Title: Reactive microglia and mitochondrial unfolded protein response following ventriculomegaly and behavior defects in kaolin-induced hydrocephalus

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Running Title: Reactive microglia and UPRmt in hydrocephalus

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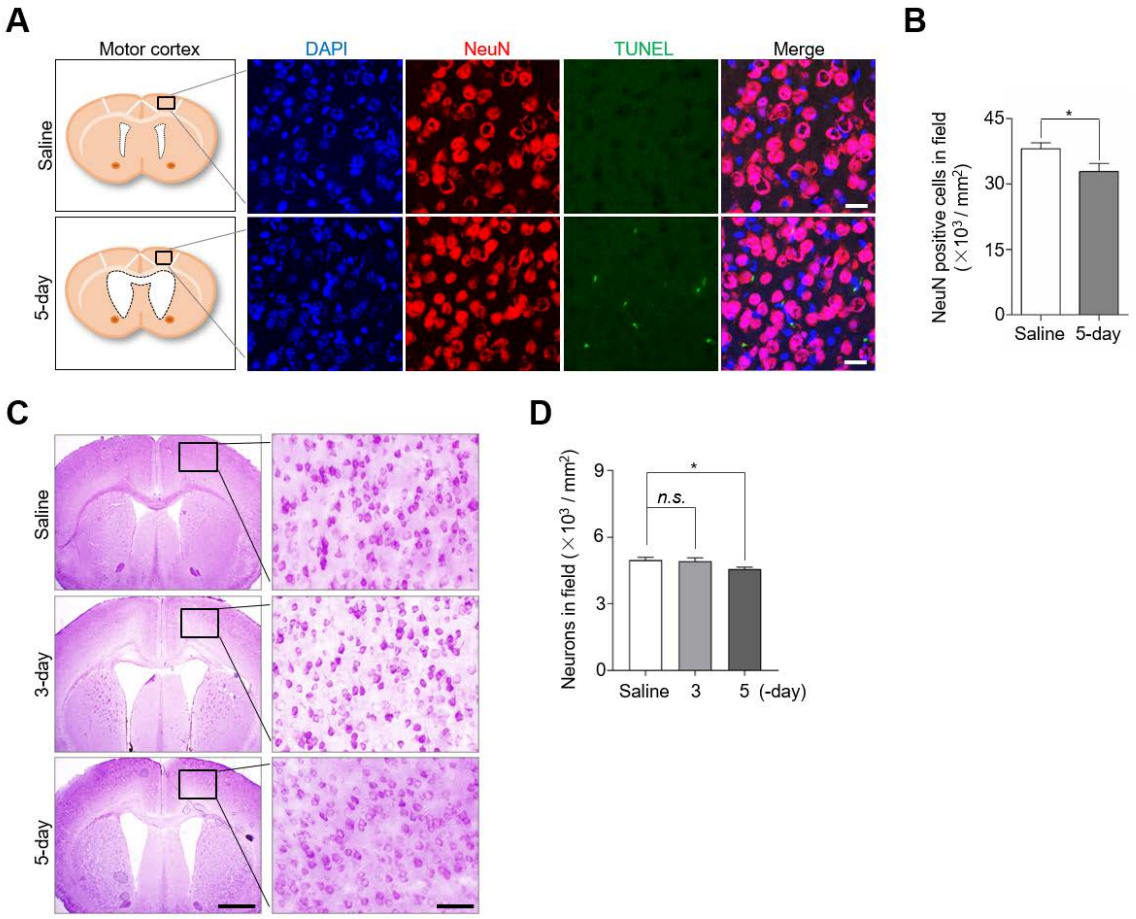
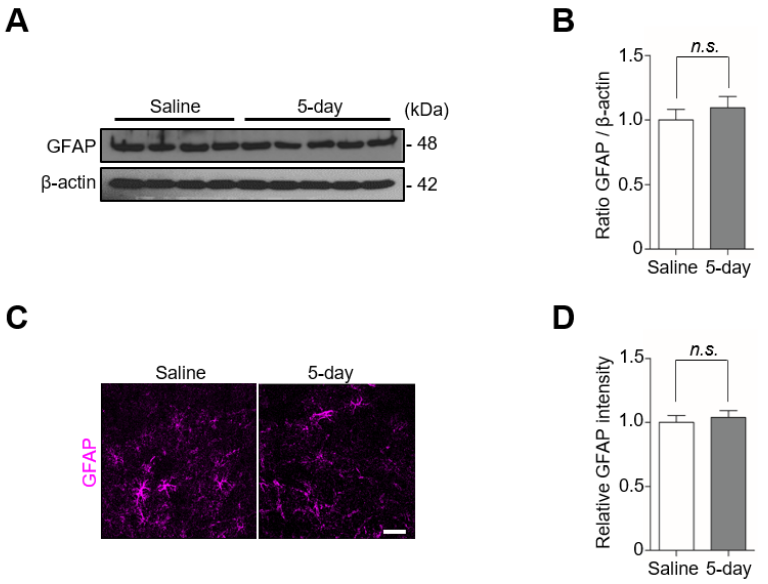
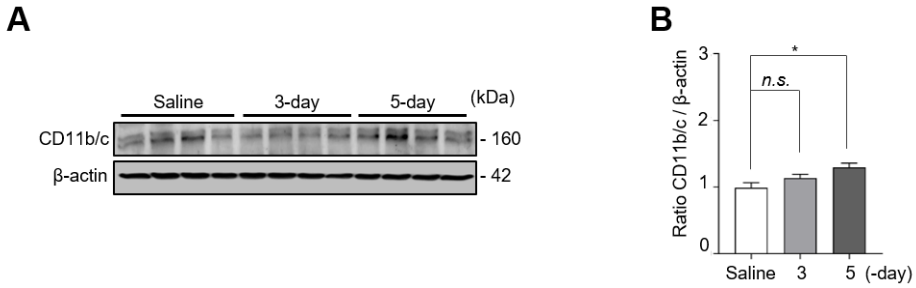
SUPPLEMENTAL MATERIALS



