Supplemental Materials

Central Biased Agonist of β-Arrestin Targeting the Angiotensin II Type 1 Receptor Alters Fluid Intake and Blood Pressure in DOCA-salt Hypertension

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Supplemental Methods

Blinding and exclusions.
As all mice were C57BL/6, there was no need to blind to genotype. Mice were randomized to each treatment. For the BP study, once hypertension was established, each mouse received an ICV injection of vehicle, losartan or TRV027 in a random manner. The operator needed to know the decided order of the treatments and thus was not blinded. No mice were excluded from the analysis. In one case where an outlier was included in the analysis, this is noted in the Figure legend.

Surgical Preparations.
All surgeries were performed in aseptic conditions and the eyes were protected with ocular ointments (Akorn®, Lake Forest, Illinois). Anesthesia was induced with 5% isoflurane in O_2 (0.8-1.5 L/min) and sedation maintained with 2% isoflurane in O_2. The hair on the top of the head was shaved and a surgical scrub was performed using povidone iodine and isopropyl alcohol. The animals were placed into a stereotaxic frame (with non-penetrating ear bars) with the head secured.

Implantation of chronic ICV pumps: An incision was made about 1.5 cm above the ears (towards the nose) and extended vertically down to 0.5 cm below the ears. The skull was exposed and the bregma localized. For the chronic infusion study, mice were implanted with 30-gauge stainless-steel cannulas (Brain infusion Kit III, Alzet®, Cupertino, California) aimed at the lateral ventricle (anteroposterior, −0.2 mm; lateral, +1.0 mm; ventral, -2.3 mm; relative to bregma). The cannula was connected to an osmotic minipump (Alzet Model 1004) for 28 day-treatment and secured to the skull with surgical glue (Vetbond 3M®, Saint Paul, Minnesota). A subcutaneous pocket was created on the left flank of the mice to accommodate the micro-osmotic pump.

A contralateral subcutaneous pocket was created for the implantation of a homemade 50 mg DOCA pellet (Sigma-Aldrich, Saint Louis Missouri).[1] The wound was sutured with 6.0 Silk and triple antibiotic ointment containing Bacitracin-neomycin-polymyxin (Vetropolycin®, Dechra Pharmaceuticals PLC, Northwich, United Kingdom) was applied to the wound.

Implantation of ICV cannulas for acute injections: A 1.0 - 1.5 mm hole was made using a hand drill to penetrate the skull and expose the brain at the same stereotaxic coordinate described above.[1] A 30-gauge stainless steel guide cannula was implanted in the lateral ventricle (same coordinates as above) and a 32-gauge inner needle was placed inside the cannula for ICV drug delivery. The cannula was fixed to the skull using dental cement. After the acrylic hardened, the guide cannula was plugged with a stainless steel stilette to seal the cannula until use. Surgical glue was applied all the way around the cement cap. Postsurgical care included subcutaneous injections of 0.9% saline and analgesics (Flunixin, 2.5 mg/Kg, i.p. or meloxicam 3-5mg/Kg subcutaneously).

Two-bottle experiment design: TRV027 was provided by one of the authors (S.K.) or was purchased from GeneScript (Piscataway, NJ). To test the effects of TRV027 in DOCA-salt model, mice were randomly divided into four groups: 1) Control sham group of mice

received ICV vehicle infusion of cerebrospinal fluid (aCSF; TOCRIS®, Bristol, United Kingdom) [0.09 µL/h] without treatment with DOCA; 2) DOCA+vehicle mice were implanted with DOCA pellets (50 mg; subcutaneous) and received ICV infusion of vehicle solution (aCSF; TOCRIS®) [0.09 µL/h]; 3) DOCA+TRV027 mice were implanted with DOCA pellets and received ICV infusion of TRV027 (TRV027 (Sar1-Arg2-Val3-Tyr4-Ile5-His6-Pro7-D-Ala8-OH) [20 µg/kg/h]; and 4) DOCA+losartan mice were implanted with DOCA pellets and received ICV infusion of losartan (Sigma-Aldrich, Saint Louis Missouri) [5 µg/kg/h]. All mice were acclimated to the experimental room for at least one week. Animals were housed in individual home cages with free access to food. Human activity in the room was restricted to the light period as were all experimental manipulations. Starting at day 14 post-surgery, we performed 3 experiments in which animals were presented with two burettes: one containing tap water and one containing varying sodium chloride concentration (0.15 M, 0.30M or 0.45 M) in a random order. The saline solution was constituted by adding 8.77g, 17.54g, and 26.31g of NaCl to 1L of tap water (non-deionized, nor distilled or chlorinated), to achieve a concentration of 0.15 M, 0.30 M and 0.45 M respectively.

