Brain

Hypothalamic Paraventricular and Arcuate Nuclei Contribute to Elevated Sympathetic Nerve Activity in Pregnant Rats

Roles of Neuropeptide Y and α-Melanocyte–Stimulating Hormone

Zhigang Shi, Priscila A. Cassaglia, Laura C. Gotthardt, Virginia L. Brooks

Abstract—Pregnancy increases sympathetic nerve activity (SNA), but the mechanisms are unknown. Here, we investigated the contributions of the hypothalamic paraventricular and arcuate nuclei in α-chloralose–anesthetized pregnant and nonpregnant rats. Baseline arterial pressure (AP) was lower, and heart rate (HR), lumbar sympathetic activity, and splanchnic SNA were higher in pregnant rats compared with nonpregnant rats. Inhibition of the paraventricular nucleus via bilateral muscimol nanoinjections decreased AP and HR more in pregnant rats than in nonpregnant rats and decreased lumbar SNA only in pregnant rats. Similarly, after arcuate muscimol nanoinjections, the decreases in AP, HR, and lumbar, renal, and splanchnic sympathetic nerve activities were greater in pregnant rats than in nonpregnant rats. Major arcuate neuronal groups that project to the paraventricular nucleus express inhibitory neuropeptide Y (NPY) and excitatory α-melanocyte–stimulating hormone. Inhibition of paraventricular melanocortin 3/4 receptors with SHU9119 also decreased AP, HR, and lumbar SNA in pregnant rats but not in nonpregnant rats. Conversely, paraventricular nucleus NPY expression was reduced in pregnant animals, and although blockade of paraventricular NPY Y1 receptors increased AP, HR, and lumbar sympathetic activity in nonpregnant rats, it had no effects in pregnant rats. Yet, the sympathoinhibitory, depressor, and bradycardic effects of paraventricular NPY nanoinjections were similar between groups. In conclusion, the paraventricular and arcuate nuclei contribute to increased basal SNA during pregnancy, likely due in part to decreased tonic NPY inhibition and increased tonic α-melanocyte–stimulating hormone excitation of presympathetic neurons in the paraventricular nucleus.

Key Words: arcuate nucleus ■ neuropeptide Y ■ paraventricular nucleus ■ pregnancy ■ proopiomelanocortin

Normal pregnancy profoundly alters fluid balance and arterial pressure (AP) regulation. Blood volume and cardiac output increase, but AP falls because of decreases in systemic vascular resistance. To partially counteract primary vasodilation and hypotension, pregnancy increases sympathetic nerve activity (SNA) to several organs, including the heart, adrenal, kidney, and skeletal muscle. In contrast, in women with preeclampsia, blood volume expansion is blunted and hypertension is produced, with further increases in SNA. Preeclampsia is a complication of considerable medical importance; it occurs in 6% to 10% of pregnant women and ranks among the top causes of maternal death. Therefore, it is imperative to understand the mechanisms that contribute, including the elevations in SNA. However, before we can understand preeclampsia, we first need to identify the mechanisms by which normal pregnancy increases SNA because preeclampsia may exaggerate these same mechanisms. Remarkably, little information is currently available.

The rostral ventrolateral medulla is a major source of basal sympathetic tone in normal individuals; yet inhibition of rostral ventrolateral medulla sympathetic neurons by gamma-aminobutyric acid (GABA) is increased during pregnancy, at variance with a major role. In contrast, although the paraventricular nucleus (PVN) of the hypothalamus normally contributes little to basal sympathetic tone, indirect evidence suggests that the PVN may underlie basal sympathoexcitation in pregnancy. More specifically, sympathoinhibitory influences in the PVN, including the considerable tonic GABAergic restraint and the expression and activity of neuronal nitric oxide synthase decreases. However, whether this decreased inhibition translates into higher tonic support of basal SNA is unknown. Therefore, the first purpose of the present experiments was to test the hypothesis that the PVN is a source of elevated basal SNA drive, by determining whether PVN inhibition, via bilateral nanoinjection of muscimol, decreases lumbar SNA (LSNA) and AP more in pregnant rats than in nonpregnant anesthetized rats.
Another tonic inhibitor of PVN presympathetic neurons is neuropeptide Y (NPY). PVN NPY expression is reduced during pregnancy, at least in mice, however, whether tonic NPY inhibition of SNA decreases during pregnancy, like GABA, is unknown. Therefore, we used immunohistochemistry to quantify PVN NPY expression in pregnant and nonpregnant rats and tested whether blockade of PVN type 1 NPY receptors (NPY1x) increases LNSA less in pregnant rats than in nonpregnant rats. Because inhibitory NPY inputs converge with excitatory α-melanocyte–stimulating hormone (α-MSH) inputs onto PVN presympathetic neurons, we next tested whether blockade of melanocortin type 3/4 receptors (MC3/4R) decreases SNA in pregnant rats. Finally, because a major source of both NPY and α-MSH inputs into the PVN is the arcuate nucleus (ArcN), we tested whether the ArcN also contributes to increased basal SNA, by determining the effects of ArcN muscimol in pregnant and nonpregnant rats.

