Full Methods

Animal experiments

Mice (C57bl6/j), 15-20 g, were obtained from Harlan Laboratories and maintained at the animal facility of the Radboud UMC. Mice were divided in 5 groups of 3 male and 3 female mice. Group 1 (control): mice received normal rodent diet (ssniff® R/M-H V1534, ssniff Spezialdiaten GmbH, Germany) for 7 days. Groups 2-5; mice received lithium chloride in a concentration of 40 mmol/kg of chow and were sacrificed after 4 (group 2), 7 (group 3), 10 (group 4) or 13 (group 5) days of lithium exposure. All mice had free access to water, food, and a sodium chloride block. For the last 48 hours of the experiment, mice were housed in metabolic cages in order to measure water intake and urine output during the last 24 hours. Mice were anaesthetized with isofluorothane and, after removal of blood by eye extraction, killed by cervical dislocation, after which the kidneys were removed.

One kidney was fixed for immunohistochemistry by overnight immersion in 4% (wt/vol) paraformaldehyde in PBS, while the other kidney was stored at -20°C for immunoblotting. These experiments were approved by the Animal Experiments Committee of the Radboud University Nijmegen Medical Centre.

Urine was centrifuged at 4000g for 5 min to remove sediment. Urine samples were then analysed for osmolality with use of Micro-Osmometer Model 3320 (Advanced® instruments Inc., Massachusetts, USA).

Cell culture and lithium treatment assay

2D filter model: Murine principal collecting duct (mpkCCD) cells were cultured as described.[1] In short, the cells were cultured in a modified defined medium containing DMEM:Ham's F12 1:1 vol/vol; 60 nM sodium selenate, 5 μg/ml transferrin, 2 mM glutamine, 50 nM dexamethasone, 1 nM triiodothyronine, 10 ng/ml epidermal growth factor, 5 μg/ml insulin, 20 mM D-glucose, 2% fetal calf serum, and 20 mM HEPES (pH 7.4). Cells were seeded at a density of $1.5x10^5$ cells/cm^2 on semi-permeable filters (Transwell, 0.4 μm pore size, Corning Costar, Cambridge, MA). After 72 hours, the cells were treated with 1 nM 1-deamino-8-Darginine vasopressin (dDAVP) at the basolateral side to induce AQP2 expression. Simultaneously, cells were incubated with 1 mM lithium chloride at the basolateral side and 10 mM lithium chloride at the apical side. After 4, 7 or 11 days of lithium exposure, the cells were either trypsinized, collected in medium and then used for the DNA image cytometry or peletted and prepared for immunoblotting.

3D spheroid model: MpkCCD cells were prepared at a concentration of $12x10^5$ cells/ml in medium containing 2 nM dDAVP with or without 20 mM lithium chloride, and mixed 1:1 (vol/vol) with Matrigel (Becton Dickinson, Bedford, United Kingdom). Then, 0.36 ml aliquots were plated into 48 well plates (Corning Costar, New York, USA). The cell/Matrigel mix was incubated at 37°C for 2 hours to allow gelling. Afterwards, the wells were filled with dDAVP-containing medium with/without lithium. After three days, the cells were collected in Recovery medium (Becton Dickinson, Bedford, United Kingdom) and used for DNA Image cytometry or peletted and prepared for immunoblotting.

DNA image cytometry

DNA image cytometry was performed as described.[2] In short, the cells were collected in medium and centrifuged at 150g for 10 minutes, after which the pelleted cells were fixed in 4% formalin for 30 minutes. After centrifugation at 150g for 10 minutes, the pellets were collected in PBS. Samples from the spheroid 3D model were then treated with 5 mg/mL pepsin (Sigma Aldrich, Zwijndrecht, The Netherlands) in PBS (pH 1.5 with HCl) for 10 minutes at 37°C. Next, the pellets of nuclei were collected by centrifugation (150g) and counted using a Coulter counter (Beckman Coulter, Woerden, The Netherlands). Subsequently, the suspensions were centrifuged (150g) and the pellets were re-suspended in PBS to a dilution of $4x10^5$ nuclei per ml. A cytospin of $4x10^4$ nuclei /100 µL was prepared (100 µL, 10 min, 100g), followed by fixation in Böhm fixative at room temperature for 60 minutes. Next, the slides were rinsed with methanol, dried to air, incubated in 1 M HCl solution for 60 min at 25°C, washed in distilled water and stained for 60 min in Schiff's reagent. After incubation, the slides were washed for 20 min in running tap water followed by increasing concentrations of alcohol and after washing in xylol mounted with permount (Fisher Scientific, Fair Lawn, USA).

Thereafter, DNA Image cytometry was performed with Q-path DNA software to determine DNA ploidy of the nuclei (Leica Imaging Systems Ltd, Cambridge, UK). The analysis was performed as described.[2] Briefly, DNA content of 5000 nuclei was calculated automatically for each sample. Images of overlapping nuclei were manually excluded from the analysis. The data were then further analyzed using the MultCycle™ AV software (Phoenix Flow Systems, Washington, USA). Cell cycle phase was

determined according to the consensus criteria of the European Society for Analytical Cellular Pathology.[3] In short, the measured Internal Optical density (IOD) of each segmented nucleus is plotted in a histogram and the G1 phase is recognized by a Gaussian peak. G2 and mitotic cells, having twice the normal G1 DNA content produce a Gaussian peak with a mean position of approximately twice of that of the G1 peak. S phase cells are observed just above the position occupied by all the G1 cells and extends to just below the position of the G2 cells. MultiCycle AV (Phoenix flow systems, San Diego, CA, USA) was used to determine the amount of cells in each phase via a deconvolution method and curve fitting.

