The role of the paraventricular nucleus of the hypothalamus in the sympathoexcitatory effects of leptin.

*Supplement*

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**Running title:** Leptin increases SNA: role of the PVN

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METHODS.

**Animals.** Eighty-five male (320-450g) Sprague Dawley rats (Charles River Laboratories, Inc) were used for these experiments. All rats were acclimated for at least 5 days before experimentation in a room with a 12-hour:12-hour light/dark cycle, with food (LabDiet 5001, Richmond, IN, USA) and water provided ad libitum. All procedures were conducted in accordance with the NIH Guide for the Care and Use of Laboratory Animals and approved by the Institutional (Oregon Health & Science University) Animal Care and Use Committee.

**Experimental preparation.** Under isoflurane anesthesia, a tracheal tube, femoral arterial (1) and venous (3) catheters, and a recording electrode around the lumbar sympathetic nerve were implanted as previously described.1,2 The rat was then placed in a stereotaxic instrument (David Kopf Instruments, Tujunga, CA). Following a midline incision, a hole was burrled in the skull near the midline to allow insertion of a lateral intracerebroventricular (icv) cannula (for leptin or aCSF infusions) or a paraventricular nucleus (PVN) or arcuate nucleus (ArcN) hypothalamic cannula (for nanoinjections). After completing surgery, a loading dose of α-chloralose (50 mg kg⁻¹; Sigma) was administered intravenously (iv) over 30 min, while isoflurane was slowly withdrawn, and this was followed by a continuous infusion of α-chloralose (25 mg kg⁻¹ h⁻¹) for the duration of the experiment. Artificial ventilation with 100% oxygen was maintained throughout the experiment, and respiratory rate and tidal volume were adjusted to maintain expired CO₂ at 3.5-4.5%. Body temperature was maintained at 37±1°C using a rectal thermister and heating pad. Anesthetic depth was regularly confirmed by the lack of a pressor response to a foot or tail pinch. After completion of surgery and the α-chloralose loading dose, rats were allowed to stabilize for at least 60 min before experimentation commenced.

**Data acquisition.** Raw sympathetic nerve activity (SNA), pulsatile arterial pressure (AP), mean arterial pressure (MAP), and heart rate (HR) were recorded using a Biopac MP100 data acquisition and analysis system (Biopac Systems, Inc., Santa Barbara, CA), sampling at 2000 Hz. SNA was band-pass filtered (100–3000 Hz) and amplified (×10,000). After data collection, post-mortem SNA was quantified and subtracted from values of SNA recorded during the experiment. The SNA signal was then rectified and integrated into 1 s bins and for the figures was normalized to basal values (% of control).

**Baroreflex function.** Baroreflex function was assessed as previously described.2 Briefly, changes in HR and SNA were induced by first dropping MAP to ~50 mmHg with iv infusion of nitroprusside followed by increasing MAP to ~175 mmHg by both reducing the iv infusion of nitroprusside and increasing an iv infusion of phenylephrine. Complete baroreflex curves were constructed by fitting a sigmoidal curve to the changes in HR and SNA during the MAP upswing using the Boltzmann equation: \( HR \text{ or } SNA = \frac{P_1 - P_2}{1 + \exp \left( \frac{MAP - P_3}{P_4} \right)} + P_2 \). \( P_1 \) is the maximum HR or SNA, \( P_2 \) is the minimum HR or SNA, \( P_3 \) is the MAP associated with the HR or SNA value midway between the maximal and minimal HR or SNA (BP50; denotes position of the curve on the x-axis), and \( P_4 \) is the width, the coefficient used to calculate maximum baroreflex gain (BRG), \( \frac{-(P_1 - P_2)}{(P_4 \times 4)} \), which is an index of the maximum slope of the most linear part of the sigmoidal baroreflex curve. Absolute values of baroreflex gain (BRG) and the mean±SEM of other sigmoidal parameters (baroreflex maxima, minima, and midpoint) are depicted in the figures.

**icv infusions.** With a flat skull and using bregma and the dorsal surface of the dura as zero, single-barreled glass pipettes drawn to a small tip were used for lateral ventricular (LV)
infusions. The icv cannulae were angled 20 degrees in the anterior–posterior direction; coordinates were as follows (mm from bregma): 0.6 rostral, 1.4 lateral, and 4.5 ventral. Leptin (R&D Systems) was 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 2.0 dextrose, pH 7.4. Correct pipette placement was confirmed at the end of the experiment by infusing 100 nL of 2.5% Alcian blue in 0.5 mM/L of sodium acetate via the same pipette, removing the brain, and verifying the presence of dye in the cerebroventricles.

