CNS EFFECTS OF BLOOD-BORNE RELAXIN ON THE PARAVENTRICULAR NUCLEUS OF THE HYPOTHALAMUS

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Master of Science

By

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MAY 2013
The undersigned, appointed by the dean of the Graduate School, have examined the dissertation entitled

**CNS EFFECTS OF BLOOD-BORNE RELAXIN ON THE PARAVENTRICULAR NUCLEUS OF THE HYPOTHALAMUS**

presented by Randall Brown,

a candidate for the degree of Master of Science

and hereby certify that, in their opinion, it is worthy of acceptance.

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# LIST OF ABBREVIATIONS

**Brain Regions**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>DP</td>
<td>Dorsal Parvocellular subregion of the PVN</td>
</tr>
<tr>
<td>IML</td>
<td>Intermediolateral cell column of the spinal cord</td>
</tr>
<tr>
<td>OVLTL</td>
<td>Organum Vasculosum of the Lamina Terminalus</td>
</tr>
<tr>
<td>PM</td>
<td>Posterior Magnocellular subregion of the PVN</td>
</tr>
<tr>
<td>PVN</td>
<td>Paraventricular Nucleus of the Hypothalamus</td>
</tr>
<tr>
<td>RVLM</td>
<td>Rostral Ventrolateral Medulla</td>
</tr>
<tr>
<td>SFO</td>
<td>Subfornical Organ</td>
</tr>
<tr>
<td>SON</td>
<td>Supraoptic Nucleus</td>
</tr>
<tr>
<td>VLP</td>
<td>Ventrolateral Parvocellular subregion of the PVN</td>
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**Peptides**

<table>
<thead>
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<tbody>
<tr>
<td>AngII</td>
<td>Angiotensin II</td>
</tr>
<tr>
<td>AVP</td>
<td>Vasopressin; anti-diuretic hormone</td>
</tr>
<tr>
<td>CRH</td>
<td>Corticotropin Releasing Hormone</td>
</tr>
<tr>
<td>RLX</td>
<td>Relaxin</td>
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**Solutions/Immunohistochemistry**

<table>
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<tbody>
<tr>
<td>-IR</td>
<td>Immunoreactive, immunoreactivity</td>
</tr>
<tr>
<td>CT-B</td>
<td>Cholera Toxin B subunit</td>
</tr>
<tr>
<td>DMEM</td>
<td>Delbecco's Modified Eagle's Medium</td>
</tr>
<tr>
<td>FG</td>
<td>Fluorogold</td>
</tr>
<tr>
<td>NDS</td>
<td>Normal Donkey Serum</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate Buffered Saline</td>
</tr>
<tr>
<td>PFA</td>
<td>Paraformaldehyde</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>------------------------------------------</td>
</tr>
<tr>
<td>ROI</td>
<td>Region of Interest</td>
</tr>
<tr>
<td>SAL</td>
<td>Saline (normal, 0.15M)</td>
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**Other**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>ica</td>
<td>Intracarotid artery</td>
</tr>
<tr>
<td>ICV</td>
<td>Intracerebroventricular</td>
</tr>
<tr>
<td>IM</td>
<td>Intramuscular</td>
</tr>
<tr>
<td>IV</td>
<td>Intravenous</td>
</tr>
<tr>
<td>NP</td>
<td>Nonpregnant</td>
</tr>
<tr>
<td>P</td>
<td>Pregnant</td>
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ABSTRACT
Pregnancy is characterized by increased blood volume and baseline sympathetic nerve activity, and profound cardiovascular adaptations. Relaxin (RLX), an ovarian hormone which is elevated in pregnancy, activates the subfornical organ (SFO) and hypothalamic regions associated with control of blood volume and sympathetic nerve activity. The current experiments phenotyped cells in the hypothalamic paraventricular (PVN) and supraoptic nuclei (SON) which were activated by RLX. Spinally projecting cells in the PVN were labeled by prior microinjection of retrogradely transported fluorescent tracers (90nl) into the intermediolateral column of the spinal cord of pregnant (P) and nonpregnant (NP) female rats. After 5 days, human RLX 2 (1 µg/hr) or saline (SAL, 1 ml/hr) was infused (1.5 hr) into the forebrain circulation (intracarotid artery) of conscious rats. RLX significantly increased heart rate (+50 ± 5) and transiently increased mean arterial pressure (+13 ± 1 mmHg) in NP rats, while SAL had no effect (+0.5 ± 2 mmHg). There were no significant hemodynamic effects in P rats with either RLX or SAL. Rats were euthanized, brains sectioned, and Fos-(index of neuronal activation), vasopressin (AVP-), and spinally-projecting neurons were evaluated. Following RLX in NP rats, cells in the lateral margins of the SFO expressed Fos-IR, consistent with activation of PVN-projecting neurons. Twenty-one ± 5% of AVP-IR cells and 19 ± 2% of spinally-projecting cells were activated (Fos-IR) by intracarotid RLX in NP rats. Fos-IR was not significantly increased in SAL treated rats, or in pregnant rats after either treatment. These data provide an anatomical substrate for a role of RLX in adaptations in regulation of blood volume (activation of AVP cells) and sympathetic nerve activity (activation of spinally projecting cells). In P rats, lack of response to exogenous RLX, might be due to pre-existing maximum activation by elevated endogenous levels of RLX.
INTRODUCTION

Relaxin (RLX) is a 6-kDa ovarian peptide hormone secreted by the corpus luteum, with highest levels achieved during pregnancy in humans, nonhuman primates, rats, and mice. Expression of RLX and its receptor has been described in various locations including the pregnant and nonpregnant female reproductive tract, in blood vessels, and parts of the CNS (5, 13). Many of the effects of RLX are species specific, but in humans and rats, RLX has been shown to exert systemic vasodilatory and angiogenic effects after long term administration. With both endocrine and autocrine/paracrine actions, and as a result of some of its protective effects in the cardiovascular system, RLX has been of interest recently in clinical trials exploring therapies for heart failure (25). Less understood are the effects of RLX in the CNS, which are the focus of the current study.

Relaxin peptides and receptors
Relaxin is one of seven members of a family of insulin like-peptides. The family includes the peptides RLX-1, -2, -3, and the insulin-like peptides-3, -4, -5, and -6. Relaxin 2 in humans is functionally equivalent to RLX-1 in all other mammals, and both are often called simply “relaxin”. The overall sequence homology in the insulin/relaxin superfamily is low. RLX, along with its family members, is first synthesized as a prohormone that includes a signal sequence and a B-C-A domain configuration (81). The biologically active peptide is created by removal of the C domain.
RLX interacts with a G-protein coupled receptor originally defined as leucine-rich repeat-containing G-protein–coupled receptor 7 (LGR7), but recently renamed RXFP1. RXFP1 is in the glycoprotein cluster of the δ family of G-protein-coupled receptors, along with receptors for luteinizing hormone, thyroid-stimulating hormone and follicle-stimulating hormone (5). Activation of RXFP1 induces coupling to Ga<sub>s</sub> and activation of adenylate cyclase, resulting in an overall intracellular accumulation of cAMP (6).

In situ hybridization and receptor autoradiography have localized RLX mRNA and RLX binding sites (presumably RLX receptor) to the reproductive organs (64, 81), vasculature (60), heart (64), and brain (5, 6)(Table 1, Fig 1). The relaxin-3 peptide and its receptor, RXFP3 (formerly called GPCR135), have been localized in brain tissue as well (5) (Table 1, Fig 1). RXFP3 is a member of the rhodopsin-like receptor (Type 1) G-protein-coupled receptors (31), and is coupled to inhibitory G-proteins (6). Specifically related to the current project, RLX and its receptors are located in specific regions in circumventricular organs (e.g., SFO, OVLT) and behind the blood-brain barrier (e.g., PVN, SON) important for regulation of the cardiovascular system.

**RLX secretion and effects on reproductive tissues**

Frederick L. Hisaw discovered RLX in 1926 after injecting serum from pregnant guinea pigs or rabbits into virgin guinea pigs after estrus and observed relaxation of the pubic ligament (81). This led to the notion that relaxin might be important in physiological adaptations during pregnancy. The highest circulating levels of RLX have been measured during pregnancy. In humans, mice, and rats, RLX is produced by the corpus luteum of the ovary of females, although it is also produced by the prostate gland of males (5). Circulating RLX reaches detectable levels by day 10 of pregnancy (~mid-term) in the rat,
after which time RLX rises as it accumulates in storage granules in luteal cells until their
degranulation 2-3 days prior to delivery, which accounts for a further increase near
parturition. This is in contrast to the patterns seen in human pregnancy, where RLX levels
follow human chorionic gonadotropin; specifically they are higher in the first trimester
coincident with corpus luteum activity, and then levels decline thereafter (81). However,
RLX remains elevated above the nonpregnant state throughout human pregnancy (13),
and likely contributes to adaptations in pregnant women.

