

(Pro)renin Receptor Knockdown in the Paraventricular Nucleus of the Hypothalamus Attenuates Hypertension Development and AT₁ Receptor-Mediated Calcium Events

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Short title: PVN (Pro)renin Receptor and Ca²⁺ Activity in Hypertension

Word count: 11780

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Abstract

Activation of the brain renin-angiotensin system (RAS) is a pivotal step in the pathogenesis of hypertension. The paraventricular nucleus (PVN) of the hypothalamus is a critical part of the angiotensinergic sympatho-excitatory neuronal network involved in neural control of blood pressure and hypertension. However, the importance of the PVN (pro)renin receptor (PVN-PRR)—a key component of the brain RAS—in hypertension development has not been examined. In this study, we investigated the involvement and mechanisms of the PVN-PRR in DOCA-salt-induced hypertension, a mouse model of hypertension. Using nanoinjection of adeno-associated virus-mediated Cre recombinase expression to knock down the PRR specifically in the PVN, we report here that PVN-PRR knockdown attenuated the enhanced blood pressure and sympathetic tone associated with hypertension. Mechanistically, we found that PVN-PRR knockdown was associated with reduced activation of ERK (extracellular signal-regulated kinase)-1/2 in the PVN and rostral ventrolateral medulla during hypertension. In addition, using the genetically encoded Ca²⁺ biosensor GCaMP6 to monitor Ca²⁺-signaling events in the neurons of PVN brain slices, we identified a reduction in angiotensin II type 1 receptor-mediated Ca²⁺ activity as part of the mechanism by which PVN-PRR knockdown attenuates hypertension. Our study demonstrates an essential

role of the PRR in PVN neurons in hypertension through regulation of ERK1/2 activation and angiotensin II type 1 receptor mediated Ca^{2+} activity.

Key words: Central nervous system, paraventricular nucleus of the hypothalamus, renin-angiotensin system, (pro)renin receptor, calcium, GCaMP6, hypertension

New and Noteworthy

PRR knockdown in PVN neurons attenuates the development of DOCA-salt hypertension and autonomic dysfunction through a decrease in ERK1/2 activation in the PVN and RVLM during hypertension. In addition, PRR knockdown reduced AT_{1a}R expression and AT_{1a}R -mediated calcium activity during hypertension. Furthermore, we characterized the neuronal targeting specificity of AAV serotype 2 in the mouse PVN and validated the advantages of the genetically encoded calcium biosensor GCaMP6 in visualizing neuronal calcium activity in the PVN.

Introduction:

Hypertension is one of the most important risk factors for cardiovascular diseases, including stroke, myocardial infarction, congestive heart failure and chronic kidney disease, and remains the number one cause of morbidity and mortality in the US(93). The mechanisms leading to hypertension are complex and only partially understood(37, 68, 70), but integrated neural, humoral, and renal mechanisms that collectively mediate increased sympathetic nervous system activity and renal sodium retention have been implicated in its pathogenesis(4, 49, 68). An increase in brain renin-angiotensin system (RAS) activity in cardiovascular regulatory nuclei is a pivotal step in the pathogenesis of hypertension(67, 91). Angiotensinergic sympatho-excitatory pathways from neurons in circumventricular organs in the forebrain project to the paraventricular nucleus (PVN) in the hypothalamus. From the PVN, these pathways project to the rostral ventrolateral medulla (RVLM) in the brainstem or directly to the intermediolateral cell column (IML) in the spinal cord(10, 50, 86). The PVN acts as a critical networking center of this angiotensinergic neural circuit in the central regulation of blood pressure (BP).

The (pro)renin receptor (PRR) is a key component of the RAS(59). *In vitro*, binding of renin or prorenin to the PRR promotes formation of angiotensin II (Ang II), the major bioactive peptide in the RAS(19, 28, 58, 72). In the central nervous system (CNS), Ang II acts through the angiotensin II type 1 receptor (AT₁R) to regulate neuronal excitability, sympathetic activity, and BP. Activation of the PRR also directly mediates downstream signaling via MAPK (mitogen-activated protein kinase)/ERK (extracellular signal-regulated kinase), phosphoinositide 3-kinase (PI3K)/AKT, Wnt, and other pathways

independent of Ang II(6, 7, 63, 87, 92). The PRR is expressed in multiple organs throughout the body, including the brain, heart, lung, pancreas, and placenta(59). We and others have previously reported that the PRR is present in brain regions involved in the central regulation of BP(53, 73), and its expression is elevated in BP regulatory brain regions, including the PVN, in the DOCA-salt hypertensive model (54) and spontaneously hypertensive animals(73). Moreover, polymorphisms of the PRR gene are associated with increased systolic BP in humans(36, 60), highlighting the clinical significance of the PRR in hypertension. However, the importance of the PVN-PRR and the specific mechanisms involved in hypertension are not fully understood. In this study, we tested the hypothesis that the PVN-PRR plays a critical role in hypertension development and explored potential underlying mechanisms.

Materials and Methods

Animals:

The PRR-floxed mouse used in these studies was generated by Dr. Atsuhiko Ichihara's laboratory(42). A colony of these mice has been established and maintained in the Feng laboratory, as described previously(54). Wild-type (WT) C57Bl/6J and CX3CR3-GFP reporter (B6.129P-Cx3cr1tm1litt/J) mice used as breeders were purchased from Jackson Laboratory and were maintained in the animal facility at the University of Nevada, Reno. Both male and female mice, age 8–10 weeks, were used. All procedures were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee, and the Institutional Biosafety Committee at the

University of Nevada, Reno. The use of adeno-associated virus (AAV) was approved by the Institutional Biosafety Committee at the University of Nevada, Reno.

Telemetry Study:

Mice were anesthetized using 4–5% isoflurane in 100% O₂, flushed at 1 L/min for 2 minutes. Anesthesia was then maintained using 0.75–1.5% isoflurane. After the neck was shaved and sterilized with alcohol swabs, an incision (~1 cm) was made to separate the oblique and tracheal muscles and expose the left carotid artery. The catheter of a radio telemetry transmitter (PA-C10; Data Science International, St. Paul, MN) was surgically implanted into the left carotid artery and secured. The body of the radio transmitter was embedded in a subcutaneous pocket under the right arm. Mice were allowed to recover for 14 days, and then baseline BP, heart rate (HR), and locomotor activity were recorded for 48 hours as described previously(51, 52). Site-specific knockdown of the PRR in the PVN in mice was achieved by bilateral injection of AAV serotype 2 expressing Cre recombinase and enhanced green fluorescent protein (AAV2-Cre-eGFP) into the PVN; control animals were injected with AAV2 expressing eGFP only (AAV2-eGFP). Mice were allowed 3 days to recover, and then were implanted with a 50 mg DOCA or sham pellet as previously described(54). Mice were returned to their cages and provided ad libitum access to standard chow and 0.9% saline or water. BP and HR were recorded daily. On days 20 and 21 following DOCA implantation, autonomic functions were assessed using a standard pharmacological method involving randomized intraperitoneal injections of a β -blocker (propranolol, 6 mg/kg), a muscarinic receptor blocker (methylatropine, 1 mg/kg), and a ganglionic

blocker (chlorisondamine, 6 mg/kg)(24). Changes in HR in response to injections of propranolol and methylatropine, and changes in BP following chlorisondamine injection, were analyzed(55).

Bilateral AAV Injections into the PVN:

Mice were anesthetized as described above and the tops of their heads were shaved and sterilized with alcohol wipes. Mice were placed in a digital stereotaxic apparatus (Stoelting, Wood Dale, IL) and held in place by ear bars secured just above the ear canal. An incision (~1 cm) in the skin along the top of the head was made to expose the skull. The skull was cleaned with 3% hydrogen peroxide using cotton swabs, after which holes were drilled into the skull at the stereotaxic coordinates, 0.6 mm posterior to bregma and 0.4 mm to either side of the midline. An outer cannula was lowered 5.1 mm from the surface of the brain and an inner injector was placed in the cannula. The outer cannula was made from a blunted 25-gauge needle, 30 mm in length, and the inner injector was made from 32-gauge tubing (Microgroup, Medway, MA), 30.1 mm in length. Mice received AAV injections bilaterally into both paraventricular nuclei, as illustrated in Figure 2A. The injector was secured to a 10- μ L Hamilton syringe (Hamilton, Reno, NV) using PE 50 tubing, and the system was charged with artificial cerebrospinal fluid (aCSF; 127 mM NaCl, 1.6 mM KCl, 1.24 mM KH_2PO_4 , 1.3 mM MgSO_4 , 2.4 mM CaCl_2 , 26 mM NaHCO_3 , 10 mM glucose). Mice then received a 100 nL (1×10^7 viral genomes [vg]/nL) injection of AAV, after which the injector and cannula were left in place for an additional 5 minutes before removing to prevent leakage of injectate. The wound was sutured and the mice were allowed to recover. PRR-floxed mice were injected with

either AAV2-Cre-GFP (hereafter, AAV2-Cre) to knock down the PRR or with AAV2-eGFP (hereafter, AAV2-Con) as a control. The cell type transduced was characterized by monitoring eGFP fluorescence. AAV2-eGFP and AAV2-Cre were purchased from the Viral Vector Core Facility at the University of Iowa. All AAV2s used in our study employed a CAG promoter.

Immunofluorescence Labeling:

For immunofluorescence labeling, mice were anesthetized with isoflurane and transcardially perfused first with ~50 mL of ice-cold phosphate-buffered saline (PBS) and then with ~50 mL of ice-cold 4% paraformaldehyde. Brains were removed, post fixed in 4% paraformaldehyde at 4°C for 2 hours, and cryoprotected by incubating in 30% sucrose in PBS for 24 hours at 4°C. Brains were then frozen in Tissue Freezing Medium (TFM; General Data, Cincinnati, OH) at -20°C, sectioned (30 µm), and floated in PBS in a 24-well plate. Sections were washed twice in PBS and blocked by incubating in PBS containing 0.3% Triton X-100 and 10% normal house serum (NHS) for 1 hour at room temperature (RT). Sections in PBS containing 0.3% Triton X-100 and 3% NHS were incubated overnight at 4°C with one or more of the following primary antibodies, as indicated in the text: guinea pig anti-NeuN (1:1000, ABN90; Millipore, Burlington MA), targeting the neuronal marker NeuN; goat anti-IBA-1 (1:200, NB100-1028; Novus, Littleton, CO), targeting the microglial marker IBA-1; chicken anti-GFAP (1:200, ab4674; Abcam, Cambridge, MA), targeting the astrocyte marker GFAP (glial fibrillary acidic protein); and rabbit anti-PRR (1:200, ab40790; Abcam, Cambridge, MA). Sections were washed four times in PBS and incubated for 2 hours at RT with Alexa

186 647-conjugated donkey anti-guinea pig (1:1000; Jackson ImmunoResearch, West
187 Grove, PA), Alexa 594-conjugated rabbit anti-goat (1:1000; Invitrogen, Carlsbad, CA),
188 Alexa 488-conjugated goat anti-chicken (1:1000; A-11039; Thermo-Scientific, Waltham,
189 MA), Alexa 594-conjugated donkey anti-rabbit (1:1000; A21207; Thermo-Scientific,
190 Waltham, MA), or Alexa 647-conjugated donkey anti-guinea pig (1:500, 706-605-148;
191 Jackson ImmunoResearch, Carlsbad, CA) secondary antibody, diluted in PBS
192 containing 0.3% Triton X-100 and 3% NHS. Sections were then incubated in PBS
193 containing 1 µg/mL DAPI (4',6-diamidino-2-phenylindole) for 5 minutes and washed four
194 times in PBS. The specificity of each primary antibody was confirmed using controls
195 lacking primary antibody and imaging with the exact same settings. The specificity of
196 the PRR antibody was further confirmed by western blotting of Neuro-2A cells
197 expressing our previously characterized, AAV2-delivered mouse PRR shRNA (53) using
198 a Wes automated western blot system (ProteinSimple, San Jose, CA). All sections were
199 mounted on Colorfrost Plus slides (Fisher Scientific, Pittsburgh, PA) and coverslip-
200 mounted with Vectashield hard-set mounting medium (Vector Labs, Burlingame, CA).
201 Sections were stored at 4°C and imaged on an SP8 confocal microscope (Leica
202 Biosystems, Wetzlar, Germany).

