Relaxin increases sympathetic nerve activity and activates spinally projecting neurons in the paraventricular nucleus of nonpregnant, but not pregnant, rats

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Coldren KM, Brown R, Hasser EM, Heesch CM. Relaxin increases sympathetic nerve activity and activates spinally projecting neurons in the paraventricular nucleus of nonpregnant, but not pregnant, rats. Am J Physiol Regul Integr Comp Physiol 309: R1553–R1568, 2015. First published September 23, 2015; doi:10.1152/ajpregu.00186.2015.—Pregnancy is characterized by increased blood volume and baseline sympathetic nerve activity (SNA), vasodilation, and tachycardia. Relaxin (RLX), an ovarian hormone elevated in pregnancy, activates forebrain sites involved in control of blood volume and SNA through ANG II-dependent mechanisms and contributes to adaptations during pregnancy. In anesthetized, arterial baroreceptor-denervated nonpregnant (NP) rats, RLX microinjected into the subfornical organ (SFO; 0.77 pmol in 50 nl) produced sustained increases in lumbar SNA (8 ± 3%) and mean arterial pressure (MAP; 26 ± 4 mmHg). Low-dose intracarotid artery infusion of RLX (155 pmol·ml⁻¹·h⁻¹; 1.5 h) had minor transient effects on AP and activated neurons [increased Fos-immunoreactivity (IR)] in the SFO and in spinally projecting (19 ± 2%) and arginine-vasopressin (AVP)-IR (21 ± 5%) cells in the paraventricular nucleus of the hypothalamus of NP, but not pregnant (P), rats. However, mRNA for RLX and ANG II type 1a receptors in the SFO was preserved in pregnancy. RLX receptor-IR is present in the region of the SFO in NP and P rats and is localized in astrocytes, the major source of angiotensinogen in the SFO. These data provide an anatomical substrate for a role of RLX in the resetting of AVP secretion and increased baseline SNA in pregnancy. Since RLX and ANG II receptor expression was preserved in the SFO of P rats, we speculate that the lack of response to exogenous RLX may be due to maximal activation by elevated endogenous levels of RLX in near-term pregnancy.

Fos; paraventricular nucleus; subfornical organ; circumventricular organs; ANG II; arginine-vasopressin

WITH ITS HIGHEST CIRCULATING levels achieved during pregnancy, relaxin (RLX) is a 6-kDa peptide hormone secreted primarily by the corpus luteum of the ovary. RLX-2 in humans is functionally equivalent to RLX-1 in all other mammals, and often both are called simply “relaxin” (2, 4, 21). Although best known for its role in growth and remodeling of the female reproductive tract during pregnancy, RLX peptide and RLX binding sites are present in male reproductive tissue and the heart, vasculature, kidney, and brain of both males and females. In regards to the cardiovascular system, in both male and female humans and rats, chronic administration of RLX causes vascular remodeling, angiogenesis, and systemic vasodilation (8). Endogenous RLX is a major contributor to peripheral vasodilation and augmented renal function seen in pregnancy, and recent clinical trials suggest that exogenously administered RLX may have therapeutic value in treating heart-failure patients (2, 8, 14).

In addition to peripheral vascular effects, circulating RLX plays an important role in the central nervous system (CNS) regulation of fluid and electrolyte balance during pregnancy via activation of projection neurons originating in forebrain circumventricular organs. In the first half of pregnancy, RLX, through an ANG II-dependent mechanism in the subfornical organ (SFO), plays a critical role in central resetting of arginine-vasopressin (AVP) secretion, contributing to the expanded plasma volume in pregnancy (18, 28, 34, 39). During the latter half of pregnancy, elevated circulating RLX promotes increased drinking behavior in rats, again via an ANG II-dependent mechanism at the SFO (25, 34, 54, 58). Thus throughout pregnancy, the CNS effects of circulating RLX are important for establishing and maintaining the expanded blood volume characteristic of normal pregnancy.

Previous studies found that acute intracerebroventricular (icv) (33) or intravenous (iv) administration of RLX to rats (59) increased Fos-immunoreactivity (IR) in neurons in the SFO, organum vasculosum of the lamina terminalis, supraoptic nucleus (SON), and paraventricular nucleus of the hypothalamus (PVN), consistent with RLX-induced activation of forebrain volume regulation pathways. Some of the activated (Fos-IR) magnocellular neurons in the SON and PVN were also immunopositive for AVP or oxytocin, although the extent of activation was not quantified (33).

Less understood is the role of RLX in pregnancy-associated adaptations in control of the sympathetic nervous system. In pregnant (P) rats, the operating pressure range for arterial baroreflex control of sympathetic nerve activity (SNA) shifts toward the lower baseline arterial pressure (AP) of pregnancy, and baroreflex-mediated sympathoexcitation is attenuated (31), likely due to increased baroreflex-independent GABAergic inhibition of the rostral ventrolateral medulla (RVLM) in the brain stem (27). These adaptations are consistent with increased orthostatic hypotension in P women and greater sensitivity to the hypotensive effects of hemorrhage in pregnancy (3). However, even though the RVLM is under greater GABAergic inhibition, baseline SNA is elevated in conscious P rats (31) and women (17, 20).

Parvocellular presynaptic neurons in the PVN receive input from the SFO (9) and send excitatory projections to preganglionic sympathetic neurons in the intermediolateral cell column of the spinal cord (IML), either directly or indirectly via a pathway that includes a synapse in the RVLM (1, 46). In other physiological and also pathophysiological states, such as dehydration, hypertension, and heart failure, activation of presynaptic parvocellular neurons in the PVN likely contrib-
utes to elevated baseline SNA (42, 46, 55). Thus it is possible that increased sympathetic outflow through a forebrain pathway may be an important adaptation of pregnancy, which could partially balance hypotension due to the profound peripheral vasodilatory effects of RLX.

Reported CNS effects of acutely administered RLX on cardiovascular control are primarily excitatory. RLX has been shown to activate SFO neurons that project to the PVN and SON (59, 60). Importantly, the pressor response, which is often seen following acute administration of relatively high doses of RLX, is attenuated by lesion of the SFO (37) or iv administration of a V1 vasopressin receptor antagonist (40). We considered that in addition to modulating pathways that increase AVP secretion, CNS effects of circulating RLX may increase SNA, which could potentially contribute to the sympathetic activation seen in pregnancy.

The present study was designed to determine effects of RLX on SNA, the forebrain pathways involved, and whether pregnancy might alter responses to RLX. We hypothesized that independent of changes in AP, RLX would result in activation of the SFO and identified spinally projecting and AVP-IR neurons in the PVN. In initial experiments in anesthetized, sinoaortic-denervated nonpregnant (NP) rats, microinjection of RLX into the SFO resulted in prolonged increases in AP and lumbar SNA (LSNA). To evaluate CNS effects of blood-borne RLX in conscious rats and to avoid confounding effects of changes in AP, we infused a low concentration of RLX into the forebrain circulation [intracarotid artery (ica)] of NP and P rats and quantitatively evaluated activation (Fos-IR) of forebrain neurons involved in control of SNA and AVP secretion. In addition, we evaluated expression of mRNA for RLX/insulin-like family peptide receptor 1 (RXFP1) and ANG II type 1a (AT1a) receptors in the SFO of NP and P rats, and immunohistochemistry (IHC) experiments identified RXFP1-IR in astrocytes in the SFO.

MATERIALS AND METHODS

Animals

NP female and timed P Sprague-Dawley rats (Harlan, Indianapolis, IN) were housed with a 12-h light/dark cycle, food and water available ad libitum, and constant room temperature (22°C) and humidity (40%). The stage of the estrous cycle was verified by vaginal smear cytology in NP females (36), and experiments were performed in late estrus or early diestrus (low estrogen and progesterone). Experiments in near-term P rats were performed on estrus or early diestrus (low estrogen and progesterone). Experiments were performed in late IN) were housed with a 12-h light/dark cycle, food and water available ad libitum, and constant room temperature (22°C) and humidity (40%). The stage of the estrous cycle was verified by vaginal smear cytology in NP females (36), and experiments were performed in late estrus or early diestrus (low estrogen and progesterone). Experiments in near-term P rats were performed on estrus or early diestrus (low estrogen and progesterone). Experiments in mice were performed on estrus or early diestrus (low estrogen and progesterone). Experiments in near-term P rats were performed on estrus or early diestrus (low estrogen and progesterone).

