ONLINE SUPPLEMENT

to

EARLY INTERFERENCE WITH P44/42 MAPK SIGNALING IN HYPOTHALAMIC PARAVENTRICULAR NUCLEUS ATTENUATES ANGIOTENSIN II - INDUCED HYPERTENSION

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Running Title: Brain p44/42 MAPK signaling in ANG II hypertension

SPECIFIC METHODS

Animals

Adult male Sprague-Dawley rats (250 to 300 g; Harlan Sprague-Dawley, Indianapolis, IN) were housed in temperature- (23±2°C) and light-controlled animal quarters and were provided with rat chow ad libitum. All experimental 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 University of Iowa Animal Care and Use Committee.

Dose-response relationship for paraventricular nucleus (*PVN) microinjection of p44/42 MAPK siRNA*

Extrapolating from doses of siRNA used *in vivo* to silence target genes in the brain in rodents, reported by us[1] and others,[2, 3] we tested the ability of three different concentrations of the pooled p44/42 siRNA to reduce p44/42 MAPK expression in the PVN of normal rats **(Figure S1)**. A dose-response curve was generated in normal rats (n=15). A 5.6 ng, 28 ng or 56 ng pooled siRNA (Thermo Fisher Scientific Inc.) targeting p44/42 MAPK in 0.5 μl 10 mM JetSI^{TM} (Polyplus-transfection, Inc) was microinjected bilaterally into PVN. Scrambled siRNA or vehicle (artificial cerebrospinal fluid) were used as controls. These siRNAs are specifically designed for *in vivo* use in animal models. JetSI^{TM} 10 mM, a cationic amphiphile molecule that has been used successfully for delivery of siRNA into the brain by intracerebroventricular or hypothalamic injection[4, 5] was used as the transfection reagent in this study. Five days later, rats were euthanized with an overdose of urethane to collect the brain for molecular study.

Western blot revealed that the smallest dose of p44/42 siRNA (5.6 ng) had no significant effect on p44/42 MAPK expression in the PVN, compared with scrambled siRNA or vehicle. A modest dose of p44/42 siRNA (28 ng) resulted in a significant reduction in both total and phosphorylated p44/42 MAPK (**Figure S1**). A higher dose of p44/42 siRNA (56 ng) had no additional effect. The p44/42 siRNA had no effects on either total p38 MAPK or c-Jun N-terminal kinase (JNK) (**Figure S1**), the two other major MAPK signaling pathways, indicating the specificity of p44/42 siRNA. We used the lower of the two effective doses of p44/42 siRNA (28 ng) to evaluate the role of p44/42 MAPK in the PVN in ANG II-induced hypertension.

PVN microinjections

PVN microinjection was performed as previously described.[1, 6] Briefly, a 29-gauge guide cannula was inserted 0.5 mm above the PVN region. A 35-gauge (128 μm OD; 51.2 μm ID) stainless steel injection cannula was attached to PE-10 tubing, which was then connected to a 0.5-μl Hamilton microsyringe. The tip of the injection cannula was inserted into the guide cannula and then adjusted to a length extending 0.5 mm beyond the tip of the guide cannula. Bilateral microinjections were made in a volume of 0.5 μl over 30 sec. The PVN injection cannulae were positioned 1.8 mm posterior to bregma, 0.4 mm from midline, 7.6 mm ventral to dura.

To verify the accuracy of the PVN microinjection, 0.5 μl 2% pontamine sky blue was microinjected bilaterally into the same stereotaxic location (n=6). One hour after the dye microinjection, the animal was perfused. The brain was removed and postfixed in 4% paraformaldehyde for 24 hours. Coronal brain sections (40 μm) were made with a microtone and the needle track was microscopically examined. In all cases, the microinjection sites were located within the PVN.

To control for the possibility that the siRNA microinjections might independently elicit an inflammatory response, untreated rats underwent bilateral PVN microinjections of p44/42 siRNA, scrambled siRNA or vehicle (n=4 for each group). One week later, rats were euthanized with an overdose of urethane to collect the brains for real-time PCR to assess pro-inflammatory cytokine expression.

Dissection of brain tissue for molecular study

The PVN tissue was obtained as previously described. [1, 7] Briefly, brain tissues were stored at -80°C. The brain was cut into 500-μm coronal sections. The PVN region was punched with a 15-gauge needle stub (ID: 1.5 mm). This method necessarily includes a small amount of surrounding hypothalamic tissue.

