DATA SUPPLEMENT

Activity of PKC-α within the Subfornical Organ is Necessary for Fluid Intake due to Brain Angiotensin

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<u>**Supplemental Methods**</u>

Experimental mice: sRA mice (line 11110/2 x 4284/1) are double transgenic mice derived from a cross of mice expressing human renin under the neuron-specific synapsin promoter (sR) with mice expressing human AGT under the control of its own endogenous promoter (A). They were generated as described previously, and littermates were used as controls.[1,2] C57BL/6 mice (stock 000664) were used for DOCA-salt and phorbol-12-myristate-13-acetate (PMA) studies. ROSA-CAG-LSL-tdTomato (stock 007914) mice were obtained from the Jackson Laboratory. We measured fluid balance in mice by either placing them in their home or a metabolic cage as described previously.[2]

Pharmacology: aCSF (Alzet Inc.), BIM (32 ng in 2 µL DMSO; Cell Signaling), H-89 (4 µg in 2 µL DMSO; Cell Signaling), FR180204 (4 µg in 2 µL DMSO; Santa Cruz), conivaptan (0.8 µg in 2 µL aCSF; Baxter Healthcare), and calphostin C (0.4 µL in 2 µL DMSO; Sigma). Gö-6976 (Millipore), and PMA (Cayman Chemical) were injected as described previously.[3]

ICV Cannula Placement: Specific cohorts of mice had cannulas (PlasticsOne, Inc.) placed into the lateral ventricle after which they recovered individually housed in a home cage on a hot pad with food and water. They were monitored for 24-hours. After 24-hours, the mice were returned to the vivarium. The mice were given an additional 7-day period in the vivarium to recover from the surgery before studies were performed. If it was evident that a mouse had ill effects of the surgery, it was killed before the study was started. The success rate for ICV cannula placement and injection was over 90%.

Acute ICV Injection: Select cohorts of mice were given isoflurane to induce (5% isoflurane in O_2) and maintain (2% isoflurane in O_2) anesthesia. The lateral ventricle was located via stereotaxic coordinates, and 1 µL of adenovirus (AdCre or Ad5CMV-DN-PKC-α, $1x10^7$ pfu/µL; University of Iowa Gene Transfer and Vector Core) was injected via a 1 µL Hamilton syringe over 5 min. The Hamilton syringe was then kept in place for an additional min before retracting it and suturing together the skin covering the cranium. Mice were then place in a home cage on a warm pad with food and water, and their recovery was monitored for 24 hours. After 24 hours, the mice were returned to a specific room in the vivarium dedicated for adenovirus. After 7 days, the mice were transferred and individually housed in a clean cage.

Specific Protocols by Figure.
Figure 1: ICV drug panel in sRA and littermate control mice.
A starting cohort of 7 sRA and 8 littermate control mice was used for this study. Eight days after placement of the ICV cannula, the mice were transferred in a home cage into a room dedicated for metabolic measurements where they were given 2 days to acclimate. Their fluid intake and body weight were monitored during the acclimation period. Ten days after ICV cannula placement, the body weight of these mice was measured, they were injected with aCSF ICV in the morning, and then 24 hours later their body weight and water intake were measured. The mice were then injected ICV

with the indicated drug following the order shown in Figure 1B, and then 24-hours later their body weight and water intake were measured. The mice were then given a two day wash out period. This procedure consisting of aCSF injection followed by 24 hour monitoring, ICV drug injection followed by 24 hour monitoring, and two day recovery was repeated as shown in Figure 1B. Concentration of drugs were: BIM (32 µg in 2 µL of DMSO), FR180204 (4 µg in 2 µL of DMSO), Conivaptan (0.8 µg in 2 µL of aCSF), H-89 (4 µg in 2 µL of DMSO), and calphostin c (0.4 µg in 2 µL of DMSO). These mice were not used for any other studies.

Figure 2: ICV BIM and Gö-6976 in sRA and littermate control mice.
Two separate cohorts of sRA (n=4 and n=3) and littermate control mice (n=5 and n=4) were used for the BIM study. Three separate cohorts of sRA and littermate control mice (2-mice per cohort) were used for the Gö-6976 study. 2 µL ICV injections of BIM or Gö-6976 at the doses listed in Figure 2. DMSO was the vehicle. The maximum change in water intake was calculated by curvilinear regression of the change in water intake due to drug from vehicle in each individual mouse. The mean±SEM of the curvilinear regression was calculated and reported. These mice were used for only one study; i.e. a mouse received either an ICV injection of BIM or Gö-6976, but not both. None of the mice were used in any other studies.

Figure 3: ICV AdCRE in ROSA-CAG-LSL-tdTomato mice.
Three separate cohorts of ROSA-CAG-LSL-tdTomato mice (n=3 each) were acutely injected with 1.0 µL of AdCRE ICV. After 4-weeks, they were intra-cardially perfused with 4% paraformaldehyde and the brain was sectioned on a vibratome for imaging.

Figure 4: ICV Ad-DN-PKC-α in sRA and littermate control mice.
Two cohorts of sRA and littermate control mice (n=7 each) were used. Mice were placed into metabolic cages in a room dedicated for metabolic measurements. They were given a two bottle choice between water and 0.15M NaCl. The mice were allowed to acclimate, and then baseline intakes of fluid and food, and body weight were measured. 1 µL of Ad-DN-PKC-α was injected ICV. The mice were placed back into metabolic cages 7 day later, were provided a two-bottle choice of water and saline, were given a day to acclimate, after which their intakes of fluid and food, and their body weight were measured for 2-days. One sRA mouse was eliminated from the study because the fluid intake was found to be an outlier (Grubb's test, $P<0.05$).

