loaded with 20 µM Calcein-AM (ThermoFisher Scientific, USA) for 90 minutes at 37°C in complete medium. After loading, the medium was replaced with 50 µl isotonic solution containing (in mM) 140 NaCl, 4 KCl, 2 $MgCl_2$, 2 $CaCl_2$, 10 HEPES and 5 Glucose (300 mOsm/kg; pH 7.4). Every well was checked for a confluent monolayer of cells without holes and successful calcein-AM loading under a fluorescent microscope before and after the experiment. Wells in which the cell layer was disrupted during recording were excluded from analysis. Fluorescent signal was measured in a BMG NOVOstar microplatereader (BMG Labtech, Germany) at 25°C using an excitation filter at 485nm and a lowpass filter at 520 nm. After a baseline period of 45 seconds, either a hypotonic or a hypertonic shock of 40% was given by adding distilled water (final osmolarity: ~180 mOsm/kg) or mannitol (~640 mM mannitol solution; final osmolarity: ~420 mOsm/kg) respectively into the well at a speed of 100 µL/s. A 1 second shake was applied to allow mixing of the fluids. Fluorescence was measured every 0.2 seconds for a total of 44 seconds.

Experiments in MDCK cells were performed as described.[13] Briefly, stably transfected MDCK cell lines were plated in black-walled clear bottom 96 well plates (Corning, USA) 24 hours before the assay. Cells were loaded with 5 µM Calcein-AM (Life Technologies, USA) and 1mM probenecid (Sigma-Aldrich, USA) for 90 minutes at 37°C in complete medium. Then, medium was exchanged for HEPES-buffered DMEM high glucose, pyruvate, supplemented with 10% fetal bovine serum. A FLUOstar Omega platereader (BMG labtech, Germany) was used for these experiments. Baseline fluorescence was measured for 4 seconds, followed by injection of either distilled water (swelling) or 400 mM mannitol in HEPES-buffered culture medium (shrinking) at a speed of 300 uL/s. Fluorescence was measured for a further 16 seconds. Cells were manually inspected for health and calcein loading before and after the experiment using a ThermoFisher EVOS microscope in either phase contrast or green fluorescence mode.

For analysis of calcein-quenching experiments, fluorescence per well was first normalized to baseline. For MDCK cells, mono-exponential rate constants were fitted to the swelling/shrinking period following the injection artefact. For display purposes, data points following injection were corrected for the fluorescence drop caused by fluid injection. For HEK293 cells, to isolate effects on swelling/shrinking that were due to AQP4 expression, and to reduce artefacts resulting from bleaching, dye leakage, fluid injection and shaking, as well as from intrinsic swelling/shrinking of HEK293T cells, the normalized fluorescence trace for each well was divided by the averaged normalized fluorescence trace for all mock transfected cells injected with either distilled water or mannitol. Resulting traces (Figure 3F) reflect the swelling/shrinking induced change in fluorescence that is due to wild-type or Ala215Thr AQP4 expression. The slope of this fluorescence change was calculated by fitting a linear function to the rising (swelling) or falling (shrinking) phase of the trace, between 20-80% of the peak value using IGOR pro software (Wavemetrics, USA).

REGULATORY VOLUME DECREASE IN LYMPHOBLASTS | Regulatory volume decrease (RVD) measurements in lymphoblasts were performed as previously described[14] with some adjustments. Lymphoblasts were loaded with 5 µM calcein-AM (ThermoFisher Scientific, USA) for 20 min at 37˚C. The coverslip was transferred to a recording bath on an inverted microscope (IX51, Olympus, Germany) equipped with a 40x objective and perfused

with isotonic solution containing: 145 mM N-methyl-D-glucamine-Cl, 5mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM Glucose and 10 mM HEPES (300 mOsm/kg, pH 7.4) running at 5ml/min at room temperature. Cells were allowed to equilibrate for 20 minutes in the bath before start of the recording. During recording, fluorescent images were acquired every 30 seconds using Streampix III software (NorPix Inc, Canada) using a Basler Scout scA1000-30gc camera (Basler AG, Germany). Following a 10 min baseline measurement in isotonic condition, the extracellular solution was exchanged to hypotonic solution (40% reduced osmolarity, 180 mOsm/kg) for 30 min, followed by a 10 min recovery period in isotonic solution. Lymphoblast cell surface area (S) was measured using ImageJ software (NIH, USA) by binarizing images with the default thresholding method and calculating the number of pixels forming the cell. Cells included in analysis were stable in surface area during baseline, i.e. the cell surface area did not deviate more than 3% between the first and last 2 minutes of the baseline period. The percentage of RVD was calculated using the following formula: $RVD = 100*((S_{end}–S_{max})/S_{max})$ (where S_{max} represents the maximum cell surface area reached during hypotonic treatment and S_{end} represents cell surface area at the end of the hypo-osmotic treatment (average of last two points in hypotonic solution).