Western blot analysis

Protein was extracted using cell lysis buffer (Cell Signaling Technology Inc, Beverly, MA). Protein level for p-p44/42 and total p44/42 were measured by Western blot analysis, as previously described,[1] using primary antibodies to p-p44/42 (Thr202/Tyr204; no. 4377; 1:250), total p44/42, p38 and JNK (no. 4695, 9212 and 9252, respectively; 1:1,000, Cell Signaling Technology, Danvers, MA) and angiotensin II (ANG II) type-1 receptors (AT_1R; SC-1173, 1: 500, Santa Cruz, CA). The density of the bands was quantified using Image Lab analysis software (Bio-Rad, Hercules, CA).

Quantification of mRNA expression

The total RNA was extracted using TRI Reagent (Molecular Research Center, Inc). mRNA levels for renin-angiotensin system components (AT_1R and ANG II type-2 receptor) and inflammatory mediators [interleukin-1β, tumor necrosis factor-α, cyclooxygenase (COX)-1 and COX-2] were analyzed with TaqMan or SYRB Green real-time PCR following reverse transcription of total RNA. The sequences for the primers and probes used are summarized in **Table S1**. Primers and probes for TaqMan GAPDH were purchased from Applied Biosystems (Foster City, CA). Real-time PCR was performed using the ABI prism 7000 Sequence Detection System (Applied Biosystems, Carlsbad, CA). The values were normalized to GAPDH and the final concentration of mRNA was calculated using the formula $x=2^{-\Delta\Delta Ct}$,[8] where x=fold difference relative to control.

Statistical Analysis

All data are expressed as mean±SEM. The significance of differences in mean values was analyzed by one-way or two-way repeated-measure ANOVA followed by Fisher's *post hoc* test. $P<0.05$ was considered statistically significant.

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Table S1. Sequences for primers and probes

Gene		**Primers and Probes**
AT_1R (SYBR)	Forward primer:	5’- GGATGGTTCTCAGAGAGAGTACAT-3’
	Reverse primer:	5'-CCTGCCCTCTTGTACCTGTTG-3'
IL-6 (SYBR)	Forward primer:	5’- TCCTACCCCAACTTCCAATGCTC-3’
	Reverse primer:	5'-TTGGATGGTCTTGGTCCTTAGCC-3'
IL-4 (SYBR)	Forward primer:	5’- ATGGGTCTCAGCCCCCACCTTG-3’
	Reverse primer:	5'-ATCCGTGGATACCGTTCCCGGT-3'
GAPDH (SYBR)	Forward primer:	5’- AAGGTCATCCCAGAGCTGAA-3’
	Reverse primer:	5'-ATGTAGGCCATGAGGTCCAC-3'
AT_2R (TaqMan)	Forward primer:	5’-CAATCTGGCTGTGGCTGACTT-3’
	Reverse primer:	5’-TGCACATCACAGGTCCAAAGA-3’
	Probe:	5’-CAACCCTTCCTCTCTGGGCAACCTATTACTCTTATA-3’
IL-1β (TaqMan)	Forward primer:	5’-CACCTCTCAAGCAGAGCACAG-3’
	Reverse primer:	5’-GGGTTCCATGGTGAAGTCAAC-3’
	Probe:	5’-TGTCCCGACCATTGCTGTTTCCTAGG-3’
TNF-α (TaqMan)	Forward primer:	5’-CCAGGAGAAAGTCAGCCTCCT-3’
	Reverse primer:	5’-TCATACCAGGGCTTGAGCTCA-3’
	Probe:	5’-AGAGCCCTTGCCCTAAGGACACCCCT-3’
COX-1 (TaqMan)	Forward primer:	5’-GAGTCTCTCGCTCCAGTTTCC-3’
	Reverse primer:	5’-AGGGAATGACTGGTGAGGGTA-3’
	Probe:	5’-TGCTGCTGCTCCTGCTGCTGCT-3’
COX-2 (TaqMan)	Forward primer:	5’-GGCACAAATATGATGTTCGCA-3’
	Reverse primer:	5’-CCTCGCTTCTGATCTGTCTTGA-3’
	Probe:	5’-TCTTTGCCCAGCACTTCACTCATCAGTTT-3’