Study 1: Contribution of Sympathetic Nervous System and Vasopressin to Central Actions of RLX: Anesthetized NP Rats

Anesthesia was induced in 10 NP rats with isoflurane (1 l/min; 5% in room air; Butler Animal Health Supply, Dublin, OH) and gradually transitioned to Inactin (100 mg/kg iv; Sigma-Aldrich, St. Louis, MO), supplemented as needed (10 mg/kg iv). The left femoral artery and vein were catheterized for measurement of AP and administration of drugs, and the trachea was cannulated for artificial ventilation with O2-enriched room air. A bipolar silver recording electrode was implanted on the left LSON (Kwik-Sil; World Precision Instruments, Sarasota, FL), a ground wire secured in adjacent muscle, and the LSON signal amplified (5,000–10,000×) and filtered (30 Hz–3 kHz; RPS 107; Grass Instruments, Quincy, MA). Pulsatile AP, mean AP (MAP), heart rate (HR), and LSNA were monitored (LabChart 7 ProV 7.1.2; ADInstruments, Colorado Springs, CO) (27). Surgical sinoaortic denervation (SAD) was performed to eliminate arterial baroreceptor afferent fibers and prevent baroreflex compensatory responses, as described previously (27, 50). Absence of baroreflex-mediated decreases in LSNA, in response to increased MAP [phenylephrine (PE), 5 μg iv], verified successful SAD. With the use of a custom-built micropressure injection system (27), a triple-barreled injection pipette (−10 μm/barrel), and stereotaxic coordinates (midline, −0.9 mm caudal to bregma; 4.7 mm ventral to dura), the SFO was first functionally identified by a transient increase in MAP (≥10 mmHg) and LSNA (≥10%) due to microinjection of ANG II (20 μM; 50 nl = 1 pmol) in the SFO. After recovery (~20 min), RLX (gift from Dr. C. Samuel, Florey Institute, Melbourne, Australia) was microinjected (15.4 μM; 50 nl = 0.77 pmol) into the SFO. The dose of synthetic human RLX for SFO microinjection was chosen to produce moderate and prolonged increases in MAP, similar to those reported with higher amounts of purified porcine RLX administered into the cerebral ventricles (37). Prolonged increases in MAP and LSNA were documented for ≥20 min following RLX in the SFO in five rats. In the other five rats, when peak responses in MAP and LSNA, due to SFO RLX, had occurred (~6–8 min), the V1 receptor antagonist, Manning compound (Sigma-Aldrich), was administered iv (20 μg/kg iv), and changes in MAP and LSNA were recorded. Chicago Sky Blue dye (2%; 50 nl) was microinjected at the end of the experiment, rats were euthanized, and brains were removed and stored in 10% formalin before sectioning. The SFO injection site was verified in forebrain sections (35 μm) compared with a standard brain atlas (43).

Recovery Surgical Procedures: Retrograde Tracer Injections and Catheters (Studies 2 and 3)

Aspects of the technique were used for all recovery surgical procedures. Rats were anesthetized with isoflurane in room air (1 l/min; induction = 5%; maintenance = 2–3%), placed in a stereotaxic apparatus, and given dexamethasone (0.2 mg/kg im; Bimeda-MTC Animal Health, Cambridge, ONT, Canada) to limit swelling of the spinal cord and brain.

In rats to be used in study 2, one of the following retrograde tracers was microinjected into the left dorsal lateral column containing preganglionic sympathetic fibers: Fluoro-Gold (FG; 2% in deionized water; Fluorochrome, Denver, CO; 5%, maintenance dosage) or a standard brain atlas (43).

Aseptic technique was used for all recovery surgical procedures. Rats were anesthetized with isoflurane in room air (1 l/min; induction = 5%; maintenance = 2–3%), placed in a stereotaxic apparatus, and given dexamethasone (0.2 mg/kg im; Bimeda-MTC Animal Health, Cambridge, ONT, Canada) to limit swelling of the spinal cord and brain.

In rats to be used in study 2, one of the following retrograde tracers was microinjected into the left dorsal lateral column containing preganglionic sympathetic fibers: Fluoro-Gold (FG; 2% in deionized water; Fluorochrome, Denver, CO; n = 2), cholester toxin β subunit (CTB; 1% in deionized water; List Biological Laboratories, Campbell, CA; n = 7), or Alexa Fluor 555-conjugated CTB (CTB-Alexa 555; 0.5% in deionized water; Molecular Probes, Grand Island, NY; n = 20). Spinal cord tracer injections (60 nl each) were made in three rostral-caudal tracks, 0.8–0.9 mm ventral to the dorsal surface along the dorsolateral sulcus between T1 and T2. Each injection was given over 30 s, with the pipette left at the site of injection for 5 min. Catheters filled with heparinized saline (500 U/ml) were inserted and secured in the left femoral artery and vein and the left common carotid artery (tip in the cephalad direction below the carotid bifurcation to target the forebrain circulation). All catheters were exteriorized between the scapula, closed with plugs, and incision sites closed.

In study 3, experiments evaluated cellular phenotype within the SFO. To label PVN-projecting SFO neurons, retrograde tracer (50 nl; CTB-Alexa 555) was microinjected bilaterally into the PVN (~1.8 mm caudal to bregma; 0.5 mm lateral from midline; and 7.4 mm ventral from the dura).

After surgery, postoperative treatment included Buprenex (50 μg/kg sc; Reckitt Benckiser Pharmaceuticals, Richmond, VA) for pain management and Baytril (2.5 mg/kg im; Bayer Health Care, Shawnee Mission, KS) to prevent infection.
their home cage, monitored until ambulatory (2–3 h), and checked daily to assess general health and condition of catheters and surgical sites.

Following euthanasia at the end of experiments in rats with spinal tracer injections (study 2), the spinal cord was removed and stored in 4% paraformaldehyde (PFA) until sectioning. Spinal cord sections (45 μm) were dry mounted on gelatin-coated slides. The injection site was viewed directly for rats injected with FG or CTB-Alexa 555. For rats injected with unconjugated CTB as a tracer, IHC for CTB was performed on mounted spinal cord sections. The IHC protocol was similar to that described below for CTB, except the following: primary antibody incubation was 2 h (goat anti-CTB; 1:200), and secondary antibody incubation was 1 h (anti-goat IgG-DyLight 549; 1:50). PVN injection sites (CTB-Alexa 555) for rats used in study 3 were viewed directly in forebrain sections (35 μm) from rats perfused as described below. Injection sites were verified by comparison with a standard brain atlas (43), and only data from rats with appropriate placement of retrograde tracers were used in data analysis.

Study 2: Infusions in Conscious Rats

Experiments were performed in 17 NP and 12 near-term P conscious rats to assess activation of AVP and spinally projecting cells in the PVN by blood-borne RLX. In addition, activation of neurons in the SFO of forebrain sections from some of these rats was assessed. Five to 6 days following surgery, rats were placed in an isolated chamber with bedding from their home cage. Pulsatile AP, MAP, and HR were recorded for a 40-min baseline period, followed by 90 min ica infusions (1 ml/h) of either synthetic human RLX-2 [RLX; 1 μg/ml (155 nM solution)/h; NP, n = 8; P, n = 6] or normal saline (SAL; 0.15 M; NP, n = 9; P, n = 6). Initial experiments in NP rats revealed that this treatment regimen did not produce a prolonged increase in MAP. Blood-borne, exogenously administered RLX would be distributed throughout the circulation, and we estimated that the maximum plasma concentrations that could be achieved due to our infusions would remain within the physiological range reported in near-term P rats (150 ng/ml on day 21) (52). (With the assumption of a plasma volume of ~10 ml for a 250-g rat, the maximum plasma concentration that could be achieved due to administration of a total of 1.5 μg RLX would be 150 ng/ml.)

After infusions, animals were rapidly anesthetized (5% isoflurane) and euthanasia solution administered (26% pentobarbital sodium, 1 ml ip; Sleepaway; Fort Dodge Animal Health, Fort Dodge, IA). The ascending aorta and vena cava were clamped, and rats were transected pericardially perfused, first with heparinized (50 U/ml) DMEM (200 ml for a 250-g rat, the maximum plasma concentration that could be achieved due to administration of a total of 1.5 μg RLX would be 150 ng/ml.)

Coronal forebrain sections containing the PVN, SON, and in most cases, SFO (35 μm) in a one in six series (separated by 175 μm) were obtained using a cryostat (CM1900; Leica, Nussloch, Germany) and stored in cryoprotectant at −20°C. IHC was performed on free-floating sections using tissue from RLX- and SAL-treated rats concurrently, and Fos-IR protein was used as a marker for neuronal activation (6, 10). Sections were rinsed, preblocked (30 min) with 10% normal donkey serum (NDS) in 0.3% Triton in PBS (0.01 M, pH = 7.4), and rinsed again. All rinsing steps used PBS (3 times, 10 min each, room temperature on a shaker). Sections were then incubated for 24 h in a solution of primary antibodies containing 3% NDS and 0.3% Triton in PBS, guinea pig anti-AVP (1:2,000; Peninsula Laboratories, Bachem, San Carlos, CA), rabbit anti-Fos (1:3,000; Calbiochem, EMD Millipore Chemicals, Gibbstown, NJ), and for those experiments using unconjugated CTB as a retrograde tracer, goat anti-CTB (1:2,000; List Biological Laboratories). Sections were rinsed and incubated (2 h) with secondary antibodies as appropriate (1:200; Jackson ImmunoResearch, West Grove, PA): donkey anti-guinea pig IgG-cyanine 2 (Cy2), anti-rabbit IgG-Cy5, and anti-goat IgG-Cy3 in 3% NDS and 0.3% Triton in PBS. Sections were rinsed, mounted on gel-coated slides, dried in the dark, coverslipped with ProLong Gold (Invitrogen, Molecular Probes, Life Technologies, Grand Island, NY), and sealed with nail polish.