203 The efficiency of PRR knockdown in the PVN by AAV2-Cre was assessed by
204 quantifying PRR labeling in images imported into ImageJ (NIH). Regions of interests
205 (ROIs) generated using the GFP channel were used to analyze average PRR
206 fluorescence intensity in the red channels for both AAV2-GFP (AAV-Con) and AAV2-
207 Cre-GFP (AAV-Cre) groups. The background fluorescence of each image was
208 subtracted from the average intensity of that image during the analysis. The average

fluorescence intensity was normalized to that in the AAV-Con group and presented as fold change.

p-ERK1/2 Immunofluorescence Labeling and Quantification:

PRR-floxed mice were bilaterally injected in the PVN with either AAV2-Cre or AAV2-Con virus and allowed to recover for 3 days. A separate set of WT mice was chronically infused with either losartan (3 mg/kg/d) or aCSF via an intracerebroventricular (ICV) cannula, as described(55). Three days after surgery, mice were implanted with a 50 mg DOCA or sham pellet and given access to 0.9% saline or water. After 14 days of treatment, mice were perfused and fixed as described above. Sections (30 μ m) were blocked by incubating in PBS containing 0.3% Triton X-100 and 10% NHS, and then incubated overnight with rabbit anti-p-ERK1/2^{Thr202/Tyr204} (1:500, 4376s; Cell Signaling, Beverly, MA) in PBS containing 0.3% Triton X-100 and 3% NHS. Sections were washed four times with PBS and incubated with Alexa 594-conjugated donkey anti-rabbit secondary antibody (1:1000; Thermo-Scientific, Waltham, MA) for 2 hours at RT. Sections were then counterstained with DAPI and mounted as described above. P-ERK1/2^{Thr202/Tyr204} staining in Figure 5 was imaged on an SP8 confocal microscope (Leica Biosystems, Wetzlar, Germany). During image acquisition, gain and offset were set at 873.3 and -0.19, respectively, for all subfornical organs (SFOs), and 831.1 and -0.19, respectively, for all PVNs and RVLMs.

P-ERK1/2^{Thr202/Tyr204} staining in both groups shown in Figure 6 was imaged on a Keyence BZ-X700 microscope with a 20x objective at the same time using the same setting (NA = 0.75). P-ERK1/2 labeling was quantified by importing images into ImageJ (NIH) and identifying the SFO, PVN, and RVLM using the DAPI channel.

ROIs defined using the DAPI channel were overlaid on the red channel (p-ERK), and the fluorescence intensity of the red channel in the ROI was measured. The fluorescence intensity of each ROI was normalized to the area to obtain the average fluorescence intensity of each brain region. The average fluorescence intensity was normalized to the same brain region in sham controls and presented as fold change.

Quantitative Reverse Transcription-Polymerase Chain Reaction:

PRR-floxed mice were bilaterally injected with either AAV2-Cre or AAV2-Con, as described above, and allowed to recover for 3 days (to assess early onset of PRR mRNA reduction) or 24 days (to assess whether PRR mRNA levels remained low at the end of the 21-day treatment protocol). Mice were sacrificed by cervical dislocation, and their brains were rapidly removed and flash frozen. Total RNA from tissue micropunches of the PVN, cortex, and brainstem was isolated with TRIzol reagent (Thermo Fisher, Waltham, MA) following the manufacturer's protocols. Briefly, tissue was homogenized in 500 μ L of TRIzol using a TissueMizer (Tekmar, Vernon, Canada) and incubated for 5 minutes at RT. Samples were centrifuged at 10,000 $\times$ g for 5 minutes, and the supernatant was transferred to a fresh tube. Chloroform (100 μ L) was added to each sample, after which samples were vortexed for 15 seconds and incubated at RT for 5 minutes. Samples were centrifuged at 12,000 $\times$ g for 15 minutes, and the clear aqueous phase was transferred to a fresh tube. RNA was precipitated by adding 250 μ L of isopropyl alcohol, and the samples were incubated for 10 minutes at RT. Samples were centrifuged at 12,000 $\times$ g for 10 minutes and the supernatant was discarded. The RNA pellet was washed twice by briefly vortexing in 500 μ L of 75%

ethanol and centrifuging at 7,500 x g for 5 minutes. Ethanol was removed and the pellet was allowed to air dry for 10 minutes at RT. RNA was dissolved in 30 μ L of DNase/RNase-free H₂O (Invitrogen, Carlsbad, CA), and its concentration and purity were determined using a NanoDrop spectrophotometer (Thermo Fisher, Waltham, MA). Samples were stored at -80°C. cDNA was reverse transcribed (RT) using an Applied Biosystems high-capacity cDNA reverse transcription kit (Thermo Fisher, Waltham, MA), and quantitative polymerase chain reaction (qPCR) was performed using Fast SYBR Green master mix (Thermo Fisher, Waltham, MA). mRNA levels of the PRR and the angiotensin receptors, AT_{1a}R, AT₂R and MAS1, normalized to those of GAPDH (glyceraldehyde-3-phosphate dehydrogenase), were determined using the $\Delta\Delta$ CT relative quantification method. The following primer pairs were used: mouse PRR, 5'-TCT CTC CGA ACT GCA AGT GCT ACA-3' (forward) and 5'-CCA AAC CTG CCA GCT CCA ATG AAT-3' (reverse); mouse AT_{1a}R, 5'-TCA CCA GAT CAA GTG CAT TTT GA-3' (forward) and 5'-AGA GTT AAG GGC CAT TTT GCT TT-3' (reverse); mouse AT₂R, 5'-CAG CAG CCG TCC TTT TGA TAA-3' (forward) and 5'-TTA TCT GAT GGT TTG TGT GAG CAA-3' (reverse); mouse MAS1, 5'-CGG TCT ACA TTA CCC ACT TGT C-3' (forward) and 5'-GAT GGC CAG AAG AGA GTT CAT AG-3' (reverse); and mouse GAPDH, 5'-AAT GTG TCC GTC GTG GAT CTG A-3' (forward) and 5'-GAT GCC TGC TTC ACC ACC TTC T-3' (reverse).

Live-Cell Ca²⁺ Imaging in PVN Brain Slices:

AAV2/9-delivered GCaMP6f (Penn Vector Core, University of Pennsylvania) was used as a genetically encoded Ca²⁺ biosensor to evaluate Ca²⁺-signaling activity in the PVN.

278 Since GCaMP6f has the same excitation and emission wavelength as eGFP, in this
279 experiment, we used an AAV2-Cre virus without the eGFP reporter to knock down the
280 PRR in PRR-floxed mice, and AAV2-mCherry (Penn Vector Core, University of
281 Pennsylvania) as the control virus (AAV-Con).

282 PRR-floxed mice were bilaterally injected into the PVN with either AAV2/9-GCaMP6 +
283 AAV2-Cre or AAV2/9-GCaMP6 + AAV2-mCherry (50 nL each virus, 2×10^7 vg/nL), as
284 described above, and allowed to recover for 3 days. Mice were then implanted with a
285 50 mg DOCA or sham pellet and given access to 0.9% saline or water for 14 days. At
286 14 days, mice were sacrificed by cervical dislocation, and their brains were removed
287 and placed in ice-cold slicing buffer (125 mM NaCl, 2.5 mM KCl, 25 mM NaHCO₃, 1.25
288 mM NaH₂PO₄, 3.0 mM 3-myo-inositol, 2.0 mM Na-pyruvate, 0.4 mM ascorbic acid, 0.1
289 mM CaCl₂, 3.0 mM MgCl₂, 25 mM glucose). The pia matter was removed and the hind
290 brain was cut away. The brain was fixed to the cutting stage and submerged in ice-cold
291 slicing buffer. Sections (175 μ m thick) through the PVN were cut with a Leica VT1200s
292 vibratome at 0.10 mm/s with 0.1 mm lateral movement of the blade. PVN sections were
293 then transferred to a chamber containing gassed, 37°C recording buffer (125 mM NaCl,
294 2.5 mM KCl, 25.0 mM glucose, 25.0 mM NaHCO₃, 1.25 mM NaH₂PO₄, 3.0 mM 3-myo-
295 inositol, 2.0 mM Na-pyruvate, 0.4 mM ascorbic acid, 1.0 mM MgCl₂, 2.0 mM CaCl₂) for
296 30 minutes. Sections were placed on a 35-mm glass-bottom dish (MatTek, Ashland,
297 MA) containing recording buffer, and Ca²⁺ events were imaged with an Olympus IX81
298 microscope (Olympus, Burlingame, CA) equipped with a 20x objective (NA = 0.45) and
299 an iXon Ultra camera (Andor, Belfast, UK), and analyzed using Andor IQ5 software. A
300 512 x 512 pixel field of view (413 μ m x 413 μ m) of the PVN along the third ventricle was

301 imaged, and Ca^{2+} events were recorded at a rate of 10 frames/s. A baseline was
302 recorded for 45 seconds, after which Ang II (100 nM) was added and events were
303 recorded for an additional 75 seconds. KCl (100 mM) was added to induce a maximal
304 Ca^{2+} response in neurons. The role of AT_1R activation in mediating Ca^{2+} events was
305 confirmed by preincubating PVN sections for 30 minutes in gassed recording buffer
306 containing losartan (1 μM) before and during incubation with Ang II and KCl. Images
307 were analyzed using ImageJ (NIH).

308 For data analysis, the frequency of Ca^{2+} events was measured as events per second,
309 and amplitude was determined as $F - F_0$, expressed as a percentage of the maximal
310 response to KCl ($(F - F_0)/F_m - F_{0m}$) as described (65, 66, 77). Briefly, F_0 was taken as the
311 least intense, uniform basal fluorescence intensity value obtained during the baseline
312 period (i.e., between spontaneous Ca^{2+} events in the absence of KCl or Ang II). In
313 cases where baseline recordings fluctuated slightly before application of KCl or AngII,
314 minimum F_0 (F_{0m}) was taken as the least intense and uniform basal fluorescence
315 intensity value immediately prior to KCl or Ang II application. Ca^{2+} events were defined
316 using a threshold intensity criterion. Dynamic Ca^{2+} events with amplitudes greater than
317 1% of the maximal response were considered valid Ca^{2+} events and were included in
318 the frequency analysis. Ca^{2+} signaling sites, defined as regions in which dynamic
319 changes in Ca^{2+} were elicited, were quantified as the number of sites per field of view
320 (FOV) during the recording period.

321 Three-dimensional (3D) reconstructions of Ca^{2+} -imaging files were created using Image
322 J (NIH, version 1.5a <http://imagej.nih.gov/ij/>)(2, 25, 26). Initially, Ca^{2+} -imaging files were
323 converted to 32-bit TIFF files to increase image quality. A color-coded look-up table

(QUBPalette.lut, Queens University, Belfast, UK) was then imported into the movie file to display intense regions of Ca^{2+} fluorescence as warm colors (red, orange) and regions of low Ca^{2+} fluorescence as cold colors (black, blue). Stacked 3D reconstructions of the movies were then created using the 'Stack 3D Surface Plot' plugin, and representative frames were selected for display using the 'Interactive 3D Surface Plot' plugin.

Statistical Analysis:

Data are presented as means $\pm$ SEM and were analyzed using Students *t* test, one-way (one variant) or two-way (two variants) analysis of variance (ANOVA) for multiple groups, followed by a Bonferroni post hoc test to compare replicate means, as appropriate. Outliers were identified using ROUT and/or Grubbs' methods, and statistical comparisons were performed using Prism7 software (GraphPad, La Jolla, CA); a *P*-value ≤ 0.05 was considered statistically significant.

Results

Cellular localization of PRR expression in the PVN of the hypothalamus in the mouse brain

We previously reported that the PRR is widely expressed in neurons located in many cardiovascular regulatory neurons, including the PVN(87). In the current study, we further characterized PRR expression in different cell types in this brain region. Using immunofluorescence labeling, we found that the PRR is mainly expressed in neurons, but not astrocytes, in the mouse PVN (Figure 1A). To determine the presence of the

PRR in microglial cells in the PVN, we performed immunolabeling for the PRR in microglia reporter mice (CX3CR1-GFP). We found that very few CX3CR1-GFP-expressing cells were positive for PRR immunostaining (Figure 1B, white arrows). Negative controls showed no fluorescence signals with any of the three secondary antibodies used in these experiments (Figure 1C), and the specificity of the primary antibody was further confirmed in PRR-knockdown and control Neuro2A cells (Figure 1D). These data indicate that, in the PVN of normotensive mice, PRR protein is primarily expressed in neurons, with potentially minor expression in microglia, but not astrocytes.