Methods
An expanded Methods section is available in the online-only Data Supplement.

Animals
Experiments were performed using female virgin or pregnant Sprague-Dawley rats. For pregnant animals, the presence of sperm was designated pregnancy day 0, and experiments were performed on pregnancy day 20. All procedures were conducted in accordance with the National Institutes of Health’s Guide for the Health and Use of Laboratory Animals and were approved by the Institutional (Oregon Health & Science University) Animal Care and Use Committee.

Surgery
Anesthesia was induced and maintained with 2% to 5% isoflurane in 100% oxygen. The rat was then surgically prepared with a tracheal tube, femoral arterial and venous catheters, and stainless steel electrodes around the lumbar, splanchic, or renal sympathetic nerves and for PVN or ArcN nanoinjections, as previously described. After surgery, isoflurane anesthesia was slowly transitioned to a continuous intravenous infusion of α-chloralose. Virgin rats received a loading dose of 50 mg kg⁻¹ for 30 minutes followed by a maintenance dose of 25 mg kg⁻¹ h⁻¹; pregnant rats received a dose equivalent to the weight of a virgin rat at a similar age. After the α-chloralose loading dose, rats were allowed to stabilize for ≥60 minutes before experimentation.

PVN and ArcN Nanoinjections
As previously described, all nanoinjections (60 nL for PVN and 30 nL for ArcN) were made bilaterally, with ≥2 minutes between injections, and each injection was conducted over ≈5 to 10 s using a pressure injection system. The following drugs and chemicals were used: muscimol (1 mM/L), SHU9119 (blocks MC3/4R; 0.5 mM/L in artificial cerebrospinal fluid [aCSF] with 10% dimethyl sulfoxide), BIBO 3304 (blocks NPY Y1R; 1 mM/L), and NPY (0.1 mM/L). In some animals, before nanoinjection of drugs, aCSF was injected into the ArcN or PVN as a vehicle control.

Immunocytochemistry
Paired pregnant and nonpregnant rats were deeply anesthetized and perfused with 4% paraformaldehyde. The brains were removed, sectioned, and processed for immunohistochemical detection of NPY as previously described. The sections were imaged in pairs, and staining was quantified using ImageJ.

Data Analysis
All data are presented as mean±SEM. Maximal responses (30 s) to PVN and ArcN nanoinjections were compared using 2- and 3-way repeated measures ANOVA. PVN NPY fiber densities were compared between pregnant and nonpregnant rats using a Student t test. All data are presented as mean±SEM. P values of <0.05 were considered statistically significant.

Results
Baseline Values
Pregnancy decreased AP and increased heart rate (HR) and LNSA (Figure S1 in the online-only Data Supplement). In the limited numbers of rats tested, we also observed increased splanchic SNA (SSNA); however, renal SNA (RSNA) was not significantly different between pregnant rats and nonpregnant rats (Figure S1).

Blockade of the PVN
As shown in Figure 1, bilateral PVN nanoinjections of muscimol significantly decreased (P<0.05) mean arterial pressure (MAP), HR, and LNSA in pregnant rats but decreased (P<0.05) only MAP and HR in nonpregnant rats; these variables began to fall immediately and reached a nadir in 7±1 minute after initiating the nanoinjections in both groups. aCSF had no effects in either group. The decreases in MAP, HR, and LNSA were greater in pregnant rats than in nonpregnant rats and also greater compared with the effects of aCSF. Collectively, these data indicate that the PVN supports elevated LSNA during pregnancy in rats.