Throughout pregnancy in rats, RLX contributes to growth and remodeling of the uterus,
cervix and vagina, endometrial thickening and vascularization (6), and elongation of the
interpubic ligament (76). In humans, RLX is associated with endometrial angiogenesis,
with evidence that it promotes endometrial thickening and vascularization (6). It has also
been shown to reduce uterine smooth muscle contractility in rodents and pigs, but not
sheep or humans. This is an example of a case where the effects of RLX are species
specific, as in the case of mammary gland development, for which RLX is essential in
pigs, but not in rats or mice. In males, RLX promotes sperm motility and penetration, and
prostate growth (6, 81). Thus, RLX plays an important role in the reproductive systems in
both males and females.

**Cardiovascular actions of RLX**
Although discovered for its actions in the reproductive tract, more recently it has been
demonstrated that RLX contributes importantly to adaptations in the cardiovascular
system through both peripheral and CNS actions. For example, chronic exogenous
(subcutaneous, osmotic mini-pump) RLX increases cardiac output and arterial
compliance and reduces systemic vascular resistance in female nonpregnant (NP) rats
Furthermore, it increases blood flow in the kidney, uterus, mammary gland, liver, mesentery and mesocecum (37). At midterm pregnancy in the rat, RLX contributes to changes in arterial compliance through vasodilation, whereas later in pregnancy RLX promotes remodeling of the vascular wall (37). These vascular adaptations during pregnancy are important for accommodating the increased needs for blood flow and nutrients to the growing placenta and fetus. The specific role of RLX in systemic hemodynamic changes is less understood in human pregnancy. However, various trials involving infusions of RLX showed that the hormone mimicked many of the adaptations of pregnancy. For example, RLX treatment in non-pregnant women increased cardiac output, decreased systemic vascular resistance, and improved renal function. Moreover, the increase in glomerular filtration rate seen in the first trimester is blunted in pregnant women who lack ovaries and thus lack endogenous RLX (13).

Interestingly, RLX utilizes different molecular pathways for vasodilation based on the duration of exposure (hours, days). The rapid pathway involves phosphoinositol 3-kinase dilatory response (51), while the latter pathways utilize matrix metalloproteinases (MMP)-9 and 2 (12) and growth factors (76)(Fig 2). These pathways are not mutually exclusive. The developing hypothesis is that longer term effects of RLX utilize angiogenic growth factors like vascular endothelial growth factor and placental growth factor in the vasodilatory pathway, however the specifics of these remain to be elucidated (12).

**Maternal Adaptations to Pregnancy**

Pregnancy is accompanied by profound adaptations in the cardiovascular system throughout gestation. These include important changes in cardiovascular hemodynamics.
resulting in a high-flow and low-resistance circulatory system (83) which ultimately supports the increased demands of gestation. The initiating event for the hemodynamic adjustments of pregnancy is a fall in systemic vascular tone likely mediated by peripheral vasodilation and increased arterial compliance, likely due to RLX (12). Vasodilation causes expansion of intravascular space which reduces “effective” volume, resulting in an initial decrease in renal perfusion (46, 78). This renal hypoperfusion results in activation of the renin-angiotensin-aldosterone system, water and sodium retention, and consequently blood volume expansion (27). In addition, decreased atrial stretch would be “perceived” as indicative of an underfilled circulation and could contribute to reflex neurohumoral compensatory mechanisms to increase plasma volume. The resulting decrease in afterload (due to increased arterial compliance) and increased preload (due to increased blood volume), coupled with tachycardia, which may also be mediated by RLX, all contribute to a substantial increase in cardiac output early in pregnancy, prior to an increase in uteroplacental blood flow (12, 27). The increase in arterial compliance could, in principle, attenuate declines in diastolic pressure which might fall too low as a result of decreased systemic vascular resistance (37).

These early adjustments in response to peripheral vasodilation (increased cardiac output and increased blood volume) early in pregnancy result in enhanced renal blood flow and filtration, a condition that persists throughout gestation. Adaptions in neurohumoral regulation in pregnancy include resetting of AVP secretion and control of sympathetic outflow (2). Despite increased blood volume and decreased plasma osmolality, which would be expected to decrease AVP secretion, plasma AVP levels in pregnant (P) humans and animals, are no different than in the nonpregnant (NP) state (26, 43, 46).
Additionally, there is a shift in the arterial baroreflex toward a lower operating pressure range an arterial baroreflex mediated sympathoexcitation is attenuated (2, 35, 50). As a result, pregnant women are more likely to experience orthostatic hypotension, and pregnant animals are more sensitive to the hypotensive effects of hemorrhage (33), likely due to attenuated arterial baroreflex mediated sympathoexcitation (2). Interestingly, baseline sympathetic nerve activity is elevated in pregnant humans (29) and rats (50).

As summarized above, RLX is believed to be critical for many of the cardiovascular adaptations during pregnancy. Studies in which RLX is removed from P animals or added to NP animals confirm this notion. For example, immunoneutralizing RLX in midterm pregnant rats prevents the pregnancy-associated increases in cardiac output, global arterial compliance, and decreased systemic vascular resistance (23). Renal adaptations seen in mid-term pregnant rats, including the increases in glomerular filtration rate, effective renal plasma flow, and decreased renal vascular resistance are also prevented by treatment with RLX immunoneutralizing antibodies (59). In addition, chronic infusion of RLX in NP rats mimics the increased cardiac output and arterial compliance, and decreased systemic vascular resistance that are seen in pregnancy (8). Similarly, chronic administration of RLX in ovariectomized rats to produce plasma levels similar to midterm pregnancy can reproduce the renal adaptations including increased renal vasodilation and hyperfiltration (18). Finally, studies using chronic subcutaneous RLX administration have shown that the increase in renal vasodilation, hyperfiltration, global arterial compliance, and the decrease in systemic vascular resistance occur in male as well as female rats (17, 22). Thus, RLX is necessary for many important maternal adaptations to pregnancy. In addition, since exogenous RLX has similar effects in males,
it is likely that effects of RLX are not dependent on the elevated estrogen and progesterone seen in pregnancy.

**RLX actions in the CNS**

RLX is also involved in central adaptations to pregnancy. During pregnancy circulating AVP is higher than might be expected based on the increased blood volume and decreased plasma osmolality (46). There is a decrease in the osmotic threshold for AVP release (46), such that, for a given plasma osmolality, pregnant animals have considerably higher plasma vasopressin levels than virgin controls (26). Thus, it appears that the low osmolality and increased plasma volume in pregnant women and animals is perceived as “normal”. Maintaining relatively high plasma AVP defends the substantial increase in plasma volume in pregnancy. RLX has been implicated in this central adaptation to pregnancy. For example, the decreased threshold for AVP secretion can be mimicked with chronic, continuous IV RLX infusions in ovariectomized rats (99). A study by Silvertown used a viral vector administered into the cerebral ventricles to promote CNS overexpression of pre-pro-RLX in NP rats, which resulted in increased plasma vasopressin secretion and decreased plasma osmolality compared to sham or control animals (82). In mid-term pregnant rats immunoneutralized for RLX, the usual osmoregulatory changes of pregnancy (decreased plasma osmolality and plasma sodium levels) are prevented (59). RLX therefore plays an important role in the central resetting of AVP secretion in pregnancy.

RLX may also modulate cardiovascular parameters such as arterial blood pressure and heart rate via actions in the CNS (6, Fig 2). While experiments utilizing lower, chronic doses of RLX have shown peripheral vasodilation, acute administration of relatively high
doses of RLX can produce a pressor effect due to a CNS action. For example, acute intravenous bolus injection of RLX (5µg—10µg) and intracerebroventricular (ICV) injections (0.068-1µg) in male and female rats increase arterial pressure, heart rate (67, 81, 100), and plasma AVP concentrations (57, 68). Circulating RLX acts at the level of the brain by activating forebrain sensory circumventricular organs which are devoid of a blood-brain barrier (52). These include the organum vasculosum of the lamina terminalis (OVLT), and the subfornical organ (SFO), in which RLX receptors have been identified by autoradiography (65), Western blot, and immunohistochemistry (47, 48). Using expression of Fos, the protein product of the immediate early gene c-fos, as an index of neuronal activation, studies have shown that ICV (250ng) RLX into male or intravenous (10-25µg) RLX in male and female rats results in activation—increased Fos-immunoreactivity (Fos-IR)—of cells in the SFO and OVLT (52, 89, 90). Additionally, in vitro electrophysiological recordings have shown that RLX activates SFO neurons (89). Importantly, the pressor response to exogenously administered RLX is greatly reduced when the SFO is ablated (57), or when vasopressin (V1) receptors in male and female NP rats are antagonized (67, 100)(IV), or in vasopressin-deficient Brattleboro rats (70). These data indicate that the SFO is critical to the actions of RLX, and that AVP contributes to the pressor response. However, although blockade of V1 receptors in female rats decreased the pressor response to RLX, a small pressor response remained (67), supporting the idea that there might be another, possibly sympathetic nervous system, component to the CNS initiated pressor response due to RLX.