PRR knockdown in PVN neurons by AAV2-mediated delivery of Cre recombinase

To determine whether the PRR in PVN neurons plays a regulatory role in neurogenic hypertension, we knocked down PRR expression in the PVN using virally mediated delivery of Cre recombinase in mice harboring floxed PRR alleles. As a Cre carrier, we used AAV2, which has been shown to primarily target neurons in cortical and hippocampal neurons(43, 69). We first characterized AAV2-mediated expression in the PVN by injecting AAV2-eGFP and monitoring eGFP fluorescence, as shown schematically in Figure 2A and 2B. Seven days after microinjection of AAV2-eGFP into the PVN, eGFP fluorescence was found to be limited to the PVN, as shown in Figure 2C. No eGFP fluorescence was detected in cell bodies in forebrain regions, such as the SFO, or brainstem regions, such as the RVLM (Figure 2D). Immunostaining for the neuron-specific marker NeuN, astrocyte-specific marker GFAP, and microglia-specific marker IBA1 showed that AAV2-mediated eGFP expression was co-localized with neurons in the PVN, but not with astrocytes or microglia (Figure 2E).

Seven days after injection of AAV-Cre or control AAV2-eGFP virus (hereafter, AAV-Con) into the PVN, PRR immunoreactivity in eGFP-positive, Cre recombinase-expressing neurons in mice injected with AAV-Cre was reduced compared with that in eGFP-positive cells in mice injected with AAV-Con virus (Figure 3A). A semiquantitative analysis of the intensity of PRR immunoreactivity confirmed this (Figure 3B), revealing a significant reduction in PRR signal intensity in PVN neurons infected with Cre (fold change: 0.38 ± 0.14) compared with those infected with control virus (fold change: 1.11 ± 0.13 , $p = 0.002$). An analysis of PRR mRNA expression in the PVN further showed that AAV-Cre injection induced a significant reduction in PRR mRNA levels, normalized to those in control virus-treated mice, that was detectable on day 3 (fold change: 0.69 ± 0.06 , $P = 0.01$) and was maintained through day 24 (fold change: 0.60 ± 0.03 , $P = 0.001$). By contrast, PRR mRNA, measured on day 24, was unchanged in the cerebral cortex (fold change: 1.08 ± 0.11) and brainstem (fold change: 1.00 ± 0.16), confirming the specificity of PVN-targeted injection (Figure 3C). These data suggest that localized injection of AAV-Cre is an efficient approach for knocking down PRR expression in PVN neurons.

PRR knockdown in the PVN attenuates DOCA-salt-induced hypertension.

To examine the functional importance of the PVN-PRR in hypertension development, we induced hypertension using DOCA-salt(3, 54, 55), and monitored BP and HR, and evaluated sympathetic and parasympathetic tone in unanesthetized mice using a telemetry recording system. As depicted schematically in Figure 4A, mice were injected with AAV-Cre or AAV-Con 3 days prior to DOCA-salt or sham treatment. We found that

PRR knockdown in the PVN did not alter baseline BP or HR in PRR-floxed mice compared with control mice injected with AAV-Con (Figure 4B and 4C). In control mice, DOCA-salt induced a gradual, and significant, increase in BP compared with sham-treated mice that reached a plateau at ~2 weeks. Importantly, BP was significantly lower throughout the protocol in DOCA-salt mice injected with AAV-Cre compared with that in DOCA-salt mice injected with AAV-Con. However, BP remained significantly elevated in AAV2-Cre DOCA-salt mice compared with that in AAV-Con Sham mice, indicating that PRR knockdown in the PVN does not completely normalize BP. Differences in HR among the three groups of mice, analyzed by two-way ANOVA, were not significant throughout the protocol (Figure 4C).

At the end of the protocol, there was a significant increase in vasomotor (ΔBP : -68 ± 4.7 mmHg) and cardiac (ΔHR : -173 ± 16.1 bpm) *sympathetic* tone in AAV2-Con mice following DOCA-salt compared with sham-treated AAV2-Con mice (ΔBP : -29 ± 5 mmHg; ΔHR : -72 ± 3.3 bpm) (Figure 4D and 4E). PRR knockdown in PVN neurons (AAV2-Cre mice) attenuated the increase in vasomotor (ΔBP : -47 ± 9.2 mmHg) and cardiac (ΔHR : -106 ± 20.2 bpm; $p < 0.05$) *sympathetic* tone induced by DOCA-salt compared with that in mice injected with AAV2-Con. Conversely, cardiac *parasympathetic* tone (Figure 4F) was impaired in AAV2-Con mice that received DOCA-salt stimulation compared with sham-treated AAV2-Con mice (ΔHR : 50 ± 12.2 vs. 156 ± 23.7 bpm; $p = 0.006$). Notably, PVN-PRR knockdown attenuated this decrease (ΔHR : 112 ± 23.6 bpm), restoring cardiac *parasympathetic* tone to levels that were not significantly different from those in sham-treated AAV2-Con mice. These data indicate

the pathophysiological importance of the PVN-PRR in neurogenic hypertension and show that it is associated with modulation of autonomic function in mice.

The PVN-PRR regulates ERK1/2 activation in the PVN and RVLM in DOCA-salt-induced hypertension

It was previously reported that ERK1/2 activation in the PVN is critical for the development of hypertension or heart failure and contributes to elevated sympathetic outflow(76, 89, 90). To examine the effect of PVN-PRR knockdown on ERK1/2 activation status, we immunostained brain slices containing the PVN for Thr202/Tyr204-phosphorylated (activated) ERK1/2 (p-ERK1/2) at 2 weeks of DOCA-salt treatment, a time point chosen because it represents the plateau of BP/hypertension. Figure 5A shows a simplified schematic of the signaling cascade linking the PRR to ERK1/2 activation and an illustration of proposed pathways for PRR deletion in the PVN that affect ERK1/2 activation in the SFO, PVN, and RVLM. As shown in Figure 5B and 5C, p-ERK1/2 signals following 2 weeks of DOCA-salt treatment were significantly increased (measured as fold change) in the SFO (1.9 ± 0.2 vs. 1.0 ± 0.1), PVN (3.3 ± 0.2 vs. 1.0 ± 0.2), and RVLM (1.3 ± 0.1 vs. 0.9 ± 0.1) of control (AAV-Con-injected) mice compared with sham-treated mice. PRR knockdown in the PVN did not alter DOCA-salt-induced changes in p-ERK1/2 levels in the SFO (2.2 ± 0.2) compared with that in mice that received AAV-Con (1.9 ± 0.2), but did significantly reduce ERK1/2 activation in both the PVN (1.0 ± 0.1 vs. 3.3 ± 0.2) and RVLM (0.8 ± 0.1 vs. 1.3 ± 0.1).

We and others have previously reported that the PRR mediates both Ang II-dependent and -independent ERK1/2 activation *in vitro* and *in vivo*(46, 62, 63). However, it is not known whether this phenomenon also occurs in the PVN of hypertensive mice. To further understand whether the reduction in ERK1/2 activation following PVN-PRR knockdown is mediated by Ang II-dependent or -independent mechanisms (or both), we examined the effects of ICV-infused losartan on p-ERK1/2 immunostaining intensity. Figure 6A illustrates targeted brain regions and the pathway linking ICV-infused losartan and ERK1/2 phosphorylation. As shown in representative images in Figure 6B and summary data in Figure 6C, ICV-infused losartan significantly reduced p-ERK1/2 immunostaining intensity in the SFO (fold change: 0.59 ± 0.05 vs. 1.0 ± 0.07), PVN (0.71 ± 0.04 vs. 1.0 ± 0.05), and RVLM (0.65 ± 0.01 vs. 1.0 ± 0.04) in WT mice following 2 weeks of DOCA-salt treatment compared with ICV infusion of artificial cerebrospinal fluid (aCSF), confirming the importance of AT₁R in this hypertension model.

To better understand whether PRR-dependent ERK phosphorylation in these brain regions is AT₁R-dependent or -independent, we reanalyzed and replotted the data by normalizing the p-ERK1/2 levels in AAV-Cre to AAV-GFP in the PVN targeting experiment or p-ERK1/2 levels in ICV losartan to ICV aCSF in the ICV infusion experiment following DOCA-salt treatment and compared the percent reduction in p-ERK1/2, as shown in Figure 6D. PVN-PRR knockdown did not affect p-ERK1/2 in the SFO, whereas ICV infusion of losartan significantly reduced p-ERK1/2 in this region as showed in original data (Figure 5C and Figure 6C), supporting the appropriateness of our analysis. In the PVN, p-ERK1/2 signal intensity was lower in PVN-PRR-knockdown

mice (0.29 ± 0.04 vs. 1.0 ± 0.15 , normalized to DOCA-salt AAV-Con) compared with that in mice that received ICV losartan (0.71 ± 0.04 , normalized to DOCA-salt aCSF). These data suggest that both Ang II-dependent and -independent signaling cascades are involved in the regulation of ERK1/2 activation by the PRR in the PVN. Interestingly, relative ERK1/2 activation in the RVLM following DOCA-salt treatment was similar between losartan-infused WT mice (0.65 ± 0.01 vs. 1.0 ± 0.04 normalized DOCA-salt aCSF) and AAV-Cre mice (0.63 ± 0.05 vs. 1.0 ± 0.09 normalized to DOCA-salt AAV-Con mice).

Knockdown of the PRR attenuates AT₁R expression and activity in the PVN in DOCA-salt hypertension

To further investigate the molecular mechanisms by which PVN-PRR knockdown reduces hypertension, we measured mRNAs for the key angiotensin receptors, AT_{1a}R, AT₂R and MasR. As shown in Figure 7, which depicts fold changes in angiotensin receptor mRNAs following PRR knockdown in the PVN, DOCA-salt treatment significantly increased AT_{1a}R (2.5 ± 0.2) and AT₂R (1.6 ± 0.1) mRNA levels, and decreased MasR mRNA levels (0.06 ± 0.01) in the PVN of AAV-Con mice compared with sham treatment (1.0 ± 0.1). Notably, AAV-Cre injection into the PVN attenuated ($P < 0.01$) the DOCA-salt-induced increase in AT_{1a}R (1.6 ± 0.1) and AT₂R (0.8 ± 0.1) mRNA levels, without affecting ($p > 0.05$) MasR (0.07 ± 0.01) mRNA levels, compared with DOCA-salt-treated AAV2-Con mice. AT_{1a}R is the major angiotensin receptor in the PVN involved in mediating BP elevation, whereas AT₂R and MasR counterbalance the action of AT_{1a}R (20, 71) (30, 50). Accordingly, we hypothesized that a reduction in AT_{1a}R

expression/activity in the PVN may be one of the key mechanisms by which PVN-PRR knockdown attenuates DOCA-salt hypertension. To functionally validate our hypothesis and more directly measure AT_{1a}R activity, we evaluated Ang II-induced Ca²⁺ activity in the PVN in brain slices from DOCA-salt or sham-treated AAV-Con mice and DOCA-salt treated AAV-Cre mice using the Ca²⁺ biosensor, GCaMP6, delivered via AAV2.

As shown in a snapshot from a live-cell Ca²⁺-imaging video (Figure 8A) and 3D-reconstructed images (Figure 8B), Ang II (100 nM) had no apparent effect on Ca²⁺ signal amplitude or number of activation sites in AAV-Con Sham mice (white bars), but increased the frequency of Ca²⁺-signaling events within a field of view (FOV). This treatment had no effect on the viability of neurons in the brain slice, as evidenced by the retention of a robust response to a depolarizing concentration of KCl (100 mM). In AAV-Con DOCA-salt animals (red bars), Ang II treatment led to a significant increase in the amplitude, number of activation sites, and frequency of Ca²⁺-signaling events. Knockdown of the PRR in the PVN (AAV-Cre-injected mice) abrogated this DOCA-salt-induced excitatory response to Ang II (blue bars). This Ang II-induced Ca²⁺ activity was blocked by pre-treatment with losartan, suggesting the involvement of AT₁Rs.

A quantitative analysis of Ca²⁺ signals in video recordings (Figure 8C, 8D and 8E) revealed that Ang II induced a significant increase in both the amplitude (11.8% ± 0.8% of maximal response) and number (13.3 ± 0.5 sites/FOV) of Ca²⁺ events in the PVN of AAV-Con mice that received 2 weeks of DOCA-salt, but had minimal effects on the corresponding sham-treated mice (*P* > 0.05), consistent with an increase in AT₁R expression with DOCA-salt treatment. Interestingly, Ang II increased the frequency of

Ca²⁺ events in the PVN of mice from both Sham (0.03 ± 0.002 Hz) and AAV-Con DOCA-salt (0.039 ± 0.003 Hz) groups. Notably, Ca²⁺ event frequency was higher in AAV-Con DOCA-salt-treated mice compared with sham-treated mice. Importantly, PVN-PRR knockdown normalized the Ang II-induced increase in the amplitude (6.5% ± 0.7% of maximal response) and number (4.8 ± 0.5 sites/FOV) of Ca²⁺ events following DOCA-salt treatment compared with the AAV-Con group, without affecting their frequency (0.036 ± 0.003 Hz) (Figure 8E), indicating that PRR knockdown specifically reduces AT₁R-mediated Ca²⁺ signal amplitude and number of activation sites.

