Supporting Information

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SI Materials and Methods

Rat. Adult male Sprague–Dawley rats (300 ± 20 g) in healthy, clean-grade condition (certification no. SCXK2008-0033) were purchased from the Experimental Animal Center of Zhejiang Province (China) and maintained under a 12/12-h light/dark cycle (lights on 06:00–18:00 h), at room temperature (20 ± 2 °C), with free access to food and water. Rats were housed in groups of six and adapted to these conditions for 1 wk before experimental manipulation. All animal procedures were performed according to National Institutes of Health guidelines under protocols approved by the Institutional Animal Care and Use Committee of Zhejiang University.

Hypoxia Model. Thirty minutes before experiments, rats were injected s.c. with 15 or 30 mg/kg corticotropin-releasing factor receptor 1 (CRFR1) antagonist CP154,526 [*N*-butyl-*N*-ethyl-4,9-dimethyl-7-(2,4,6-trimethylphenyl)-3,5,7-triazabicyclo[4.3.0]nona-2,4,8,10-tetraen-2-amine; provided by Pfizer] or intraperitoneally with 150 mg/kg NF-κB inhibitor pyrrolidine dithiocarbamate (PDTC) (Sigma). The doses of CP154,526 and 5-chloro-4-[*N*-(cyclopropyl)methyl-*N*-propylamino]-2-methyl-6-(2,4,6-trichlorophenyl) aminopyridine (NBI) were used according to published papers (1–3). The control group received the same volume of vehicle. Rats were placed into a ventilated and controllable hypobaric chamber (FLYDWC-50-IIC; Avic Guizhou Fenglei Aviation Armament Co., Ltd.) with the same light/dark cycle, nutrition, and temperature. An altitude of 7,000 m (equivalent to 7.8% O_2 at sea level, 200 m/min) was simulated for 8 h. Control groups were set at sea level (20.9% O_2) in the same type of chamber at the same time. In vitro, cultured cerebral cortex astrocytes and mixed microglia were incubated with 1% hypoxia in the Proox Model P110 and ProCO_2 Model P120 hypoxia systems (BioSpherix).

Magnetic Resonance Imaging. Rats were anesthetized with pentobarbital sodium (30 mg/kg i.p.) before MRI, fixed in a body restrainer, and placed in an MRI spectrometer (GE Medical System; 3T). T2-multiecho images were acquired with a spin-echo sequence with the following parameters: 10 contiguous coronal slices at 2-mm thickness, field of view 24 × 24 mm, matrix 128 × 128, repetition time (TR) = 3,950 ms, echo time (TE) = 102 ms. To map the apparent diffusion coefficient (ADC) of water, diffusion-weighted images were acquired with a spin-echo sequence. Ten contiguous coronal slices were acquired (2-mm thick, field of view 24 × 24 mm, matrix 128 × 128, TR = 6,300 ms, TE = 78.4 ms). ADC maps were automatically calculated according to the following equation: $ADC = \ln(S_0/S_1)/(b_1 - b_0)$ (mm^2/s). After hypoxic exposure, rats were immediately placed into a MRI scanner (GE) for ADC measurement and imaging. After MRI, rats were rapidly decapitated and the brain was immediately removed, frozen in liquid nitrogen, and stored at −80 °C.

Western Blot. Aquaporin-4 (AQP4), inducible nitric oxide synthase (iNOS), PKC, and PKG protein expression was determined by Western blot. Briefly, protein samples from cortex were separated by 10% (wt/vol) SDS–polyacrylamide gel elctrophoresis and transferred to PVDF membranes. The membranes were reacted with a rabbit polyclonal antibody against AQP4 (Chemicon; 1:3,000), iNOS (Chemicon; 1:3,000), and PKG (Chemicon; 1:3,000) at 4 °C overnight. After washing, the membranes were sequentially reacted with a horseradish peroxidase-conjugated goat anti-rabbit antibody (Zhongshan; 1:3,000) at room temperature for 1 h. After repeat washes, the membranes were reacted with ECL reagents and exposed to X-ray film to show the bands; AQP4, iNOS, PKC, and PKG were normalized to protein-loading controls (β-actin: 1:4,000, 43 kDa; GAPDH: 1:1,000, 36 kDa) and quantified by densitometry with the Gel Image System (Bio-Rad).