Paraventricular Nucleus NPY
As depicted in representative PVN sections in Figure 2, NPY immunoreactivity was lower in pregnant rats compared with nonpregnant rats, as in mice. In 4 experiments, the intensity of staining in pregnant rats was 61±8% of nonpregnant rats (P<0.05). To test whether the reduced NPY expression is functionally significant, we determined the effects of acute NPY1x in nonpregnant and pregnant animals. NPY1x increased (P<0.05; maximum reached at 9.3±0.5 minutes) MAP, HR, and LNSA more in nonpregnant rats than in pregnant rats (P<0.05; Figure 3). Indeed, this treatment was ineffective in pregnant rats. Nevertheless, the sympathoinhibitory, bradycardic, and depressor responses (all P<0.05) to NPY injections were similar between groups (Figure 4).

Paraventricular Nucleus MC3/4R
We have shown that PVN NPY inputs directly inhibit presympathetic neurons also activated by α-MSH. Therefore, because pregnancy reduced tonic PVN NPY inhibition of SNA, we next determined whether elevated basal SNA is mediated in part by increased MC3/4R drive of PVN neurons. As shown previously, PVN nanoinjection of SHU9119 had no effects in nonpregnant rats. In contrast, PVN SHU9119 significantly decreased (P<0.05) MAP, HR, and LNSA in pregnant rats, reaching a nadir in 5±1 minute. Thus, the decreases in MAP, HR, and LNSA were greater in pregnant rats than in nonpregnant rats. These data support the hypothesis that elevated LSNA is supported at least in part by PVN MC3/4R (Figure 5).
Blockade of the ArcN

PVN NPY inputs originate largely from the ArcN and brain stem, and α-MSH inputs primarily arise from the ArcN, suggesting that the ArcN contributes to SNA support during pregnancy. Therefore, we quantified the effects of acute ArcN blockade. A key feature of this protocol is that we studied not only LSNA but also RSNA and SSNA, given the diverse effects of the ArcN on reproduction, energy balance, and AP regulation, as well as the limited information on ArcN control of SNA. Although ArcN aCSF had no significant effects, ArcN muscimol injections decreased (P<0.05) MAP in both nonpregnant and pregnant rats, with the maximum response occurring after 6.1±0.4 minutes in pregnant rats and 6.5±0.4 minutes in nonpregnant rats. However, the depressor response was significantly greater in pregnant rats. ArcN muscimol also decreased (P<0.05) HR in both groups, but the nadir was delayed compared with the decreases in MAP (pregnant: 8.5±0.4 minutes; nonpregnant: 6.9±0.6 minutes), and the responses were not significantly different between groups. On the other hand, ArcN muscimol decreased (P<0.05) LSNA, SSNA, and RSNA in pregnant rats more than either ArcN muscimol injections in nonpregnant rats or aCSF injections in either group, and the falls occurred simultaneously with the decreases in MAP. Collectively, these data suggest that the ArcN plays a critical role in the modulation of SNA during pregnancy.

Figure 1. Acute inhibition of the paraventricular nucleus with bilateral nanoinjections of muscimol (Musc) decreases mean arterial pressure (MAP), heart rate (HR), and lumbar sympathetic nerve activity (LSNA) more in late pregnant (P) rats (n=7) than in nonpregnant (NP) rats (n=7) and compared with artificial cerebrospinal fluid (aCSF) nanoinjections in P rats (n=5) and NP rats (n=7). Left and middle, Representative experiments. Right, Group data. Double arrows indicate the time of injections. Triangles above the integrated LSNA tracings indicate times at which sections of raw LSNA, shown below, were obtained. *P<0.05 compared with all other groups.

Figure 2. Representative images showing that paraventricular nucleus neuropeptide Y immunoreactivity is less in pregnant (P) rats than in nonpregnant (NP) rats. Scale bar, 100 μm.
indicate that the ArcN supports SNA and MAP during pregnancy (Figure 6).

**Histological Verification of Injection Sites**
Figures S2 and S3 summarize the location of PVN and ArcN injection sites.