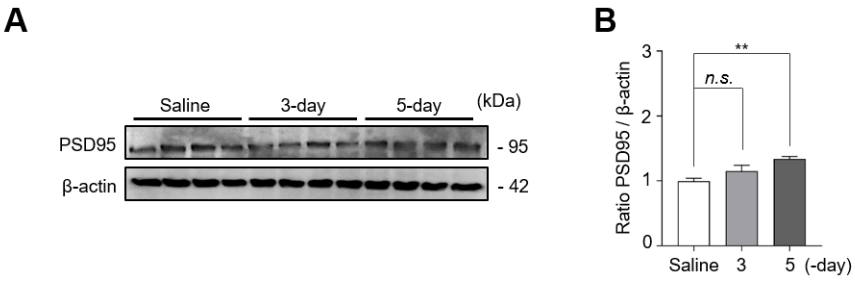
Supplemental fig. 1. The percentage of apoptotic neurons increased in the 5-day kaolin group. (A) The motor cortex above the ventricles was stained for DAPI (blue), NeuN (red), and TUNEL (green). (B) The NeuN positive cells in field were quantified, n = 6. (C) The motor cortex above the ventricles was stained by Nissl. (D) The neurons in field were quantified, n = 3 (* P ⟨ 0.05; n.s., not significant). Scale bar: A: 20 μm, C: 200 μm, the enlarged images: 36 μm.