WESTERN BLOT AND QPCR ANALYSIS | For western blot analysis on patient lymphoblasts, cells were grown in a T175 flask for 1-2 weeks until clumps formed. Cells were re-suspended and washed with PBS, spinning down in between washes (400g). Living cells were counted using trypan blue, 1 mL of cell suspension at $2 \cdot 10^6$ cells/mL was transferred to a cryo-vial, and PBS was removed. Cell pellets were frozen until use. Total protein extracts from control or patient lymphoblast cell lines were obtained as previously described.[10] Briefly, cell pellets were lysed in buffer containing 1% Triton X-100, 0.5% sodium deoxycholate, 150 mM NaCl, 10 mM Hepes (pH 7.4) and protease and phosphatase inhibitor cocktail. Lysates were passed through a 26-gauge needle, incubated on ice for 20 min and centrifuged at 14.000 rpm for 20 min at 4°C. The resulting total protein extracts in the supernatants were collected. The protein concentrations of the samples were determined using a BCA protein assay kit (ThermoFisher Scientific, USA).

Equal amounts of proteins (30 µg) were resolved on SDS–PAGE using gradient (4–12%) pre-casted gels (ThermoFisher Scientific, USA), and transferred onto a nitrocellulose membrane. Nitrocellulose membranes were blotted overnight (ON) at 4°C using anti-GPRC5B mAb (1:500; Santa Cruz Biotechnology, USA), anti-MLC1 pAb (1:1500, in-house generated[15]), anti-TRPV4 pAb (1:500, Santa Cruz Biotechnology, USA) and anti-β-actin mAb (1:2000; Santa Cruz Biotechnology, USA) in PBS+3% BSA. After washings in tris buffered saline (TBS), membranes were incubated with horseradish peroxidase-conjugated anti-mouse or anti-rabbit Abs (1:5000; Bio-Rad Laboratories, USA) for 1h at RT. Immunoreactive bands were visualized using an enhanced chemiluminescence reagent (Pierce, ThermoFisher Scientific, USA), according to the manufacturer's instructions, and exposed on a Bio-Rad ChemiDoc XRS system (Bio-Rad Laboratories, USA). Densitometric analyses of WB experiments were performed using ImageJ software (NIH, USA) and a Bio-Rad ChemiDoc XRS system.