Two-day-acclimatization was given between every sodium chloride concentration. Given that rodents tend to prefer drinking from the bottle closer to the food source, the bottles positions were alternated every 24 hours and all drinking data was calculated as the average between 2 consecutive days. To account for side preference, we performed a control experiment at day 10 post-surgery, where we presented mice with two bottles, both containing tap water. At the end of the experimental protocol, mice were given free access to food and tap water for 48h prior to sacrifice with pentobarbital (90mg/kg). Ninety-five microliters of whole blood collected with heparinized syringes, were loaded into a CHEM8+ I-STAT cartridge (Abbott Point of Care Inc., Abbott laboratories, Illinois). The cartridges were run on a VetScan I-STAT1 (Abbott Point of Care). Plasma osmolality (mOsm/Kg H_2O) was assessed using a freezing-point depression osmometer. Body weight was measured prior to the surgical implantation of ICV pumps and at the end of the experiment. The weight of the kidneys, heart, lung and liver was measured for each mouse. The relative organ weight was calculated as the organ weight divided by the body weight.

In a separate cohort, we tested the effect of TRV027 in the absence of DOCA infusion. Mice were randomized into the following 3 groups:1) Control sham group of mice received ICV vehicle (aCSF infusion at 0.09 µL/h); 2) mice that received ICV TRV027 (20 µg/kg/hl), and 3) mice that received ICV Ang II (22.8 µg/kg/h; Sigma-Aldrich, Saint Louis Missouri). Mice then underwent the same 2-bottle test experiments described above. Of note, this experiment was performed at a different institution (MCW) and the only relevant difference we noted is a higher level of chlorine in tap water.

Water- and salt-intake measurements: Glass graduated burettes (0.1ml resolution) were employed to accurately measure daily water and/or saline intake. Measurements were obtained every day at 0800 AM. During the experiment, mice had free access to food. The burettes with attached stainless-steel spouts containing water and/or saline were inserted through the bars at the top of the cage with the spouts protruding ~5 cm into the cage. Daily volume of water intake, saline intake and total fluid intake were expressed in

milliliters (mL). Percent saline preference was calculated as volume of fluid intake from the burette containing the saline solution (mL) divided by total fluid intake (mL).

Surgical implantation of telemeters: Anesthesia was induced and maintained by intraperitoneal injection of 100mg/kg of ketamine and 10 mg/kg of xylazine. The neck was shaved and scrubbed with povidone iodine and isopropyl alcohol. An incision of approximately 1-2 cm was made in the skin and the internal carotid artery was exposed using blunt dissection. Mice were then implanted with radiotelemeters (TA11PA-C10; Data Sciences International, New Brighton, Minnesota) by inserting the catheter into the common carotid artery.[2] The wound was closed in a simple, continuous pattern with 5.0 sutures. Vetropolycin (Dechra Pharmaceuticals PLC, Northwich, United Kingdom) was applied to the wound. Postsurgical care included subcutaneous injections of 0.9 % saline and analgesics (Flunixin, 2.5 mg/kg, i.p.). Mice recovered for 10 days before behavioral testing.

Telemetry study: Mice underwent implantation of ICV cannulas, telemeters and DOCA pellets infusion as described above. Mice had free access to food and (0.9%) NaCl solution (0.15 mol/L). After 10 days of recovery, BP and heart rate (HR) were recorded every 10s for 5 min using Ponemah software (Data Sciences International, New Brighton, Minnesota). The quality control for the correct placement of the radiotelemetry cannula was a pulse pressure above 15 mmHg, a systolic blood pressure (SBP) below 200 mmHg and above 80 mmHg (indicative of a non-occluded catheter). Mice that did not pass the quality control were excluded. Three acute ICV injections (2 µL) were tested in each animal: vehicle solution (aCSF:2µL), losartan (14µg) and TRV027 (2µg) (Figure 6A). At least 48 hours after each ICV injection were given before a new drug was tested to allow the complete elimination of the preceding drug. The SBP, diastolic blood pressure (DBP), MBP and HR were recorded for the duration of 1-hour post-injection during which they did not have access to food or water. Delta (Δ) SBP at time t(i) was calculated by subtracting the SBP at time t(i) from the SBP at time t(0) (moment of injection). ΔDBP, ΔMBP and ΔHR were calculated using the same method. Peak responses in ΔSBP, ΔDBP, ΔMBP and ΔHR were defined as the maximum absolute value of ΔSBP, ΔDBP, ΔMBP and ΔHR respectively.