Interestingly, under baseline (Vehicle) conditions, we observed a small, but significant, increase in the amplitude of Ca²⁺ events in the PVN of DOCA-salt mice (9.1% ± 0.5% of maximal response) that received AAV-Con compared with the corresponding Sham mice (5.3% ± 0.6% of maximal response), whereas there was no difference in the number of active sites (4.6 ± 0.6 vs. 4.0 ± 1.2 sites/FOV) or frequency (0.01 ± 0.004 vs. 0.02 ± 0.003 Hz) between these two groups. To our surprise, we found that pretreatment of brain slices from the vehicle group with losartan prevented the DOCA-salt-induced increase in Ca²⁺ event amplitude, indicating that basal Ca²⁺ activity in the PVN is losartan sensitive and possibly depends on the AT₁R.

Discussion

The PRR is a recently discovered receptor for both renin and prorenin that increases renin activity or non-proteolytically activates prorenin(59). The importance of the PRR is underscored by recent reports of its role in mediating endogenous Ang II formation in

the CNS(38, 53, 54, 73). It is well established that the PVN is part of neural circuits that are sensitive to Ang II(10, 11, 44, 50). Expression of the PRR in the PVN and the functional role of the PVN in modulating neuronal activity have been demonstrated previously(54, 64, 87). For example, we showed that PRR knockdown globally in neurons using a neuron-specific, synapsin1-promoter–driven Cre-loxP system decreases Ang II formation in the brain, improves autonomic function, and blunts development of DOCA-salt hypertension(54). However, the brain regions that are key to this effect and the mechanism by which the PRR contributes to the DOCA-salt hypertension remained incompletely understood. The present study provides mechanistic insight into this unresolved question, specifically establishing that the PRR is important in the development of DOCA-salt hypertension through regulation of AT₁R-dependent Ca²⁺ activity in PVN neurons. In summary, our findings indicate that 1) PVN delivery of AAV2 primarily targets neurons; 2) knockdown of the neuronal PVN-PRR in adult mice attenuates the development of DOCA-salt-induced hypertension and is associated with improved autonomic function; 3) knockdown of the PVN-PRR diminishes ERK1/2 activation in the PVN and RVLM neurons; and 4) knockdown of the PVN-PRR reduces AT_{1a}R mRNA expression levels and functionally attenuates AT₁R-dependent Ca²⁺ activity in the PVN, providing a mechanistic basis for the role of the PRR in angiotensinergic signaling during DOCA-salt–induced hypertension.

The PRR is expressed in many cardiovascular regulatory regions of the brain and is localized to neurons and microglia in rat, primate, and human brains(1, 12, 13, 54, 64, 73, 79, 82). In the mouse brain, the PRR is expressed in neurons, but not in

astrocytes(54, 87). Although evidence for PRR expression in a mouse microglial cell line was reported previously(74), whether PRR protein is also expressed in microglia in the mouse PVN was not known. Using microglia reporter mice (CX3CR1-GFP), we showed here that the PRR is expressed in a very small number of microglial cells in the PVN at the normotensive stage. Similarly, we showed that the PRR is primarily expressed in neurons and not astrocytes. Accordingly, we chose to investigate the role of the neuronal PRR in the PVN in this study. AAVs have come to be used as common tools for genetic manipulation owing to their lack of immunogenicity and effective neuron targeting in the CNS(39). The cell-targeting specificity is determined by the serotype of the AAV. In this study, we characterized AAV2 infection in the PVN and showed that the AAV2 serotype infects mostly neurons, and not astrocytes or microglia, a tropism similar to that reported in previous studies on neurons in other brain regions(43, 69). We further validated that AAV2-Cre sufficiently knocked down the PRR in neurons (Figure 3). Accordingly, the effect of PRR knockdown in the current study can be inferred to reflect neuronal PRR knockdown. However, considering the importance of brain hypothalamic inflammation in cardiovascular metabolic diseases(27, 74, 81), we recognize that PRRs in microglia may also have a role in the PVN, a question of interest for our future studies.

To evaluate the neuronal activation states that might be altered by PVN-PRR knockdown, we examined the levels of p-ERK1/2^{Thr202/Tyr204} —a marker of neuronal activation(15, 89)—in three key brain regions: the SFO, PVN, and RVLM. The SFO, which is located outside the blood-brain barrier, senses inputs from the circulation and

receives projections from other brain regions. In this latter context, the SFO lies upstream of and projects to the PVN in the hypothalamus(4, 23, 80, 83). Thus, it is not surprising that PRR knockdown in the PVN does not affect ERK1/2 activation in the SFO. Interestingly, however, we found that PVN-PRR knockdown reduced ERK1/2 activation in both the PVN and RVLM, the latter of which is the presympathetic center of BP regulation(14, 45, 47). This finding is consistent with our observation that PVN-PRR knockdown reduces BP, as well as cardiac and vasomotor sympathetic tone (Figure 4). Following ICV infusion of losartan, ERK1/2 phosphorylation was uniformly reduced in the SFO, PVN and RVLM, an observation in agreement with a previous finding on the importance of AT₁R in mediating ERK1/2 activation in the brain(63). Interestingly, ERK1/2 activation in the PVN was lower with PVN-PRR knockdown than with ICV infusion of losartan. Our data suggest that both Ang II-dependent and direct PRR signaling independent of Ang II are involved in mediating ERK1/2 activation in DOCA-salt hypertension. Surprisingly, however, the degree of ERK1/2 activation in the RVLM was similar between PRR knockdown and losartan treatment. Although the underlying mechanism is not clear, we speculate that angiotensinergic projections from the PVN to the RVLM are dominant in ERK1/2 activation in the RVLM in this hypertension model.

We noted that BP was not completely normalized in PVN-targeted PRR-knockdown mice during DOCA-salt hypertension development. In this context, it is important to highlight that, although the PVN PRR contributes to increased BP, neural autonomic and neural endocrine dysregulation during hypertension(8, 54, 61, 87), a role for the PRR in other important cardiovascular regulatory areas of the brain, including the

SFO(9, 53), supraoptic nucleus (SON)(73) and nucleus of the solitary tract (NTS)(92), in regulating BP has been reported. Moreover, other key BP regulating systems in addition to RAS also contribute to DOCA-salt induced hypertension in mice(3, 31, 88). Thus, it is not surprising that knockdown of PRR in the PVN did not completely normalize BP and autonomic function.

In this study, we also showed that PVN-PRR knockdown differentially affected sympathetic and parasympathetic input to the heart, attenuating the former while increasing the latter, thus producing beneficial effects. It is well known that PVN projections to the RVLM are predominantly excitatory, whereas those to the NTS are primarily inhibitory(18). We showed that PVN-PRR knockdown decreased the levels of p-ERK1/2, a marker of neuronal activity, in the PVN and RVLM, supporting the inference that PVN-PRR knockdown decreases neuronal activity in the PVN, and thus its excitatory effect in the RVLM, which explains the decreased sympathetic activity to the heart and the periphery. It is possible that PVN-PRR knockdown also decreases inhibitory output from the PVN to the NTS, likely leading to increased neuronal activity in the NTS, and thus increased cardiac parasympathetic activity.

To decipher the mechanisms of the PRR in hypertension development, we took advantage of the DOCA-salt-induced hypertensive mouse model, which is a model of neurogenic hypertension with upregulated brain RAS activity(3, 54, 55). Accumulating evidence indicates that angiotensin receptors in the PVN, including AT₁R, AT₂R and MasR, play key roles in modulating cardiovascular function by interacting with

angiotensin peptides(16, 17, 32, 34, 41, 84). Here, we showed that PRR knockdown in the PVN attenuates hypertension and is associated with improved autonomic function. Furthermore, PRR knockdown in the PVN reduced mRNA levels of both AT_{1a}R and AT₂R mRNA, but interestingly had no effect on MasR. AT₂R was recently reported to localize in peri-PVN GABAergic neurons that project to both autonomic and AVP-positive neurons in the PVN(22). Activation of these AT₂R neurons leads to inhibition of PVN-AVP-positive neurons(20). In rodent models of Ang II-induced hypertension or chronic heart failure, AT₂R levels are decreased in the PVN(33, 75); however, in DOCA-salt-induced hypertension, AT₂R expression levels in the PVN were reported to be elevated(17), a result similar to our findings (Figure 7B). The increase in AT₂R expression in DOCA-salt hypertensive mice may be a compensatory mechanism that protects against elevated BP in this model.

Since Ang II/AT₁R represents the major vasoconstrictor axis of the brain RAS, whereas Ang II/AT₂R and Ang (1-7)/MasR constitute the protective vasodilator axis(20, 21), we proposed that PRR knockdown exerts its beneficial effects on BP, in part, by reducing AT₁R signaling in this brain region and tested this hypothesis using Ca²⁺ imaging. Ca²⁺ signaling is a critically important aspect of neuronal activity as it participates in the transmission of depolarizing signals and thereby contributes to synaptic activity(5, 35). By acting on AT₁Rs, Ang II increases intracellular Ca²⁺ concentration through various signaling pathways(40). Using the genetically encoded Ca²⁺ biosensor GCaMP6(56), we confirmed that PRR knockdown in the PVN alters AT₁R-mediated Ca²⁺ activity in neurons. Because of the cell-specific targeting of AAV2, discussed above (see also

Figure 2), AAV2-mediated GCaMP6 expression is superior to conventional Ca^{2+} -binding fluorescent dyes(56), because it allows intracellular Ca^{2+} to be recorded in neurons without interference from other cell types in the brain, such as astrocytes and microglia. Importantly, using this Ca^{2+} biosensor, we showed that PRR knockdown eliminated the augmented AT_1R -mediated Ca^{2+} activity induced by Ang II in PVN neurons of DOCA-salt-treated mice, supporting the conclusion that PRR knockdown reduces AT_1R activity.

Because the amplitude of Ca^{2+} events was increased in the PVN of DOCA-salt hypertensive mice compared with sham-treated normotensive mice at baseline (vehicle-treated group), and this change was inhibited by treatment with losartan, we speculate that this is an Ang II/ AT_1R -sensitive mechanism. Among the best-characterized Ca^{2+} signaling pathways activated by G-protein coupled receptors is the release of intracellular Ca^{2+} stored in the endoplasmic reticulum (ER) (78). The release of Ca^{2+} from the ER causes a depletion of Ca^{2+} in this cell compartment that can be sensed by Stim1 (stromal interaction molecule 1), which triggers influx of extracellular Ca^{2+} by store-operated Ca^{2+} channels (SOCs), such as the Ca^{2+} release-activated Ca^{2+} modulators, Orai1–3, and members of the canonical transient receptor potential cation channel (TRPC) family. Some groups have shown that this store-operated Ca^{2+} entry (SOCE) mechanism plays an important role in astrocyte activity(48) and dorsal root ganglion neuron excitability(85). The expression of Orai channels has not been investigated in PVN neurons or microglia, but TRPC expression in PVN neurons has been previously demonstrated(29, 57). Further studies are needed to determine if these

mechanisms play a role in PVN neurons and are responsible for increased excitability and sympathetic activity in hypertension, and to assess the importance of PVN-AT₁R in this process.

In summary, we reported here that knockdown of PRR in the PVN attenuates the development of DOCA-salt-induced hypertension and is associated with improved autonomic function. Mechanistically, PVN-PRR knockdown reduces ERK1/2 activation in the PVN and RVLM. In addition, PRR knockdown reduces AT₁R-mediated Ca²⁺ activity in PVN neurons. We conclude that, in the PVN, the PRR makes an important contribution to DOCA-salt-induced hypertension, in part, by regulating AT₁R expression and function.

Perspectives and Limitations

This study demonstrates an essential role of the PRR in PVN neurons in the development of neurogenic hypertension. The PVN is a complex brain nucleus composed of excitatory and inhibitory neurons, and neurosecretory neurons. Although we observed an overall beneficial effect of PRR knockdown in PVN neurons, the contributions and roles of specific types of neurons in hypertension remain incompletely understood. Further studies should examine the roles and mechanisms of the PRR in specific neuron types.