Figure 5: ICV infusion of BIM in DOCA-salt and control mice.
Two-cohorts of C57BL/6 mice were used (n=22 for DOCA; n=10 for the sham procedure). 10 DOCA-salt and 5 sham mice were infused with aCSF ICV and 12 DOCA-salt and 5 sham mice were infused with BIM ICV. Osmotic mini-pumps were subcutaneously implanted 2 weeks after implantation of the DOCA pellet or sham operation, and an ICV cannula kit was used to infuse drug into the lateral ventricle. Mice were placed into metabolic cages with a two-bottle comparator choice of water and 0.15M NaCl after the 3rd week after DOCA implantation or sham operation. Fluid intakes were measured for 3 days with the first day counted as an acclimation period.

Figure 6: SFO Cultures: Cultures of the SFO were obtained from rat newborn pups as previously described with minor modifications.[4,5] Briefly, cells from the SFO were incubated in Dulbecco's modified eagle medium (Life Technologies, Inc.) with 10% bovine serum at 37°C for 9-10 days. PD123319 (an AT2 receptor, 7 μM; Sigma), was applied to the media during treatment. Cultured cells were pre-treated for 30 minutes with vehicle (DMSO in aCSF). Afterwards, the cells were treated for 15 minutes with either DMSO or 10 μM PMA. The cells were fixed and incubated with primary antibody for phosphorylated-PKC-α (1:200; Abcam, Inc., ab76016). Goat anti-rabbit DyLight 488 (1:200; VectorLabs, DI-1488-1.5) in 5% NGS and PBS was used for secondary labeling. The cultures were mounted with media containing DAPI (VectaShield; VectorLabs, H-1200), and were viewed with a Zeiss LSM710.

Figure 7: ICV PMA in C57BL/6 mice.
Two cohorts of C57BL/6 mice (n=5 and n=6) underwent surgery for ICV cannula placement. After recovery, these mice were individually housed in a home cage and transferred to acclimate in a room dedicated for metabolic measurements where body weight and water intake was monitored for 2 days. After acclimation, the 1st cohort was ICV injected with vehicle (aCSF) in the morning, and then cumulative water intake at 1-, 5-, 15-, 120-, and 300-minutes and 24-hours was measured. PMA was injected ICV the next morning, and cumulative water intake was measured at the same time periods.

The 2nd cohort of mice were pre-treated with an ICV injection of vehicle (DMSO for BIM), and then 30 minutes were injected ICV with vehicle (aCSF for PMA). Cumulative water intake at 1-, 5-, 15-, and 30-minutes and 24 hours was measured. Next, these mice were pre-treated with DMSO, and then 30 minutes later PMA was injected and cumulative water intake at the same time points was measured. The next morning, the same mice were pre-treated with BIM ICV. 30-minutes later PMA was injected and cumulative water intake at the same time points was measured. A final group received BIM then aCSF to assess the effects of BIM alone.

Supplemental References

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Supplemental Figures

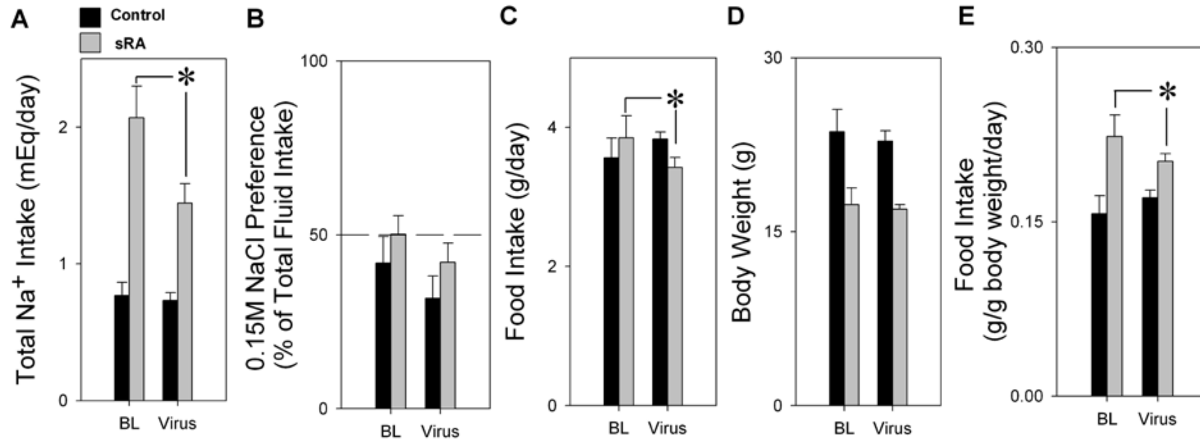

Figure S1: Total sodium (A), preference for 0.15 M NaCl (B), food intake (C), body weight (D), and food intake normalized for body weight (E) were measured in sRA (n=13) and littermate control mice (n=14) at baseline (BL) and 9-10 days after ICV Ad-DN-PKC-α. *P<0.05, genotype x virus interaction.