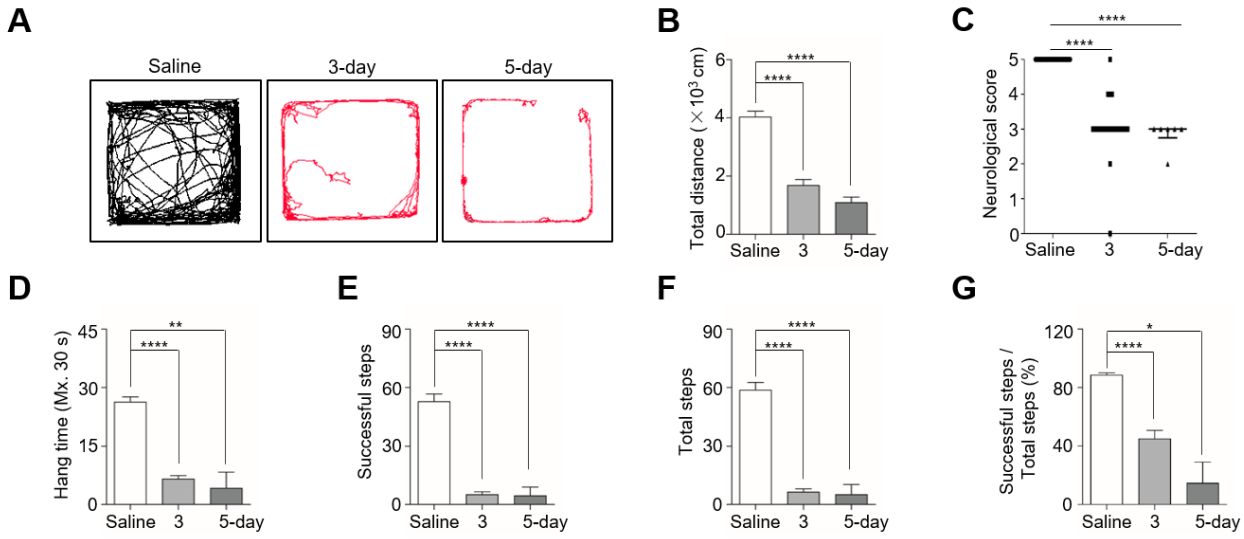
Supplemental fig. 2. The expression of astrocyte was not changed in the 5-day kaolin group. (A) The expression of GFAP in the motor cortex was analyzed by western blotting. (B) The intensity value of GFAP was shown. (C) The motor cortex was stained for the marker of astrocyte GFAP. (D) The immunofluorescence intensity of GFAP was quantified. Western blotting n (saline) = 4, n (5-day) = 5, immunofluorescence n = 5 (n.s., not significant). Scale bar: 20 μm.



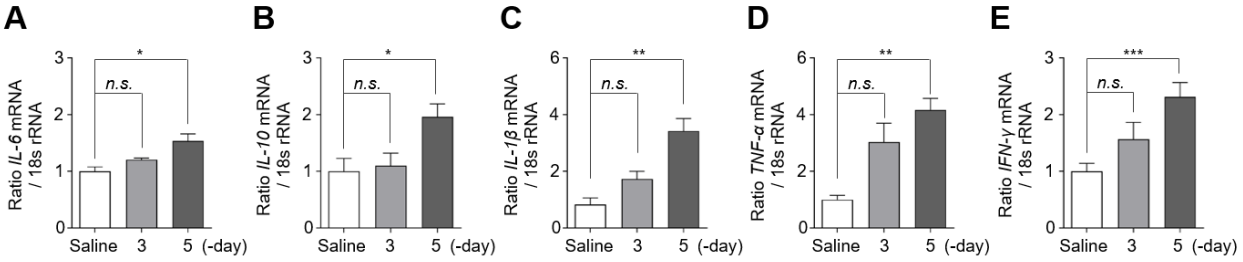
Supplemental fig. 3. Microglia activated in the 5-day kaolin group. (A) The expression of CD11b/c in the motor cortex was analyzed by western blotting. (B) The intensity value of CD11b/c was shown, n = 4 (* P ⟨ 0.05; n.s., not significant).



Supplemental fig. 4. PSD95 upregulated in the 5-day kaolin group. (A) The expression of PSD95 in the motor cortex was analyzed by western blotting. (B) The intensity value of PSD95 was shown, n = 4 (** P ⟨ 0.01; n.s., not significant).



Supplemental fig. 5. Kaolin-induced hydrocephalus mice show motor disturbances in both 3-day and 5-day groups. (A, B) Movement activity was measured for 10 min in the open-field test. (C) The neurological function was scored by a 5-point paradigm and plotted. (D - G) Bar plots showed the results were calculated for 30 s in a horizontal grid test. Behavior test n (saline) = 16, n (3-day) = 24, and n (5-day) = 6. For the neurological score, n (3-day) = 24. For the open-field test and horizontal grid test, n (3-day) = 23 (* P ⟨ 0.05, ** P ⟨ 0.01, **** P ⟨ 0.0001).



Supplemental fig. 6. Inflammatory cytokines expression increased in the 5-day kaolin group. (A - E) The expression of *IL-6, IL-10, IL-1β, TNF-α,* and *IFN-γ* in the motor cortex was analyzed by qPCR, n = 4 (* P ⟨ 0.05, ** P ⟨ 0.01, *** P ⟨ 0.001; n.s., not significant).