**Discussion**
The purpose of this study was to test whether the PVN contributes to increased basal SNA in pregnant rats and to begin to investigate PVN inputs that drive this elevated sympathetic tone. We confirm that pregnancy decreases MAP and profoundly elevates basal SNA and HR. Our major new findings are (1) acute PVN inhibition decreases MAP, HR, and LSNA more in pregnant rats than in nonpregnant rats; (2) PVN NPY expression is reduced in pregnant rats; blockade of PVN NPY1R increases MAP, HR, and LSNA in nonpregnant rats but not in pregnant rats, yet responses to PVN NPY are preserved during pregnancy; (3) blockade of PVN MC3/4R decreases MAP, HR, and LSNA more in pregnant rats than

**Figure 3.** Bilateral nanoinjections of the neuropeptide Y (NPY) Y1R antagonist BIBO3304 (NPY1x) into the paraventricular nucleus increase mean arterial pressure (MAP), heart rate (HR), and lumbar sympathetic nerve activity (LSNA) in nonpregnant (NP) rats (n=8) but not in late pregnant (P) rats (n=5). **Left and middle,** Representative experiments. **Right,** Group data. Double arrows indicate the time of injections. Triangles above the integrated LSNA tracings indicate times at which sections of raw LSNA, shown below, were obtained. *P<0.05, P rats compared with NP rats.

**Figure 4.** Bilateral paraventricular nucleus nanoinjections of neuropeptide Y (NPY) decreases mean arterial pressure (MAP), heart rate (HR), and lumbar sympathetic nerve activity (LSNA) similarly in nonpregnant (NP) rats (n=5) and late pregnant (P) rats (n=4). **Left and middle,** Representative experiments. **Right,** Group data. Double arrows indicate the time of injections. Triangles above the integrated LSNA tracings indicate times at which sections of raw LSNA, shown below, were obtained.
in nonpregnant rats; and (4) acute ArcN inhibition decreases MAP, LSNA, RSNA, and SSNA more in pregnant rats than in nonpregnant rats. Collectively, these data indicate that both the PVN and the ArcN contribute to pregnancy-induced increases in basal SNA, likely in part due to decreased tonic PVN NPY inhibition and also to increased tonic PVN α-MSH stimulation.

Previous studies have documented increased SNA to multiple organs during pregnancy, and we show for the first time that SSNA is also markedly elevated. Increased RSNA has been detected in 1 study but not in others; we also did not observe such an elevation. This inconsistency may be explained by methodological differences and also the difficulty in detecting small between-group differences in SNA because of variations in experimental preparation. In support, we found that acute ArcN inhibition produced a small fall in RSNA, as it did in LSNA and SSNA, suggesting that at least ArcN drive of RSNA is increased in pregnant rats.

A key finding was that acute PVN inhibition profoundly decreases MAP, HR, and LSNA more in pregnant rats than in nonpregnant rats. A major contributor to this increased SNA may be reduced tonic inhibition of PVN presympathetic neurons. In addition to the previously reported decreases in GABAergic and neuronal nitric oxide synthase–mediated SNA suppression, our data suggest that tonic PVN NPY inhibition of SNA is also lessened. First, similar to the mouse, we found that PVN NPY expression is decreased in pregnant rats. Second, PVN NPY–induced sympathoinhibition was preserved, but the normal sympathoexcitatory response to PVN NPY Y1R blockade was abolished in pregnant rats. The failure of NPY1x to increase SNA in pregnant rats cannot be because the elevated basal SNA has reached a maximal level because baroreceptor unloading and PVN nano-injection of the GABA	extsubscript{A} antagonist, bicuculline, can both increase SNA further in pregnant rats. Nevertheless, our immunohistochemistry data seem to conflict with studies showing that the expression of NPY (and the coexpressed agouti-related peptide) in the ArcN, one major source of PVN NPY inputs, is increased or unchanged during late pregnancy and that PVN NPY Y1R may be elevated. In late pregnant women, CSF concentrations of agouti-related peptide also rise. However, ArcN and PVN NPY regulate multiple modalities, including food intake, which is increased during pregnancy in association with the elevations in the orexigenic peptides, NPY, and agouti-related peptide. Thus, the lack of correlation between whole ArcN NPY expression and the ability of PVN NPY to inhibit SNA during pregnancy may be explained by a differential regulation of ArcN neurons that influence SNA versus those that modulate, for example, energy balance. In support, it has been reported that increased ArcN NPY/agouti-related peptide and proopiomelanocortin expression during pregnancy vary within the nucleus. Moreover, the altered ArcN regulation of SNA versus energy balance is divergent in another state, obesity. Alternatively, because the PVN also receives a significant NPY projection from the brain stem, it may be that a decrease in this component largely accounts for the decreases in PVN NPY expression and tonic sympathetic inhibition. Future experiments are required to delineate the source of NPY inputs that are diminished during pregnancy. Regardless, collectively, the data support the hypothesis that reduced tonic NPY inhibition of PVN presympathetic neurons supports elevated SNA during pregnancy.