AT_1R: angiotensin II type-1 receptors; IL-6: interleukin-6; IL-4: interleukin-4; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; AT_2R: angiotensin II type-1 receptors; IL-1β: interleukin-1β; TNF-α: tumor necrosis factor-α; COX-1: cyclooxygenase-1; COX-2: cyclooxygenase-2

Table S2. Effects of p44/42 MAPK activity in PVN on ANG II-induced cardiac remodeling

Anatomical Variables	***Control***	***ANG II + vehicle***	***ANG II + p44/42 siRNA***	***ANG II + scrambled siRNA***
Two weeks after early siRNA treatment	*(n=8)*	*(n=10)*	*(n=10)*	*(n=10)*
BW (g)	383 ± 3	379 ± 4	378 ± 4	382 ± 5
HW (mg)	997 ± 23	1093 ± 23*	1029 ± 24	1085 ± 20*
HW/BW (mg/g)	2.60 ± 0.06	2.88 ± 0.12*	2.72 ± 0.06†	2.84 ± 0.06*
Two weeks after late siRNA treatment	*(n=10)*	*(n=9)*	*(n=9)*	*(n=9)*
BW (g)	396 ± 4	385 ± 6	390 ± 7	387 ± 8
HW (mg)	996 ± 21	1124 ± 30*	1119 ± 35*	1127 ± 33*
HW/BW (mg/g)	2.52 ± 0.04	2.92 ± 0.07*	2.87 ± 0.06*	2.91 ± 0.07*

BW: body weight; HW: heart weight. Values are expressed as mean ± SEM. *P< 0.05, vs. Control; †P< 0.05, ANG II + p44/42 siRNAs vs. ANG II + vehicle or ANG II + scrambled siRNA