Acknowledgement

Research reported in this publication used imaging core facility supported by the National Institute of General Medical Sciences of the National Institutes of Health (NHI/NIGMS P20 GM103650), and the Transgenic Genotyping and Phenotyping Core facility supported by National Institute of General Medical Sciences of the National Institutes of Health (NHI/NIGMS 1P20GM130459)

Sources of Funding

This work was supported, in part, by grants from the National Heart, Lung, and Blood Institute of the National Institutes of Health (NIH/NHLBI) R01HL122770, R01HL091905, and NIH/NIGMS (1P20GM130459) and the American Heart Association National Center (17IRG33370128) to Y. Feng. The content of this manuscript is solely the responsibility of the authors and does not necessarily represent the official views of the granting agencies.

Disclosures

None

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Figure Legends

Figure 1. Expression and cellular localization of the PRR in the PVN of the mouse hypothalamus. The PVNs of WT C57Bl/6J mice were immunofluorescently labeled for **(A)** the PRR (red), GFAP (green), and NeuN (blue). **(B)** The PVNs of CX3CR1-GFP reporter mice were immunofluorescently labeled for the PRR (red) and counterstained with DAPI (blue); white arrows indicate co-localization of the PRR with GFP. **(C)** No primary antibody controls using secondary antibodies conjugated with Alexa 594 (red), Alexa 488 (green), or Alexa 647 (blue). **(D)** Validating the specificity of the PRR antibody by western blotting of whole-cell extracts of Neuro2A cells infected with scrambled (SC) or PRR shRNA. PVNs are outlined. 3V, third ventricle.

Figure 2. Characterization of AAV2 targeting in the PVN of the hypothalamus. AAV2-eGFP was bilaterally injected into the PVN of PRR-floxed mice; 7 days later, mouse brains were collected for imaging. **(A, B)** Schematic showing the bilateral PVN injection site and stereotaxic injection coordinates. **(C)** Representative images of eGFP fluorescence following bilateral injection of AAV2-eGFP into the PVN. **(D)** Representative images showing the specificity of PVN injection in slices from the SFO and RVLM. **(E)** Representative images of immunolabeling for the neuronal cell marker NeuN (red), astrocyte marker GFAP (red) and microglial marker IBA-1 (red), and their colocalization with eGFP (green). 3V, third ventricle.

Figure 3. PRR knockdown in the PVN by AAV2-delivered Cre recombinase. (A) Representative images of mouse PRR (red) immunostaining and eGFP fluorescence (green) in the PVN. **(B)** Semi-quantitative analysis of the relative intensity of PRR fluorescence signals in eGFP-positive cells. **(C)** Number of eGFP-positive cells in each PVN (n = 7 PVNs from 3 mice/group). **(D)** PRR mRNA levels in the PVN, cortex and brainstem (BS), determined by RT-qPCR (**P* < 0.05 vs. AAV-Con; ***P* < 0.01 vs. AAV-Con; n = 3 mice/group, 2 PVNs from each mouse were combined). 3V, third ventricle; PaPo, paraventricular hypothalamic nucleus, posterior part; PaMP, paraventricular hypothalamic nucleus, medial parvicellular part.

Figure 4. Knockdown of the PRR in the PVN attenuates the development of DOCA-salt–induced hypertension and dysautonomia. (A) A schematic of the experimental protocol. **(B, C)** Mean arterial pressure (MAP) and HR at baseline and during 21 days of DOCA-salt treatment. **(D)** Vasomotor sympathetic tone, measured as changes in MAP in response to intraperitoneal injection of 6 mg/kg chlorisondamine. **(E)** Cardiac sympathetic tone, measured as changes in HR in response to intraperitoneal injection of 5 mg/kg propranolol. **(F)** Cardiac parasympathetic tone, measured as changes in HR in response to intraperitoneal injection of 1 mg/kg methylatropine. N = 4–8 mice/group; **P* < 0.05 vs. AAV-Con Sham; ***P* < 0.01 vs. AAV-Con Sham; [§]*P* < 0.05 vs. AAV-Con DOCA-salt.

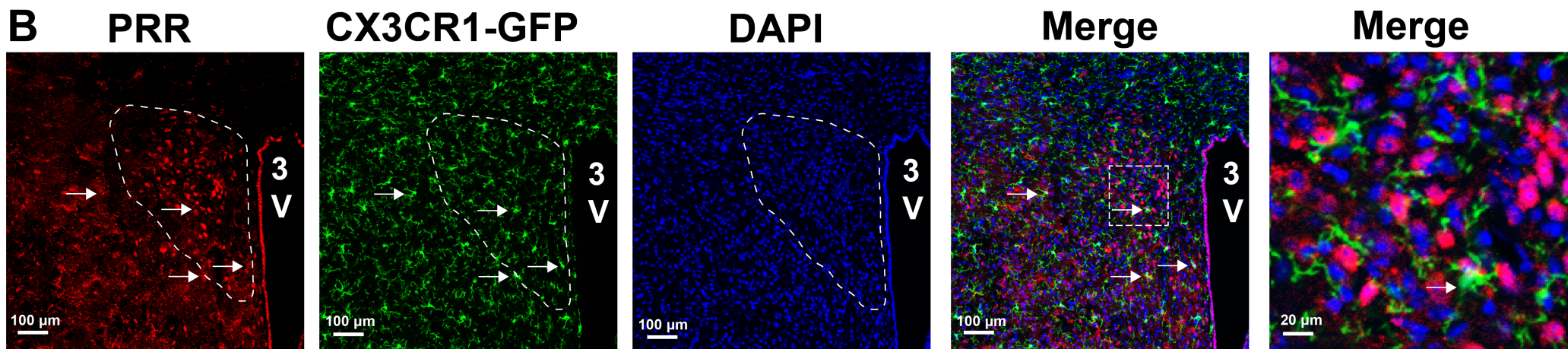
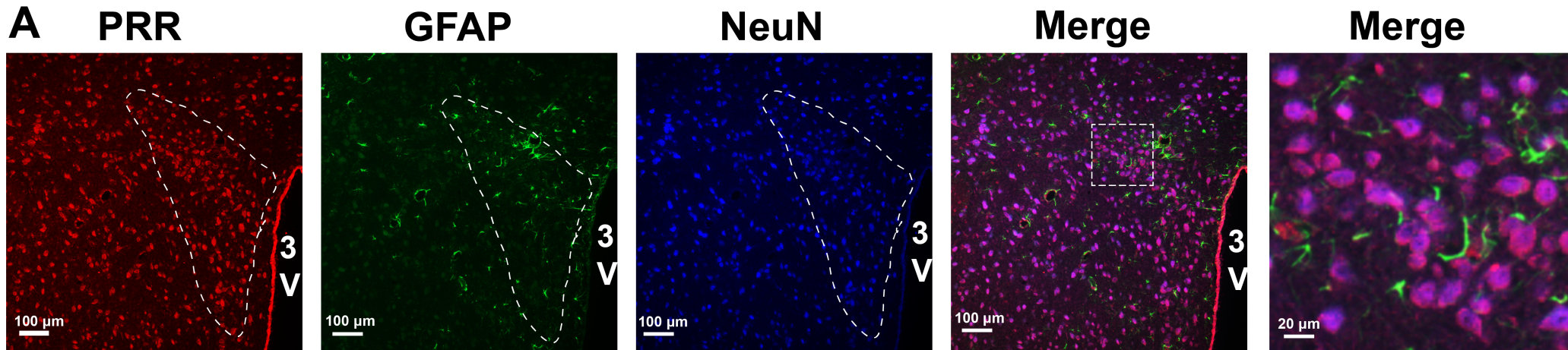
Figure 5. PVN-targeted knockdown of the PRR reduces ERK1/2 activation in the PVN and RVLM. (A) Schematics illustrating the activation of ERK1/2 by the PRR and

effect of PVN-PRR knockdown on ERK1/2 activation. **(B)** Representative images and **(C)** summary data quantifying p-ERK1/2 immunostaining intensity in the SFO, PVN, and RVLM of AAV-Con Sham, AAV-Con DOCA-Salt, and AAV-Cre DOCA-Salt groups following 2 weeks of DOCA-salt or sham treatment. $*P < 0.05$ vs. AAV-Con Sham; $**P < 0.01$ vs. AAV-Con Sham; $***P < 0.001$ vs. AAV-Con Sham; $$$$P < 0.001$ vs. AAV-Con DOCA-Salt ($n = 2-5$ brain sections from each mouse, 3–5 mice/group).

Figure 6. Effects of ICV-infused losartan on ERK1/2 activation in the SFO, PVN, and RVLM. **(A)** Schematics illustrating activation of ERK1/2 by the PRR and the pathways by which chronically ICV-infused losartan inhibits ERK1/2 activation. **(B)** Representative images and **(C)** summary data quantifying p-ERK1/2 immunostaining in the SFO, PVN, and RVLM following 2 weeks of chronic ICV infusion of aCSF or losartan (3 mg/kg/d) ($n = 3-5$ brain sections per mouse, 3 mice/group). **(D)** Data normalized and replotted from Figure 5C (dotted gray squares) and Figure 6C. $*P < 0.05$ vs. ICV aCSF; $**P < 0.01$ vs. ICV aCSF; $****P < 0.001$ vs. ICV aCSF. $$$$P < 0.001$ vs. AAV-Con DOCA-Salt; $##P < 0.01$ vs. ICV aCSF; $###P < 0.001$ vs. PVN AAV-Cre DOCA-salt.

Figure 7. PVN-PRR knockdown regulates angiotensin receptor expression. **(A)** $AT_{1a}R$, **(B)** $AT_{2}R$, and **(C)** MasR mRNA levels. $**P < 0.01$ vs. AAV-Con Sham; $***P < 0.001$ vs. AAV-Con Sham; $$$P < 0.01$ vs. AAV-Con-DOCA-Salt; $$$$P < 0.001$ vs. AAV-Con-DOCA-Salt ($n = 3-4$ mice/group).

Figure 8. PVN-PRR knockdown regulates AT₁R-mediated Ca²⁺-signaling events in the PVN. (A, B) Representative snapshot images (A) and 3D images from 3D FOV reconstructions (B) of Ca²⁺ activity within PVN slices of AAV-Con Sham, AAV-Con DOCA-Salt, and AAV-Cre DOCA-Salt mice under control conditions and following treatment with Ang II (100 nM) or KCl (100 mM). (C–E) Summary data for live-cell Ca²⁺ imaging showing amplitude (C), sites/FOV (D), and frequency (E) in response to Ang II, detected using the Ca²⁺ biosensor GCaMP6, in slices containing the PVN from AAV-Con Sham, AAV-Con DOCA-Salt, and AAV-Cre DOCA-Salt mice. **P* < 0.05 vs. AAV-Con Sham in the same treatment group (Vehicle or Ang II); *****P* < 0.0001 vs. AAV-Con Sham in the same treatment group (Vehicle or Ang II); \$\$\$*P* < 0.0001 vs. AAV-Con DOCA-salt in the Ang II group; #*P* < 0.05 Ang II vs. Vehicle or Ang II vs. Ang II + Losartan within the same treatment group; ###*P* < 0.01 Ang II vs. Vehicle or Ang II vs. Ang II + Losartan within the same treatment group; #####*P* < 0.0001 Ang II vs. Vehicle or Ang II vs. Ang II + Losartan within the same treatment group (AAV-Con Sham, AAV-Con DOCA-salt, AAV-Cre DOCA-salt) (n = 4 mice/group).