This study also identified 1 source of excitatory support of PVN presympathetic neurons during pregnancy, α-MSH. The ArcN is the primary if not the sole source of PVN α-MSH inputs. However, many studies have reported unchanged or decreased ArcN proopiomelanocortin expression during late pregnancy. Therefore, in parallel to the results with NPY, it may be that ArcN proopiomelanocortin expression is not

\[ \text{Figure 5. Bilateral nano-injections of the melanocortin type 3/4 receptor antagonist SHU9119 (SHU) into the paraventricular nucleus decrease mean arterial pressure (MAP), heart rate (HR), and lumbar sympathetic nerve activity (LSNA) in pregnant (P) rats (n=5) but not in nonpregnant (NP) rats (n=7). Left and middle, Representative experiments. Right, Group data. Double arrows indicate the time of injections. Triangles above the integrated LSNA tracings indicate times at which sections of raw LSNA, shown below, were obtained. *P<0.05, P rats compared with NP rats.} \]
relevant to its sympathoexcitatory actions in PVN. Alternatively, because PVN SHU9119 blocks the excitatory effects of PVN NPY1x and withdrawal of tonic NPY inhibition is required to unmask α-MSH excitation,37 the decrease in tonic NPY inhibition may be sufficient to explain the increased α-MSH drive of PVN presympathetic neurons during pregnancy.

Because pregnancy decreased tonic NPY inhibition and increased α-MSH excitation in PVN and the ArcN is a major source of NPY and α-MSH inputs into PVN, we next tested whether the ArcN, like the PVN, contributes to increased basal SNA. Indeed, inhibition of the ArcN decreased MAP, LSNA, SSNA, and RSNA more in P than in NP rats and more than aCSF injections. On the other hand, while ArcN muscimol decreased HR in both groups, these responses were not different. Double arrows indicate time of injections. Triangles above the integrated LSNA tracings indicate times at which sections of raw LSNA, shown below, were obtained. *P<0.05 compared to all other groups.

Figure 6. Effects of acute inhibition of the arcuate nucleus (ArcN) with bilateral nanoinjections of muscimol (musc) on mean arterial pressure (MAP: n=13, NP: n=13, P), heart rate (HR: n=13, NP: n=13, P), lumbar sympathetic nerve activity (LSNA: n=4, NP: n=5, P), splanchnic SNA (SSNA: n=5, NP: n=4, P), and renal SNA (RSNA: n=4, NP: n=4, P) in late pregnant (P) and nonpregnant (NP) rats. The effects of artificial cerebrospinal fluid (aCSF) nanoinjections in NP (LSNA: n=5; SSNA: n=4; RSNA: n=4) and P (LSNA: n=4; SSNA: n=4; RSNA: n=4) rats are also shown. Left and middle, Representative experiments. Grouped data (right) show that ArcN muscimol decreased MAP, LSNA, SSNA, and RSNA more in P than in NP rats and more than aCSF injections. On the other hand, while ArcN muscimol decreased HR in both groups, these responses were not different. Double arrows indicate time of injections. Triangles above the integrated LSNA tracings indicate times at which sections of raw LSNA, shown below, were obtained. *P<0.05 compared to all other groups.

The ArcN, likely mediated in part by α-MSH, predominate. To our knowledge, this is the first demonstration that the ArcN is capable of driving increased basal SNA in any physiological or pathophysiological state. We did not investigate the factors that mediate the changes in ArcN activity during pregnancy, but several candidates emerge. First, insulin and leptin are increased in pregnant women and rats, and both hormones are sympathoexcitatory at least in part by binding to receptors in the ArcN and triggering a pathway that includes the PVN.20,21,38,39 Second, angiotensin II, via actions at AT1 receptors, supports AP and RSNA during pregnancy,40–42 and stimulation of ArcN AT1 receptors increases SNA43. Future experiments are required to test these possibilities. Nevertheless, it is noteworthy that although basal raw LSNA and
SSNA were more than double in pregnant rats than in nonpregnant rats, complete PVN inhibition or blockade of PVN MC3/4R reduced LSN by about only 20%, and ArcN muscimol only decreased LSNA, SSNA, and RSNA by slightly >10%. Thus, it would seem that other brain regions are also involved in setting elevated basal SSA tone during pregnancy.