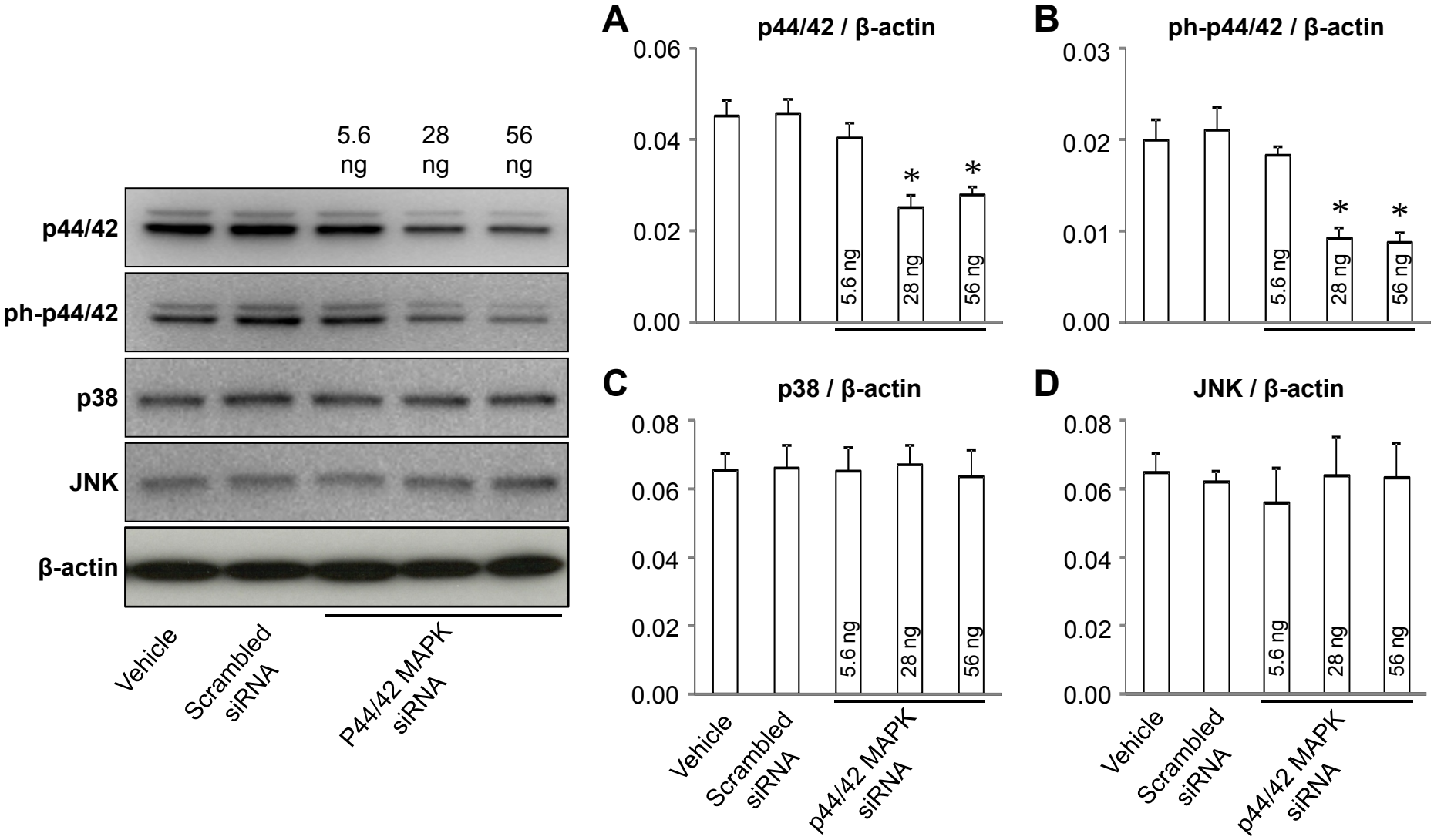

Figure S1. Dose-response relationship and specificity for p44/42 MAPK siRNAs. Pooled siRNAs targeting p44/42 at doses of 5.6 ng, 28 ng or 56 ng in 0.5 μl delivery reagent were microinjected bilaterally into PVN of normal rats. Non-targeting scrambled siRNA or vehicle (aCSF) were used as controls. 5 days later, Western blot from PVN, including some immediately surrounding tissue, revealed significant reduction in both total and phosphorylated p44/42 MAPK with the 28 ng dose. Notably, p44/42 siRNA had no effects on total p38 or JNK, two other major components of MAPK family. Values are mean ± SEM (n = 3 for each group). *P< 0.05 vs. vehicle or scrambled siRNA.