Study 3: IHC For RLX Receptors in the SFO.

IHC for RLX receptor RXFP1, also known as leucine-rich, repeat-containing G protein-coupled receptor 7 (GPCR; LGR7), was performed on forebrain sections containing the SFO in 11 NP and 3 P rats with prior retrograde tracer (CTB-Alexa 555) in the PVN. Five to 7 days following PVN tracer injections, general procedures for IHC experiments were as described above, except that to evaluate RXFP1, biotin tyramide signal amplification (TSA) was performed, according to the manufacturer’s instructions (Perkin-Elmer, Waltham, MA). Briefly, rinsed sections were blocked initially with 0.3% H2O2 (30 min) to quench endogenous peroxidase, followed by preblock with NDS and then incubation (24 h) in rabbit anti-RXFP1 primary antibody (anti-GPCR LGR7, 1:5,000; Abcam, Cambridge, MA). Day 2 sequential incubations included donkey anti-rabbit biotinylated IgG (1:200; 2 h; Jackson ImmunoResearch), streptavidin horseradish peroxidase (1:200; 1 h), TSA biotin (1:100; 15 min), and streptavidin-Alexa Fluor 647 conjugate (1:200; 2 h; Jackson ImmunoResearch).

In addition, sections from NP rats containing the SFO were used to identify the cellular location of RXFP1-IR. In rats with prior injections of retrograde tracer in the PVN, IHC for RXFP1 was as described above except that in some experiments, primary antibody for one of the following was included in the incubation solution: goat anti-ionized calcium-binding adapter molecule 1 (Iba1; microglia marker; 1:1,000; Abcam; n = 4), guinea pig anti-angiotensin II type 1 receptor (AT1; 200; 2 h; Jackson ImmunoResearch), streptavidin horseradish peroxidase (1:200; 1 h), TSA biotin (1:100; 15 min), and streptavidin-Alexa Fluor 647 conjugate (1:200; 2 h; Jackson ImmunoResearch). IHC for RLX receptor RXFP1 was then performed as described above. Slides were dried, dehydrated in 3 × 5-min washes in ethanol (70, 90, and 100%), followed by 2 × 5-min washes in xylene, and coverslipped with Cytoseal (Thermo Fisher Scientific, Waltham, MA). Under bright light, Fos-IR was visualized as a dark-brown reaction product in the nucleus, whereas RXFP1-IR was visualized on the cell body using fluorescence.

Antibody Specificity

As a negative control in all IHC trials, primary antibodies were eliminated from the incubation in one forebrain section from each rat. Specificity of the rabbit anti-Fos antibody was verified previously in rat brain tissue by lack of staining following preabsorption with either the Fos antigen (15) or the synthetic peptide immunogen (51). Guinea pig anti-AVP specificity was confirmed previously in mouse brain by preabsorption experiments using (Arg8)-AVP (47). The specificity of CTB antibody was confirmed by distribution of staining in regions of
the PVN with known projections to the spinal cord and an absence of staining in other forebrain regions. There was no apparent difference in distribution of retrogradely labeled neurons using the antibody to CTB, FG, or CTB-Alexa Fluor 555. Specificity of Abscam goat anti-IBa1 antibody for microglia (57) and guinea pig anti-GFAP (Synaptic Systems GmbH) and mouse anti-S100 (32) for astrocytes was demonstrated previously by preabsorption with the peptide immunogen.

Specificity of the RLX receptor antibody (anti-GPCR LGR7) was verified previously in cardiac tissue by the manufacturer (Abscam) as a single band in Western blot analysis, which was eliminated by preabsorption with the synthetic peptide immunogen (http://www.abs-cam.com/GPCR-LGR7-antibody-ab72159.html). In addition, we demonstrate in both standard Western blot analyses (23) using NIH/3T3 lysate (Santa Cruz Biotechnology, Santa Cruz, CA; manufacturer recommended positive control for RXFP1) and in IHC on forebrain sections containing the SFO that overnight preincubation of the Abscam RXFP1 antibody with a 200 (immunoblot)- or 10 (IHC)-fold higher concentration of the peptide immunogen (New England Peptide, Gardner, MA) blocked RXFP1 staining (see Fig. 8). To the best of our knowledge, this is the first demonstration of specificity of this RXFP1 antibody in brain tissue.

Study 4: Real-time RT-PCR: Expression of mRNA for AT1a and RXFP1 in the SFO of NP and Near-Term P Rats

Seven NP and five P rats were deeply anesthetized with Sleepaway euthanasia solution, as described above; brains were rapidly removed and snap frozen in a dry-ice/isopentane bath; and tissue punches of the SFO from 1 mm-thick coronal sections were collected (20G needle). RNA was isolated using the RNAqueous-Micro RNA isolation kit (Ambion, Life Technologies) and RNAse-Free DNase Set (Qiagen, Valencia, CA). cDNA SuperScript III First-Strand synthesis kit (Invitrogen, Life Technologies) was used to synthesize cDNA. RT-negative aliquots (3 μl) were analyzed to verify no genomic DNA contamination. Real-time RT-PCR (SmartCycler II, SYBR Mix; Cepheid, Sunnyvale, CA) was performed in triplicate for the housekeeping gene Gapdh, Rxfp1, and AT1a. PCR primer sequences were the following: GAPDH (forward AGA CAG CCG CAT CTT CTT GT; reverse CTT GCC GTG GGT AGA GTC AT), Rxfp1 (forward CTT GCC TAT CAA CAG TGC TT; reverse ATT TCC ACC CAG ATG AAT GA), and AT1a (forward AAA GGG CAA GGA ACC TTT GT; reverse CAG ATG CAG AAT AAC GCA GA). Expression of mRNA for RXFP1 and AT1a receptors in the P group was expressed relative to the NP group using the comparative cycle threshold (2−ΔΔCT) method for analysis of relative changes in gene expression (29). To verify specificity of PCR primers, PCR products were electrophoresed (Horizon 11.14 apparatus; GIBCO-BRL Life Technologies) on a 2% agarose gel, visualized with ethidium bromide, and photographed (Kodak EDAS 290). A single band of appropriate molecular weight verified target amplification.

Data Analysis

Hemodynamics. In study 1, anesthetized rats receiving RLX in the SFO, MAP, and LSNA were recorded at baseline (last 10 min of stabilization period), peak response to RLX (~6–8 min), and again at 20 min. In study 2, in conscious rats, MAP and HR were recorded at baseline, the time of the initial peak response to iCA RLX (~9 min), and every 10 min thereafter. Values over the 90-min infusion period were averaged to obtain the overall response to iCA RLX and SAL.

IHC Image Analysis

Z-Stack images (every 2 μm) at a magnification of ×20 were obtained for brain regions of interest (ROIs): four rostral-caudal levels of the PVN, defined as levels 1–4, that contain the majority of AVP and spinally projecting cells (approximately −1.4 to −2.0 mm caudal to bregma) (1, 61); the SON on either of the lateral edges of the optic tract (approximately −0.8 to 1.88 mm caudal to bregma); and the SFO (midline, approximately −0.8 to −1.4 mm caudal to bregma) (43). An Olympus BX51 with a disk-scanning unit (spinning disk), equipped with a three-axis motorized stage (Ludl Electronic Products, Hawthorne, NY), was used. Filter sets for Cy2 (excitation λ480 nm; emission λ510 nm), Cy3 (excitation λ550 nm; emission λ570 nm), Cy5 (excitation λ650 nm; emission λ670 nm), and FG (excitation λ330 nm; emission λ515 nm) were used for identifying positively labeled cells. Image stacks were obtained with each filter set appropriate to the experiment using a cooled monochrome digital camera (ORCA-AG; Hamamatsu, Bridgewater, NJ) and Neurolucida software (version 9; MicroBrightField, Willston, VT) and then merged (ImageJ software, version 1.45b; NIH, Bethesda, MD). Only the contrast and brightness of digital image stacks were adjusted for clarity. For the PVN, unilateral digital images on the side ipsilateral to the spinal retrograde tracer injection were captured, and separate image stacks within each level of the PVN were stitched together using FIJI three-dimensional plugin (45, 49). In ImageJ, version 1.44m, NIH. For the SON, Z-stack images were obtained for the two ipsilateral sections with the greatest AAV-IR labeling and results averaged for final analysis. Subregions of the PVN, including posterior magnocellular (PM), dorsal parvocellular (DP), and ventrolateral parvocellular (VLP), and the region of the SON were outlined using ImageJ (version 1.45b; NIH), and cells were counted manually using a custom plugin (GAIA Group, Novato, CA; http://gaiaig.net/index.html).