One limitation of this work is that experiments were performed in rats under anesthesia, which can influence autonomic control of the cardiovascular system. However, MAP and HR levels measured in the pregnant and virgin rats used in this study were essentially identical to values obtained using telemetry in conscious rats. In addition, the effect of pregnancy to impair baroreflex control of HR is similar in conscious animals, and this is the pregnancy-induced elevation in basal SSA. Finally, recent studies reveal that the influence of neurotransmitters and neuromodulators, such as GABA and nitric oxide, in the PVN on MAP and HR, is similar in conscious and anesthetized rats. Thus, the effect of anesthesia may be minimal.

Perspectives

Although pregnancy clearly increases basal sympathetic tone, preeclampsia increases it even more. However, the mechanisms are unknown. One hypothesis is that the factors that mediate increased SSA during normal pregnancy contribute to this further elevation. Indeed, preeclampsia is associated with even greater increases in insulin and leptin, and the balance of pressor versus depressor components of the renin–angiotensin system is shifted toward the hypertension arm. If true, then before we can identify the cause of sympathetic hyperactivity with sympathetic pregnancy, the mechanisms underlying increased SSA in normal pregnancy must first be established. The present studies, identifying key roles for the PVN and ArcN, is one step toward this goal.

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Disclosures

None.

References

Novelty and Significance

What Is New?

• Inhibition of the paraventricular nucleus (PVN) with muscimol decreases lumbar sympathetic nerve activity (LSNA), mean arterial pressure (MAP), and heart rate more in pregnant rats than in nonpregnant rats.

• Blockade of PVN melanocortin type 3/4 receptors decreases LSNA, heart rate, and MAP in pregnant rats but not in nonpregnant rats.

• PVN neuropeptide Y (NPY) expression is reduced in pregnant rats, blockade of PVN NPY Y1 receptors fails to increase LSNA, heart rate, and MAP in pregnant rats, unlike nonpregnant animals, yet the decreases in LSNA, heat rate, and MAP after PVN NPY injections are preserved during pregnancy.

• Inhibition of the arcuate nucleus with muscimol decreases LSNA, splanchnic SNA, renal SNA, and MAP more in pregnant rats than in nonpregnant rats.

What Is Relevant?

• Our partial identification of the neurocircuitry through which normal pregnancy increases SNA may contribute toward an understanding of the mechanisms by which preeclampsia increases SNA even more, because preeclampsia may exaggerate the same processes that elevate SNA during normal pregnancy.

• We show for the first time in any physiological or pathophysiological study that a decrease in tonic PVN NPY inhibition of SNA contributes to basal sympathoexcitation, which may lead to novel pharmacological approaches to treat excess SNA in other conditions, such as hypertension.

• These are the first data implicating the arcuate nucleus in increased basal SNA in any physiological or pathophysiological state, which may open the door to a new therapeutic arena.

Summary

The PVN and the arcuate nucleus contribute to increased basal SNA during normal pregnancy, at least in part due to decreased tonic PVN NPY inhibition and also increased stimulation of PVN melanocortin type 3/4 receptors.
Hypothalamic Paraventricular and Arcuate Nuclei Contribute to Elevated Sympathetic Nerve Activity in Pregnant Rats: Roles of Neuropeptide Y and α-Melanocyte–Stimulating Hormone

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The hypothalamic paraventricular and arcuate nuclei contribute to elevated sympathetic nerve activity in pregnant rats: roles of Neuropeptide Y and α-melanocyte stimulating hormone.