Fig. 3 is a schematic of forebrain pathways that may be affected by CNS RLX. The SFO which is activated by circulating RLX, projects to magnocellular neurosecretory neurons
in forebrain sites, including the paraventricular (PVN; Fig 3-1) and supraoptic nuclei (SON; Fig 3-2) of the hypothalamus. The magnocellular neurons, located in the posterior magnocellular (PM) group in the PVN and the SON, project to the posterior pituitary gland (Fig 3-3) and release AVP and oxytocin into the circulation. In addition, efferent projections from the SFO (and OVLT, not shown) also terminate on parvocellular neurons in regions of the PVN important for autonomic function (53, 54) (Fig 3-4). Some parvocellular neurons located dorsal (DP) and ventrolateral (VLP) to the PM and in more caudal regions of the PVN project to the rostral ventrolateral medulla in the brainstem (RVLM; Fig 3-5) and/or the intermediolateral cell column of the spinal cord (IML; Fig 3-6) and participate in regulation of efferent sympathetic nerve activity (1). Physiological challenges, such as water deprivation (86) or hemorrhage (1), activate both RVLM and IML projecting parvocellular neurons in the PVN. In addition, there can be subregional specificity in activation of these projecting cells within the PVN. For example, during water deprivation the ventrolateral parvocellular neurons exhibit greater activation compared to neurons in the dorsal parvocellular subregion (86).

Studies evaluating ICV RLX in male and IV RLX in male and NP female rats found increased Fos-IR in magnocellular neurons in the SON and PVN, indicating activation of these neurons (52, 89). Although not quantified, it has been shown that some of the Fos-IR magnocellular neurons in the PVN and SON also were immuno-positive for vasopressin or oxytocin (52). Activation of parvocellular neurons associated with pre-sympathetic activity has not been evaluated, and thus, one of the goals of the current study was to determine if RLX results in activation of pre-sympathetic parvocellular neurons in the PVN and if activated cells are preferentially distributed within the PVN.
From the discussion above, it is clear that RLX has multiple actions in the periphery and on the CNS and the overall effects of RLX will be dependent on a balance between these varying effects. Figure 2 summarizes literature on the complex actions of RLX in rats.

The immediate response to a relatively high bolus dose of RLX includes CNS actions to increase AVP secretion and possibly increase sympathetic nerve activity. Direct actions of RLX on the atria and possibly increased cardiac sympathetic nerve activity produce tachycardia. These early effects of RLX would account for the immediate pressor responses that have been reported. RLX also is a potent vasodilator and with continued exposure multiple vasodilator pathways come in to play and predominate, contributing to decreased arterial pressure. It is not known if central actions of RLX persist during chronic RLX exposure. However, while pregnant rats (chronic endogenous RLX) exhibit decreased arterial pressure, AVP secretion is higher than would be expected, and baseline sympathetic nerve activity is elevated, suggesting that the overall profile following chronic RLX exposure incorporates both peripheral and CNS actions of RLX.

**Effects of RLX administration in pregnant rats**

Studies have reported that some central and peripheral effects of exogenously administered RLX are attenuated in late pregnancy in the rat. While acute RLX treatment evokes AVP release and a pressor response in the early stages of pregnancy in rats, these responses are reduced by day 14 (~mid-term) of pregnancy. By gestation day 19 (near term), MAP, HR, and AVP secretion are not increased by RLX compared to saline treated rats (96). Furthermore, a cross sectional study by Parry et al. (68) examined the effects of IV RLX (5µg, a “typical” IV dose expected to produce a pressor response)
during gestation and also found that the pressor response and the release of AVP and oxytocin gradually diminished as parturition approached.

As discussed above, although responses to exogenous RLX are attenuated in mid- to late-term pregnancy in the rat, it is clear that endogenous RLX is important to adaptations of normal pregnancy. Novak et al. (59) used two methods to chronically decrease endogenous RLX during pregnancy: ovariectomized rats supplanted with progesterone and estradiol; and RLX immunoneutralized rats. Both of these preparations revealed that pregnancy-induced changes in renal function and osmoregulation in day 14 P rats did not occur in the absence of functional endogenous RLX.

Interestingly, studies which have shown effects of exogenous RLX in P rats performed manipulations to decrease endogenous RLX prior to testing. Heine et al. (36) eliminated endogenous RLX by surgical ovariectomy and maintained pregnancy with exogenously administered progesterone and estradiol. At late term (gestation day 19), an IV RLX bolus intended to recreate the naturally occurring late term surge in RLX levels produced an increase in Fos-IR in the SFO, SON, and magnocellular regions of the PVN. Although not quantified, some oxytocinergic cells appeared to be co-labeled with Fos. The current experiments evaluated the effects of exogenous RLX on Fos expression in the PVN and SON in intact pregnant rats to determine if the CNS activation by RLX might be maintained even in the presence of high circulating endogenous RLX.

Specifically in regard to control of sympathetic nerve activity, previous work from our laboratory and others (2, 50) demonstrated that arterial baroreflex sympathoexcitation, which is due to disinhibition of the RVLM, is attenuated in P compared to NP rats. This
attenuated baroreflex sympathoexcitation is likely due to increased tonic GABAergic inhibition of the RVLM in the brainstem of term pregnant rats (44). Despite increased GABAergic inhibition in the RVLM, baseline sympathetic nerve activity is elevated in P compared to NP rats (2, 50). Therefore, we considered that IML projecting neurons from the PVN might be activated in pregnancy. Interestingly, preliminary data from our laboratory indicated that active sympathoexcitation due to activation of the SFO by microinjection of angiotensin II (AngII), a response that requires the PVN, is actually augmented in intact near-term P compared to NP rats (34). In vitro electrophysiological experiments have demonstrated that the same neurons in the SFO are activated by both RLX and AngII (89). Pilot experiments in our laboratory demonstrated that microinjection of RLX directly into the SFO produced prolonged increases in arterial pressure and lumbar sympathetic nerve activity in female NP rats (3). Taken together, the results of these studies led us to consider that: 1.) blood-borne RLX contributes to sympathoexcitation by activating the sympathoexcitatory SFO-PVN-IML pathway, and CNS effects of AngII may be important for this response (Fig 3-4, 6); and 2.) sympathoexcitatory responses to exogenously administered RLX might be potentiated in intact P compared to NP rats.

**Rationale for current experiments**

Based on the literature discussed above, it is clear that RLX increases secretion of AVP and oxytocin through an action at the SFO which in turn projects to the PVN and SON in the hypothalamus. Immunohistochemical studies have demonstrated that some of the magnocellular neurons activated (Fos-IR) by RLX also expressed AVP and oxytocin (52). However, none of these studies quantified the extent of activation of AVP cells, nor
was the effect of RLX on Fos expression examined in brain tissue from intact pregnant rats. Indirect evidence (67) and our preliminary data in which efferent sympathetic nerve activity was directly recorded (3), suggest that RLX might result in activation of pre-sympathetic neurons in the forebrain. In addition, other sympathoexcitatory responses mediated through the SFO-PVN pathway appear to be enhanced in term-pregnant rats (34). However, the CNS effects of RLX on pre-sympathetic parvocellular neurons in the PVN have not been evaluated in either nonpregnant or pregnant animals.

Previous studies evaluating CNS effects of RLX administered relatively high intravenous doses, or injected RLX into the cerebral ventricles (52, 57, 69) (ICV). However, it has been suggested that a barrier exists that separates the circumventricular organs from the cerebrospinal fluid (52), and thus RLX administered ICV could have different effects than blood borne RLX (53). We therefore considered that examining responses to centrally directed blood-borne RLX would provide a more physiological assessment of effects on the SFO. In the majority of previous studies, relatively high doses of RLX produced significant increases in arterial pressure. In our experiments we administered low doses of RLX into the carotid artery of conscious rats to evaluate the effects of blood-borne RLX directed to the forebrain circulation. Low doses were given to circumvent major hemodynamic effects which could confound interpretation of our results.

We hypothesized that subpressor intracarotid artery (ica) RLX would result in activation of vasopressin containing neurons in the PVN and SON and identified spinally projecting neurons in the PVN of NP rats. Possibly, similar to actions of central AngII, the actions of RLX to activate the sympathetically related neurons in the PVN may be potentiated in
P compared to NP rats. We infused low dose (~pressor threshold) synthetic Human RLX-2 (equivalent to Rat RLX-1) intracarotid artery directed toward the forebrain circulation of NP (estrus stage) and term P conscious rats, and used Fos-immunoreactivity to quantitatively evaluate activation of identified vasopressinergic neurons in the PVN and SON and spinally projecting neurons in the PVN.
METHODS

**Animals**
Seventeen nonpregnant (NP) normally cycling adult female Sprague-Dawley rats (Harlan, Indianapolis, IN, USA) weighing 170-250g were used. Stage of the estrous cycle was verified by vaginal smear cytology (55) and experiments were performed in the late estrus or early diestrus phase, a time when plasma levels of estrogen and progesterone are low. Twelve timed-pregnant rats (P) were purchased from Harlan-Sprague-Dawley. Day 1 of pregnancy was defined by the presence of a vaginal plug in the cage. Experiments were performed on day 21 in near term pregnant rats (rat gestation = 22-23 days)(42).

Animals were caged on a 12-h light/dark cycle with food and water available *ad libitum*. Room temperature and humidity were maintained at 25.5°C and 40%, respectively. Experiments were performed according to the guidelines of the American Physiological Society for research involving animals and all experimental protocols were approved by the Animal Care and Use Committee at the University of Missouri.