MATERIALS AND METHODS

Animals

Eight weeks old male C57BL/6J mice (Damul Science, Daejeon, Korea), provided with a standard chow diet (Research Diets, AIN-76A, New Brunswick, NJ, USA) and water *ad libitum*. Mice were maintained at 22˚C, 12 h light/dark cycle (light phase: 6:00 to 18:00, dark phase: 18:00 to 6:00). All the experimental procedures were approved by the Institutional Animal Care and Use Committee of Chungnam National University (ethical approval number, 202103A-CNU-022).

Mice model of kaolin-induced hydrocephalus

The mouse head was fixed in the stereotactic frame (KOPF, CA), anesthetized with 2.5% sevoflurane (Ilsung, Seoul, Korea) in an O_2 air mixture (2:1) delivered by loosely snout mask, anesthesia was verified by touching the footpad. Wipers tissue to protect mouse eyes, 70% ethanol sterilized mouse head. Followed midline made 1 cm incision, separated the soft tissue and muscle, exposed the cisterna magna. In the saline group, cisterna magna was injected with 10 μL saline. In the kaolin group, cisterna magna was injected with 10 μL 25% kaolin (250 mg/mL in saline; Sigma, K7375), through a 0.5 mL insulin syringe (Becton-Dickinson, 328821). After surgery, mice have sutured the muscle and skin, allowed to recover from anesthesia on a heated surface for 30 min. Returned the mice to their cages, kept them at room temperature, and provided them with standard chow and water *ad libitum*.

Measurement of ventricular size

Mice were randomly divided into 3 experimental groups, 6 mice per group: (a) saline group (the fifth day after 10 μL saline injection), (b) 1-day group (the first day after 10 μL 25%

kaolin injection), (c) 3-day group (the third day after 10 μL 25% kaolin injection), and (d) 5-day group (the fifth day after 10 μL 25% kaolin injection). All the mice were euthanized with sevoflurane and then brains were collected. Mice brains were immersed in 4% paraformaldehyde for 48 h and dehydrated in 30% sucrose solution at 4˚C for another 48 h. Then samples were frozen and sliced into coronal sections, from the anterior horn of the lateral ventricle (Lv) to the 4th ventricle (4v), the thickness of 100 μm using a cryotome (Leica). Slices were mounted on glass slides and imaged at 20 magnifications under a microscope (Olympus, Japan). The size of the Lv, the dorsal part of the 3rd ventricle (d3v), the ventral part of the 3rd ventricle (v3v), and the 4v were analyzed by the Image J software.

Behavioral analysis

Mice were randomly divided into 2 experimental groups: (a) 16 mice in the saline group (the third day after 10 μL saline injection), (b) 24 mice in the 3-day group (the third day after 10 μL 25% kaolin injection), and (c) 6 mice in the 5-day group (the fifth day after 10 μL 25% kaolin injection). Mice were handled 3 days before behavioral tests, to reduce the effects that handling stress might have on the tests. All tests were carried out from 9:00 to 18:00 during the light phase, in the same low-intensity lightroom, and analyzed by the same experimenter. After the test, mice were returned to their cage and the boxes were cleaned with 70% ethanol. EthoVision XT 11.5 software to analyze the mice behavior.

Open-field test

The general activity was recorded by placing the mice in a 40 × 40 × 40 cm box for 10 min. To start the test, a mouse was placed at the center of the box. And the travel distance was recorded.

Neurological score

Mice were scored for global neurologic function, using a modified neurological scale as follows: normal (5), decreased scavenging activity and scatter reflex (4), no spontaneous scavenging, loss of scattering reflex, ataxia (3), non-purposeful movements (2), loss of righting reflex (1), dead (0) (1).

Horizontal grid test

The hang time, successful steps, and total steps were recorded, by placing the mouse in a 12 × 12 cm horizontal square grid box. The box includes the bottom clear plexiglass walls, a height of 20 cm. The top black plexiglass walls, the height of 8 cm, with 0.8 × 0.8 cm wire mesh. Placed the grid side on the floor, put the mouse in the box. When the mouse grabbed the grids with four paws, the box inverted slowly, the mouse would hang on the grid. The camera recorded for 30 s and replayed the videos for analysis.