Confocal Imaging. Confocal double immunofluorescence was used to investigate the coexistence of CRFR1 and AQP4 in cortex, mixed hippocampus neurons, and primary astrocyte culture. Before the reaction with primary antibodies, sections were fixed in 4% (wt/vol) buffered paraformaldehyde, incubated in 0.4% Triton X-100 in PBS for 30 min, and then blocked with 10% (vol/vol) normal goat serum in PBS (pH 7.4) for 30 min at 37 °C. Consecutive sections were incubated overnight at 4 °C simultaneously with CRFR1 (Proteintech Group; 1:1,000) or AQP4 (Chemicon; 1:3,000) antibody and glial fibrillary acidic protein (GFAP; Abcam; 1:500) or DAPI (Invitrogen; 1 μg/mL). After incubation, sections were rinsed with PBS three times for 10 min and incubated with secondary antibodies for 1 h at 37 °C. Immunofluorescence was analyzed by confocal microscopy (TE-2000; Nikon; Ultra-ViewERS; PerkinElmer) with Volocity 4D image analysis software. Negative controls for the immunofluorescence reaction were performed without primary antibody or with preadsorption immunizing peptide, and these sections yielded only background staining.

Cell Cultures and Transfection. Primary astrocytes were prepared from cerebral cortices of Sprague–Dawley rats born within 24 h according to the published method (4). Briefly, cortical cells were trypsinized and plated into flasks containing growth medium [high-glucose DMEM supplemented with 10% (vol/vol) FBS]. The medium was changed 3 d later and then every 3 d for a total of 10–12 d. Oligodendrocytes and microglial cells were removed by shaking at 200 rpm for 12 h at 37 °C. More than 90% of the cells were astrocytes as confirmed by immunocytochemical examination with an anti-GFAP antibody. We harvested the cells before purification at 200 rpm for 12 h as unpurified cultures (including 10% microglia).

CHO Cell Transgenic Method. Rat AQP4 plasmid (rAQP4) was generated by PCR with kpnI on the 5′ end and XhoI on the 3′ end and then inserted into the multiple cloning site of pcDNA3.1(+) plasmid (Invitrogen). The rat CRFR1α plasmid (P-R1R) was generated by PCR and constructed by inserting the BamHI and HindIII fragments spliced from pMD19-T (TaKaRa) into the multiple cloning site of pcDNA3.1(−) plasmid (Invitrogen). The CRFR1 cDNA clone was a gift from W. Vale (Salk Institute for Biological Studies, La Jolla, CA). CHO cells were cultured in 1640 medium supplemented with 10% (vol/vol) FBS at 37 °C under 5% CO_2. Cells were seeded on 24-well plates at 70–80% density 1 d before transfection. Transfection was carried out by using a modified liposome-mediated method according to the manufacturer's instructions. Briefly, plasmid DNA (0.67 μg for each of rAQP4 and P-R1R) and cotransfection of rAQP4/P-R1R was done at a ratio of 1:1 mixed with 2 μL of Lipofectamine 2000 (Invitrogen) in 50 μL of serum-free Opti-MEMI. After incubation for 20 min at room temperature, 100 μL of the mixture was layered onto the cells cultured in 1640 medium supplemented with 10% (vol/vol) FBS without antibody. Six hours later, the medium was replaced with serum-containing medium as described. At 24 h after transfection, water permeability was measured in CHO cells.

Drug Treatment. CRF (Phoenix Pharmaceuticals), CRFR1 antagonist, CP154,526 (provided by Pfizer), NBI (provided by Pfizer or NBI-27914, Sigma, N3911), NF-κB inhibitor, PDTC, PKG inhibitor, KT5823, and PKC inhibitor bisindolylmaleimide (Sigma), as well as LPS (*Escherichia coli* 055:B5, Sigma) were used for astrocyte cultures or CHO cell.

Osmotic Water Permeability Measurements. Osmotic water permeability was measured by using a published method (5) with modifications. Briefly, primary astrocytes or transfected cell lines were mounted in a closed perfusion chamber (Warner DH-35i; Live Cell System) on the stage of a TE-2000 inverted confocal laser scanning microscope (Nikon) in isotonic, 300 mOsm HBSS (HBSS buffer; Gibco). The cells were loaded with calcein by incubation with 20 μM calcein-AM (Molecular Probes, Invitrogen) for 5 min at 37 °C. In some experiments, cells were preincubated with 100 nM CRFR1 antagonist CP154,526 (Pfizer) or 100 nM NBI (Pfizer) for 30 min at 37 °C in the corresponding culture medium before calcein-AM loading. Only culture medium was added to the control cells. A series of images was recorded on a confocal laser scanning microscope by using a 40× objective every 2 s with excitation at 488 nm. The emitted fluorescence signal was collected at 515–525 nm with the focal plane set at 1/3 of the cell height from the bottom of the cell monolayer. After 10 s of recording, the perfusate was rapidly switched to hypoosmotic 200 mOsm HBSS with or without CRF. The recorded images were analyzed off-line to obtain the time course of calcein fluorescence in individual cells. Regions of interest (ROIs) were drawn along the inside of the cell membrane, and the mean intensity within the ROI was recorded every 2 s. The parts of the curves obtained immediately after the solution switch (6–10 s) were used for calculations. During these first seconds of exposure to the hypoosmotic solution, the rate of cell swelling is proportional to the permeability of the cellular membrane to water and is not influenced by mechanisms involved in regulatory volume decrease. The initial parts of the fluorescence curves were fitted with a single exponential function, and the time constant was used as a measure of the rate of cell swelling.