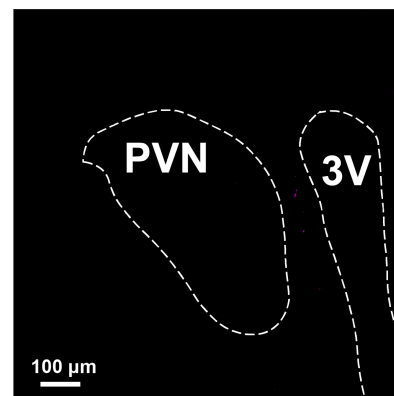
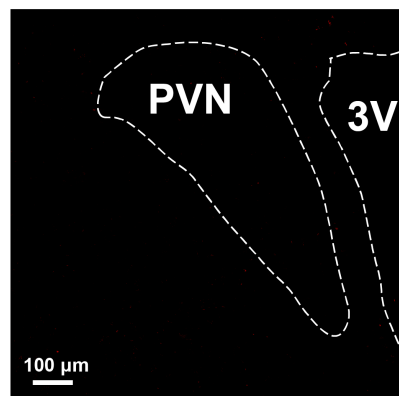
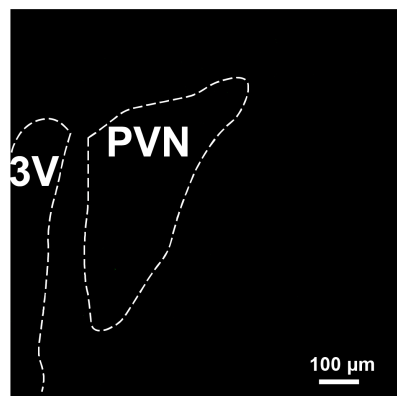


C
Negative Controls (no 1°Ab)

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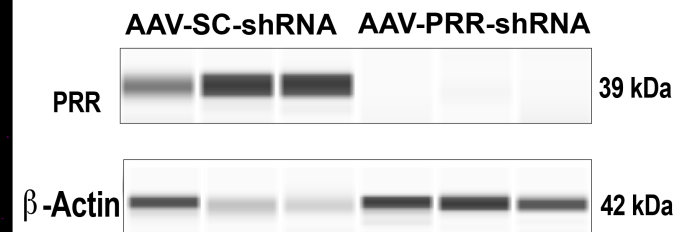
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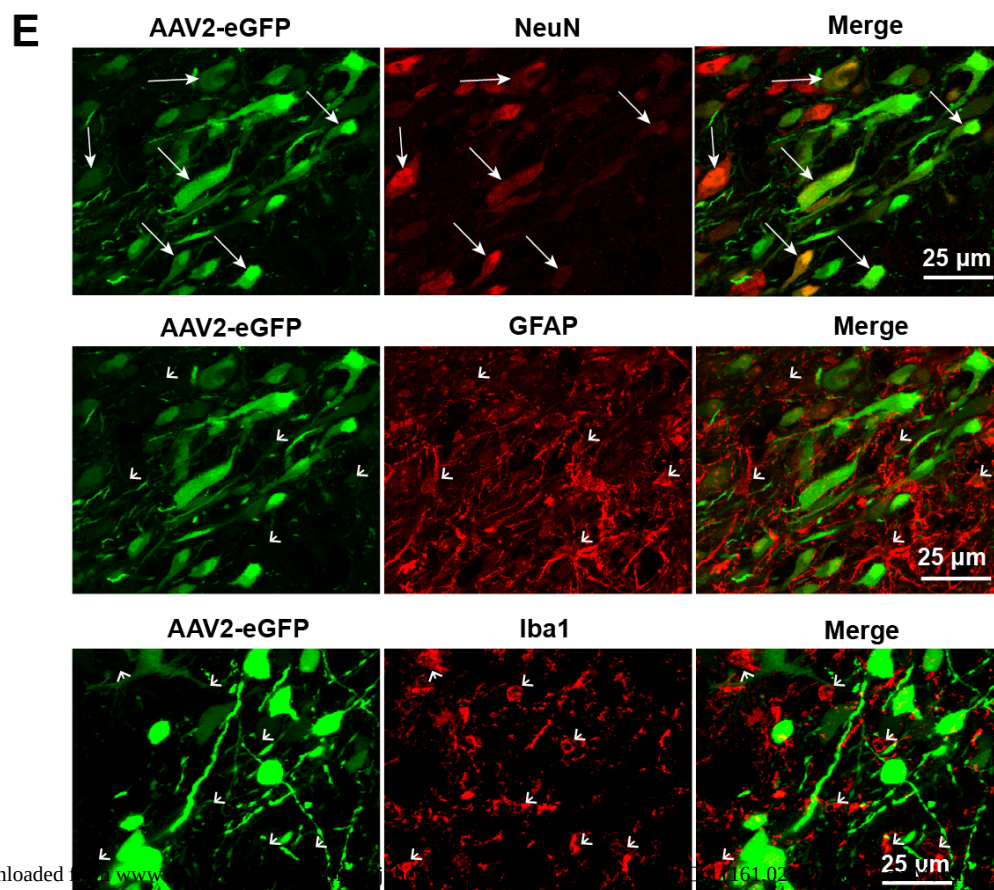
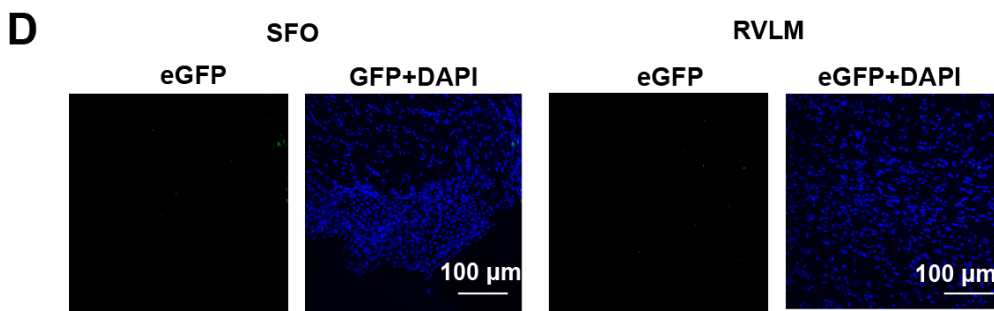
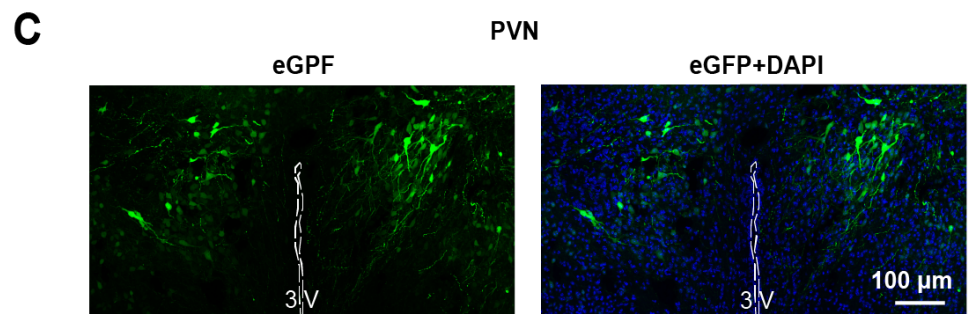
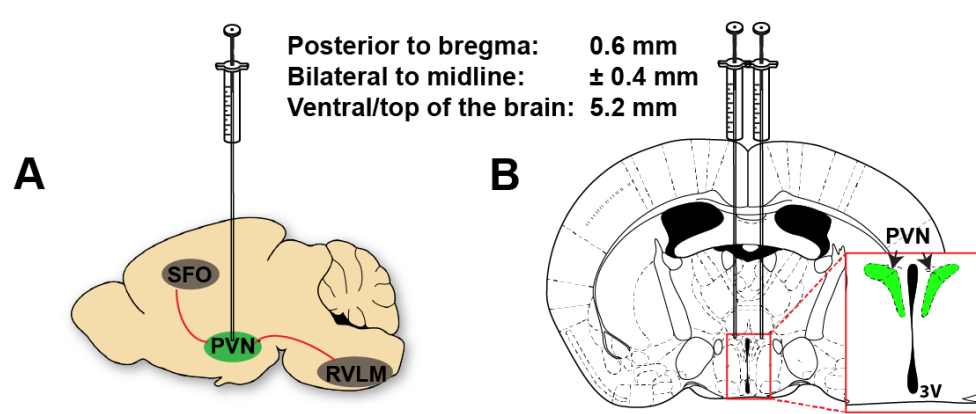
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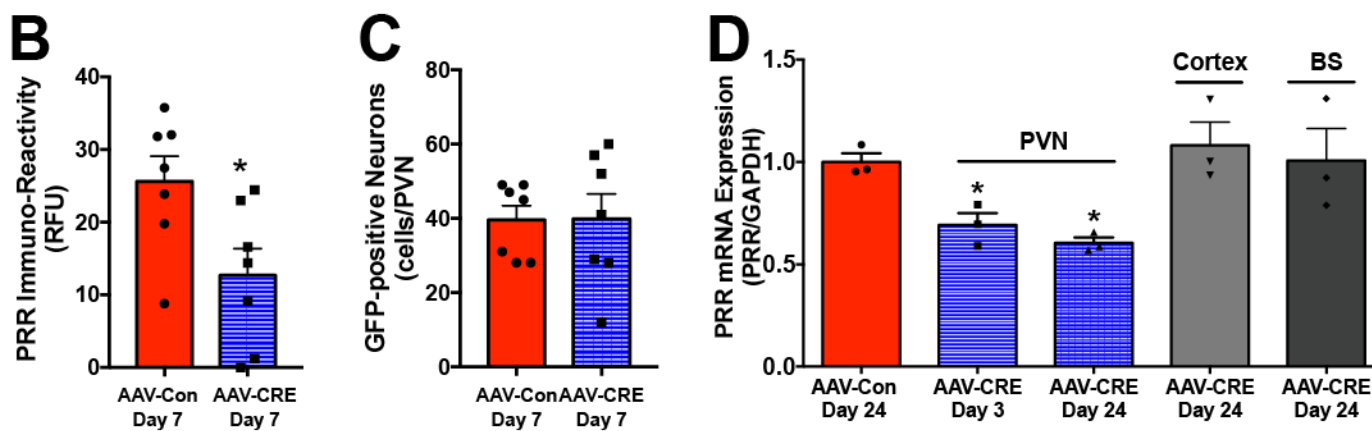
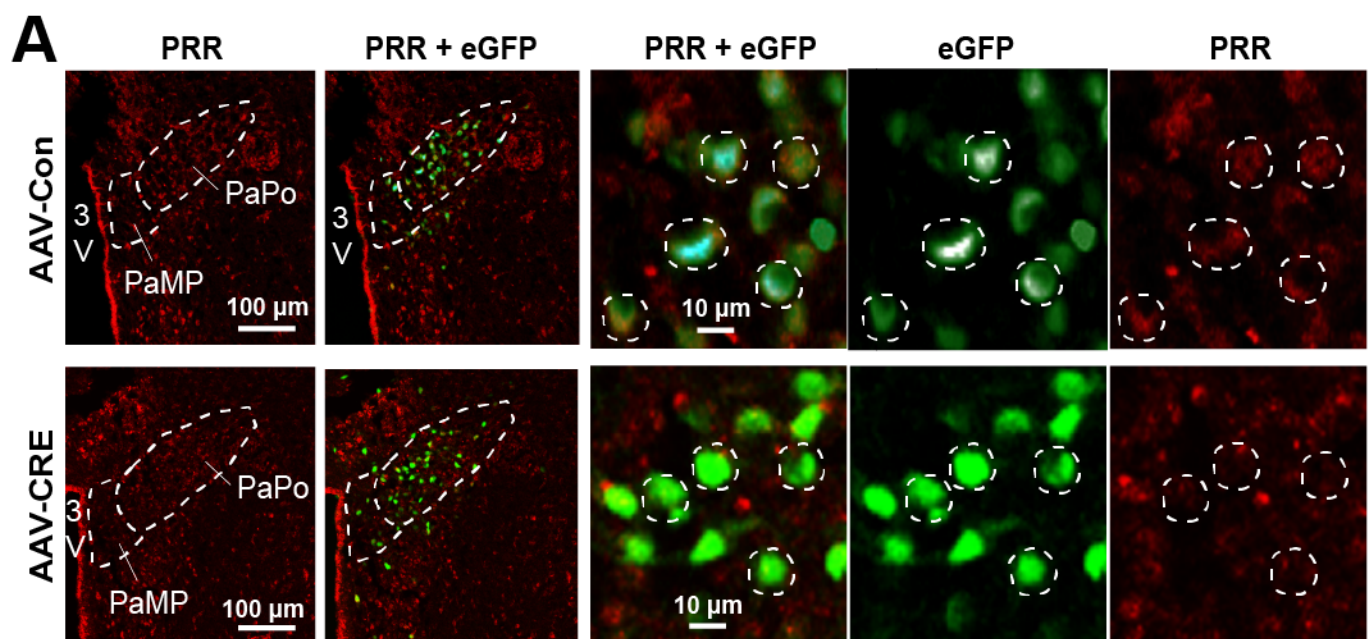