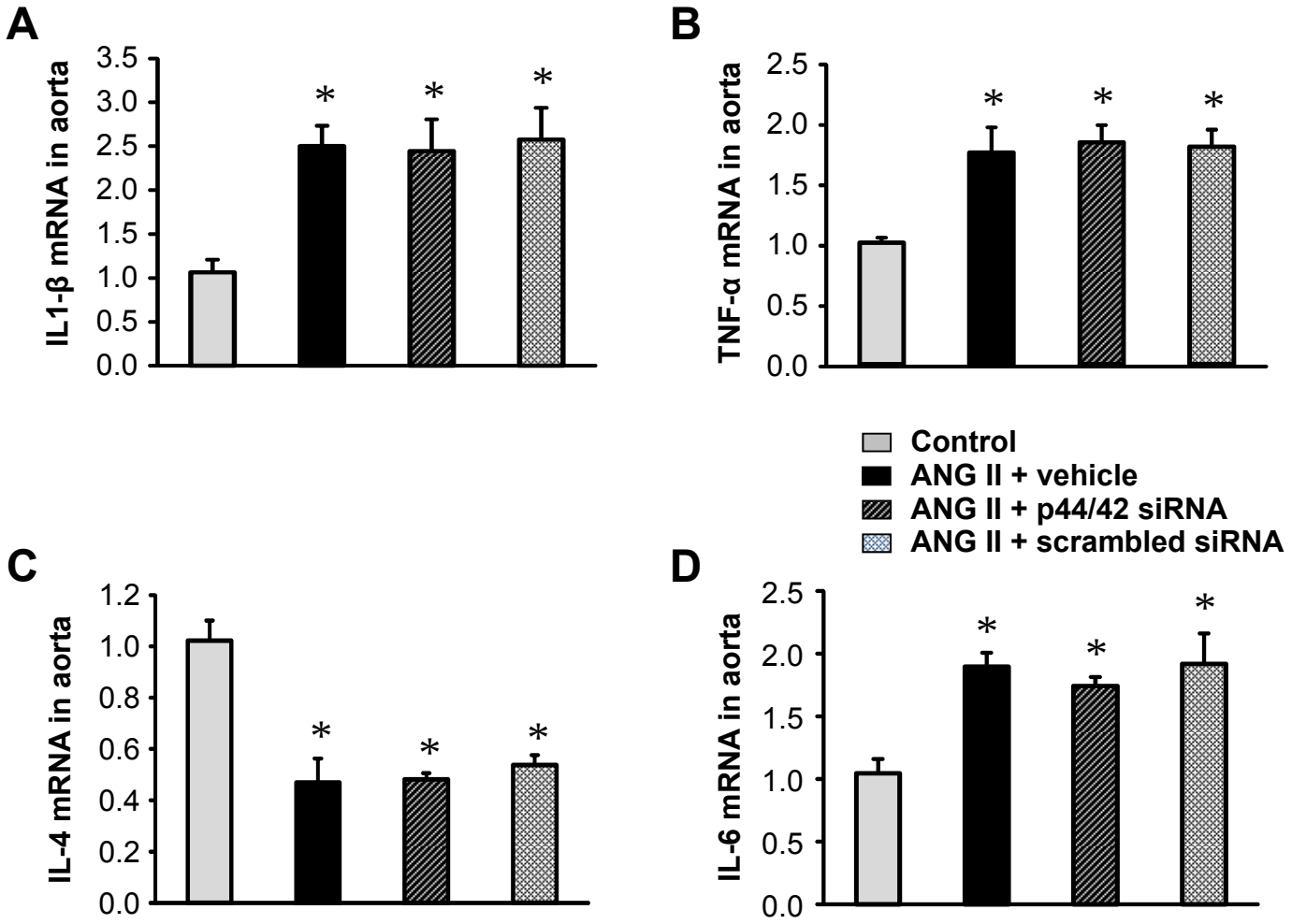

Figure S2. The effect of bilateral PVN microinjection of p44/42 MAPK siRNA on mRNA expression for IL-1β, TNF-α, IL-4 and IL-6 in thoracic aorta of ANG II-infused rats treated early with p44/42 MAPK siRNA, a scrambled siRNA, or vehicle. Data was obtained at 2 weeks after PVN microinjection. Untreated rats served as Control. Values are mean ± SEM (n = 4 for each group). *P< 0.05, vs. Control.