Identification of Immunopositive Cells

The presence of Fos-IR was used as an indicator of neuronal activation (6, 11). Fos-positive cells contained fluorescence in the cell nucleus, with a blank region representing the nucleolus (see Fig. 3, B2 and C2). Neurons in the PVN (study 2) or SFO (study 3) were identified as retrogradely labeled from the spinal cord or PVN, respectively, when cytoplasmic labeling with a void nuclear region was visible (see Figs. 3C1, 8, and 9). Similarly, cells were positively identified for AVP in the presence of dense staining of the cytoplasm with an empty nuclear region (see Fig. 3B1). IBa1 (microglia), GFAP (astrocytic processes), and S100 (astrocyte cell bodies) labeled cellular elements other than neurons. When the criteria above for a positively labeled cell were met under more than one filter set in the same focal plane, cells were identified as double or triple labeled. In all IHC protocols for negative control sections, tissue was examined with identical camera settings for brightness and contrast, and the absence of fluorescent labeling indicated lack of nonspecific labeling.

In study 2, quantitative analysis of IHC data was performed. For the PVN, cell counts were recorded as Spinal (CTB-Alexa Fluor 555, CTB-IR, or FG), AVP-IR, Fos-IR, Spinal-Fos (“activated spinal”), AVP-Fos (“activated AVP”), AVP-Spinal, and triple labeled. For the SON, cell counts were for Fos-IR, AVP-IR, or AVP-Fos. Counting was performed by two individuals independently, and results were averaged.

Statistical Analyses

Student’s t-tests were used to compare body weight, baseline MAP and HR, and relative expression of RXFP1 and AT1a receptor mRNA in the SFO between NP and P groups. Two-way repeated-measures (RM) ANOVA was used to compare hemodynamic (studies 1 and 2) and nerve activity (study 1) responses. In study 2 IHC: group = NP and P; treatment = SAL or RLX; PVN level = respective rostral-caudal PVN sections (1–4; −1.4 to −2.0 mm caudal to bregma); and ROI = PM, VLP, and DP subregions of the PVN. Three-way RM ANOVA was performed on cell counts for each phenotype in the PVN to determine interactions among group, treatment, and PVN level using the SAS/STAT software general linear model. If three-way RM ANOVA revealed a significant interaction involving group (NP or P),...
Fig. 1. Intravenous (iv) arginine-vasopressin (AVP) blockade reverses the increase in mean arterial pressure (MAP) but not lumbar sympathetic nerve activity (LSNA) due to relaxin (RLX) in the subfornical organ (SFO) of sinoaortic-denervated nonpregnant (NP) rats. A: representative example of Chicago Sky Blue dye (2%; 50 nl) marking an injection site (white arrow) in 1 NP rat (bottom). Original scale bar, 1 mm. Comparison of a trace of the brain section (Fig. 1. Intravenous (iv) arginine-vasopressin (AVP) blockade reverses the increase in mean arterial pressure (MAP) but not lumbar sympathetic nerve activity (LSNA) due to relaxin (RLX) in the subfornical organ (SFO) of sinoaortic-denervated nonpregnant (NP) rats. A: representative example of Chicago Sky Blue dye (2%; 50 nl) marking an injection site (white arrow) in 1 NP rat (top). Original scale bar, 1 mm. Comparison of a trace of the brain section (Fig. 1). B: prolonged increases in AP and LSNA following microinjection of RLX (0.77 pmol) into the SFO of 1 rat. int. LSNA, integrated LSNA. C: responses to iv Manning compound (AVP-X) to block AVP receptors, following RLX in the SFO of 1 rat. D and E: mean data: RLX produced sustained increases in MAP and LSNA in rats receiving RLX only (open circles); Manning compound after RLX in the SFO (RLX + AVP-X; filled squares) returned MAP to baseline levels, whereas LSNA continued to increase. *Different from baseline within group; **different from maximum response to RLX [RLX(max)] within group; #different from RLX-only group.

RESULTS

Study 1: RLX in the SFO of NP Sinoaortic-Denervated Rats

Increased Both MAP and LSNA

In 10 inactin-anesthetized NP rats, adequate SAD was verified by the absence of arterial baroreflex-mediated sympathetic inhibition. Before SAD, increased MAP, due to iv PE (+42 ± 3 mmHg), reflexly decreased LSNA (−50 ± 7%). Following SAD surgery, PE increased MAP (+36 ± 3 mmHg), but LSNA was no longer inhibited (+5 ± 4%). Microinjection of ANG II into the SFO, which was used to identify the SFO functionally, transiently increased MAP (+20 ± 3 mmHg) and LSNA (+12 ± 1%).

Figure 1 contains a representative example of an SFO injection site (Fig. 1A) and responses to RLX in the SFO in individual rats (Fig. 1, B and C). RLX microinjected into the SFO resulted in increased MAP and LSNA, which peaked at ~6–8 min and was sustained for >20 min (Fig. 1B). Administration (iv) of the AVP-V1 antagonist, Manning compound (AVP-X), at the peak pressor response to SFO RLX returned MAP to baseline, whereas LSNA remained elevated (Fig. 1C). Mean data are in Fig. 1, D and E. Two-way RM ANOVA revealed significant interactions between group and time for both MAP and LSNA. In rats receiving only RLX (n = 5), increases in both MAP and LSNA, due to RLX in the SFO, were sustained over the 20-min recording period. Within the RLX + AVP-X group (n = 5), iv Manning compound returned the initial increase in MAP, due to RLX in the SFO, to a level not different from baseline, whereas LSNA increased progressively. Twenty minutes following RLX, LSNA was greater in the RLX + AVP-X group compared with the RLX-only group. HR responses were minimal and insignificant in these anesthetized rats.
Initially, relaxin (RLX) increased mean arterial pressure (MAP) and tended to increase heart rate (HR; \( P = 0.06 \)), and responses were greater in nonpregnant (NP) compared with pregnant (P) rats (peak, ~9 min). Over the 90-min infusion period, there was no effect of RLX on MAP, and HR was increased in the RLX-treated NP group only. Results of 2-way repeated-measures (RM) ANOVA analysis of IHC data from the PVN revealed no interaction terms for group (NP or P) with treatment or PVN level for spinal cell counts. Subsequent two-way RM ANOVA, with group (NP or P) no longer included as a factor, revealed no effect of RLX and similar to previous reports (55), indicated that spinally projecting cells were located caudally, with the highest number in level 3 (Tables 2 and 3). For all other cell phenotypes, three-way RM ANOVA revealed a significant interaction of group with either treatment or level, and thus two-way RM ANOVA was performed within each group. Within both NP (Table 2) and P (Table 3) groups, consistent with other studies (24, 34), the majority of AVP cells was located in levels 1 and 2 of the PVN (Tables 2 and 3).

### Table 1. Hemodynamic responses to intracarotid artery infusion of SAL and RLX in conscious rats

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Peak Δ MAP, mmHg</th>
<th>90 min Δ MAP, mmHg</th>
<th>Peak Δ HR, beats/min</th>
<th>90 min Δ HR, beats/min</th>
</tr>
</thead>
<tbody>
<tr>
<td>NP</td>
<td>NP &gt; P</td>
<td>NS</td>
<td>NP &gt; P</td>
<td>NP: RLX &gt; P</td>
</tr>
<tr>
<td>RLX</td>
<td>RLX &gt; P</td>
<td>NS</td>
<td>NS</td>
<td>RLX: NP &gt; P</td>
</tr>
<tr>
<td>Group</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NP</td>
<td>0.5 ± 2</td>
<td>4 ± 2</td>
<td>16 ± 4</td>
<td>12 ± 13</td>
</tr>
<tr>
<td>SAL</td>
<td>((n = 8))</td>
<td>((n = 8))</td>
<td>((n = 6))</td>
<td>((n = 6))</td>
</tr>
<tr>
<td>RLX</td>
<td>13 ± 1</td>
<td>7 ± 2</td>
<td>42 ± 12</td>
<td>50 ± 5</td>
</tr>
<tr>
<td>P</td>
<td>SAL ((n = 6))</td>
<td>0 ± 2</td>
<td>-7 ± 2</td>
<td>6 ± 8</td>
</tr>
<tr>
<td>RLX ((n = 6))</td>
<td>2 ± 4</td>
<td>-5 ± 2</td>
<td>8 ± 5</td>
<td>8 ± 8</td>
</tr>
</tbody>
</table>

Immediately after the start of the infusion and included a small increase in MAP and a trend for increased HR \(( P = 0.06 \)). MAP returned to preinfusion levels within 10–20 min in these rats. Average responses over the 90-min infusion period revealed that over time, HR, but not MAP, was elevated by RLX in the NP group only.