AT1R internalization assay: The gene encoding $AT_{1a}R$ was subcloned into pBiT3.1-N vector (Catalog #: N2361, Promega) to introduce the HiBiT tag to the extracellular, N-terminus of $AT_{1a}R$ (AT1R NT). N43/5 cells were transfected with $AT_{1a}R$ NT or control pBiT3.1-N vector with Lipofectamine 3000 according to manufacturer protocol (Catalog #: L3000015, ThermoFisher). To detect the capacity of TRV027 to internalize AT1aR, N43 immortalized hypothalamic mice neurons [3] were transfected with $AT_{1a}R$ tagged with HiBiT or control plasmid.[4] Cells were pre-treated with losartan (1 µM), Dynasor (Dyno) [50 µM] or vehicle (phosphate-buffered saline) prior to stimulation with Ang II (0.1 µM), TRV027 (1 µM) or vehicle. Nano-Glo® HiBiT Extracellular Detection reagent (Promega, Madison, Wisconsin) was added 30 min after stimulation. Cells were then lysed (Mammalian Lysis Buffer, Catalog #: G9381, Promega) to measure total luminescence signal, which was used to normalized cell surface localized $AT_{1a}R$ luminescence. The extracellular surface tagged $AT_{1a}R$ expression was measured then by luminescence. Cells were then lysed to measure total luminescence signal (surface + intracellular

$AT_{1a}R$). Non-transfected cells served as background noise. To calculate the percentage of surface $AT_{1a}R$ expression, the extracellular surface signal luminescence was divided by total luminescence signal (after extracting background noise). The ratio of surface signal luminescence for each treatment was then divided by that of the treatment with vehicle solution for comparison. Thus, vehicle treatment served as control with surface signal emission of 100%.

Supplemental References

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4. Schwinn MK, Machleidt T, Zimmerman K, Eggers CT, Dixon AS, Hurst R, Hall MP, Encell LP, Binkowski BF and Wood KV. CRISPR-Mediated Tagging of Endogenous Proteins with a Luminescent Peptide. *ACS Chem Biol*. 2018;13:467-474.

Supplemental Figures

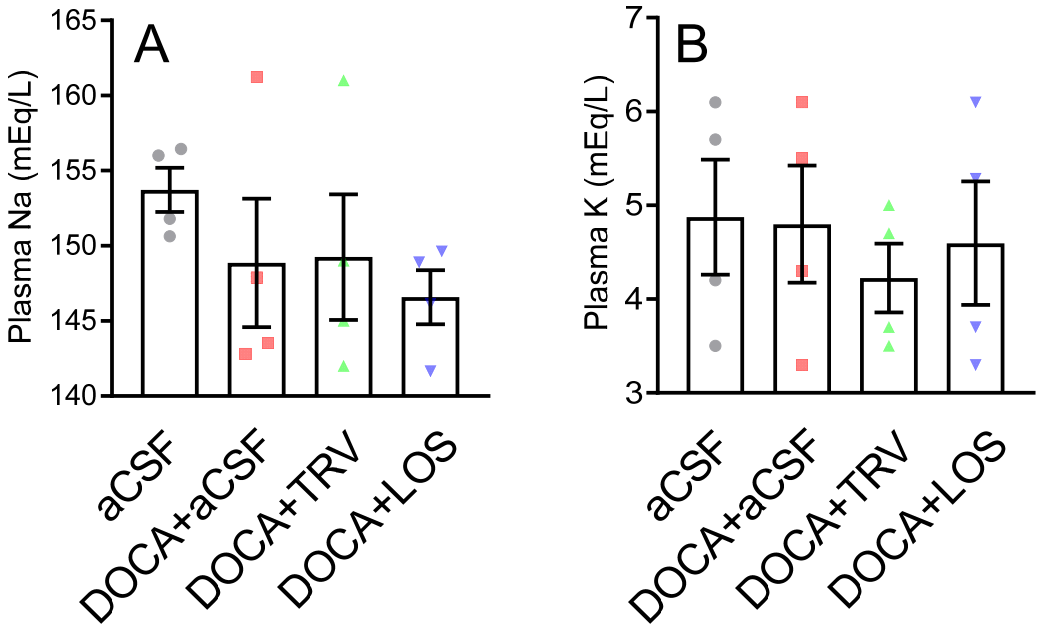

Figure S1: *Plasma electrolytes*.
At the end of the 2-bottle choice experiments, mice had free access to water and food for 48h prior to sacrifice with pentobarbital (90 mg/kg). A) Plasma sodium [Na] (mEq/L). B) Plasma potassium (mEq/L). Data were analyzed by one-way ANOVA with Tukey's multiple comparisons procedure. CSF (n=4), DOCA + aCSF (n= 4), DOCA + TRV027 (n=4), DOCA + losartan (n=4).