**PVN and ArcN nanoinjections.** Nanoinjections into the PVN and ArcN were conducted with single-barreled glass micropipettes, as described previously.² Briefly, with a flat skull and using bregma and the dorsal surface of the dura as zero, the micropipette (20–40 μm tip o.d.) was positioned in the PVN using the following coordinates: 1.8–2.0 mm caudal, 0.5 mm lateral and 7.4–7.6 mm ventral. For the ArcN, the following coordinates were used: 3.5-3.6 mm caudal, 0.3 mm lateral and 9.8–10.0 mm ventral. Muscimol (Tocris Bioscience, Bristol, UK; 1 mM/L),³,⁴ SHU9119 (Tocris Bioscience; 0.5 mM/L in aCSF with 10 % DMSO),⁵,⁶ kynurenate (KYN; Sigma; 27 mM/L),⁶,⁷ the NPY Y1R antagonist BIBO 3304 (NPY1x; Tocris Bioscience; 1 mM/L),² the MC3/4R agonist melanotan-II (MTII; 1 mM/L; Tocris Bioscience),⁸ or vehicle (aCSF with 10 % DMSO) were injected (all 60 nL) bilaterally into the PVN. Leptin (30 ng in 30 nL) or aCSF was injected into the ArcN. In all cases, ~2 min were allowed between injections on each side of the brain, and each injection was conducted over approximately 5–10 s using a pressure injection system (Pressure System Ile, Toohey Company, Fairfield, NJ). At the end of the experiment, ~30-60 nl of 2.5% Alcian Blue were injected into the ArcN and PVN using the same pipette and coordinates to identify injection sites using a standard anatomical atlas.⁹

**Experimental Protocols.** After stabilization, basal baroreflex function was established by producing at least two baroreflex curves, 30 min apart, with similar gains; the final control curve was used for data analysis. **Protocol 1:** After reestablishment of basal values, LSNA and HR BRG were measured 1 hr after icv injection of a dose of leptin that has been shown to inhibit food intake when administered in conscious rats¹⁰,¹¹ and that we have previously shown increases LSNA and its baroreflex regulation¹ (3 μg in 3 μL, followed by 5 μg/hr) or the aCSF vehicle (0.6 μL/min). Thirty min later one of the following injections was made bilaterally into the PVN: aCSF, muscimol, SHU9119, KYN, SHU+KYN, or NPY1x. For combined SHU9119 and KYN injections, 5 min were allowed between injections. After 10 min, baroreflex function was reassessed. Basal data were taken as the average of the 30 sec period prior to each baroreflex assessment. The icv leptin followed by PVN aCSF control set of data are duplicated in the supplemental figures. **Protocol 2:** After establishing basal hemodynamic and SNA values, leptin (30 ng in 30 nL) or aCSF was injected bilaterally into the ArcN. Then, either aCSF or NPY1x was injected into PVN and measurements continued for 30 min. **Protocol 3.** After baseline measurements of LSNA, MAP, and HR were made, either aCSF or NPY1x was injected into the PVN. Thirty min later, either aCSF or the α-MSH agonist MTII was injected into the PVN, and measurements were continued for an additional 30 min. Response values were the difference between the averages of 2 min bins following injections and the 2 min averages of baseline values before injections.
REFERENCES