**Drugs and Solutions**
A gift of human relaxin 2 (RLX) was provided by Dr. Chrishan Samuel at the Florey Institute (Melbourne, Australia). Stock solution of RLX was made in double distilled water (1mg/ml), stored in aliquots at – 70 °C and diluted (1µg/ml) with normal saline (SAL; 0.15 M) on the day of the experiment. All other drugs were dissolved in 0.01M
phosphate buffered saline (PBS; 0.387 M NaCl, 0.02 M monobasic NaH₂PO₄ (anhydrous), and 0.8 M dibasic Na₂HPO₄ in distilled H₂O; pH 7.4) unless otherwise noted. Dexamethasone was purchased from Bimeda-MTC Animal Health Inc. (Cambridge, Ontario, Canada) and Isothesia (99.9% isoflurane, USP) was purchased from Butler Animal Health Supply (Dublin, OH). Heparin was purchased from Abraxis Pharmaceutical Products Inc, (Schaumburg, IL). Buprenex was purchased from Reckitt Benckiser Pharmaceuticals (Richmond, VA), and Baytril was purchased from Bayer Health Care (Shawnee Mission, KS). Euthanasia solution Sleepaway (26% sodium pentobarbital) was purchased from Fort Dodge Animal Health (Fort Dodge, IA). Chemicals for post mortem perfusion solutions, Dulbecco’s Modified Eagle Medium (DMEM), and paraformaldehyde (PFA), were purchased from Sigma Life Science (Sigma-Aldrich, St. Louis, MO).

Retrograde spinal tracers used were Fluoro-Gold (FG; 2% in deionized water, Fluorochrome Inc., Denver, CO, USA), cholera toxin B subunit (CT-B; 1% in deionized water, List Biological Laboratories, Inc., Campbell, CA), and Alexa Fluor 555 conjugated CT-B (0.5% in deionized water, Molecular Probes, Grand Island, NY).

**Immunohistochemistry:** Normal donkey serum (NDS) and the secondary antibodies: donkey anti-guinea pig Cy2, donkey anti-rabbit Cy5, and donkey anti-goat Cy3 were purchased from Jackson ImmunoResearch Inc. (West Grove, PA, USA). Primary antibodies used included guinea pig anti-arginine vasopressin (anti-AVP; Peninsula Laboratories, Bachem, San Carlos, CA), rabbit anti-cFos (Calbiochem, EMD Millipore Chemicals, Inc., Gibbstown, NJ), and goat anti-CT-B (List Biological Laboratories, Campbell, CA). Triton X-100, gelatin, chromium potassium sulfate, sucrose,
polyvinylpyrrolidone-40 (MW 40,000), and ethylene glycol were purchased from Sigma-Aldrich (St. Louis, MO). ProLong Gold (P36930) was purchased from Invitrogen (Molecular Probes by Life Technologies Grand Island, NY). The solution used to gel coat slides contained 5g/L gelatin and 1.76mM chromium potassium sulfate in distilled H2O. Cryoprotectant solution contained 1M sucrose, 0.05M polyvinylprrolidone-40 (MW 40,000), and 5.4M ethylene glycol in PBS.

**Surgical Procedures**

**Retrograde tracer injections**

Surgery was performed under aseptic conditions. Animals were anesthetized with Isoflurane (1 liter per minute; 5% in room air for induction; maintenance at 2-3%) and given dexamethasone (0.2mg/kg, IM) to limit swelling of the brain. Body temperature was maintained at 37ºC with a water blanket, (Model K-20; American Pharmaceal Company, Valencia, CA).

Rats were placed in a stereotaxic apparatus (Kopf, Tujunga, CA) and an incision was made over the second thoracic vertebrae (T2) and the process trimmed. Tissues were blunt dissected to expose the spinal cord between T1 and T2. Injection of retrograde tracer was performed using a single barrel glass pipette (OD 10-20µm) filled with 2% FG (n=2), 1% CT-B (n=7), or 0.5% Alexa Fluor 555 conjugated CT-B (n=20). The pipette was advanced 0.8mm-0.9mm ventral to the dorsal surface along the dorsolateral sulcus of the spinal cord. Injections were made in three rostral-caudal tracks between T1 and T2. A total of 60nl was injected into each tract. Volume of the spinal tracer microinjected was quantified by measuring the movement of the meniscus in the micropipette using a 150x microscope (Rolyn Optics, Corvina, CA) with an eyepiece micrometer calibrated for the
volume. Each injection was given over 30 seconds, with the micropipette left at the site of injection for 5 minutes.

**Catheters**
Following spinal cord injections, an incision was made in the groin and catheters filled with 10 units/ml heparin in saline were inserted and secured in the left femoral artery (PE50 connected to microrenethane, i.d. = 0.84 mm, Braintree Scientific, Inc., Braintree, MA) and vein (PE 10 connected to PE 50) for future measurement of arterial blood pressure and intravenous access, respectively. A midline incision was made on the ventral surface of the neck, the left carotid artery was isolated and a catheter (PE10) was inserted in the cephalad direction and secured. All catheters were exteriorized at the nape of the neck, filled with heparinized saline (500 units/ml) and closed with plugs. Incision sites were closed with suture.

Post-operative treatment included Buprenex (0.5mg/kg, subcutaneous) for pain management and Baytril (2.5mg/kg; IM) to prevent infection. Rats were returned to their home cage and monitored following surgery until ambulatory (2-3 hours). In the days preceding the experiment, rats were monitored to assess general health and the condition of catheters.

**Infusion in Conscious Rats/Transcardial Perfusion**
Animals were given 5 days to recover from surgery and to allow for transport of the retrograde tracer. On the day of the experiment, body weights were recorded and rats were placed in an isolated chamber with bedding from their home cage. Implanted catheters were flushed with heparinized saline (10 units/ml) prior to connecting to extension tubing. The femoral arterial catheter was connected to a blood pressure
transducer (ADInstruments, Colorado Springs, CO); the venous catheter was connected to a 1ml syringe filled with heparinized saline (10 units/ml); and the carotid artery catheter was connected to a syringe containing either RLX (1ug/ml) or saline (SAL; 0.15M). Arterial pressure was recorded using LabChart 7 Pro (V 7.1.2; ADInstruments) for a 40-min baseline period and then for 90 min during the infusion of either RLX (1 µg/ml; NP, n=8; P, n =6) or SAL (0.15M; NP, n=9; P, n=6) for 1.5 hrs at a rate of 1ml/hr (Razel Syringe Pump, Razel Scientific Instruments, Stamford, CT). Initial experiments in NP rats determined that intra-carotid artery administration of this dose of RLX (1ug/hr) was near the threshold for a pressor response.

Following infusions animals were rapidly anesthetized with isoflurane (5%), and the euthanasia solution, Sleepaway (260mg sodium pentobarbital in 1mL) was administered intraperitoneally. Heparin was either given to the rat intravenously (200 Units) prior to perfusion, or it was added to the DMEM solution (final concentration = 50 Units/ml) prior to bubbling with 100% O₂ for 5 minutes before perfusion. The descending aorta and vena cava were clamped and rats were transcardially perfused first with 200 ml DMEM, immediately followed by 450-500 ml of 4% PFA (pH=7.4). Brains and spinal cords were removed, post-fixed in 4% PFA overnight, and then placed in 30% sucrose in PBS and stored at 4º C until sectioning.

In pregnant rats, the number of fetuses was counted. Four fetuses were randomly selected from each rat and their individual weights were recorded and averaged.
**Immunohistochemistry**

Brains were rinsed three times with PBS and the forebrains sectioned on a cryostat (CM1900; Leica, Germany) at 35µm in a 1 in 6 series (separated by 175µm) and stored in cryoprotectant at -20° C.

Immunohistochemistry was performed on free-floating sections. Primary and secondary antibody concentrations were optimized in preliminary experiments and immunohistochemistry protocols were conducted concurrently on brain tissue from RLX and SAL treated rats. Sections from each rat were placed into mesh plates, washed and then pre-blocked for 30 minutes with 10% NDS in 0.3% Triton in PBS (0.01M, pH 7.4). All washes were performed 3 times for 10 minutes each with 0.01M PBS (pH 7.4) at room temperature on a shaker. Following pre-block, sections were washed and incubated for 24 hours in a solution of primary antibodies containing 3% NDS, 0.3% Triton in PBS; guinea pig anti-AVP (1:2,000); rabbit anti-cFos (1:3,000); and for those experiments utilizing unconjugated CT-B as a retrograde tracer, Goat anti-cholera toxin β subunit (CT-B; 1:2,000). As a control, one section from each rat was incubated with 3% NDS and 0.3% Triton in PBS without primary antibodies. Following incubation in the primary antibodies, sections were washed and placed into secondary antibody solution containing donkey anti-guinea pig Cy2 (1:200), donkey anti-rabbit Cy5 (1:200), and donkey anti-goat Cy3 (1:200), in 3% NDS, and 0.3% Triton in PBS for 2 hours. For rats with spinal microinjections of FG or Alexa Fluor 555 conjugated CT-B, the intrinsic fluorescence of these compounds were visualized, and sections were not exposed to CT-B primary and secondary antibodies. After incubation in secondary antibody solution (2hrs), sections were washed and mounted on gel-coated slides (0.5% gelatin in dH2O), allowed to dry in
the dark, protected with the anti-fading agent Prolong Gold, coverslipped and the slides sealed with nail polish.