### Study 2: Effects of Intracarotid Artery RLX in Conscious Rats

**Hemodynamic responses.** Before ica infusions of SAL or RLX and similar to our previous studies in conscious rats (31), pregnancy was associated with increased body weight \(( P = 298 ± 9 \text{ g}; \text{NP} = 229 ± 6 \text{ g})\), decreased MAP \(( P = 87 ± 3 \text{ mmHg}; \text{NP} = 110 ± 3 \text{ mmHg})\), and increased HR \(( P = 418 ± 10 \text{ beats/ min}; \text{NP} = 389 ± 11 \text{ beats/ min})\). During the stabilization and 90-min ica infusion periods, rats were left undisturbed with infrequent flushes of the arterial catheter. Data from rats with reliable readings of MAP and HR at the time points indicated in Table 1 were compared. Peak responses to RLX, which were measurable in four NP rats, occurred ~9 min after the start of the infusion and included a small increase in MAP and a trend for increased HR \(( P = 0.06 \)). MAP returned to preinfusion levels within 10–20 min in these rats. Average responses over the 90-min infusion period revealed that over time, HR, but not MAP, was elevated by RLX in the NP group only.

**Rostral-Caudal Comparisons Within the PVN**

Figure 2 contains representative photomicrographs of the tracer injection site in the spinal cord (Fig. 2 A) and the rostral-caudal levels of the PVN containing the two cellular phenotypes examined—AVP-IR and spinoally projecting neurons—from one NP rat. The majority of AVP-containing cells was located more rostrally, primarily in the PM subregion (Fig. 2, B and C), whereas the majority of spinally projecting cells was located more caudally (Fig. 2, D and E). Three-way RM ANOVA analysis of IHC data from the PVN revealed no interaction terms for group (NP or P) with treatment or PVN level for spinal cell counts. Subsequent two-way RM ANOVA, with group (NP or P) no longer included as a factor, revealed no effect of RLX and similar to previous reports (55), indicated that spinally projecting cells were located caudally, with the highest number in level 3 (Tables 2 and 3). For all other cell phenotypes, three-way RM ANOVA revealed a significant interaction of group with either treatment or level, and thus two-way RM ANOVA was performed within each group. Within both NP (Table 2) and P (Table 3) groups, consistent with other studies (24, 34), the majority of AVP cells was located in levels 1 and 2 of the PVN (Tables 2 and 3).

![Image](http://ajpregu.physiology.org/)

**Fig. 2.** Distribution of AVP-immunoreactivity (IR) and spinally projecting neurons in the paraventricular nucleus of the hypothalamus (PVN). A: representative example of the spinal Alexa Fluor 555-conjugated cholera toxin β subunit (CTB-Alexa 555) injection site in 1 rat (top; total volume = 180 nl), which included the intermediolateral cell column (IML; bottom; arrow). Original scale bar, 0.5 mm. B–E: examples of 4 rostral-caudal levels of the PVN (caudal to bregma skull suture) in 1 NP rat. Images are pseudocolored: AVP-IR, green; spinally projecting, white. PM, posterior magnocellular; DP, dorsal parvocellular; VLP, ventrolateral parvocellular regions; f, fornix. Original scale bars, 125 μm. Traces of IHC sections indicate injection site and regions of interest. Approximate coordinates were determined by comparison with a standard rat brain atlas (43).
RELAXIN ACTIVATES PRESYMPATHETIC HYPOTHALAMIC NEURONS

Table 2. PVN cell counts by rostral-caudal level in NP rats

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Spinal*</th>
<th>AVP</th>
<th>Fos</th>
<th>Spinal-Fos</th>
<th>AVP-Fos</th>
<th>Fos Only</th>
</tr>
</thead>
<tbody>
<tr>
<td>Level</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Level 1</td>
<td>3 &gt; 4</td>
<td>2 &gt; 1</td>
<td>3 &gt; 4</td>
<td>1 &gt; 4</td>
<td>3 &gt; 4</td>
<td>2 &gt; 1</td>
</tr>
<tr>
<td>SAL</td>
<td>13 ± 2</td>
<td>42 ± 8</td>
<td>52 ± 10</td>
<td>0.7 ± 0.4</td>
<td>1 ± 1</td>
<td>50.10</td>
</tr>
<tr>
<td>RLX</td>
<td>15 ± 2</td>
<td>27 ± 7</td>
<td>98 ± 29</td>
<td>3 ± 0</td>
<td>4 ± 1</td>
<td>91 ± 29</td>
</tr>
<tr>
<td>Level 2</td>
<td>32 ± 5</td>
<td>103 ± 7</td>
<td>45 ± 12</td>
<td>2 ± 0</td>
<td>2 ± 1</td>
<td>41 ± 12</td>
</tr>
<tr>
<td>SAL</td>
<td>37 ± 7</td>
<td>98 ± 10</td>
<td>180 ± 53</td>
<td>7 ± 1</td>
<td>26 ± 8</td>
<td>147 ± 46</td>
</tr>
<tr>
<td>RLX</td>
<td>77 ± 8</td>
<td>16 ± 5</td>
<td>38 ± 6</td>
<td>6 ± 2</td>
<td>0.2 ± 0.1</td>
<td>32 ± 6</td>
</tr>
<tr>
<td>Level 3</td>
<td>89 ± 6</td>
<td>17 ± 5</td>
<td>70 ± 14</td>
<td>18 ± 3</td>
<td>4 ± 2</td>
<td>48 ± 13</td>
</tr>
<tr>
<td>SAL</td>
<td>62 ± 10</td>
<td>8 ± 2</td>
<td>15 ± 3</td>
<td>7 ± 1</td>
<td>0 ± 0</td>
<td>13 ± 3</td>
</tr>
<tr>
<td>RLX</td>
<td>67 ± 7</td>
<td>12 ± 2</td>
<td>37 ± 4</td>
<td>11 ± 3</td>
<td>2 ± 1</td>
<td>26 ± 3</td>
</tr>
</tbody>
</table>

Three-way RM ANOVA revealed a significant interaction of group (NP or P) with treatment or level for all cell phenotypes other than Spinal. *For consistency, spinal values shown are for NP rats only, but marked significances for the spinal phenotype represent 2-way RM ANOVA for combined data from NP and P rats (Tables 2 and 3). Results of 2-way RM ANOVA within the P group are indicated for all other cell phenotypes. Values are means ± SE. SAL, n = 8; RLX, n = 8. NP, paraventricular nucleus of the hypothalamus; AVP, arginine-vasopressin. NS, not significant.

RLX Activated Cells in the PVN of NP, But Not P, Rats

Fos-IR cells were most prevalent in level 2 of the PVN in both NP (Table 2) and P (Table 3) rats, and RLX treatment increased the number of FOS-IR cells in the NP group (Table 2). Figure 3 contains representative examples of level 2 in one RLX-treated (Fig. 3A1) and one SAL-treated (Fig. 3A2) NP rat, illustrating that Fos-IR nuclei were more prevalent in the RLX-treated rat. In contrast to NP rats, RLX had no significant effect on Fos-IR in the PVN of P rats (Table 3).

RLX Activated Spinally Projecting Cells in the PVN of NP, But not P, rats

In both NP (Table 2) and P (Table 3) rats, co-labeling of Fos and spinal tracer (Sinal-Fos) was greatest in level 3, where the majority of Spinal cells was located. However, RLX increased activation of spinally projecting cells only in the NP group. Figure 3C contains an example of Fos-IR in spinally projecting cells in the PVN (level 3) of an RLX-treated NP rat.

In the current study, very few vasopressinergic spinally projecting cells were identified in the PVN of either NP or P rats. The two or three AVP-Spinal cells found per rat were located more caudally in levels 3 and 4 (not shown). Occasionally, one of these cells also expressed Fos, but there was no apparent difference in the number of these rarely seen, triple-labeled cells between RLX- and SAL-treated rats, and no further analysis was performed.