Measurement of Intracellular Ca^{2+} and Signal Imaging. The intracellular Ca^{2+} ($[Ca^{2+}]_i$) was measured according to a published method with modifications (6). Briefly, cultured astrocytes on coverslips were incubated with 3 μM fura-3/AM for 45 min in physiological buffer in the dark at room temperature. Cells were then washed with buffer and stored in the dark for an additional 30 min to ensure fura-3/AM hydrolysis. Coverslips with loaded cells were transferred to a perfusion chamber and visualized under the microscope. Photon readings were taken by using a multifunctional microplate reader (Tecan; Infinite M200) at an excitation wavelength of 488 nm and emission at 526 nm. The $[Ca^{2+}]_i$ was computed from the equation $[Ca^{2+}]_i = k_d \times [F - F_{min}]/[F_{max} - F]$, where k_d is the ionic dissociation constant.

Immunoprecipitation. The lysate from 1×10^7 cells was used for one immunoprecipitation. The cells were washed twice with ice-cold PBS and lysed in radioimmunoprecipitation assay lysis buffer. The lysates were cleared by centrifugation at $16{,}000 \times g$ for 15 min at 4 °C. The supernatant was incubated with 10 μL of protein A/G agarose beads and incubated rocking for 1 h at 4 °C. The bead-associated, nonspecifically absorbed proteins were removed by centrifugation for 15 s at $16{,}000 \times g$. The supernatant was then mixed with 20 μL of PPKAS, PPKCS (Cell Signaling; 1:50), and the mixture was incubated rocking for 1 h at 4 °C. The samples were then transferred to protein A/G beads and incubated rocking overnight at 4 °C. The beads were collected by centrifugation and washed three times with ice-cold PBS. After the final wash, the beads were resuspended in 50 μL of SDS/PAGE sample buffer and boiled for 3 min. Then, AQP4 was separated by SDS/PAGE (10% gels) and ECL and analyzed as Western blot.

Quantitative Real-Time PCR. Total RNA was prepared by using TRIzol reagent (Life Technologies). RNA (2 μL) was reverse-transcribed with oligo(dT) 12-18 primer by using the TransScript TM RT enzyme mix and then stored at 20 °C.

The primer sequences were as follows:

CRF: 5′-AAAATGTGGATCCAAGGAGGA-3′ (F),

5′-TAGCCACCCCTCAAGAATGAA-3′ (R);

CRFR1: 5′-CACTACCATGTTGCAGTCATC-3′ (F),

5′-CGAACATCCAGAAGAAGTTGG-3′ (R);

AQP4: 5′-TGGTCCTCATCTCCCTCTGCTT-3′ (F),

5′-TGAACCGTGGTGACTCCCAATCC-3′ (R);

Endothelin-1 (*ET-1*): 5′-CACCTGGACATCATCTGG-3′ (F),

5′-CTGGCATCTGTTCCCTTG-3′ (R);

Bim: 5′-AGTCTCAGGAGGAACCTGAAGATCT-3′ (F),

5′-TCCGATCCTCCGCAGCT-3′ (R);

Bax: 5′-GGCTGGGAAGGCCTCCT-3′ (F),

5′-AGCCACAAAGATGGTCACTGTCT-3′ (R);

18S RNA: 5′-GTAACCCGTTGAACCCCATT-3′ (F),

5′-CCATCCAATCGGTAGTAGCG-3′(R);

GAPDH: 5′CCTCAAGATTGTCAGCAAT-3′ (F),

5′-CCATCCACAGTCTTCTGAGT-3′ (R).

Quantitative real-time PCR was carried out on a 7500 Real-Time PCR System. A reaction solution consisting of 2 μL of cDNA product, 0.5 μL of PCR forward/reverse primer, 1 μL of ROX Reference DyeII, 8.5 μL of ddH_2O, and 12.5 μL of SYBR Premix Ex TaqTM (TaKaRa) was used as the detection dye. Quantitative real-time PCR conditions were optimized in preliminary experiments to achieve a linear relationship between initial RNA concentration and PCR product. The temperature range to detect the melting temperature of the PCR product was set from 60 °C to 95 °C. The 18S or GAPDH was measured for each sample as an internal PCR control for sample loading and normalization. The specificity of the primers was verified by examining the melting curve as well as subsequent sequencing of the quantitative real-time PCR products.