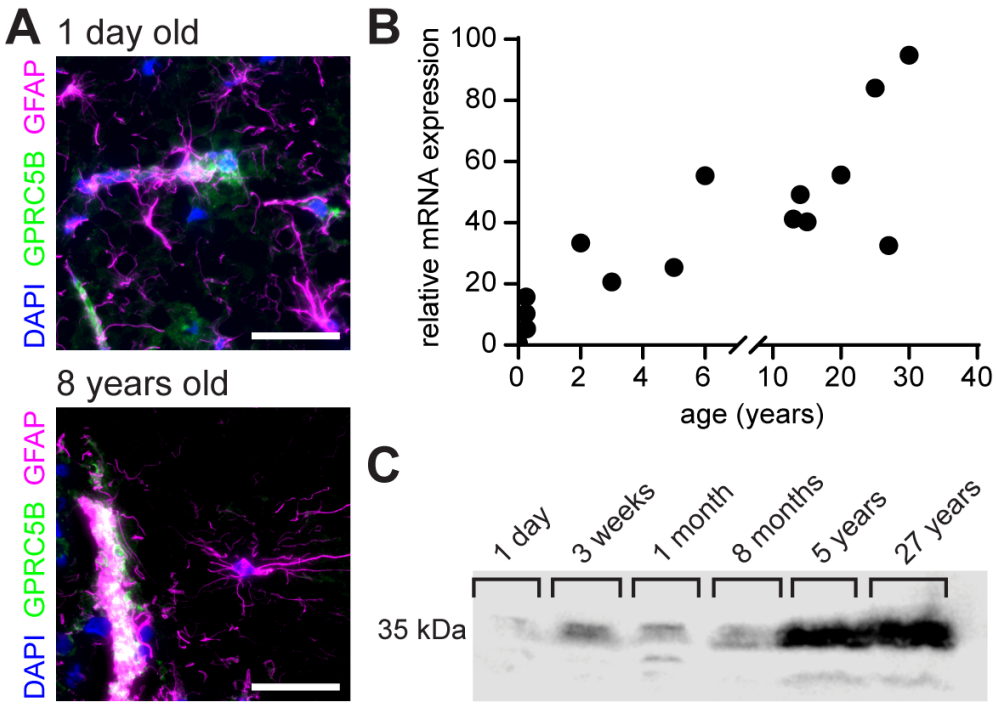
Western blots from AQP4-GFP transfected HEK293T and MDCK cells were performed as described above with the following modifications: gels were handcast 4–16% gradients, 0.2 µm PVDF membrane was used, and washes were with phosphate-buffered saline with 0.1% v/v Tween-20 (PBS-T). The antibodies used were anti-GFP (Abcam ab6556, 1:5000), anti-AQP4 (Abcam ab128906, 1:5000), anti-beta actin for canine loading control (Merck A2228, 1:3000), and anti-alpha tubulin for human loading control (Abcam ab52866, 1:10,000).

Western blot and qPCR on human brain lysates (Supplementary Figure 1) was performed as described earlier.[16] Samples were obtained from donors without any neuropathology. For western blot anti-GPRC5B pAB (1:200, PA5-32853, ThermoFisher Scientific, USA) was used. For qPCR *AKT* was used as reference. Primers were: *GPRC5B* - FW: 5'-> 3': GCAACGTGTATCAGCCAACTG, REV: 5'-> 3': CAGGGAGGCAAATCGGTAAGA; *AKT* - FW: 5'->3': TTGTGAAGGAGGGTTGGCTG, REV: 5'-> 3': TTGAGGAGGAAGTAGCGTGG.