**Verification of Microinjection Sites.** Spinal cords were sectioned at 45µm on a cryostat and dry mounted on gelatin coated slides. The injection site was viewed directly for rats injected with FG or CT-B conjugated to Alexa-555. Immunohistochemistry for CT-B was performed on mounted sections from rats injected with unconjugated CT-B as a tracer. The protocol for CT-B immunohistochemistry was the same as that described above with the following exceptions: Immunohistochemistry was performed on slides, and primary incubation was 2 hours at 1:200 in goat anti-CT-B (List Biological Laboratories, Inc.) with 3% NDS in 1mL total; secondary incubations were one hour in 3% Triton PBS with a 1:50 Donkey anti-Goat Dylight 549 (Jackson) in 1mL total volume. After coverslipping, tissue sections were viewed microscopically and fluorescent labeling in the intermediolateral cell column verified the injection site.

**Antibody specificity.** In each protocol, primary antibody was withheld from one tissue section per rat which served as a control. Specificity of rabbit anti-Fos was previously verified by Western blot(24). Guinea pig anti-AVP specificity was confirmed by preabsorption experiments(61). The specificity of CT-B antibody was confirmed by distribution of staining in regions with known projections to injection sites and an absence of staining in other brain regions. In the current experiments, the distribution of neurons labeled using the antibody to CT-B was not different from the distribution of FG or Alexa Fluor 555 conjugated CT-B.
Data analysis

Hemodynamics. Baseline MAP and HR readings were one-minute recordings during the last ten minutes of the baseline period averaged. The initial peak response to RLX was the maximal response recorded after the start of the infusion and occurred at approximately 9 minutes after RLX treatment. For the mean values during the 90 minute infusion period, MAP and HR were recorded every 10 minutes for a period of one minute and these values were averaged.

Image analysis. Z stack images (every 2 µm) were obtained for sections at four levels of the PVN using an Olympus BX51 with DSU (spinning disk) equipped with a three-axis motorized stage (Ludl Electronic Products Ltd., Hawthorne, NY, USA). Filter sets for Cy2 [ex. λ480nm; em. λ510nm], Cy3 [ex. λ550 nm; λ570 nm], Cy5 [ex. λ 650nm; em. λ 670nm], and FG [ex. λ330 nm; em. λ515 nm] were utilized as appropriate for identifying positively labeled cells. Digital images were captured at a magnification of 20x with each filter in the same focal plane using a cooled monochrome digital camera (ORCA-AG, Hamamatsu, Bridgewater, NJ, USA) and the software package Neurolucida (version 9, MicroBrightField, Willston, VT, USA). Image stacks for separate fluorophores were merged with Image J software (version 1.45b, NIH). Background was subtracted from digital image stacks and the contrast and brightness were adjusted for clarity. The separate image stacks for a section were stitched together using FIJI 3D plug-in(72, 77) (ImageJ; version 1.44m).

Four levels of the PVN which contain the majority of AVP and spinally projecting cells (-1.4 to -2.0 mm caudal to bregma) were evaluated and defined as levels 1, 2, 3, and 4, consistent with previous convention(1, 86, 91). Spinally projecting immunofluorescent
cells and AVP-immunoreactive (AVP-IR) cells were used to orient the specific
topography of the PVN. Levels 1 and 2 are characterized by the distribution of AVP-IR
cells of the posterior magnocellular (PM) group, while the more caudal levels 3 and 4 are
distinguished by the dorsolateral arching distribution of spinally projecting cells (Fig 5).
Immunopositive cells were counted on the side ipsilateral to the spinal retrograde tracer
injection. The region of the supraoptic nucleus (SON; Fig 9) and PVN (Fig 5) including
subregions of posterior magnocellular (PM), dorsal and ventrolateral parvocellular (DP
and VLP, respectively), were outlined using Image J (ver. 1.45b, NIH) and cells were
counted manually using a custom plugin (GAIA Group, Novato, Ca, USA,
http://gaiag.net/index.html).

Identification of Immunopositive Cells. The presence of Fos immunoreactivity was used
as an indicator of neuronal activation (7, 56). Cells were counted positive for Fos if cell
fluorescence was present in the nuclear region, with a dark region representing the
nucleolus (Fig. 6Bii & 6Cii). Neurons were identified as retrogradely labeled (positive for
FG, CT-B or Alexa Fluor 555 conjugated CT-B) when a dense and granular labeling of
the cytoplasm was present, or total cytosolic labeling with a void nuclear region was
visible (Fig 6Ci). Similarly, cells were positively identified for AVP in the presence of
dense staining of the cytoplasm with an empty nuclear region (Fig 6Bi). 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. Cell counts were recorded as
Fos-IR, AVP-IR, Spinal (Alexa Fluor 555 conjugated CT-B, CTB-IR or FG), AVP-Fos
(“activated AVP”), spinal-Fos (“activated spinal”), AVP-Spinal, and triple labeled. Counts
were made by two people independently and the results were averaged. For control
sections, tissue was examined under the microscope with identical exposure and contrast and the absence of fluorescent labeling indicated minimal non-specific labeling.

Neurons in the SON were counted using the same method as above. The SON (Fig 9A) was identified on either of the lateral edges of the optic tract (Bregma -0.8-1.88mm). For each rat, the Fos- and AVP-IR counts from the two tissue sections of the SON with the greatest AVP-IR labeling were averaged.

Statistical Analysis. The designation of “Group” is used to describe nonpregnant (NP) and pregnant (P), while “Treatment” describes SAL or RLX, region of interest (ROI) describes the PM, VLP, and DP subregions, and “Level” describes the respective Rostral/Caudal sections of the PVN (1 through 4; -1.4 to -2.0 mm caudal to bregma). Student’s t-tests were used to compare body weight, baseline MAP, HR, and changes in hemodynamics between groups (NP and P), as well as fetal counts and fetal weights between RLX and SAL treated pregnant rats. Two-way repeated measures ANOVA was used to compare baseline MAP and HR values to peak or 90-minute averages within pregnant or nonpregnant groups. Within each group, cell counts in the PVN were compared using 2-way repeated measures ANOVA, with treatment and level (repeated measure) as factors. Cell counts of the individual levels were also summed to create a “total” value that reflects counts of the entire counted PVN, and pregnant and nonpregnant values for SAL and RLX treated rats were compared using 2-way ANOVA with Group and Treatment as factors. Counts for SON and PVN magnocellular neurons (PM), and the percent activated AVP or Spinal cells were compared using 2-way ANOVA with Group and Treatment as factors. Counts in VLP and DP subregions were analyzed within each Group using 2-way repeated measures ANOVA where ROI and
Treatment were factors. In some instances log, square root, or reciprocal transforms were performed to meet criteria of normality and equal variance prior to statistical comparison. ANOVAs were followed by post hoc analysis using Student-Newman-Keuls test when appropriate. Statistical analyses were completed with SigmaPlot (v11.0, Systat Software, San Jose, CA, USA). Data are presented as mean ± SE, and significance was accepted at $p \leq 0.05$. 
RESULTS

Baseline values
Similar to our previous studies in conscious rats (50), pregnancy was associated with increased body weight (P = 298 ± 9g; NP = 229 ± 6g), decreased MAP (P = 87 ± 3mmHg; NP =110 ± 3mmHg), and increased HR (P =418 ± 10bpm; NP = 389 ± 11bpm) (Figure 4). Within each group (NP or P), there were no differences in these baseline values between rats assigned to receive SAL or RLX and therefore data were pooled. Within the P group, neither number of fetuses nor fetal weights were different between rats which received SAL versus RLX. In P rats, the average number of fetuses was 13 ± 1 and the average fetal weight was 3.4 ± 0.1 g, consistent with near-term pregnancy.

Effects of Intracarotid Artery RLX in NP Rats
Hemodynamic responses.
During intracarotid artery (ica) infusions, effort was made not to disturb the rats once they were connected to the infusion pump in order to minimize unnecessary stress and nonspecific Fos expression. In four NP rats we were able to obtain reliable readings for peak changes in MAP and HR which occurred approximately nine minutes after starting ica infusion of 1µg/hr of RLX. In NP rats, MAP peaked at 13±1mmHg above baseline, while SAL infusion (n=8) had no apparent effect on MAP at this time point. The peak increase in MAP and HR were significantly greater in RLX compared to SAL treated rats (Table 2). In RLX treated rats, MAP gradually declined over 30 minutes, although this
time varied. Reliable 90 minute averages were available for all 8 rats in the RLX treatment group. Over the 90 minute RLX infusion, changes in MAP were not different from baseline, while HR remained significantly elevated. The average change over 90 minutes in HR was greater in RLX compared to SAL treated NP rats (Table 2). Absolute values for MAP and HR before and after RLX are contained in Table 3. Two-way ANOVA evaluating cardiovascular parameters at baseline and during RLX infusion revealed early (9 min) main effects of both treatment (RLX > SAL) and time (After > Before) on MAP in NP rats. However, the average MAP over the course of the 90 minute infusion (RLX = 113±4.2; SAL = 114±2.1 mmHg) was not significantly different from baseline in either RLX (107±5mmHg) or SAL (110±2mmHg) treated NP rats (Table 3).