Supplement

By

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Running title: Pregnancy: role of PVN and ArcN in increased SNA

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METHODS

Animals. Experiments were performed using female virgin (260–320 g; n=44) or pregnant (400–460 g; 12-17 pups; n=38) Sprague-Dawley rats (Charles River Laboratories, Inc., Raleigh, NC, USA). After arrival, all rats were housed in a room with a 12:12-h light-dark cycle and had free access to food (LabDiet 5001, Richmond, IN) and water; at least 5 days were allowed before any experimentation. For pregnant animals, the presence of sperm was designated pregnancy day 0, and experiments were performed on pregnancy day 20. All procedures were conducted in accordance with the National Institutes of Health’s Guide for the Health and Use of Laboratory Animals and were approved by the Institutional (Oregon Health & Science University) Animal Care and Use Committee.

Surgery. Anesthesia was induced and maintained with 2–5% isoflurane in 100% oxygen. Body temperature was maintained at 37 ± 1°C using a rectal thermistor and heating pad. A tracheal tube, femoral arterial and venous catheters, and stainless steel electrodes around the lumbar, splanchnic or renal sympathetic nerve were implanted, as previously described.\(^1,2\) The rat was then placed in a stereotaxic instrument (David Kopf Instruments, Tujunga, CA, USA), and, following a midline incision, the skull was prepared for nano-injections into the paraventricular nucleus (PVN) or arcuate nucleus (ArcN) by burring a hole in the skull near the midline. After completion of surgery, isoflurane anaesthesia was slowly withdrawn over 30 min, and a continuous intravenous infusion of α-chloralose (Sigma-Aldrich, St. Louis, MO, USA) was initiated and continued for the duration of the experiment. Virgin rats received a 50 mg•kg\(^{-1}\) loading dose over 30 min followed by a 25 mg•kg\(^{-1}•h\(^{-1}\) maintenance dose; pregnant rats received a dose equivalent to the weight of a virgin rat at a similar age. Throughout the experiment, artificial ventilation with 100% oxygen was maintained, and respiratory rate and tidal volume were adjusted to maintain expired CO\(_2\) at 3.5–4.5%. Anesthetic depth was regularly confirmed by the lack of a pressor response to a foot or tail pinch; if necessary, additional α-chloralose was administered. After completion of surgery and the α-chloralose loading dose, rats were allowed to stabilize for ≥60 min before experimentation.

After experimentation, all rats were killed with an overdose of pentobarbital. The post-mortem nerve activity was recorded in all animals.

Data acquisition. Pulsatile and mean arterial pressure (MAP), heart rate (HR), and raw sympathetic nerve activity (SNA) were continuously recorded throughout the experiment with Grass amplifiers (Model 79D, Grass Instrument Co., Quincy, MA, USA) and a Biopac MP100 data acquisition and analysis system (Biopac Systems, Inc., Santa Barbara, CA, USA), sampling at 2000 Hz. SNA was band-pass filtered (100–3000 Hz) and amplified (×10,000). The SNA signal was then rectified, integrated in 1 s bins and corrected for post-mortem background activity. Except in Figure S1, in which raw baseline SNA values are depicted, SNA was normalized to the baseline (or control) SNA, which was defined as the average of the 30 sec period before the first nano-injections into the PVN or ArcN and expressed as percentage of control (% Control).
**PVN and ArcN nanoinjections.** Nanoinjections into the PVN and ArcN were conducted with single-barreled glass micropipettes, as described previously. Briefly, with a flat skull and using Bregma and the dorsal surface of the dura as zero, single-barreled glass micropipettes (20–40 μm tip OD) were positioned using the following coordinates: 1.7–1.9 mm caudal, 0.5 mm lateral and 7.3–7.5 mm ventral for PVN and 3.5–3.7 mm caudal, 0.3 mm lateral and 9.5–9.7 mm ventral for ArcN. All nanoinjections (60 nl for PVN and 30 nl for ArcN) were made bilaterally, with ~2 min between injections, and each injection was conducted over approximately 5–10 s using a pressure injection system (Pressure System IIE, Toohey Company, Fairfield, NJ, USA). In experiments involving ArcN nanoinjections, the ArcN was functionally identified prior to the beginning of the experiment by observing a least a 10 mmHg decrease in MAP following unilateral microinjection of 10 mM/L NMDA. The following drugs and chemicals were used: Muscimol (Tocris Bioscience, Bristol, UK; 1 mM/L), SHU9119 (Tocris Bioscience; 0.5 mM/L in aCSF with 10 % DMSO), the NPY Y1R antagonist BIBO 3304 (NPY1x, Tocris Bioscience; 1 mM/L), and NPY (0.1 mM/L, Tocris). Drugs for microinjection were dissolved in artificial cerebrospinal fluid (aCSF) containing (in mM): 128 NaCl, 2.6 KCl, 1.3 CaCl2, 0.9 MgCl2, 20 NaHCO3, 1.3 Na2HPO4 and 2 dextrose, pH 7.4. In some animals, prior to nanoinjection of drugs, aCSF was injected into the ArcN or PVN as vehicle control. At the conclusion of the experiment, 2.5% Alcian Blue was injected into the PVN or ArcN using the same pipette and coordinates as for microinjections. The brain was removed and placed in 4% paraformaldehyde for at least 48 h. The hypothalamus was subsequently cut into 25 μm sections using a cryostat and mounted on gelatin-coated glass microscope slides. Correct placement into the PVN or ArcN (without breaching the ventral brain surface) was confirmed using a standard anatomical atlas. Immu