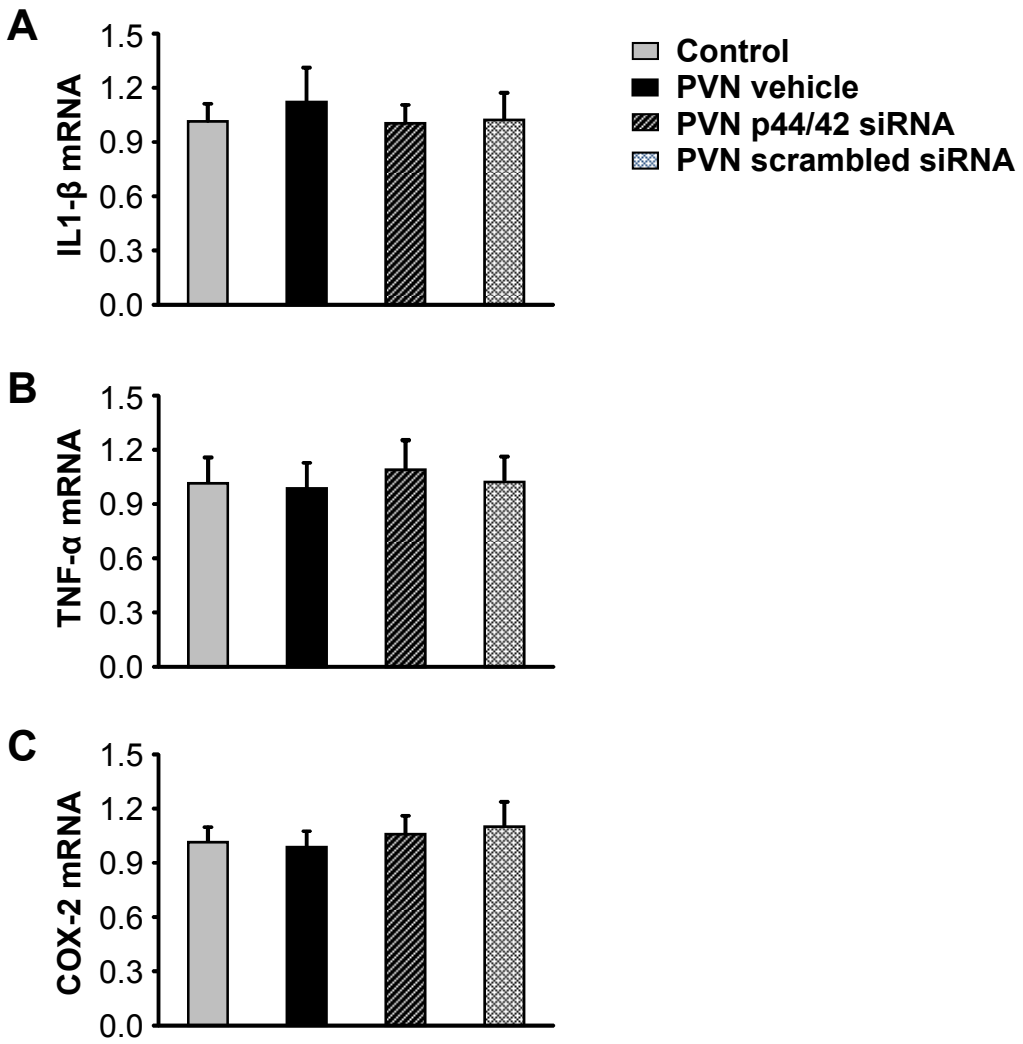

Figure S3. The effect of bilateral PVN microinjection of p44/42 MAPK siRNA, a scrambled siRNA, or vehicle alone on mRNA expression for IL-1β, TNF-α and COX-2 in the PVN in rats without ANG II infusion. Untreated rats served as Control. Values are mean ± SEM (n = 4-8 for each group).

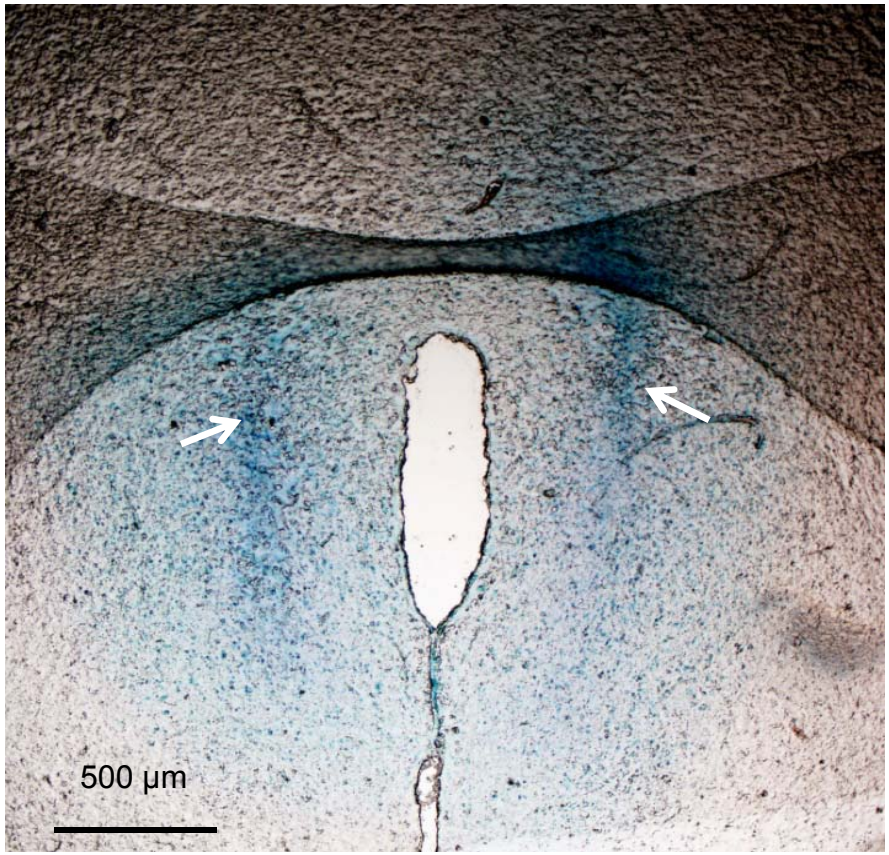

Figure S4. A representative photomicrograph of bilateral PVN microinjections of 2% pontamine sky blue dye in a rat. The arrows indicate needle tracts penetrating the PVN. Technical artifact is present.