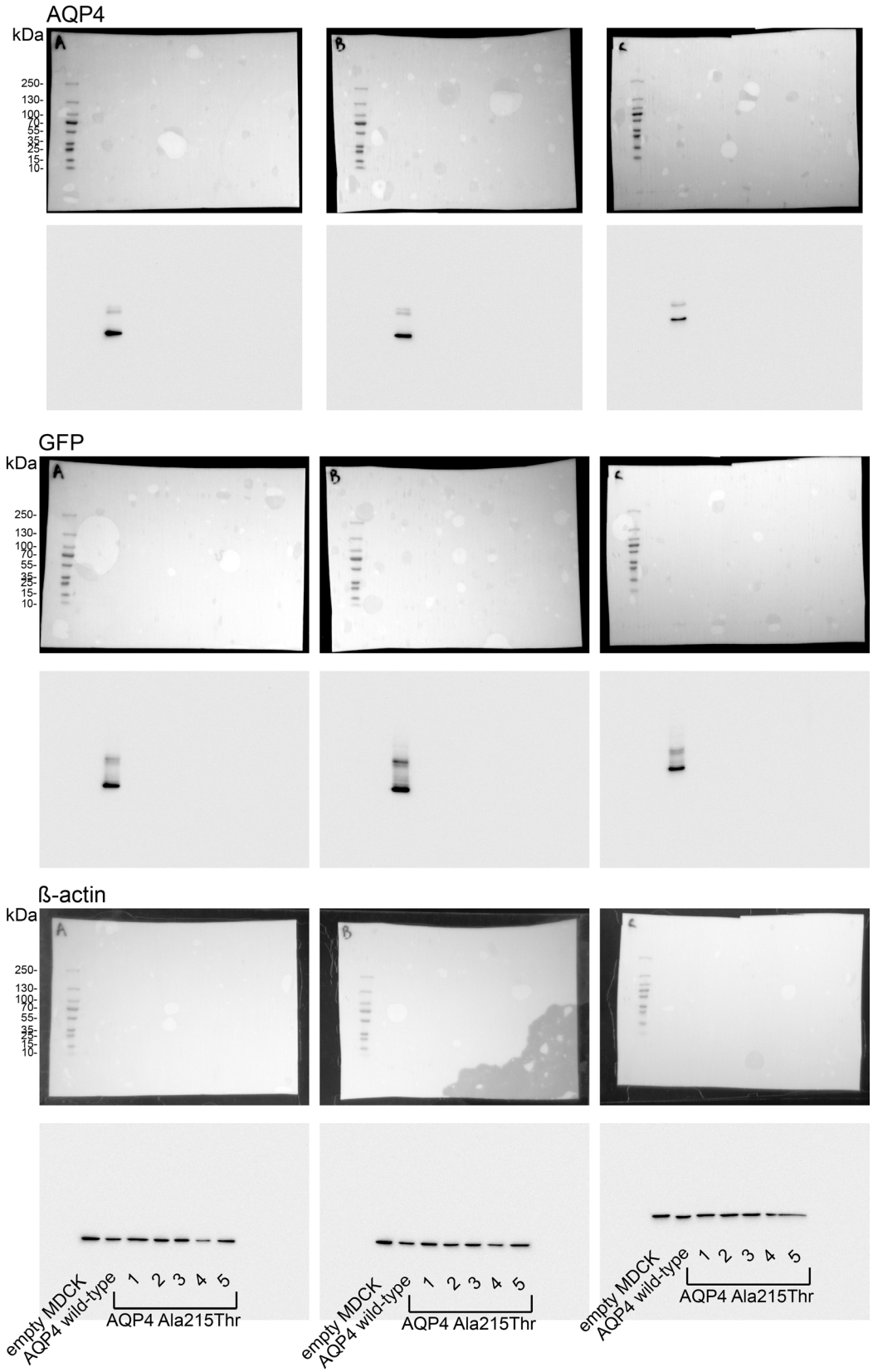
WHOLE CELL PATCH-CLAMP RECORDINGS | Whole cell patch-clamp recordings were performed in varying U251 cell lines[10] at room temperature. Coverslips with cells were placed in isotonic bath solution containing in mM: 102 NMDG, 2 KCl, 1 $MgCl_2$, 2 $CaCl_2$, 10 HEPES, 10 glucose, 100 mannitol (pH adjusted to 7.35 with HCl and osmolality adjusted to 310 mOsm/kg). The 30% hypotonic solution (217 mOsm/kg) was the same as isotonic solution, but without mannitol. Whole cell patch-clamp recordings were made using borosilicate glass micropipettes (pipette resistance 2.5-4.5 MΩ) filled with an internal solution containing in mM: 137 CsCl, 10 HEPES, 4 Mg-ATP, 0.5 Na_3-GTP, 1 EGTA (pH adjusted to 7.2 with CsOH and osmolality adjusted to 290-300 mOsm/kg). Cells were kept at a holding potential of -40 mV, and three minutes after achieving whole cell configuration anion currents in isotonicity were measured by applying voltage steps from -100 to +100 mV (averaged over three runs, Δ_{step}=10 mV, 30 s start-to-start interval 1 s step duration) with an AXOPATCH 200B amplifier and pCLAMP 10.2 software (Molecular Devices, USA). Thereafter, hypotonic solution was applied through a capillary positioned in close proximity to the cell. To account for the possible effect of mechanic stress of the application flow from the capillary, isotonic solution was applied in the same manner during the baseline recording. After 5 minutes of hypotonic solution application, the response to the same voltage step protocol as in isotonicity was measured. VRAC currents were pharmacologically isolated by application of the VRAC-specific inhibitor 4-(2-butyl-6, 7-dichloro-2-cyclopentyl-indan-1-on-5-yl) oxobutyric acid (DCPIB, 20 μM) for 5 minutes from a third capillary, followed by the recording of the remaining anion current response to the voltage step protocol. Only recordings in which series resistance remained <20 MΩ were analysed. VRAC current density was determined by subtracting current in isotonic solution from that hypotonic solution and normalizing to whole cell capacitance.