Immunofluorescence

Mice were randomly divided into 2 experimental groups, 6 mice per group: (a) saline group (the fifth day after 10 μL saline injection) and (b) 5-day group (the fifth day after 10 μL 25% kaolin injection). All the mice were euthanized with sevoflurane and then brains were collected. Mice were perfused and fixed with 4% paraformaldehyde and dehydrated with 30% sucrose solution at 4˚C for 48 h. Then samples were frozen and sliced into coronal sections, at a thickness of 30 μm using a cryotome. The sections were stored in tissue stock solution and blocked in 2% donkey serum (Gene Tex), 0.3% Triton X-100 with phosphate-buffered saline (PBS) for 1.5 h and then incubated with anti-TNF-α (1:100; Abcam, ab6671), ionized calcium-binding adaptor molecule-1 (Iba-1) (1:200; Novus Biologicals, NB100-1028), GFAP (1:600, Abcam, 4674), at 4˚C overnight. Washed with PBS, and incubated Alexa Fluor® 488 AffiniPure Donkey Anti-Rabbit IgG (H+L) (1:100; Jackson Immuno, 711-545-152), Alexa Fluor® 594 AffiniPure Donkey Anti-Goat IgG (H+L) (1:200;

Jackson Immuno, 705-585-147), Alexa Fluor® 647 AffiniPure Donkey Anti-Chicken IgG (H+L) (1:600; Jackson Immuno, 703-605-155) for 1.5 h at room temperature, Hoechst 33342 Trihydrochloride (1:5000; MedChemExpress, HY-15559A) for 3 min at room temperature. Using fluorescent mounting solution (Dako) mounted tissue on slides, imaged by the confocal microscope (Leica). Fluorescence integrated density (IntDen) was quantified with the Image J software.

ELISA measurements

Mice were randomly divided into 4 experimental groups, 6 mice per group: (a) saline group (the fifth day after 10 μL saline injection), (b) 1-day group (the first day after 10 μL 25% kaolin injection), (c) 3-day group (the third day after 10 μL 25% kaolin injection), and (d) 5-day group (the fifth day after 10 μL 25% kaolin injection). All the mice were euthanized with sevoflurane and then brains were collected. The motor cortex above the ventricles was homogenized in 200 μL PBS, centrifuged at 3000 rpm for 5 min at 4 °C. The supernatants were stored at -70°C until performed mouse TNF-α enzyme-linked immunosorbent assay (ELISA) (KOMA, K0331186) according to the manufacturer's instructions.

Western blotting

Mice were randomly divided into 4 experimental groups, 3 mice per group: (a) saline group (the fifth day after 10 μL saline injection), (b) 1-day group (the first day after 10 μL 25% kaolin injection), (c) 3-day group (the third day after 10 μL 25% kaolin injection), and (d) 5-day group (the fifth day after 10 μL 25% kaolin injection). All the mice were euthanized with sevoflurane and then brains were collected. The motor cortex above the ventricles was separated and lysed by radioimmunoprecipitation assay (RIPA) buffer with phosphatase inhibitor and protease inhibitor cocktail (Roche) to extract the protein. The extracted protein (each 20 μg protein) was run on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then were transferred to polyvinylidene fluoride (PVDF) membrane (Millipore). The PVDF membranes were blocked with 2.5% bovine

serum albumin (BSA) (GenDEPOT) for 1 h at room temperature, then incubated 4˚C, overnight with anti-TNF-α (1:1000; Abcam, ab6671), p65 (1:500; Cell Signaling Technology, 8242), phosphorylated p65 (P-p65) (1:500; Cell Signaling Technology, 3033), Iba-1 (1:500; Novus Biologicals, NB100-1028), LONP1 (1:1000; Abcam, 103809), HSP60 (1:5000; Abcam, 46798), CLPP (1:1000; Sigma, HPA010649), PARP-1 (1:1000; Cell Signaling Technology, 9532), GFAP (1:5000, Abcam, 7260), CD11b/c (1:500, Neuromics, RA25012), PSD95 (1:2000, Thermo, MA1-046), and β-actin (1:2000; Santa Cruz Biotechnology, sc-47778) in 1% BSA. The PVDF membranes were washed with Tris-buffered saline with Tween (TBST) and incubated with secondary antibodies IgG horseradish peroxidase antibody (HRP, Pierce Biotechnology), for 1.5 h at room temperature. The protein band was visualized by the enhanced chemiluminescence (ECL) reagent (Thermo). Then used medical X-ray film blue (AGFA CP-BU NEW), developer solution, and fixer solution for the ECL detection. Band intensity was quantified with the Image J software.