Figure S1. Baroreflex control of LSNA was increased (increased BRG, maximum, minimum, and BP50) 1 hr after icv leptin injection, and these changes were completely reversed by bilateral PVN nanoinjection of muscimol 30 min later. *: P<0.05 compared to baseline control values (CON); #: P<0.05 compared to 1 hr leptin within group; †: P<0.05 compared to icv aCSF at the same time; ‡: P<0.05, icv leptin plus PVN muscimol compared to icv leptin plus PVN aCSF.
Figure S2. Baroreflex control of HR was enhanced (increased baroreflex maximum, minimum, BP50) following icv injection of leptin, and these changes were completely reversed by bilateral nanoinjection of muscimol into the PVN. *: P<0.05, compared to baseline control values (CON); #: P<0.05 compared to 1 hr leptin within group; †: P<0.05 compared to icv aCSF at the same time; ‡: P<0.05, icv leptin plus PVN muscimol compared to icv leptin plus PVN aCSF.
Figure S3. Baseline LSNA, heart rate, and mean arterial pressure, and baroreflex control of LSNA and heart rate, were increased 1 hr after icv leptin injection, and these changes were not altered by bilateral nanoinjection of muscimol outside of the PVN 30 min later (i.e. not different from icv leptin plus PVN aCSF). See Figure S13 for location of injection sites (n=4). *: P<0.05 compared to baseline control values (CON); #: P<0.05 compared to 1 hr leptin within group; †: P<0.05 compared to icv aCSF at the same time.
Figure S4. Baroreflex control of LSNA was enhanced (increased BRG, maximum, minimum, BP50) following icv injection of leptin, and these changes were partially reversed by bilateral nanoinjection of SHU9119 into the PVN. *: P<0.05, compared to baseline control values (CON); #: P<0.05 compared to 1 hr leptin within group; †: P<0.05 compared to icv aCSF at the same time; ‡: P<0.05, icv leptin plus PVN SHU9119 compared to icv leptin plus PVN aCSF (as in Figure S1).
Figure S5. Baroreflex control of HR was enhanced (increased baroreflex maximum, minimum, BP50) following icv injection of leptin, and these changes were partially reversed by bilateral nanoinjection of SHU9119 into the PVN. *: P<0.05, compared to baseline control values (CON); #: P<0.05 compared to 1 hr leptin within group; †: P<0.05 compared to icv aCSF at the same time; ‡: P<0.05, icv leptin plus PVN SHU9119 compared to icv leptin plus PVN aCSF (as in Figure S2).
Figure S6. Baroreflex control of LSNA was enhanced (increased BRG, maximum, minimum, BP50) following icv injection of leptin, and these changes were partially reversed by bilateral nanoinjection of KYN into the PVN. *: P<0.05, compared to baseline control values (CON); #: P<0.05 compared to 1 hr leptin within group; †: P<0.05 compared to icv aCSF at the same time; ‡: P<0.05, icv leptin plus PVN KYN compared to icv leptin plus PVN aCSF (as in Figure S1).
Figure S7. Baroreflex control of HR was enhanced (increased baroreflex maximum, minimum, BP50) following icv injection of leptin, and these changes were partially reversed by bilateral nanoinjection of KYN into the PVN. *: P<0.05, compared to baseline control values (CON); #: P<0.05 compared to 1 hr leptin within group; †: P<0.05 compared to icv aCSF at the same time; ‡: P<0.05, icv leptin plus PVN SHU9119 compared to icv leptin plus PVN aCSF (as in Figure S2).
Figure S8. LSNA, HR, and MAP were increased 1 hr following icv injection of leptin, and these changes were partially reversed by bilateral nanoinjection of SHU+KYN into the PVN 30 min later. **Top**: Representative experiment showing icv leptin + PVN SHU+KYN. **Bottom**: icv leptin + PVN SHU+KYN (n=5). *: P<0.05, compared to baseline control values (CON); ‡: P<0.05, icv leptin plus PVN SHU9119+KYN compared to icv leptin plus PVN aCSF (Figure 1A).
Figure S9. Baroreflex control of LSNA (increased BRG, maximum, minimum, BP50) and HR (increased baroreflex maximum, minimum, BP50) were enhanced following icv injection of leptin, and these changes were partially reversed by bilateral nanoinjection of SHU+KYN into the PVN. *: P<0.05, compared to baseline control values (CON); #: P<0.05 compared to 1 hr leptin within group; ‡: P<0.05, icv leptin plus PVN SHU9119+KYN compared to icv leptin plus PVN aCSF (as in Figures S1 and S2).
Figure S10. Baroreflex control of LSNA was enhanced following icv injection of leptin (top; increased BRG, maximum, minimum, BP50) or bilateral PVN nanoinjection of NPY1x 90 min after icv aCSF injection (bottom; increased BRG and maximum). However, NPY1Rx failed to further increase baroreflex control of LSNA following icv leptin (middle). As a result, the baroreflex curve after icv leptin + PVN NPY1x (middle) was not different from that following icv leptin + PVN aCSF (top). *: P<0.05, compared to baseline control values (CON). #: P<0.05 compared to 1 hr leptin within group; †: P<0.05 compared to icv aCSF at the same time.
Figure S11. Baroreflex control of HR was enhanced following icv injection of leptin (top; increased maximum, minimum, BP50) or bilateral PVN nanoinjection of NPY1x 90 min after icv aCSF injection (bottom; increased maximum). However, NPY1Rx failed to further increase baroreflex control of HR following icv leptin (middle). As a result, the baroreflex curve after icv leptin + PVN NPY1x (middle) was not different from that following icv leptin + PVN aCSF (top). *: P<0.05, compared to baseline control values (CON). *: P<0.05 compared to 1 hr leptin within group; †: P<0.05 compared to icv aCSF at the same time.
Figure S12. Two hr following ArcN aCSF, bilateral PVN nanoinjection of NPY1x increased LSNA and MAP (top). ArcN injection of leptin increased LSNA and MAP 2 hr later (middle, bottom); however, subsequent PVN nanoinjections of neither aCSF nor NPY1x produced further increases in LSNA and MAP. *: P<0.05, compared to basal control values (Basal). †: P<0.05 Post PVN nanoinjection compared to immediately before PVN nanoinjections (Pre).
Figure S13. Histological placement of PVN nanoinjection sites (vehicle, muscimol, SHU9119, KYN and SHU+KYN). Anatomical images modified from the Paxinos and Watson brain atlas.⁹
Figure S14. Histological placement of ArcN and PVN (NPY1x and MTII) nanoinjection sites. Anatomical images modified from the Paxinos and Watson brain atlas.\textsuperscript{9}