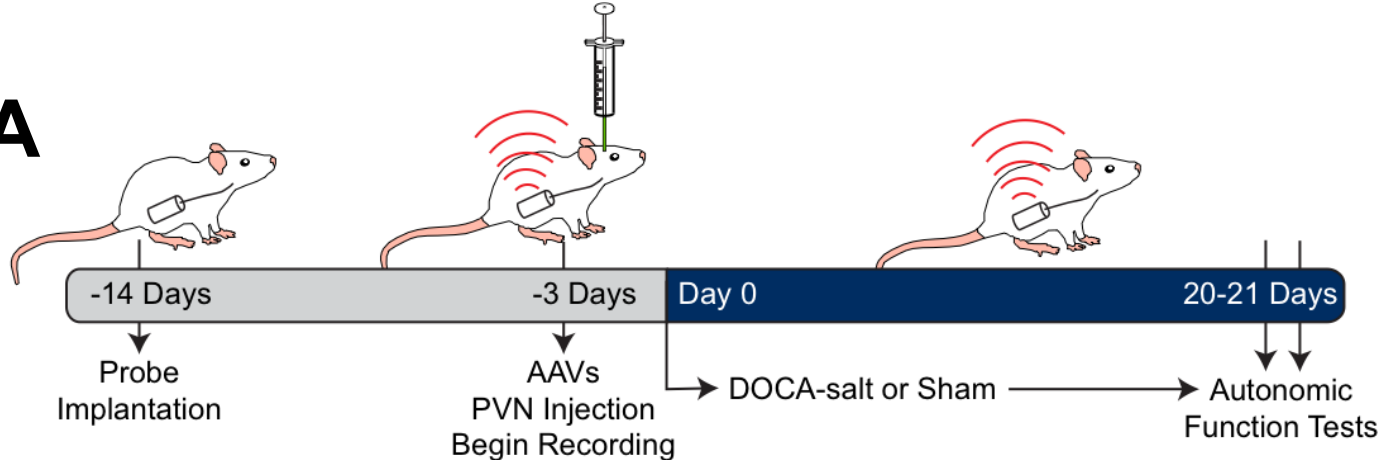
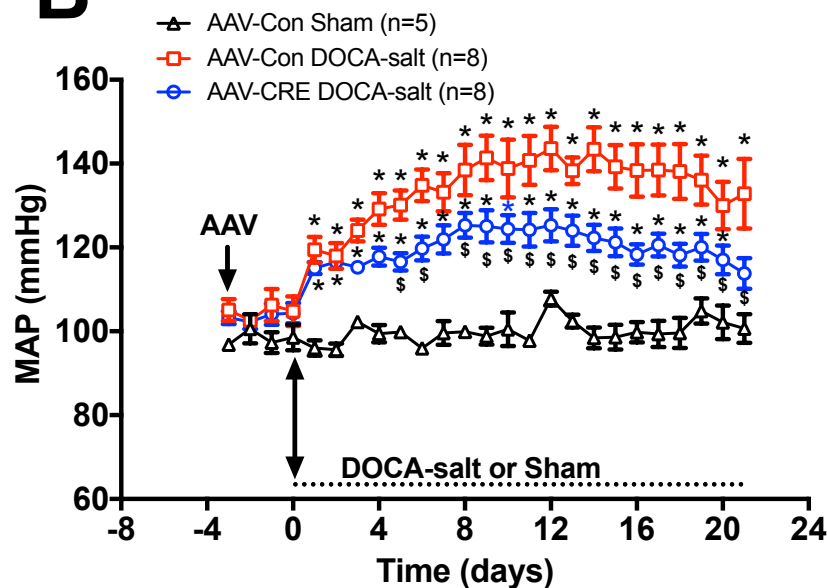
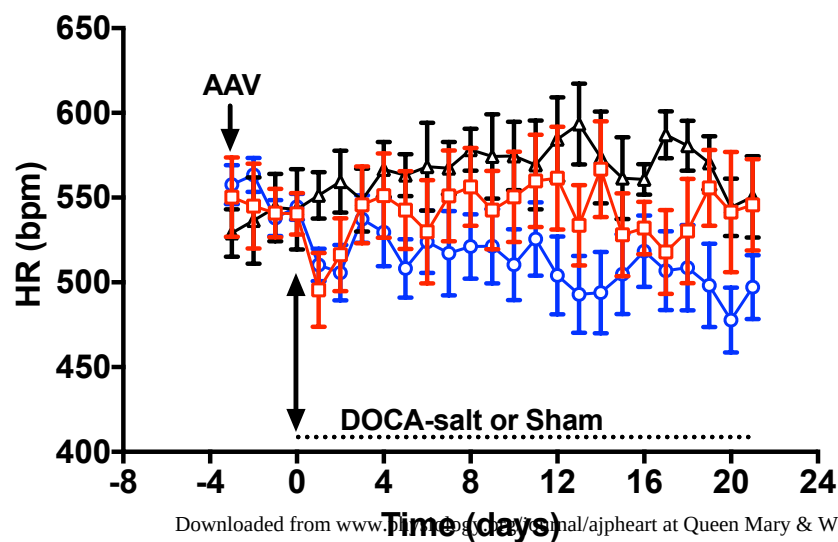
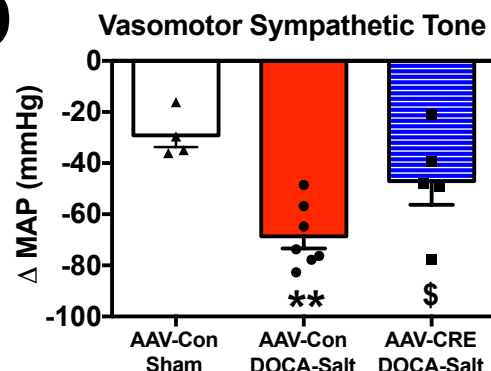
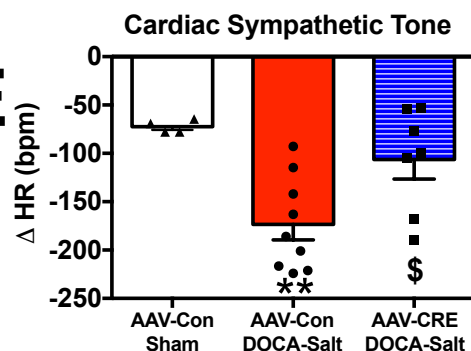
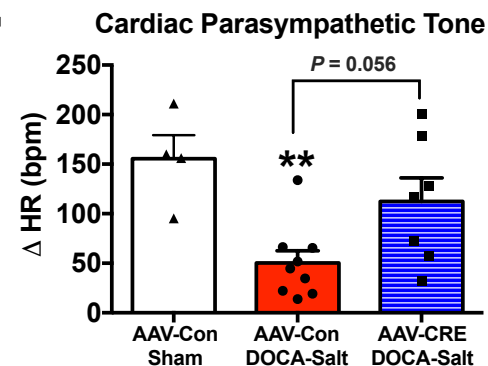
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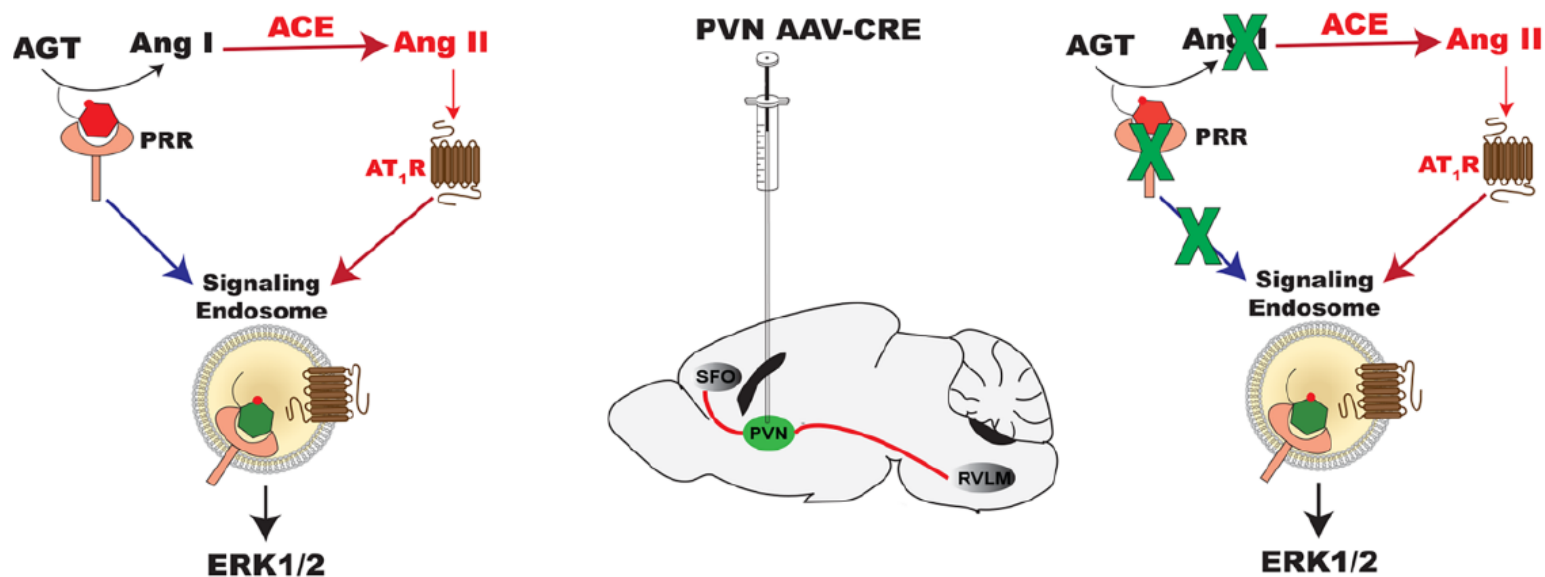
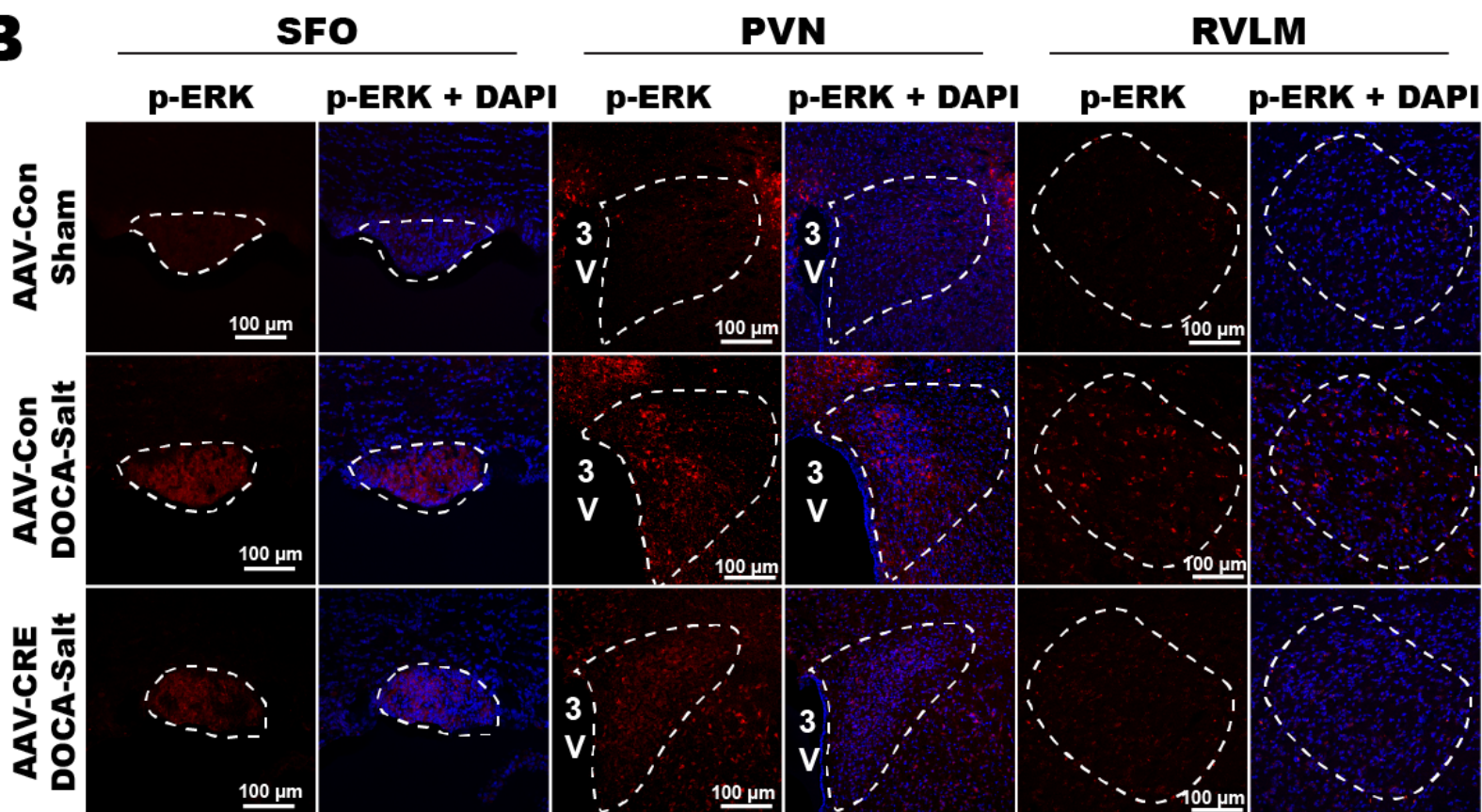
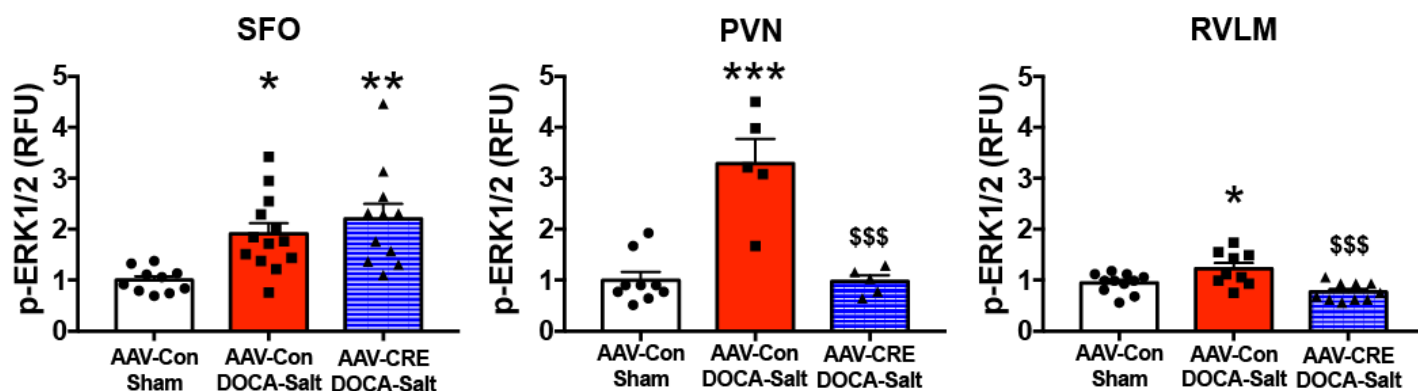
Neuro2A cells