TUNEL staining

Mice were randomly divided into 2 experimental groups, 6 mice per group: (a) saline group (injected with 10 μL saline as vehicle solution) and (b) 5-day group (the fifth day after 10 μL 25% kaolin injection). All the mice were euthanized with sevoflurane and then brains were collected. The brain tissues were embedded in 4% paraformaldehyde, dehydrated brain sections were stained with terminal-deoxynucleotidyl transferase-mediated nick end labeling (TUNEL) assay (Roche, 11684795910) according to the manufacturer's instructions. Double-label with neuronal nuclear antigen (NeuN) (1:200; Abcam, ab104224). Finally, the sections were covered with 4'6-diamidino-2-phenylindole (DAPI) (1:5000; Thermo, H3570). The slides were imaged using the confocal microscope (Leica). Fluorescence IntDen was quantified with the Image J software.

Nissl staining

Mice were randomly divided into 3 experimental groups, 3 mice per group: (a) saline group (injected with 10 μL saline as vehicle solution), (b) 3-day group (the third day after 10 μL 25% kaolin injection), and (c) 5-day group (the fifth day after 10 μL 25% kaolin injection). All the mice were euthanized with sevoflurane and then brains were collected. The brain tissues were embedded in 4% paraformaldehyde, dehydrated brain sections were stained with 0.1% cresyl violet for 3 min, dehydrated through graded alcohols (70, 95, 100%). Using fluorescent mounting solution (Dako) mounted tissue on slides, imaged by the confocal microscope (Leica). Neurons in field were quantified with the Image J software.

Quantitative Real-time PCR (qPCR)

Mice were randomly divided into 4 experimental groups, 3 mice per group: (a) saline group (the fifth day after 10 μL saline injection), (b) 1-day group (the first day after 10 μL 25% kaolin injection), (c) 3-day group (the third day after 10 μL 25% kaolin injection), and (d) 5-day group (the fifth day after 10 μL 25% kaolin injection). Total RNAs were isolated by TRIzol reagent (Thermo) and cDNA was prepared with reverse transcription master premix (5 × Rnase H+). The qPCR was performed with cDNA, SYBR green PCR master mix (PhileKorea, Korea), and primers. *Lonp1* primer (Forward: 5`-GACAGAGAACCCGCTAGTGC-3`, Reverse: 5`-CTCAGTGGTTCTGGGATGGT-3`), *Hspd1* primer (Forward: 5`-GAGCTGGGTCCCTCACTCG-3`, Reverse: 5`-AGTCGAAGCATTTCTGCGGG-3`), *Clpp* primer (Forward: 5`-GCCATTCACTGCCCAATTCC-3`, Reverse: 5`-TGCTGACTCGATCACCTGTAG-3`), *IL-6* primer (Forward: 5`-ACAACCACGGCCTTCCCTACTT-3`, Reverse: 5`-CACGATTTCCCAGAGAACATGTG-3`), *IL-10* primer (Forward: 5`-ATAACTGCACCCACTTCCCA-3`, Reverse: 5`-GGGCATCACTTCTACCAGGT-3`), *IL-1β* primer (Forward: 5`-TGACGGACCCCAAAAGATGA-3`, Reverse: 5`-AAAGACACAGGTAGCTGCCA-3`), *TNF-α* primer (Forward: 5`-CCCACGTCGTAGCAAACCAC-3`, Reverse: 5`-GCAGCCTTGTCCCTTGAAGA-3`), *IFN-γ* primer (Forward: 5`-AGACATCTCCTCCCATCAGCAG-3`, Reverse: 5`-TAGCCAAGACTGTGATTGCGG-3`), and *18s rRNA* primer (Forward: 5′-CGACCAAAGGAACCATAACT-3′, Reverse: 5′-

CTGGTTGATCCTGCCAGTAG-3′). Results were analyzed with the Rotor-Gene 6000 real-time rotary analyzer system (Corbett Life Science).

Statistical analysis

All results are acquired from at least three independent experiments and are presented as the mean ± SEM. Data were compared using a student's *t*-test or a one-way ANOVA (Prism software). P-values 〈 0.05 were deemed statistically significant.

Reference:

1. Bloch O, Auguste KI, Manley GT and Verkman AS (2006) Accelerated progression of kaolin-induced hydrocephalus in aquaporin-4-deficient mice. J Cereb Blood Flow Metab 26, 1527-1537