**RLX did not affect the number of vasopressinergic or spinally projecting cells.**

Figure 5 contains representative photomicrographs from one rat to illustrate the rostral-caudal distribution of AVP-IR and Spinal neurons in the PVN. Two-way repeated measures ANOVA revealed a main effect of level for the number of vasopressin containing cells, with more rostral regions of the PVN (Levels 1 & 2; bregma – 1.4mm to – 1.6mm) having the greatest number of AVP-IR cells (Table 4), primarily in the posterior magnocellular subregion (Fig 5). There was no difference in number of AVP-IR cells between RLX and SAL treated rats.

The number of spinally projecting cells in NP rats differed by rostral-caudal level, with most of the cells located caudally and the highest number in Level 3 (Bregma – 1.8mm; Table 4, Fig 5) There was no effect of treatment with RLX on the number of spinally projecting cells.
Very few vasopressinergic-spinally projecting cells (AVP-Spinal) were identified. 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 difference in the number of these triple-labeled cells between RLX and SAL treatments.

**RLX activated cells in the PVN.** Figure 6 contains representative examples of level 2 in RLX treated (Fig 6Ai) and saline treated (Fig 6Aii) NP rats. Fos-IR (red nuclei) was greater in the RLX compared to the SAL treated rat. Two-way repeated measures ANOVA revealed main effects of both treatment and level on the number of Fos-IR cells in the PVN. Compared to SAL, RLX increased Fos expression, which was greatest in Level 2 (Table 4).

**RLX activated AVP-IR cells predominantly within the posterior magnocellular (PM) subregion of the PVN.** Figure 6B contains high magnification pseudocolored immunofluorescent images demonstrating co-labeling of AVP-IR and Fos-IR in cells of the PM subregion of the PVN (Level 2) of a RLX treated NP rat. Whether expressed as absolute number of AVP-Fos colabeled cells or as a percentage of total AVP-IR cells, there were main effects of both treatment and level. RLX increased the number of AVP- and Fos-IR colabeled cells primarily in Level 2 (Table 4, Fig 7A, 10A). There was a main effect of RLX to increase the percentage of AVP cells activated [(AVP-Fos/AVP * 100)] and there was a trend (p=0.055) for the percentage of AVP-Fos cells to be higher in Level 2 (RLX = 24 ± 6%; SAL = 2 ± 1%) compared to Level 4 (RLX = 12 ± 3%; SAL = 0 ± 0
%; Table 5). Consistent with this finding, analysis of the PM subregion of the PVN alone revealed that RLX increased both the number of AVP-Fos colabeled cells (RLX = 25 ± 6; SAL = 2 ± 1) and the % of AVP cells colabeled with Fos-IR (RLX = 22 ± 5%; SAL = 2 ± 1%) in NP rats (Table 7).

**RLX activates spinally projecting cells in the PVN of NP rats.**

RLX also increased activation of spinally projecting cells. An example of immunofluorescent colabeling of Fos-IR spinally projecting cells in the PVN (Level 3) of a RLX treated NP rat is shown in Fig. 6C. Two-way repeated measures ANOVA revealed main effects of both treatment and level on the number of activated spinally projecting cells. Colabeling of Fos and spinal tracer was greater in RLX compared to SAL treated rats, with the majority of activated cells located in Level 3 (Table 4, Fig 7B). Expressed as a percentage of spinally projecting cells (Table 5), activation by RLX was greater than in SAL treated rats at all levels of the PVN (Levels 1 -4 respectively: RLX = 20 ± 3%, 22 ± 4%, 20 ± 3%, 15 ± 3%; SAL = 3 ± 2%, 7 ± 1%, 8 ± 2%, 4 ± 1%).

**RLX activates spinally projecting cells in DP and VLP subregions of the PVN of NP rats.** Whether expressed as number of tracer and Fos colabeled cells (Fig. 8A) or as percent of spinally projecting cells that were activated within these subregions (Fig. 8B), two-way ANOVA revealed a main effect of treatment only. Thus, RLX treatment increased activation of spinally projecting cells, but this was not specific to the DP or VLP subregion.
Unphenotyped activated cells in the PVN. Although across all levels of the PVN, 21±5% of AVP-IR cells and 19±2% of spinally projecting cells were activated by RLX in NP rats (Fig 10, Table 9), the majority of Fos expressing cells in the PVN of RLX treated rats (77±3%) were neither vasopressinergic nor spinally projecting (Table 9). Fos only cells in the PVN were found predominantly in the more rostral regions of the PVN (Levels 1 and 2; Table 4) medial to the PM subregion (Fig 6Ai). The total number of Fos only cells was greater in RLX (311±74) compared to SAL (136±23) treated NP animals (Table 8).

RLX activates AVP-IR magnocellular neurons in the SON

Figure 9A is a representative photomicrograph of the SON from a RLX treated NP rat. Arrows indicate examples of cells colabeled for AVP-IR and Fos-IR. Student’s t-test revealed that compared to SAL (8±3%), RLX significantly increased the percentage of AVP-IR cells activated in the SON (25 ± 7%) of NP rats.

Effects of Intracarotid Artery RLX in P Rats

Hemodynamic responses. Compared to baseline values, neither ica SAL nor RLX changed MAP and HR in P rats (Table 3). In addition, there were no differences in either the early (9 min) or longer term (average of 90 min) changes in MAP and HR between SAL and RLX treated P rats (Table 2).

Distribution of vasopressinergic, spinally projecting cells, and Fos-IR cells. The general distribution of AVP-IR, spinally-projecting, and Fos-IR cells in P rats was similar to NP rats (additional Fig 2). RLX had no effect on number of AVP-IR nor spinally
projecting cells in the PVN (Table 6). As in NP rats, in P rats, two-way repeated measures ANOVA revealed a main effect of level, with the majority of AVP-IR cells located more rostrally (Levels 1 and 2), the majority of spinally projecting cells was located more caudally (Levels 3 and 4), and the majority of Fos-IR cells located in Level 2 (Table 6). Also similar to NP rats, the majority of Fos-IR cells in the PVN of P rats were neither AVP-IR nor spinally projecting (RLX = 84 ± 3%; SAL = 90 ± 1%) (Table 9). Very few vasopressinergic spinally projecting cells were identified irrespective of treatment with RLX or SAL and no further analysis was performed. Different from NP rats (Table 4), RLX treatment had no significant effect on Fos expression or colabeling of Fos with spinally projecting cells (Table 6). There was a trend (p = 0.08) for RLX to increase the number of activated AVP-IR cells in P animals. Student’s t-test comparison of the PM subregion of RLX and SAL treated P rats also revealed a trend (p = 0.09) for an increase in the number of AVP-Fos colabeled cells in RLX treated rats (SAL = 1±1; RLX = 7±4).

**Activation of spinally projecting cells in PVN subregions in P rats was limited.**

Although the absolute number of Spinal-Fos cells was low, VLP (SAL = 4±2; RLX = 8±2) and DP (SAL = 2±0; RLX = 7±2) subregions were compared in P rats. Two-way ANOVA for repeated measures revealed an effect of subregion whereby activation was greater in the VLP compared to the DP region, but there was no effect of RLX treatment on Spinal-Fos colabeling in P rats (Figure 11).
Comparisons between NP and P rats

PVN. Two-way ANOVA comparisons of total cell counts across all levels of the PVN between NP and P rats revealed that the total number of AVP-IR cells was less in P compared to NP rats (Table 8). However, when the PM subregion of the PVN, which contains the majority of AVP-IR cells, was evaluated there was no effect of pregnancy or RLX treatment on the number of AVP cells (NP-SAL = 120±11; NP-RLX = 109±16; P-SAL=94±8; P-RLX=92±12) (Table 7). The total number of spinally projecting cells was not different between the groups. RLX increased Fos-IR in the NP group only (Table 8). Whether expressed as absolute number of colabeled cells (Table 8) or percentage of AVP or spinally projecting cells that were colabeled (Table 9, Fig 10), RLX treatment resulted in significant activation of vasopressinergic and spinally projecting cells in the PVN of NP, but not in P rats.

SON. Two-way ANOVA revealed that the number of AVP-IR cells in the SON was not different among groups (Table 10). Fos-IR was greater in NP compared to P rats. There was a main effect of group, such that NP rats had greater colabeling of AVP- and Fos-IR neurons. In addition, there was a trend (p = 0.059) for RLX to increase the number of AVP-Fos colabeled cells. When expressed as a percentage of AVP-IR cells, 2-way ANOVA revealed main effects of both group and treatment, such that the NP state and RLX treatment were associated with increased activation of AVP-IR cells in the SON (Table 10; Fig 12). Student’s t-tests comparing RLX to SAL within the NP and P groups revealed an increase in the percent of AVP cells activated only in the NP group, suggesting that the main treatment effect was primarily due to changes in the NP rats. For comparison, Fig 13 contains data for the percentage of activated AVP cells in the PM
region of the PVN and magnocellular cells in the SON and demonstrates similarities between the effects of RLX in the two magnocellular regions.