**Immunocytochemistry.** Four pairs of pregnant (gestational day 20) and virgin rats were overdosed with sodium pentobarbital and perfused transcardially with heparinized saline and 4% paraformaldehyde [in 0.1 M phosphate buffer (PB)]. Brains were dissected and postfixed overnight in the same fixative at 4°C. The hypothalamus was coronally sectioned (40 μm) on a vibrating microtome and collected into 0.1M PB. As described previously, free-floating sections were pretreated with 1% NaBH4 (Sigma-Aldrich, St Louis, MO, USA) and 0.5% bovine serum albumin (BSA, Sigma-Aldrich). Subsequently, the sections were incubated with a polyclonal rabbit antibody to NPY (1:1,000; Immunostar, Hudson, WI) overnight at 4°C and visualized with donkey anti-rabbit conjugated to Alexa Fluor 488 (1:800; Invitrogen, Carlsbad, CA, USA). Control slices in which either the primary or secondary antibody was excluded exhibited no staining. Staining is completely eliminated by preabsorption with NPY. Each pregnant rat and its virgin control were processed simultaneously and under identical conditions. Immunohistochemical (IHC) staining was examined using a Zeiss Axiophot 2 Plus fluorescence microscope (Carl Zeiss Microscopy, LLC, Thornwood, NY) with a 10x objective. Sections at two distinct rostrocaudal levels of PVN corresponding to approximately −1.8 and −2.0 mm from Bregma were imaged with a Zeiss AxioCam MRm digital camera. The density of NPY-immunoreactive staining in PVN was determined by threshold discrimination using ImageJ. The images in each pair of NP and P rats were analyzed identically.
Data analysis. Baseline values were taken as the average of data obtained 30 sec before the first injection. Maximal responses to PVN and ArcN nanoinjections were identified and averaged over a 30-second period. The changes in MAP and HR depicted in Figures 1 and 3-6 were the difference between these two values. To determine within and between group differences following muscimol or aCSF injections into the PVN or ArcN, responses were compared using 3-way repeated measures ANOVA [factors were group (P and NP), treatment (muscimol or aCSF), and time (control and post-treatment)]. However, because this analysis revealed differences in the baseline levels of MAP and HR between NP and P rats, between group differences in the changes in MAP and HR were compared using 2-way ANOVA. Similarly, within and between group differences following PVN SHU9119, NPY1x, or NPY were first determined with 2-way repeated measure ANOVA (factors were group and time), followed by t-tests to test for between group differences in responses. PVN NPY fiber densities were compared between P and NP rats using t-tests. All data are presented as means ± SEM. P values < 0.05 were considered statistically significant.
REFERENCES


Figure S1. Effects of pregnancy on basal levels of mean arterial pressure (MAP), heart rate (HR), lumbar sympathetic nerve activity (LSNA), splanchnic sympathetic nerve activity (SSNA), and renal sympathetic nerve activity (RSNA). Left and middle panels depict representative tracings; the right panel shows grouped data. *: P<0.05, pregnant (P) compared to nonpregnant (NP) rats.
Figure S2. Histological placement of PVN nanoinjection sites in pregnant and nonpregnant rats. Anatomical images modified from the Paxinos and Watson brain atlas.7
Figure S3. Histological placement of ArcN nanoinjection sites in pregnant and nonpregnant rats. Anatomical images modified from the Paxinos and Watson brain atlas.\textsuperscript{7}