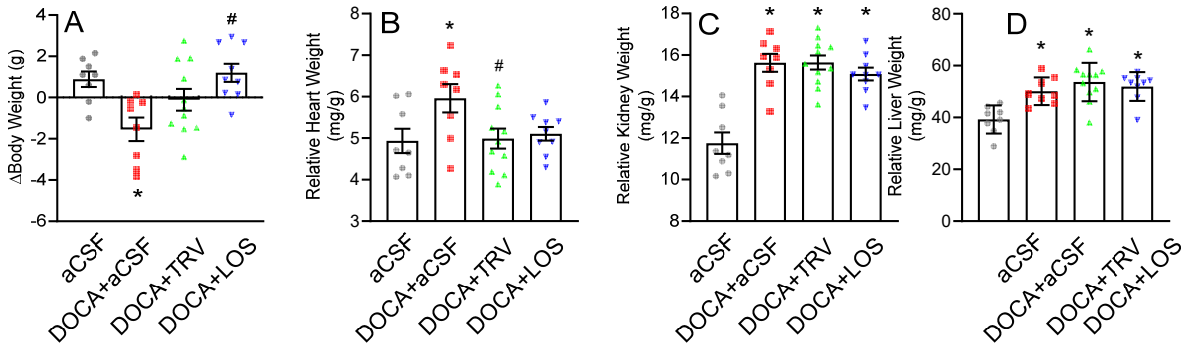

Figure S2: *Body weight and organs weight*.
Body weight was measured prior to the surgical implantation of intracerebroventricular (ICV) pumps and at the end of the experiment. The weight of the kidneys, heart, lung and liver was measured for each mouse. The relative organ weight was calculated as the organ weight divided by the body weight. A) ∆bodyweight (g) calculated by subtracting the body weight at the end of the experiment from the body weight prior to the surgical implantation of ICV pumps. B) Relative heart weight (mg/g). C) Relative kidney weight (mg/g). D) Relative liver weight (mg/g). Data were analyzed by one-way ANOVA with Tukey's multiple comparisons procedure. *$p<0.05$ compared with aCSF; $^{\#}p<0.05$ compared with DOCA+aCSF. aCSF (n=8), DOCA + aCSF (n= 8), DOCA + TRV027 (n=11), DOCA + losartan (n=9).