RLX equivalently activated spinally projecting cells in DP and VLP subregions of the PVN of NP rats. RLX activation of spinally projecting cells in NP rats was further evaluated to determine the distribution of activated cells within the DP and VLP parvocellular subregions (see Fig. 2C). Two-way RM ANOVA revealed main effects of treatment (RLX > SAL) but not subregion. The number of tracer and Fos-colabeled cells was increased by RLX in both the DP (RLX, 7 ± 2 > SAL, 1 ± 0.4) and VLP (RLX, 8 ± 2 > SAL, 4 ± 2) subregions. Results were similar when data were expressed as the percentage of spinally projecting cells activated within these subregions ([Spinal-Fos]/Spinal×100; DP: RLX, 19 ± 3% > SAL, 6 ± 1%; VLP: RLX, 23 ± 3% > SAL, 8 ± 3%]. Thus RLX treatment activated spinally projecting cells in both the DP and VLP subregions of the PVN in NP rats, and activation was not subregion specific.

RLX Activated AVP-IR Cells in the PVN of NP, But Not P, Rats.

Coexpression of Fos with AVP cells was most prevalent in level 2 of NP rats and levels 1 and 2 of P rats, where the majority of AVP cells is located. However, RLX increased the number of activated AVP cells (AVP-Fos) in the PVN of NP (Table 2) but not P (Table 3) rats. Figure 3B contains high-magnification immunofluorescent images, demonstrating co-labeling of AVP- and Fos-IR in cells in the PM subregion of the PVN in an RLX-treated NP rat.

Table 3. PVN cell counts by rostral-caudal level in P rats

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Spinal*</th>
<th>AVP</th>
<th>Fos</th>
<th>Spinal-Fos</th>
<th>AVP-Fos</th>
<th>Fos Only</th>
</tr>
</thead>
<tbody>
<tr>
<td>Level</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Level 1</td>
<td>3 &gt; 4</td>
<td>2 &gt; 1</td>
<td>3 &gt; 4</td>
<td>1 &gt; 4</td>
<td>3 &gt; 4</td>
<td>2 &gt; 1</td>
</tr>
<tr>
<td>SAL</td>
<td>8 ± 3</td>
<td>37 ± 11</td>
<td>36 ± 7</td>
<td>0.7 ± 0.6</td>
<td>0.8 ± 0.7</td>
<td>35 ± 6</td>
</tr>
<tr>
<td>RLX</td>
<td>14 ± 4</td>
<td>25 ± 6</td>
<td>23 ± 8</td>
<td>0.2 ± 0.1</td>
<td>1 ± 1</td>
<td>22 ± 7</td>
</tr>
<tr>
<td>Level 2</td>
<td>35 ± 8</td>
<td>65 ± 5</td>
<td>46 ± 5</td>
<td>4 ± 1</td>
<td>0.3 ± 0.2</td>
<td>42 ± 5</td>
</tr>
<tr>
<td>SAL</td>
<td>30 ± 4</td>
<td>72 ± 11</td>
<td>43 ± 15</td>
<td>2 ± 1</td>
<td>6 ± 4</td>
<td>35 ± 14</td>
</tr>
<tr>
<td>RLX</td>
<td>73 ± 9</td>
<td>12 ± 6</td>
<td>24 ± 6</td>
<td>6 ± 2</td>
<td>0.3 ± 0.2</td>
<td>18 ± 5</td>
</tr>
<tr>
<td>Level 3</td>
<td>81 ± 10</td>
<td>12 ± 4</td>
<td>32 ± 10</td>
<td>7 ± 3</td>
<td>0.8 ± 0.4</td>
<td>25 ± 7</td>
</tr>
<tr>
<td>SAL</td>
<td>59 ± 10</td>
<td>5 ± 2</td>
<td>27 ± 3</td>
<td>1 ± 1</td>
<td>0 ± 0</td>
<td>26 ± 3</td>
</tr>
<tr>
<td>RLX</td>
<td>75 ± 7</td>
<td>6 ± 2</td>
<td>19 ± 5</td>
<td>3 ± 1</td>
<td>0 ± 0</td>
<td>16 ± 4</td>
</tr>
</tbody>
</table>

Three-way RM ANOVA revealed a significant interaction of group (NP or P) with treatment or level for all cell phenotypes other than Spinal. *For consistency, spinal values shown are for P rats only, but marked significances for the spinal phenotype represent 2-way RM ANOVA for combined NP and P rats (Tables 2 and 3). Results of 2-way RM ANOVA within the P group are indicated for all other cell phenotypes. Values are means ± SE. SAL, n = 6; RLX, n = 6. NS, not significant.
Comparisons by level within the PVN (Tables 2 and 3) verify our IHC techniques compared with previously published work (1, 24, 34, 55). Furthermore, they provide information on anatomical distribution of cells activated by RLX. However, a certain amount of variability among rats in identification of sections representing the four rostral-caudal levels of the PVN is unavoidable, and an important goal of the current study was to evaluate potential differences between NP and P rats regarding RLX-induced activation of presympathetic and AVP-containing cells within the PVN as a whole. Table 4 contains comparisons of total cell counts across all levels of the PVN.

The total number of Spinal cells was not different between NP and P rats. However, RLX increased both the absolute number (Spinal-Fos; Table 4) and the percentage of spinally projecting cells activated (Fig. 4A) in NP, but not P, rats. Interestingly, the total number of AVP-IR cells counted in the PVN was less in P compared with NP rats. Nevertheless, whether expressed as absolute number of AVP-Fos-colabeled cells (Table 4) or as a percentage of AVP cells that was colabeled with Fos (Fig. 4B), significant activation of AVP cells, due to RLX treatment, occurred in the PVN of NP rats only. Separate analysis of the number of AVP-Fos-colabeled cells in the PM subregion (P: SAL = 1 ± 1; RLX = 7 ± 4; NP: SAL = 2 ± 0; RLX = 25 ± 6) yielded similar results. There were main effects of group and treatment, such that NP > P, and RLX > SAL.

Unphenotyped Fos-IR cells in the PVN of NP and P rats. The total number of Fos-only cells in the PVN was greater in RLX-treated NP rats compared with P-RLX and SAL-treated NP and P rats (Table 4). Comparisons by level within the NP

### Table 4. Total cell counts of 4 levels of the PVN in NP and P rats

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
<th>Spinal</th>
<th>AVP</th>
<th>Fos Only</th>
</tr>
</thead>
<tbody>
<tr>
<td>NP</td>
<td>SAL</td>
<td>183 ± 20</td>
<td>169 ± 11</td>
<td>133 ± 8</td>
</tr>
<tr>
<td></td>
<td>RLX</td>
<td>207 ± 6</td>
<td>154 ± 17</td>
<td>12 ± 4</td>
</tr>
<tr>
<td>P</td>
<td>SAL</td>
<td>175 ± 10</td>
<td>118 ± 11</td>
<td>12 ± 4</td>
</tr>
<tr>
<td></td>
<td>RLX</td>
<td>200 ± 13</td>
<td>115 ± 14</td>
<td>12 ± 4</td>
</tr>
</tbody>
</table>

Results of 2-way ANOVA are indicated at the top (Group, Treatment). Values are means ± SE. *Different from SAL within NP group; †different from P within RLX treatment (P ≤ 0.05). NP-SAL, n = 9; NP-RLX, n = 8; P-SAL, n = 6; P-RLX, n = 6. NS, not significant.
group revealed that Fos-only cells were located predominantly in the more rostral regions of the PVN (Table 2). Although across all levels of the PVN, a substantial portion of spinally projecting and AVP-IR cells was activated by RLX in NP rats (Fig. 4), the majority of Fos-expressing cells in the PVN of RLX-treated rats was neither vasopressinergic nor spinally projecting (77/11006 3%), and many were located medial to the PM subregion (Fig. 3A).

RLX Activated AVP-IR Magnocellular Neurons in the SON of NP, But Not P, Rats

There was no effect of group or treatment on the number of AVP-IR cells in the SON (NP-SAL = 82 ± 5; NP-RLX = 76 ± 7; P-SAL = 73 ± 8; P-RLX = 85 ± 6). Figure 5 contains representative photomicrographs of the SON from NP and P SAL- and RLX-treated rats. Fos-IR appears most prevalent in RLX-treated NP rats. Mean data indicate that compared with P rats, RLX resulted in greater Fos-IR in NP rats, and there was a trend (P = 0.06) for RLX to increase the number of AVP-Fos-colabeled cells (NP-SAL = 8 ± 3; NP-RLX = 21 ± 6; P-SAL = 2 ± 1; P-RLX = 5 ± 1). Expressed as a percentage of AVP-IR cells, two-way RM ANOVA revealed main effects of both the NP state and RLX treatment to increase activation of AVP-IR cells in the SON (Fig. 5).

Study 3: RLX Receptors in the SFO Are Located in Astrocytes but Not in PVN-Projecting Cells or Microglia

Examination of RLX receptors (RXFP1) in the SFO of an NP (Fig. 6B2) and a P (Fig. 6D2) RLX-treated rat from study 2 indicated a generally annular pattern of expression. However, distribution of RXFP1-IR was distinct and located mainly dorsal and lateral to RLX-induced Fos expression in the SFO of the NP rat.