H&E and TUNEL Staining. The protocol was carried out as suggested by the manufacturer (Chemicon). Briefly, rats were killed after hypoxia. Brains were harvested, frozen, and stored at −80 °C. Brain sections (20 μm) were prepared and fixed in 4% (wt/vol) paraformaldehyde in PBS for 10 min at room temperature. Then sections were permeabilized with ethanol:acetic acid (2:1) for 5 min at −20 °C. Terminal deoxynucleotidyltransferase enzyme was diluted in reaction buffer, and slices were incubated for 1 h at 37 °C. Sections were then placed in stop/wash buffer and incubated for 10 min at room temperature. Antidigoxygenin conjugate was diluted in blocking solution, and slices were incubated for 30 min at room temperature. Exposure to light was avoided. Sections were washed four times in PBS (2 min each). Antifade containing 1 μg/mL DAPI was applied to sections, mounted under glass coverslip, and examined with confocal microscopy.

Assays for CRF, ET-1, and Corticosterone. Levels of CRF in the cortex were measured with a commercial enzyme immunoassay kit (Bachem) according to the manufacturer's protocol. ET-1 (Cayman

Chemical) and plasma corticosterone (Cayman) were estimated with commercial ELISA kits for rats. The sensitivity of the assay was 0.40 ng/mL, and interassay and intraassay coefficients of variation were 6.5% and 4.5%, respectively. The antibody cross-reacted 100% with corticosterone and <0.5% with other steroids. Samples repeated from individual rats were analyzed within the same assay.

Brain Water Content. In another group, rats were rapidly decapitated, and brains were immediately removed and weighed after hypoxia exposure, dried at 120 °C in a constant temperature oven, and reweighed until a constant dry weight was achieved. Water content of the brain was calculated as follows: brain water content = [(wet weight − dry weight)/wet weight] × 100%.

Statistical Analysis. All statistical analyses were performed by using SPSS software (Version 16.0). Data are presented as means ± SD. Statistical significance compared with control was determined with a two-tailed, unpaired Student's *t* test. Comparisons among multiple variables were determined with one-way ANOVA, followed by the Fisher least significance difference test.

1. Chen C, et al. (1996) Design and synthesis of a series of non-peptide high-affinity human corticotropin-releasing factor1 receptor antagonists. *J Med Chem* 39(22):4358–4360.
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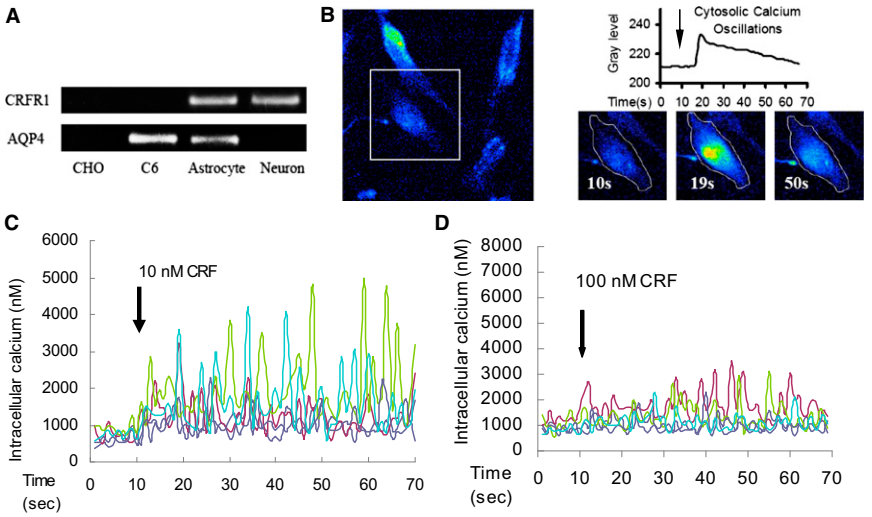

Fig. S1. CRFR1-activated Ca^{2+} signaling pathways in primary cerebral astrocytes. (*A*) CRFR1 expression in astrocytes and neurons. AQP4 expression in C6 glioma cells and astrocytes by PCR; absence of CRFR1 and AQP4 in CHO cells, as negative control. (*B*) Video showed a representative recording of Ca^{2+} transients in a single cultured primary astrocyte after 10 nM CRF challenge; the curve shows the maintenance of transients in a single astrocyte. (*C*) Shown is 10 nM CRF induced elevations of $[Ca^{2+}]_i$ in Ca^{2+}-containing medium. (*D*) Shown is 100 nM CRF reduced induction of elevations of $[Ca^{2+}]_i$ in Ca^{2+}-free medium.