Immunoblotting

Cells were lysed in Laemmli buffer, incubated at 37°C for 30 minutes, sonicated with a Branson Sonifier (Branson Ultrasonics Corporation, Danbury, USA) and analyzed by SDS-PAGE and immunoblotting as described.[4] As primary antibodies, affinity-purified rabbit R7 AQP2 (1:1500),[5] mouse cyclin B1 (1:1000, Abcam, Cambridge, UK), rabbit Histone-H3 (1:5000, Abcam, Cambridge, UK), rabbit phosphoSer10-Histone-H3 (pHistone-H3; 1:4000, Cell Signaling, Massachusetts, USA), mouse PCNA (1:1000, Abcam, Cambridge, UK), mouse Cdc2 (CDK1; 1:1000, Santa Cruz Biotechnology, Santa Cruz, USA), rabbit phosphoTyr15-cdc2 (p-CDK1; 1:1000, Cell Signaling, Massachusetts, USA), mouse β-actin (1:10000, Sigma, St. Louis, USA), and rabbit cyclin D1 Sp4 (1:800, ImmunoLogic, Duiven, Netherlands) were used. As secondary antibodies 1:10000-diluted goat anti-rabbit or sheep anti-mouse peroxidase-conjugated antibodies were used. Proteins were visualized using enhanced chemiluminescence (ECL; Thermo

scientific, Rockford, USA). Pictures of the blots were taken using BioRad Universal Hood II and Quantity I software package (Bio-rad Laboratories Inc., Hercules, USA). Later, blots were stained with coomassie blue G250 (Serva, Heidelberg, Germany) to confirm loading of protein equivalents.

Immunocytochemistry

Culture medium was removed from matrigel-cultured mpkCCD cells and cells were subsequently washed 3 times with ice-cold PBS/0.5 mM $CaCl_2$/1.0 mM $MgCl_2$ and fixed for 30 min at room temperature with 4% w/v paraformaldehyde in PBS. After washing twice with PBS, cells were treated with permeabilization buffer (0.5% v/v Triton X-100 and in PBS with 0.7% w/v gelatin) for 30 min. Then, cells were incubated at 4°C overnight with primary antibodies (1:50 Rb5 AQP2 and 1:500 α-tubulin (Invitrogen, Camarillo, USA) in permeabilization buffer and after washing for 3 times with secondary antibodies conjugated to Alexa dyes (Invitrogen, Paisly, UK) (1:500) for 2-3 hours at 37°C. Finally, cells were mounted in Vectashield containing DAPI (Vector Laboratories, Burlingame, USA) and imaged by confocal microscopy (Olympus FV1000, Center Valley, PA, USA).

Immunohistochemistry

Immunohistochemistry was performed as described.[6] In short, the kidneys were removed and incubated O/N in paraformaldehyde (4%) in PBS at 4°C. After fixation, kidneys were dehydrated and embedded in paraffin. Five-micrometer sections were cut, stretched in 37°C water, and dried on gelatin-coated object glass (Menzel Gläser, Braunschweig,

Germany) for at least 1 hour at 37°C. After the sections were deparaffinized with xylol and rehydrated with ethanol and water, sections were boiled in 10 mM sodium citrate (pH 6.0) for 30 min. After cooling down in TN buffer (0.15 M NaCl, 0.1 M Tris/HCl, pH 7.5) for 3 hours, sections were incubated for 30 min in 20mM Glycine in TN buffer and subsequently, after washing, incubated for 30 min with 0.3% H_2O_2 in TN buffer. Next, sections were incubated for 30 min in TNB buffer (TN buffer containing 0.5% (w/v) blocking reagent from NEN Life Science Products, Zaventem, Belgium) and incubated O/N at 4°C with 1:1000 rabbit anti-pHistone-H3 in TNB buffer. Sections were then washed three times with TNT buffer (TN buffer containing 0.05% (v/v) Tween 20) and incubated for 60 min at room temperature with biotin-labeled secondary anti-rabbit antibody (1:1000). After washing with TNT, sections were incubated for 30 min with 1:100 Streptavidin-HRP (TSA Fluorescein System, PerkinElmer, Waltham, USA) in TNB, washed again and incubated O/N at 4°C with 1:100 mouse PCNA and 1:400 rabbit H^+-ATPase (gift from Dr. S. Nielsen, Denmark) in TNB. After washing, sections were incubated with secondary antibodies conjugated to Alexa dyes (1:1000) for 1 hour at 37 °C, washed again and incubated O/N at 4°C with 1:100 guinea pig AQP4. Next day, sections were washed, incubated with an Alexa-conjugated secondary antibody against guinea pig for 1 hour at 37°C, washed again, and incubated for 7 min with 1:50 Fluorescein Tyramide in Amplification Diluent (TSA Fluorescein System, PerkinElmer, Waltham, USA). After washing and incubation with DAPI (1:10.000 in TN) for 30 min, sections were embedded in Fluoromount G (Southern Biotech, Birmingham, USA).

Statistical analysis

The difference between groups was tested by student t-test and one-way ANOVA corrected by the Newmans-Keuls multiple-comparisons procedure. A p-value < 0.05 was considered statistically significant.

References

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