**RLX activates the SFO in NP but not P rats.** Sections containing the SFO were available for 5 NP-RLX, 6 NP-SAL, 4 P-RLX, and 4 P-SAL treated rats. Figure 14 contains representative photomicrographs of Fos-IR in the SFO of RLX and SAL treated NP and P rats. NP rats receiving ica RLX displayed an annular pattern of Fos expression around the lateral and dorsal edges of the SFO (Fig. 14B), while SAL treated NP rats showed no visible Fos-IR or limited scattered labeling near the core of the SFO (Fig. 14A). In P rats, neither RLX nor SAL resulted in Fos expression in the SFO.
DISCUSSION

Relaxin is a peptide secreted from the corpus luteum of the ovary and has been implicated in many of the cardiovascular adaptations of pregnancy. Although many effects are due to peripheral actions, there is evidence, supported by the current study, that RLX has important actions within the CNS which contribute to control of blood volume and possibly blood pressure. The current experiments quantitatively evaluated effects of centrally directed, blood-borne RLX to activate vasopressinergic neurons in the forebrain and were the first to specifically evaluate spinally projecting neurons in the PVN likely to be involved in control of efferent sympathetic outflow. The present study showed that infusion of a relatively low concentration of RLX into the carotid artery of NP rats, which produced only a small transient increase in MAP, activated vasopressin containing magnocellular neurons in the SON and PVN and spinally projecting parvocellular neurons in the PVN. Thus, independent of blood pressure effects, RLX activates forebrain regions important for vasopressin secretion and sympathetic outflow. In contrast, infusion of the same concentration of RLX did not change MAP or HR and did not produce significant activation of these forebrain regions in near-term pregnant rats.

Endogenous relaxin and relaxin treatment

Plasma concentrations of RLX
The highest plasma concentration of endogenous RLX is found during pregnancy, when it is secreted from the corpora lutea. In rats, it is first detectable around day 10 of a 22-23
day pregnancy, and plasma levels increase until a final surge when luteal cells
degranulate 2-3 days before birth, producing maximal levels between 70-140ng/ml in
near term pregnant rats (80, 81). In contrast, although plasma RLX increases in pregnant
women, maximum levels are much lower than in the rat, reaching only around 0.7-
1.1ng/ml (13, 95). In humans, plasma RLX is highest when the corpus luteum is most
active, during the first trimester. RLX does not accumulate in human luteal cells, and
luteolysis does not occur, and so the antepartum surge in RLX levels seen in rats does not
occur in humans (81). However, RLX remains elevated above nonpregnant levels
throughout human pregnancy (13). Most studies report that RLX is undetectable in
peripheral plasma in nonpregnant women; however, during the luteal phase of the
menstrual cycle, the plasma RLX concentration in the ovarian vein increases to ~ 0.4
ng/mL (41). Thus, in both humans and other mammals, circulating levels of RLX
fluctuate during the reproductive cycle.

**Relaxin homologues**

There are a few homologues of relaxin which have been used in studies examining the
effects of RLX. While they bind the same receptor, some differences in their effects have
been noted. Porcine (pRLX), recombinant human (rhRLX), and synthetic human relaxin
(H2RLX) are commonly used in studies examining the effects of exogenously
administered RLX. While Danielson and Conrad have shown that both pRLX and rhRLX
are equally effective on renal circulation (increased glomerular filtration rate and
effective renal plasma flow) after chronic subcutaneous administration (18), pRLX was
less potent than rhRLX after acute administration (16). In an *in vitro* study by Tan et al.
in which acute effects of rat, human and porcine RLX were compared, they found that rat
RLX was less potent than either synthetic H2RLX or pRLX on rat atrial tissue in
measurements of chronotropic and inotropic responses (93). In order to apply our data to the growing knowledge of the effects of centrally administered RLX, it is important to recognize that subtle differences in effects of the homologues of relaxin exist.

In the current experiments H2RLX (synthetic) was administered acutely into the carotid artery at an infusion rate of 1µg/hr for 1.5 hrs. This treatment regimen was chosen to be near threshold for a central pressor response, therefore avoiding confounding effects of reflex compensation for changes in arterial pressure (baroreflex compensation) on CNS responses. We did not measure plasma levels of RLX in our experiments, but reasoned that, even with no metabolism and assuming a blood volume of 40 ml/kg (73, 84), the maximum blood concentrations that could be achieved with this treatment regimen would be ~120 ng/ml in a 250 g rat. However, RLX is metabolized with a reported half-life of ~22 min. to 2 hrs over this time period (14, 80) with no difference in the pharmacokinetics of RLX between pregnant and nonpregnant rats (14). This allows for the assumption that RLX will be metabolized similarly in the P and NP groups. Furthermore, Danielson & Conrad (16) administered a bolus dose of rhRLX (2µg), followed by a 6 hr IV infusion (4µg/h) in NP rats and measured RLX plasma levels of 6.8±1 ng/mL. Given our shorter infusion period and lower dose, it is likely that the treatment regimen used in the current experiments produced plasma levels of RLX well within the physiological range (0-140ng/ml) (81).

**Peripheral cardiovascular effects of RLX**

Studies that examined the cardiovascular effects of RLX have used both chronic (days) and acute (minutes to hours) administration of RLX. An important consideration in evaluating the literature is that the effects of exogenous RLX differ based on the duration
of exposure, the dose, and the route of administration. These details are critical when comparing and contrasting previous studies with new data from the current study.

**Chronic RLX**

Normal pregnancy is characterized by cardiovascular adaptations including increases in HR (10, 83), blood volume and cardiac output, vasodilation, and decreased baseline MAP (2, 37). Interestingly, normal pregnancy is accompanied by increased baseline sympathetic nerve activity in both humans (29) and rats (50). RLX is chronically elevated in pregnancy and contributes to these adaptations in critical ways.

Immunoneutralizing endogenous RLX in mid-term pregnant rats abrogates the maternal cardiovascular adaptations (23), indicating that RLX contributes importantly to these changes during pregnancy. These effects of pregnancy can be mimicked by chronic administration of RLX to nonpregnant animals and experiments, primarily in rats, have provided insight into potential mechanisms. One study performed in conscious, chronically instrumented NP rats using osmotic minipumps to provide long term (10 days) subcutaneous infusion of RLX reproduced cardiovascular adaptations similar to those seen in mid-term pregnancy. Compared to vehicle treated control rats, heart rate, stroke volume, cardiac output, and global arterial compliance were greater, and systemic vascular resistance was less in RLX treated rats (8). Taken together, these data support the conclusion that the chronic presence of RLX dictates the course of many cardiovascular maternal adaptations.

One of the first reports of the effect of RLX on MAP was performed in male rats by St-Louis and Massicote (85). Chronic infusion (rat RLX 1.8µg/day, IV) for 5-6 days reduced blood pressure in conscious spontaneously hypertensive rats, but had no effect in
normotensive Wistar-Kyoto rats. The effects of chronic RLX to increase arterial compliance and decrease vascular resistance are associated with peripheral vasodilation and vascular remodeling. In the kidneys for example, RLX administration (rhRLX; 4µg/h, >6hrs) causes vasodilation of renal arteries and increased renal plasma flow and glomerular filtration rate (8, 12, 16). One of the suspected mechanisms for the drop in systemic vascular resistance and vasodilation is maternal angiogenesis (23). After hours or days of exposure, RLX increases the activity of matrix metalloproteinases (MMPs) 9 and 2, respectively, in the vascular wall (12). These MMPs are believed to be endothelin converting enzymes, which ultimately serve to activate an ET_{B} receptor-nitric oxide synthase pathway and subsequently produce vasodilation (51). Growth factors such as vascular endothelial growth factor and placental growth factor also likely contribute to long term vascular effects of RLX by inducing arterial remodeling and angiogenesis (23, 51, 76). For example, subcutaneous infusions for five days at doses intended to produce plasma RLX levels similar to mid-term pregnancy, increase elastic properties and compliance in isolated rat arteries (8). Importantly, doses of RLX administered to NP rats to produced plasma levels similar to those seen in mid-pregnancy (measured after chronic infusions (~10 days)) produce cardiovascular and angiogenic effects similar to those seen in mid-term pregnant rats.

Thus, there is ample evidence that chronically elevated RLX contributes importantly to many of the cardiovascular adaptations of pregnancy, including increased peripheral vasodilation, cardiac output, stroke volume, arterial compliance and decreased systemic vascular resistance. Moreover, RLX has the abilities to modify the extracellular matrix and elicit vascular remodeling, which are essential components of its effects during
pregnancy. The chronic pathway and effects differ from those seen after acute RLX administration, which is discussed below.

**Acute RLX**

In contrast to chronic administration, short term RLX treatment (1-6 hours) has non-significant effects on cardiac output, global arterial compliance and systemic vascular resistance (22). Many of the studies, either used a short term infusion (similar to the present study), or a bolus of RLX. Within minutes, RLX induces a rapid relaxation response on arteries in different vascular beds and nonreproductive organs like the heart and liver (12, 81). Similar to vasodilation during chronic RLX exposure, the ultimate mechanism for acute vasodilation is nitric oxide dependent, but the pathways differ. One of the mechanisms for rapid RLX-induced vasodilation depends on Gαi/o coupled to phosphatidylinositol 3-kinase, Akt, and eNOS (12). In addition, the exact timelines for these pathways are not exclusive, and so it is possible there is overlap in the acute and chronic vasodilatory paths. Apart from vasodilation, studies have also reported CNS and cardiovascular effects including vasopressin release and tachycardia, following acute RLX administration of boluses greater than ~5µg (57).