STATISTICS | Statistical analysis was performed using GraphPad Prism 9 (GraphPad, USA). Data was tested for normality with a Kolmogorov-Smirnov test. Normally distributed data was compared using an unpaired t-test, while not-normally distributed data was compared using a Mann-Whitney test. Data on cell surface biotinylation, shrinking and swelling rate constants in different MDCK lines were compared using ANOVA. Cell surface biotinylation in HEK293T cells was compared using a ratio paired t-test. For analysis of lymphoblast data (RVD and western blot), a nested t-test was performed, where lymphoblast line was taken along as a nest. For analysis of U251 cells data (VRAC current density), the nonparametric Kruskal-Wallis test was performed, followed by Dunn's multiple comparison tests. Statistically significant differences were defined as $P \leq 0.05$. Data are represented as mean ± SEM.

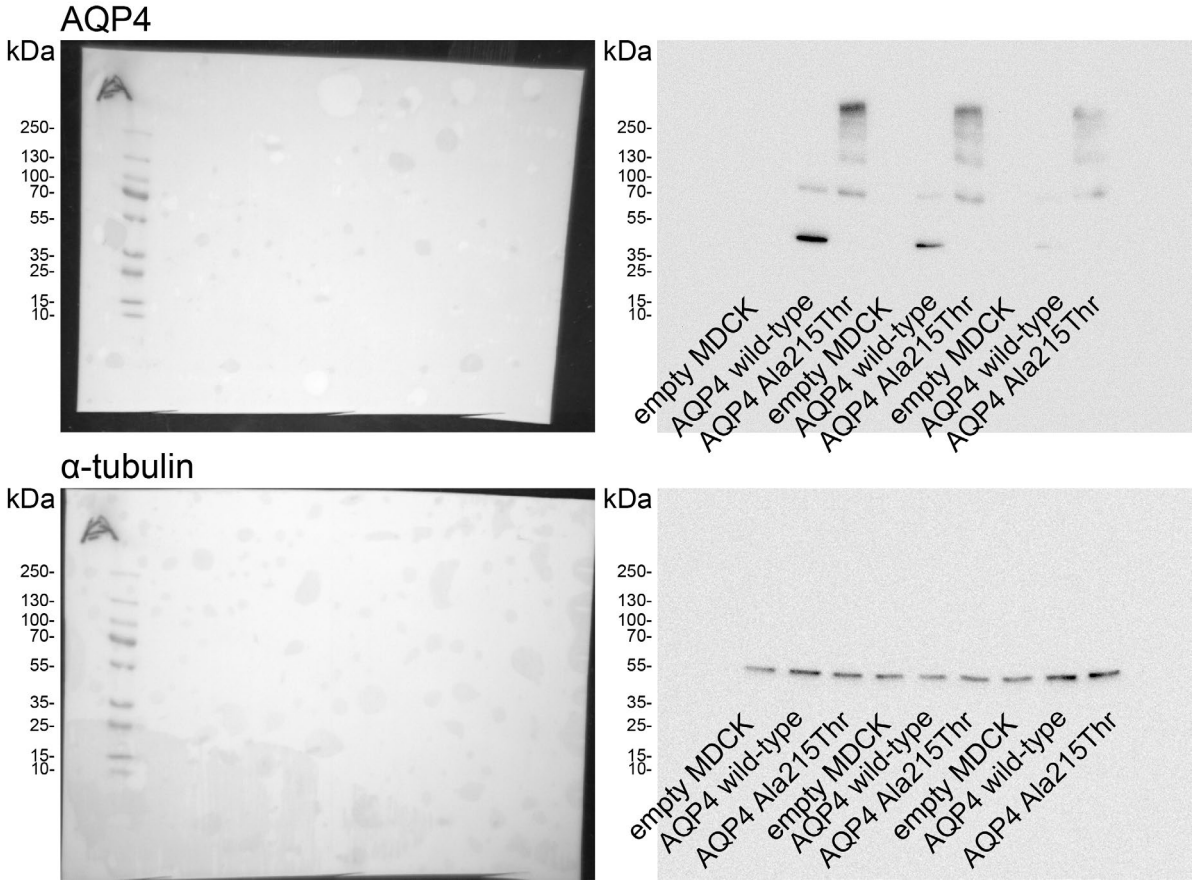
Supplementary Figures



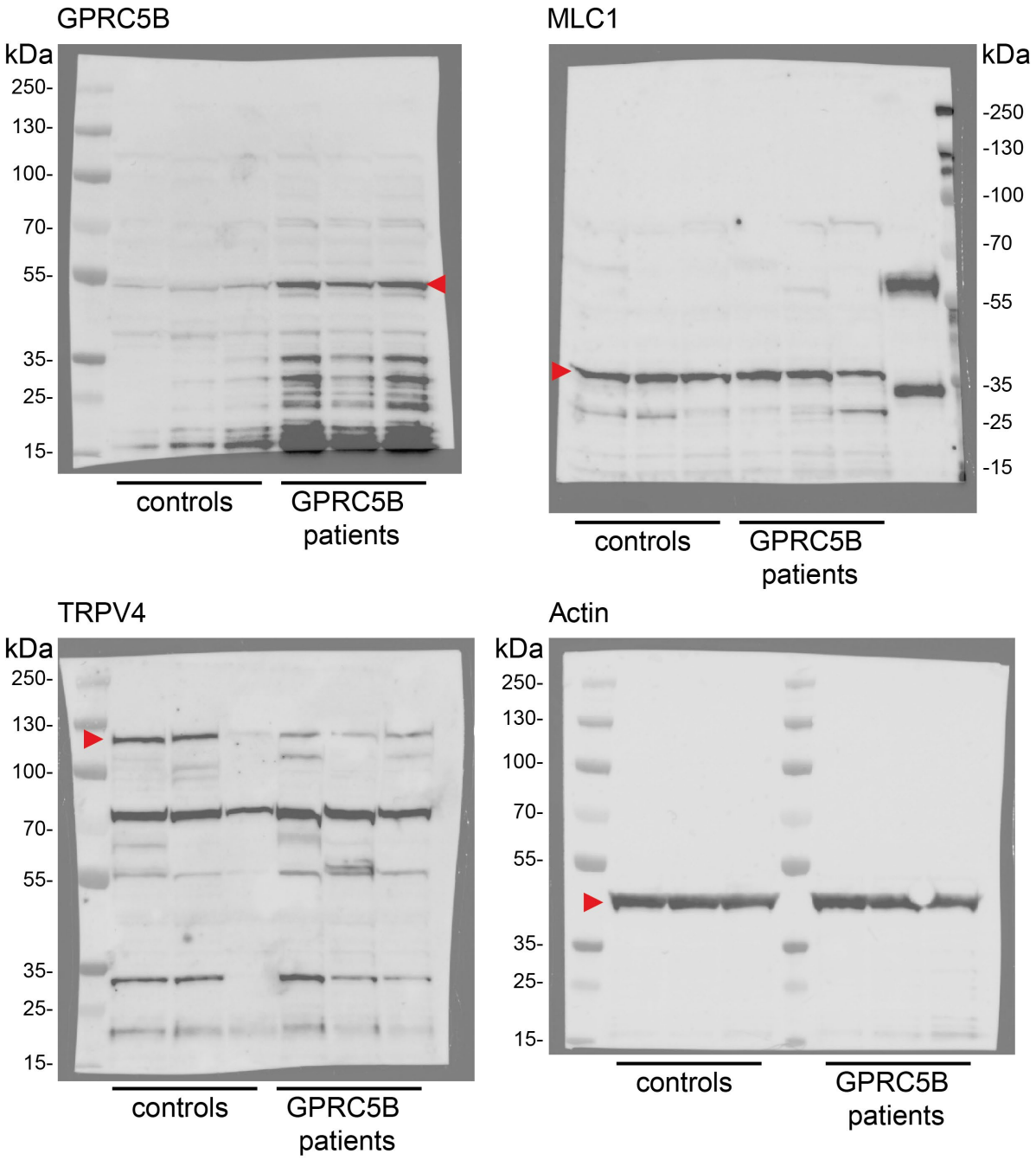
Supplementary Figure 1. Developmental localization and expression of GPRC5B in the human brain. (**A**) Example immunofluorescence images from human frontal white matter from a 1 day old (top) and an 8 year old (bottom) donor. Staining for the astrocyte protein GFAP (magenta) and GPRC5B (green). In the developing brain, GPRC5B localization is more restricted to perivascular regions, when compared to adult brain (Figure 4). Scale bars: 50 µm. (**B**) *GPRC5B* mRNA expression in human white matter lysates was quantified using qPCR (*AKT* as reference). *GPRC5B* mRNA levels increase with age.(**C**) Similarly, western blot analysis on human white matter lysates shows an increase in GPRC5B protein levels with age.