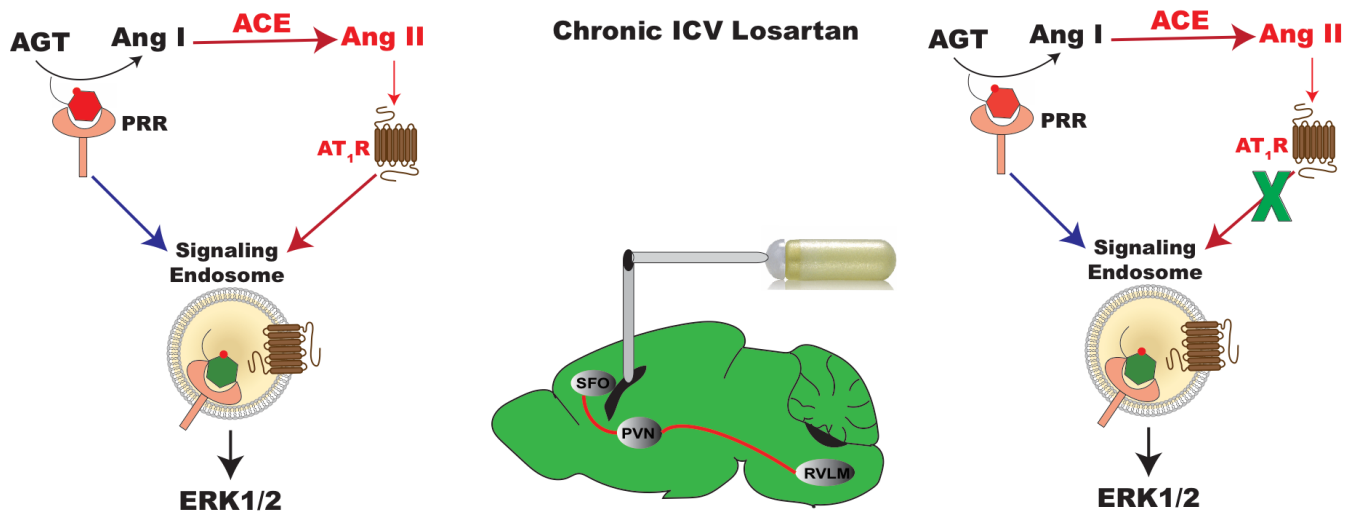
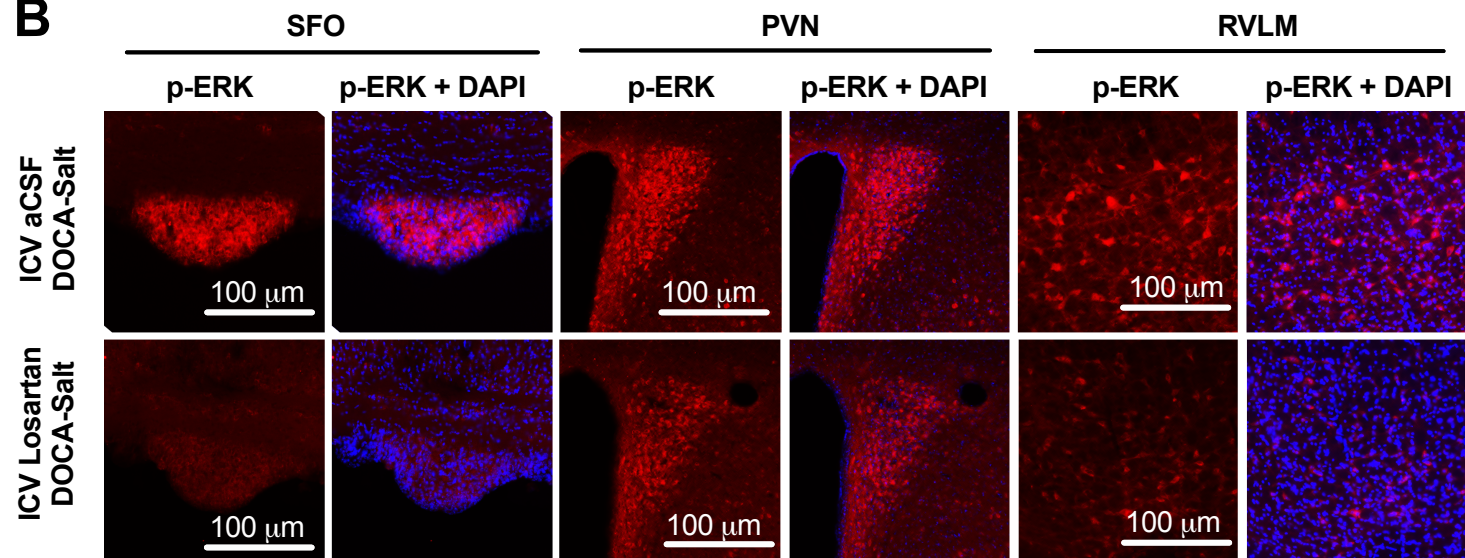
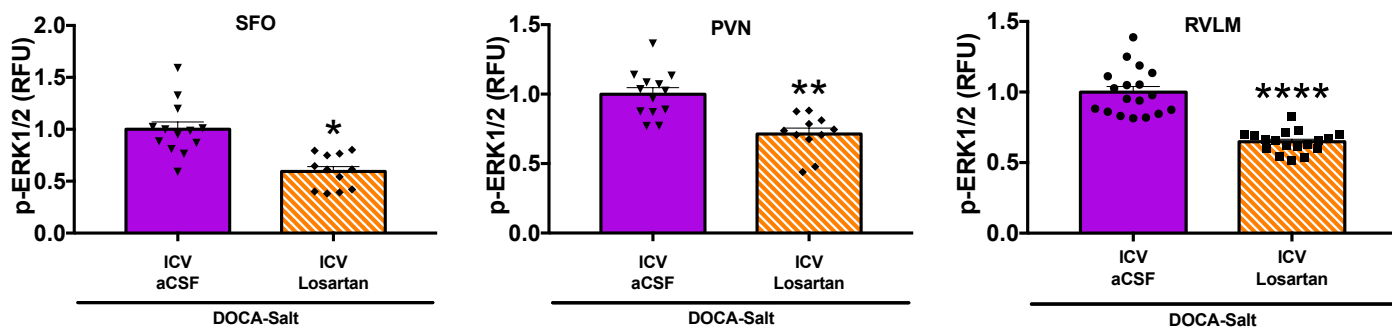
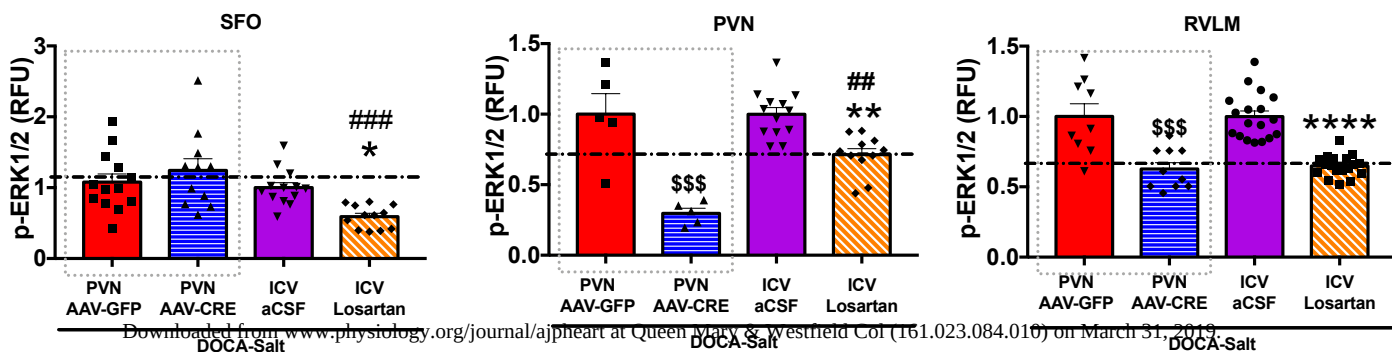




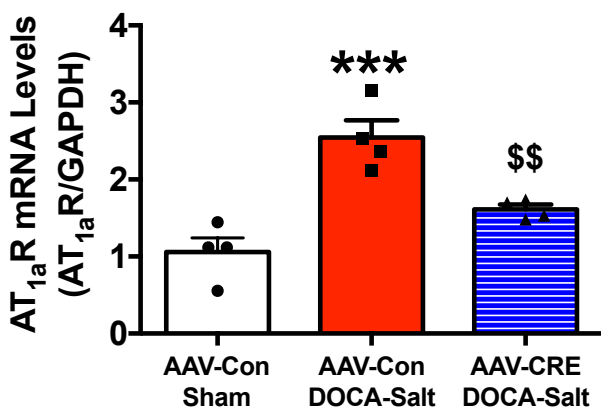


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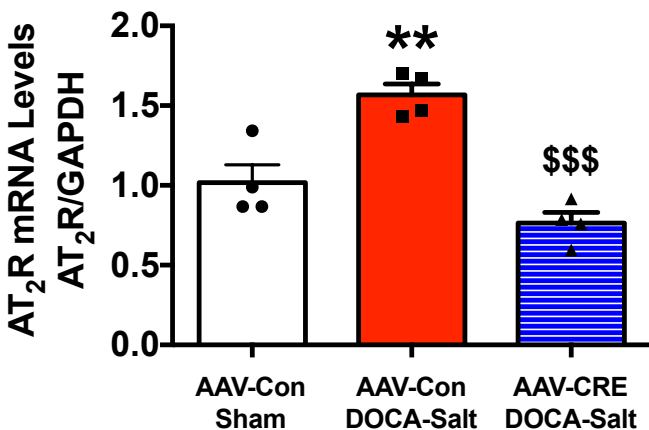
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