Additional experiments revealed that RXFP1-IR was distinct from PVN-projecting cells in the SFO of both NP (n = 54).
Study 4: Expression of Rxfp1 and AT1a Receptor mRNA Was Preserved in the SFO of Near-Term P Rats

Figure 9A is an example of an agarose gel of PCR products for the housekeeping gene Gapdh, the RLX receptor (Rxfp1), and the AT1a receptor (AT1a) from tissue punches of the SFO in one NP and one P rat. Mean data (Fig. 9B) indicate that relative expression of mRNA for the RLX and AT1a receptors was preserved. Although not statistically significant, there was a trend (P = 0.1) for pregnancy to increase mRNA for AT1a receptors in the SFO.

DISCUSSION

RLX has been implicated in many of the cardiovascular adaptations of pregnancy. Increased circulating RLX contributes importantly to peripheral vasodilation (7) and via an ANG II-dependent action at forebrain circumventricular organs, including the SFO, is responsible for resetting of AVP secretion, which supports the expanded blood volume in P animals (34, 39). Less understood is a potential role for RLX in the control of efferent SNA and possible effects in pregnancy. In the current experiments, we microinjected RLX directly into the SFO and demonstrated for the first time that through a CNS action, RLX increased efferent SNA. Consistent with this observation, quantitative assessment of neuronal activation, using Fos IHC, revealed that physiologically relevant concentrations of blood-borne RLX administered to conscious NP female rats activated spinally projecting parvo- cellular neurons in the PVN. In addition, RLX activated a substantial percentage of vasopressin-containing magnocellular neurons in the SFO and SON, supportive of known effects of RLX to increase AVP secretion (37, 39). Interestingly, similar infusion of RLX in near-term P rats did not activate forebrain spinal ly projecting or AVP-IR neurons. Furthermore, ica RLX activated neurons in the SFO of NP, but not P rats, despite the fact that RLX and AT1a receptor expression in the SFO was preserved in pregnancy. Results are consistent with a CNS action of blood-borne RLX, through a pathway involving the SFO and PVN, to increase SNA as well as vasopressin secretion. We speculate that elevated endogenous RLX may be providing ongoing and maximal activation of these pathways to maintain increased baseline SNA and vasopressin secretion in term P rats.

Several RLX family peptides and their respective receptors have been identified within the CNS (2, 4). In particular, RLX-IR and Rxfp1 receptor binding and mRNA have been localized to various regions of the brain involved in CNS hemodynamic control, including structures of the lamina terminalis, the PVN, and the SON (4, 30). It appears that CNS actions of blood-borne RLX, the focus of the current experiments, are mainly initiated by activation of Rxfp1 receptors on neurons in circumventricular organs of the lamina termina-

![Figure 6](http://ajpregu.physiology.org/)

![Figure 7](http://ajpregu.physiology.org/)
lis, primarily the SFO. For example, lesion of the SFO elimi-
nates the pressor response due to injection of RLX into the
dorsal third cerebral ventricle (37) and prevents water drinking
induced by iv administration of RLX (59). In vitro bath
application of RLX in brain slice experiments produces a
profound and sustained increase in discharge of SFO neurons,
and the majority of the RLX-responsive cells also responded to
ANG II (59). Following iv RLX in intact rats, PVN-projecting
SFO neurons are activated (60). Thus both anatomical and
physiological evidence supports a pathway, whereby circulat-
ing RLX results in activation of neurons in the SFO with
subsequent neuroendocrine and autonomic effects mediated by
projections to structures within the blood-brain barrier, such as
the PVN and SON (4). Relatively little is known about the
function of CNS RXFP1 receptors inside the blood-brain
barrier, although it has been suggested that RLX peptide
systems within the brain may subserve a local neuromodula-
tory function (21).

Whereas both immediate and long-term peripheral vascu-
lar effects of RLX are vasodilatory, ultimately mediated by
a nitric oxide pathway (8), the most commonly reported
CNS effect of acutely administered RLX is an increase in
AP (18, 19, 39). This is at least partially due to peripheral
vasoconstrictor actions of released vasopressin (19, 37, 40).
Indeed, in experiments in which we microinjected RLX
directly into the SFO, the observed pressor response was
reversed by iv administration of Manning compound, a V1
vasopressin receptor antagonist. However, RLX in the SFO
also produced sustained increases in LSNA, which were not
attenuated by peripheral V1 receptor blockade. In fact,
following iv Manning compound, LSNA increased even further so that LSNA was greater in rats receiving both SFO RLX and iv Manning compound compared with rats receiving SFO RLX alone. Because these rats were sinoaortic denervated, the increased LSNA following peripheral blockade of AVP receptors was not mediated by baroreflex compensation. The further increase in LSNA following Manning compound could be related to the fact that high circulating levels of AVP limit increases in efferent SNA through a CNS action on V1 receptors in another circumventricular organ, the area postrema (22). Blockade of V1 receptors in the current protocol would eliminate any effects of released AVP to blunt increases in LSNA due to RLX in the SFO. Data from these physiological experiments support a pivotal role of the SFO in sympathoexcitatory responses to circulating RLX.

In our experiments in conscious rats, the low concentration of RLX (1 μg/h; 1.5 h) that was infused into the forebrain circulation (ica) produced a small and transient initial pressor response in NP rats. However, it is unlikely that this contributed to the increased Fos-IR observed in the PVN and SON. Fos expression as an index of neuronal activation requires a “strong and sustained” (25 min or longer) stimulus, with peak Fos expression occurring at ~90 min (10). Others have demonstrated that PE-induced increases in blood pressure of greater magnitude and duration than those produced by RLX in our experiments did not result in activation of neurons in the hypothalamus (10, 11, 34).

Similar to reported effects of iv RLX (19, 52), we saw a sustained increase in HR over the 90-min ica RLX infusion in NP rats. RLX peptide and RXFP1 receptors have been identified in the atria and ventricles of rat heart (2, 4), and RLX produces positive chronotropic effects in isolated heart preparations (62, 63). Thus direct effects of circulating RLX on the heart may have contributed to the tachycardia observed in our experiments. However, injection of RLX into the third cerebral ventricle was reported to produce a sustained increase in HR (37), and in our experiments, we recorded an increase in efferent SNA due to microinjection of RLX into the SFO. Thus it is possible that in addition to direct effects on the heart, blood-borne RLX could activate cardiac symp-
pathetic nerves through a CNS pathway that might contribute to the increase in HR.

As indicated by colabeling of spinal tracers with Fos-IR, we demonstrated that RLX produced substantial activation of identified presympathetic spinoally projecting parvocellular neurons in the PVN of NP rats (19%), consistent with our observation of increased SNA following microinjection of RLX into the SFO. These data provide, for the first time, evidence that physiological concentrations of RLX in the circulation may play a role in CNS control of sympathetic outflow. Potential subregion-specific activation of presympathetic parvocellular neurons was evaluated, since another condition characterized by increased activation of the PVN—water deprivation—results in preferential activation of the VLP compared with the DP subregion (55). However, in response to ica RLX, we found that neurons in the DP and VLP subregions of the PVN were activated similarly, suggesting that the pattern of activation of presympathetic neurons may differ between these two stimuli. Although not quantified, previous IHC studies have demonstrated that acute administration of RLX to rats, either iv or icv, activated magnocellular neurons in the forebrain, some of which were immunopositive for vasopressin or oxytocin (33). Quantitative evaluation in the current experiments supports these earlier observations and indicates that RLX activated vasopressin-containing magnocellular neurons in both the PVN (21%) and SON (25%).

As noted above, the majority of Fos-IR cells in the PVN in the current experiments was otherwise unphenotyped. Neurosecretory oxytocinergic neurons are located around the periphery of the vasopressin containing neurons in the PM region of the PVN (61) and can be activated by RLX (33). Thus it is likely that some of the unidentified, activated cells in this region were oxytocin-containing neurons. Some IML-projecting parvocellular neurons in the PVN (14–30%) send collaterals to the RVLM, whereas other neurons with a similar pattern of distribution project to the RVLM alone (1, 55). Thus it is possible that a portion of the neurons that contained only Fos was RVLM-projecting neurons, which similar to IML-projecting presympathetic neurons, were activated by RLX. Lastly, many of the unphenotyped, activated neurons were found in the medial parvocellular region of the PVN where neurosecretory corticotropin-releasing factor (CRF) neurons are prevalent (61) (see Fig. 3A1). Within the brain, it has been proposed that RLX-3 peptide from the nucleus incertus may interact with the CRF system within the PVN and participate in responses to stress (4). An action of circulating RLX to activate CRF-containing neurons has not been reported previously. However, the SFO sends projections to this region of the PVN, and activation of the SFO-to-PVN pathway results in elevation of hypothalamic portal plasma CRF (44). Therefore, it is possible that some of the activated PVN neurons, not further identified in our experiments, could be CRF neurosecretory neurons.