Supplementary Figure 2. Full-length blots belonging to Figure 2. Full western blot gels for characterization of AQP4-GFP expression (as assessed using anti-AQP4 (top) or anti-GFP (middle) antibodies) in different stably transfected MDCK cell lines, performed in triplicate. β-actin was used as loading control (bottom). Cropped gels shown in Figure 2B.



Supplementary Figure 3. Full-length blots belonging to Figure 3. Full western blot gels for characterization of AQP4-GFP expression (as assessed using anti-AQP4 (top) antibody) in transiently transfected HEK293T cells, performed in triplicate. α-tubulin was used as loading control (bottom). Cropped gels shown in Figure 3D.



Supplementary Figure 4. Full-length blots belonging to Figure 5. Full western blot gels for determination of protein levels for GPRC5B, MLC1 and TRPV4 in patient lymphoblast cell lines. Actin was used as loading control. Cropped gels shown in Figure 5D.

Supplementary Tables

Supplementary Table 1: Genetic and clinical details.

	Patient, family				
	Pt 1, F1	**Pt 2, F2**	**Pt 3, F3**	**Pt 4, F4**	**Pt 5, F4**
General					
Number	EL104	EL919	EL138	EL788	EL950
Sex	male	male	female	male	female
Current age	19 y	27 y	24 y	16 y	13 y
Consanguinity of parents	-	-	-	+	+
Genetic defect	*GPRC5B* c.526_528dup, p.(Ile176dup) heterozygous *de novo*	*GPRC5B* c.526_528dup, p.(Ile176dup) heterozygous *de novo*	*GPRC5B* c.528_530dup, p.(Ala177dup) heterozygous *de novo*	*AQP4* c.643G>A, p.(Ala215Thr) homozygous	*AQP4* c.643G>A, p.(Ala215Thr) homozygous
Medical history					
Initial cognitive development	normal	mildly delayed	normal	highly delayed	mildly delayed
Initial motor development	normal	mildly delayed	normal	highly delayed	mildly delayed
Age at					
determination of increased head circumference	9 mo	7 mo	6 mo	6 mo	6 mo
unsupported walking	never	3 y	13 mo	4 - 5 y	2 y
start motor decline	4 y	4 y	8 y	n.a.	n.a.
loss of unsupported walking	n.a.	5 y	8 y	n.a.	n.a.
full wheelchair dependency	6 y	6 y	12 y	n.a.	n.a.
start of cognitive decline	4 y	15 y	14 y	-	-
Behavioural problems	rage	-	-	in childhood agitation, impulsivity, hyperactivity and sleep disorder, later improvement	impulsivity, hyperactivity in early childhood, later normalization
(History of) psychiatric diagnoses	-	-	depression since age 14 y	-	-

Epilepsy – age first seizure	refractory generalized seizures – 4 y	occasional generalized seizures – 14 y	2 single seizures after a fall – adulthood	severe epilepsy – 1 y	occasional generalized seizures – 1 y
Antiepileptic drugs used	oxcarbazepine and clobazam	levetiracetam	no drugs	valproic acid, lamotrigine, clobazam and cannabinol	lamotrigine and valproic acid
Status epilepticus	-	unknown	-	frequent	-
Current status					
Head circumference	exact measurement unknown: > + 2SD	62 cm: > + 2SD	61.5 cm: > + 2SD	57.5 cm: normal	58 cm: > + 2SD
Clumsiness	+	+	+	+	+
Spasticity of arms	+	+	+	-	-
Spasticity of legs	+	+	+	-	-
Gait / truncal ataxia	-	+	+	+	-
Appendicular ataxia	-	+	+	+	-
Dystonia	-	+	-	+	-
Rigidity	-	unknown	+	+ (mild)	-
Dysarthria	+	+	+	+	-
Dysphagia	-	+	-	-	-
Cognition	severe cognitive deficit	mild cognitive deficit	mild cognitive deficit	severe cognitive deficit	mild cognitive deficit
Autistic features	unknown	-	-	-	-
GMFCS level	V	IV	V	II	I
MACS level	V	IV	III	IV	II
CFCS level	V	IV	II	IV	II

y: years, mo: months, + present, - not present

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