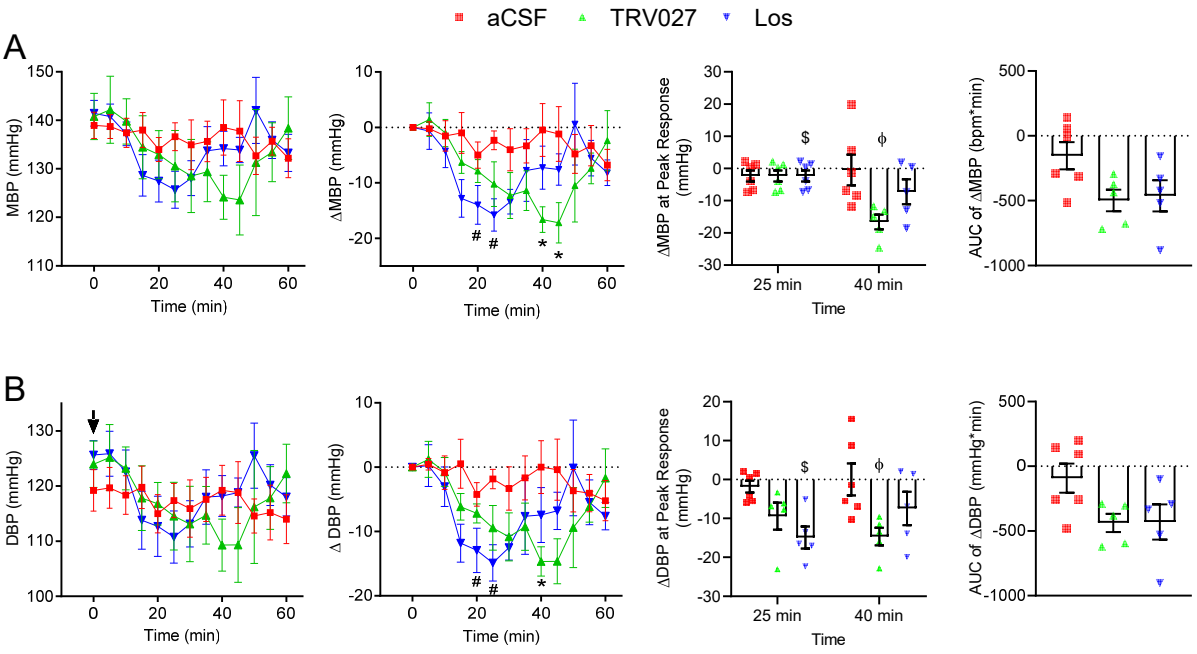

Figure S3: *Effect of TRV027 on blood pressure*.
Panel 1: Mean blood pressure (MBP; A) and diastolic blood pressure (DBP; B) were recorded for one-hour post-injection of either vehicle solution (aCSF), TRV027, or losartan (Los) as defined in the legend to Figure 6. Data was analyzed by mixed effects model and post-hoc Dunnett's test for multiple comparisons procedure. Panel 2: ΔMBP and ΔDBP were calculated by subtracting the baseline MBP and DBP at t=0 min from the MBP and DBP at any given time t(i). A Mixed effects model with repeated measure and post-hoc Dunnett test was performed to compare the ΔMBP at each t(i) compared with the control ΔMBP at t=0 and to compare the ΔDBP for each t(i) compared with the control ΔDBP at t=0 for each experimental group. Panel 3: Peak responses for TRV027 and Los injections were defined as the drop in ΔMBP or ΔDBP that were significantly different from vehicle injection. Peak ΔSBP and ΔDBP occurred at 25 min for Los and 40 for TRV027 injections. Mixed effects model with post-hoc Tukey-Krammer test was performed to compare the ΔMBP and the ΔDBP between TRV027, Los and vehicle injection at each timepoint. Panel 4: The area under the curve (AUC) for ΔMBP or ΔDBP are shown. One-way ANOVA with Tukey's post hoc test was performed. $^{\#}p<0.05$ compared with Los injection at t=0, *p<0.05 compared with TRV027 at t=0. $^{\$}p<0.05$ compared with aCSF at 30 min; $^{\phi}p<0.05$ compared with aCSF at 40 min.

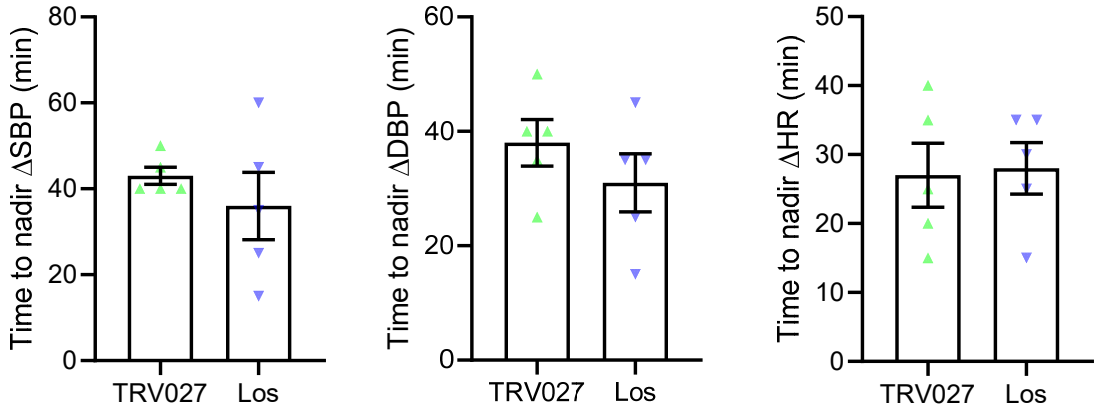

Figure S4: *Timing of TRV027 on blood pressure and heart rate*.
Time to nadir for SBP, DBP, and HR is shown. A Wilcoxon test was performed (paired data) but no statistical differences were found. TRV027 (n=5), losartan (n=5).