In regard to the role of RLX in pregnancy, chronic treatment with RLX (days to weeks) mimics many of the effects of pregnancy, including decreased plasma osmolality and increased cardiac output, stroke volume, and global arterial compliance (8). Functional studies clearly demonstrate that endogenous RLX contributes to cardiovascular adaptations throughout pregnancy. Immunoneutralization of RLX from days 8 to 15 of gestation blocks the increases in stroke volume, cardiac output, and global arterial compliance and prevents the decrease in vascular resistance that normally occurs between days 11 and 15 of gestation in rats (13). The decrease in plasma osmolality and osmotic threshold for AVP secretion normally seen in the first half of pregnancy occurs simultaneously to increased plasma RLX (52), and a decrease in osmolarity does not occur in ovariectomized or RLX-immunoneutralized P rats (38). Immunoneutralization of RLX (icv) in the second half of rat pregnancy (days 12–22) blocks the increase in water consumption that is normally mediated by an effect of RLX in the forebrain at this time (58).

These previous studies provide convincing evidence that endogenous RLX contributes to both peripheral- and centrally mediated adaptations in cardiovascular and blood volume regulation throughout pregnancy. However, others have reported that responses to exogenous RLX are attenuated by day 14 of gestation and remain suppressed throughout the second half of gestation (39, 52). In the current study, we found that different from NP rats, acute administration of RLX to near-term P rats did not increase HR or result in significant activation of the SFO, spinally projecting neurons in the PVN, or AVP containing magnocellular neurons in the PVN and SON.

Data from our experiments in NP rats are consistent with activation of the SFO by blood-borne RLX, which then leads to activation of the PVN and SON (4). In P rats, ica RLX did not activate neurons in the SFO, which likely accounts for the lack of Fos expression in the PVN. The RLX receptor in the SFO, RXFP1 (30), is a GPCR, and one possible explanation is that the receptors could have become desensitized as a result of long-term exposure to elevated endogenous RLX over the course of pregnancy. However, different from regulatory mechanisms for most GPCRs, the RXFP1 receptor undergoes minimal RLX-dependent phosphorylation, and receptor internalization is unlikely since activation of RXFP1 does not result in recruitment of β-arrestins to the cell surface (5). These nontraditional features of the RXFP1 receptor have been proposed as the mechanism for the prolonged responses observed following RLX administration (4). With the use of adenovirus technology, Silvertown et al. (53) delivered human gene 2 (H2) prepro-RLX into the lateral cerebral ventricles of female rats and verified overexpression of recombinant human H2 RLX in the peri-SFO region. Increased plasma AVP was evident by day 2 and remained elevated on day 21. Thus long-term exposure to the ligand RLX does not appear to result in deactivation of CNS RLX receptors.

Another possibility is that other hormones that are elevated in pregnancy, such as estrogen and progesterone, may contribute to decreased responsiveness to RLX in late P rats, but this does not appear to be the case. P rats that have been ovariec- tomized at mid-term (to reduce endogenous circulating RLX) and treated with exogenous estrogen and progesterone to maintain pregnancy respond to acute RLX administration at near-term (day 19) with activation of neurons in the SFO, PVN, and SON (24), suggesting that CNS RLX receptors in P rats are functional and respond to exogenous RLX if endogenous levels are reduced.

The current experiments provide indirect evidence supportive of a continued action of endogenous RLX in P animals. Through a CNS action, RLX contributes to resetting of the osmotic threshold for AVP secretion throughout pregnancy,
such that magnocellular neurons continue to be stimulated to secrete AVP, despite reduced plasma osmolarity. During conditions of chronic stimulation of forebrain pathways responsible for AVP secretion, the content of AVP in the PVN decreases (66), which would be consistent with the decreased AVP-IR observed in P rats in the current experiments. Furthermore, we demonstrated that expression of mRNA for RLX and AT1 receptors in the SFO was preserved in P rats. Others have concluded that RLX promotes AT1 receptor expression in the SFO during pregnancy, since icv administration of immunoneutralizing RLX antibody blocked expression of AT1 receptor mRNA in the SFO of P rats (25). We verified AT1 receptor expression in the SFO of near-term P rats, and although not statistically significant, there was a trend for increased AT1a mRNA expression in pregnancy, suggesting that RLX receptors in the SFO were functional.

IHC experiments revealed that RXFP1-IR was present in the SFO of both NP and P rats. Interestingly, RXFP1-IR did not colabel with RLX-induced Fos-IR in the SFO of NP rats. Since 90% of PVN-projecting cells in the SFO express Fos in response to RLX (60), it was not surprising that RXFP1-IR was also distinct from PVN-projecting neurons in both NP and P rats. CNS action of RLX is mediated by ANG II (18, 41, 54), and we found that RXFP1-IR colocalized with markers for astrocytes, which are the major source of angiotensinogen in the brain (56). Based on experiments in transgenic mice expressing a floxed version of angiotensinogen and conditional ablation of angiotensinogen in the SFO, Sakai et al. (48) proposed that in the SFO, locally produced ANG II activated ANG II receptors on neurons that project to hypothalamic nuclei, such as the PVN. Astrocytes in culture respond to RLX (65), and activation of AT1 receptors excites isolated SFO neurons in vitro (35). Thus we propose that the process by which blood-borne RLX increases SNA depends on RLX binding to RXFP1 receptors on astrocytes (which are often found surrounding blood vessels in the SFO), the subsequent formation of ANG II, and activation of PVN-projecting SFO neurons.

Regarding the lack of responsiveness to exogenously administered RLX in intact P animals, we speculate that the most likely explanation is that RLX receptors in the SFO are maximally occupied by endogenous RLX in pregnancy. In this case, long-term activation of the SFO could be mediating ongoing excitation of spinally projecting parvocellular and AVP magnocellular neurons, but exogenous administration of RLX would have no additional effect.

It is important to note that in these experiments, we evaluated expression of Fos (c-Fos) protein, which is a commonly used tool to assess acute neuronal activation in response to a variety of stimuli (6, 10). However, a limitation of the c-Fos technique is that expression is maximal within 90 min, after which time, it becomes progressively downregulated and thus does not allow for direct comparisons of the baseline level of neuronal activation in P vs. NP rats. To assess whether endogenously secreted RLX chronically activates neurons in the SFO and PVN of P rats and to evaluate the time course of neuronal activation during gestation, future experiments could evaluate rats at different stages of gestation using an antibody that detects multiple members of the Fos family (some with induction times over weeks) (12). In addition, future experiments, in which RLX receptors are blocked or downregulated throughout pregnancy, would provide functional data on the potential role of endogenous RLX in elevated SNA in P rats.

**Perspectives and Significance**

Based primarily on its angiogenic and vasodilatory actions that decrease cardiac afterload, the therapeutic potential of exogenously administered RLX in disease states, such as heart failure and preeclampsia, is receiving attention (2, 8). It is important to consider that both preeclampsia (20) and heart failure (2, 42) are characterized by a hyperactive sympathetic nervous system, and increased SNA is an independent predictor of cardiac mortality in heart-failure patients (16). However, to the best of our knowledge, effects of RLX on sympathetic outflow in these particular disease states have not been evaluated. Given results of the current experiments, which demonstrate that through a CNS action, RLX increased SNA, careful evaluation of effects of RLX on SNA in humans will be important.

Early results of clinical trials using RLX, especially related to hospitalized patients presenting with acute heart failure, have been promising (2, 14). It may be very important for outcomes that the patients in these trials have, for the most part, remained on medications that suppress the renin-angiotensin system, such as converting enzyme inhibitors and AT1 blockers. ANG II is sympathoexcitatory (9), and CNS effects of RLX appear to be mediated by ANG II (18, 41, 54) and are blocked by AT1 antagonists (41, 54). Thus prior blockade of CNS sympathoexcitatory effects of ANG II could have contributed to positive outcomes of clinical trials in heart-failure patients to date. Inclusion of converting enzyme inhibitors or AT1 antagonists in treatment regimens in which RLX is used in these patients may be very important therapeutically, at least partially, due to an action that would limit potential increases in sympathetic outflow due to RLX.

Although safety trials were proposed for the use of RLX to reduce elevated AP in preeclamptic women (64), further clinical trials do not appear to be in progress. Since preeclamptic women have elevated baseline SNA, well above the moderate elevation seen in normal pregnancy (20), and the mechanisms for these untoward effects are not known, extreme caution in administering exogenous RLX to these patients would be warranted.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

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