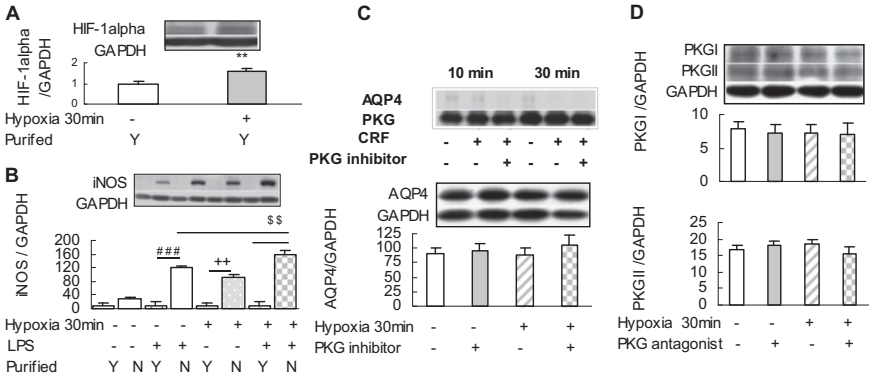

Fig. S2. Intracellular hypoxia-activated hypoxia inducible factor 1α (HIF-1α) expression was not involved in astrocyte swelling. (*A*) Hypoxia (1% O_2) for 8 h significantly elicited HIF-1α expression in cultured primary astrocytes in a hypoxic chamber ($n = 3$). $^{**}P < 0.01$ (vs. control). (*B*) Hypoxia (1.0% O_2) for 30 min did not change iNOS expression in cultured purified (Y) astrocytes but significantly increased iNOS level in unpurified (N) astrocytes (with ∼10–15% microglia), suggesting the increased iNOS expression might result from activation of microglia ($n = 3$). $^{###}P < 0.001$ (purified astrocytes + LPS vs. unpurified astrocytes + LPS); $^{++}P < 0.01$ (purified astrocytes + hypoxia vs. unpurified astrocytes + hypoxia); $^{\$\$}P < 0.01$ (purified astrocytes + LPS + hypoxia vs. unpurified astrocytes + LPS + hypoxia) and (unpurified astrocytes + LPS + hypoxia vs. unpurified astrocytes + LPS). (*C* and *D*) This hypoxia did not change the expression of PKGI, PKGII isoforms, and AQP4 protein in cultured purified astrocytes, nor did a PKG inhibitor affect AQP4 expression.

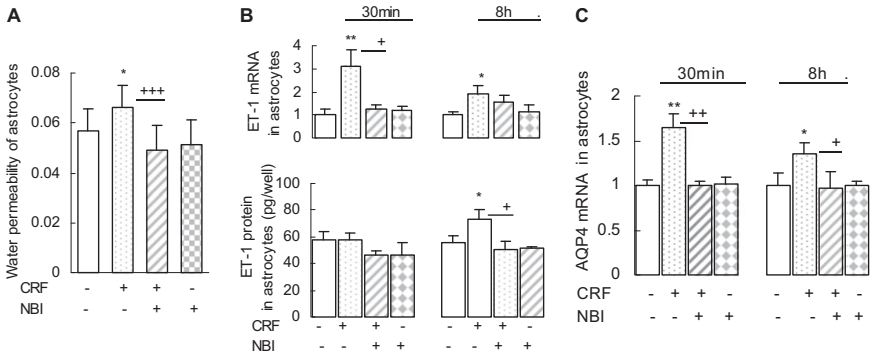

Fig. S3. CRFR1 involved in astrocytes swelling edema via ET-1 and AQP4. (*A*) CRF (10 nM) significantly increased water permeability in cultured rat primary astrocytes, and the increase of water permeability was blocked by NBI (100 nM). (*B*) CRF significantly increased ET-1 mRNA and protein in cultured rat primary astrocytes, and the increase of ET-1 mRNA and protein was blocked by NBI. (*C*) CRF significantly increased AQP4 mRNA in cultured rat primary astrocytes, and the increase of AQP4 mRNA was blocked by NBI (n = 30–35 cells for each). $^{*}P < 0.05$; $^{**}P < 0.01$ (CRF vs. control); $^{+++}P < 0.001$ [CRF vs. CRF + CRFR1 antagonist (NBI)].