A primary hemodynamic effect reported in response to acute RLX is an increase in heart rate (22, 67) likely due to a direct action on the heart. In isolated rat atria RLX increases the rate and strength of contraction (97). However, while radiolabeled RLX binds strongly to the adult rat atria (64), RLX receptors are not evident in either the atria or ventricles of the human heart (9). In the current study, acute RLX treatment increased HR significantly in the NP-RLX treated group which was evident at 9 minutes and persisted for the duration of the experiment. The increase in HR seen in our experiments
in conscious rats could be the result of RLX directly acting on receptors in the heart as mentioned above, or it could involve activation of pre-ganglionic cardiac sympathetic neurons in the IML of the spinal cord (58, 88) by pre-sympathetic parvocellular neurons in the PVN, as discussed below.

Early studies in anesthetized female rats showed that acute IV bolus injection of relatively high doses of RLX (5µg, pRLX) produced a pressor response associated with an increase in AVP secretion (38, 67). Vasopressin release is an important component of the pressor effect to both centrally and peripherally administered relaxin. For example, peripheral AVP receptor V1 blockade (IV) greatly attenuates the pressor response to IV pRLX (10ug) in anesthetized rats (67). However, in that study vasopressin V1 receptor blockade did not completely abrogate the pressor response to RLX. It is possible that a portion of the pressor response to RLX is the result of sympathetic nervous system activation. We explored this concept via immunohistochemical evaluation of activation of IML projecting neurons in the PVN following RLX and demonstrated that pre-sympathetic neurons in the PVN were activated by blood-borne RLX.

Studies that demonstrated a pressor response to RLX typically utilized a higher doses (>5 µg) and shorter administration times (e.g., IV bolus), with continuous cardiovascular measurements. Additionally, the onset of the pressor response to IV RLX is often immediate (38, 57, 67), and the duration is typically greater than 10 minutes, so immediate peripheral vasodilatory effects would be obscured by the CNS mediated pressor response.
In another study examining the pressor effect of RLX, Brattleboro rats—which lack endogenous AVP—exhibited a small but non-significant increase in arterial pressure after a typical IV dose of pRLX (5µg, IV) (70). Brattleboro rats did however, exhibit a significant increase in HR after IV RLX, although slightly less than in rats with normal AVP levels (67). This increase in HR might be due to direct tachycardic actions of RLX on the heart, and/or via RLX mediated activation of cardiovascular control centers in the brain via the sensory circumventricular organs. The current study supports the possibility that RLX could be activating parvocellular neurons in the PVN that project to cardiac sympathetic preganglionic neurons in the IML in order to induce tachycardia.

The current study also monitored changes in arterial pressure in conscious rats and found an initial small, but transient increase in MAP in the NP group. Over the course of the infusion period (1.5hr), there was no difference in MAP between RLX and SAL treatments. This was expected with our low dose, and consistent with our aim of limiting confounding effects of changes in blood pressure on the expression of Fos. A study by Danielson et al. similarly infused a lower dose of pRLX (4µg per hour for 4 hours) into conscious female rats and also found no changes in MAP (18).

The measurement of the effects of RLX is likely impacted by the use of anesthesia. Studies reporting a pressor response to RLX after either IV or ICV administration were performed mainly in anesthetized rats, whereas the current study utilized conscious rats. Jones and Summerlee showed that RLX (5µg, IV bolus) increased MAP and AVP levels in anesthetized rats (38). Studies examining IV and ICV RLX administration in anesthetized female (69) and IV RLX in anesthetized male and female early pregnant (68) and nonpregnant (67) rats reported an increase in blood pressure and a tachycardic
response. The current study was performed in conscious rats in order to avoid adverse effects of anesthesia on Fos-IR and cardiovascular measurements and found a small transient pressor response and prolonged tachycardia with a low dose intracarotid infusion of RLX (1µg/hr).

Figure 2 summarizes potential mechanisms for the multiple effects that have been reported following RLX administration. CNS effects, through increased AVP secretion and possibly increased sympathetic nerve activity, would produce pressor effects. Direct actions on the vasculature, both short-term and longer term favor vasodilation and decreased arterial pressure. The overall balance of these various mechanisms will determine the effects of RLX on arterial blood pressure. Thus, the presence of anesthesia, the dose, and the route and time course of administration likely all determine the effect that RLX will have on arterial pressure.

**CNS RELAXIN**

RLX receptors are present in the sensory circumventricular organs (SFO, OVLT, 65) and also in the brain parenchyma, inside the blood brain barrier including the PVN, SON, basolateral amygdala, and the neocortex (5). RLX is a relatively large peptide that would not be expected to cross the blood brain barrier, and SFO ablation studies have demonstrated that without the SFO the central effects of RLX are greatly attenuated (57). Therefore, although RLX receptors have been identified in the PVN and SON, it is likely that activation of SFO neurons that project to these regions mediate responses observed after ica RLX in the current experiments.

Similar to previous reports using IV (89, 90) or ICV (52) routes of administration, intracarotid RLX administration to NP rats resulted in a distinct annular pattern of Fos
expression in the SFO. In one study examining the effects of RLX in the forebrain, Sunn et al. (90) microinjected a tracer into the PVN or SON to retrogradely label PVN and SON projection neurons in the SFO. In response to subsequent IV RLX, the distribution of Fos-IR in the SFO was similar to the distribution of PVN and SON projecting neurons, such that approximately 90% of the projecting neurons co-labeled with Fos. Therefore based on the similar, distinct anatomical distribution of Fos-IR in the SFO in the current experiments, it is likely that the SFO neurons activated in our study project to the PVN and SON. This supports our hypothesized pathway of centrally acting RLX from the SFO to the forebrain regions of the PVN and SON.

**Activation of AVP Neurons by RLX**
As discussed above, studies which have reported a pressor response following acute administration of RLX have implicated increased circulating vasopressin as an important factor. Typical doses of RLX increase plasma levels of both vasopressin and oxytocin (68). Furthermore, although not quantitated, previous experiments evaluating CNS effects of RLX have shown that RLX activates vasopressinergic as well as oxytocinergic forebrain magnocellular neurons in the PVN and SON (52).

The current experiments were designed to evaluate the effects of centrally directed blood-borne RLX on the activation of forebrain neurons involved in control of AVP secretion and sympathoexcitation. Overall, relaxin treated NP rats showed higher levels of activation in the PVN and SON than all other groups. This supports other studies which demonstrated that IV (89) and ICV (52) RLX administration produced Fos-IR in AVP containing cells in areas consistent with the location of the PM subregion. In our study, compared to SAL, RLX produced greater activation of AVP-IR neurons, consistent with
relaxin’s noted effect to increase plasma AVP (21, 68). The current data are supportive of existing literature and further provide quantitative information showing that ~21% of magnocellular vasopressin containing neurons in the SON and PVN are activated by non-pressor doses of RLX. Activation of AVP neurons by exogenously administered RLX in NP rats is consistent with maintained AVP secretion in pregnancy, a state characterized by elevated endogenous RLX levels.

**Activation of Spinally Projecting Cells by RLX**
Previous immunohistochemistry studies evaluating the effects of IV (89) and ICV (52) RLX noted that parvocellular neurons in several subregions of the PVN (predominantly in the medial subregion) appeared to express Fos-IR after RLX. However, Fos labeling was also present in parvocellular subregions of the PVN of vehicle treated rats in these experiments (89) and thus the parvocellular cells were neither phenotyped nor evaluated further. In our study, pre-sympathetic parvocellular neurons were identified by prior injection of retrograde tracer into the IML of the spinal cord. Compared to SAL, RLX activated a higher percentage of spinally projecting cells (~19%) across all levels of the PVN. Functionally speaking, the activation of cells in the PVN that project to the IML suggests a possible mechanism for RLX to mediate cardiovascular changes through the sympathetic nervous system. In addition to increased AVP secretion, an increase in SNA might further contribute to the pressor response observed by others following acute administration of high doses of RLX(67, 68, 100). It is possible that exogenous RLX, which is elevated in pregnant rats, could contribute to elevated baseline sympathetic nerve activity and tachycardia characteristic of normal pregnancy. In pregnant rats with chronically elevated RLX, profound peripheral vasodilation (12) likely predominates and
masks any pressor effects that could result from CNS mediated vasopressin secretion and sympathetic activation (Fig 2).

The increased Spinal-Fos colabeling seen in the current experiments following ica RLX is consistent with preliminary experiments in our laboratory that measured a sustained increase of 10±1% in lumbar sympathetic nerve activity following microinjection of RLX into the SFO of NP rats (3). These data along with the current immunohistochemical evidence support the idea that RLX increases sympathetic nerve activity through an SFO-PVN-IML pathway.

Although other stimuli have been shown to produce preferential activation of the VLP subregion of the PVN (86), in the current study, RLX activated the DP and VLP spinally projecting subregions of the PVN equally. It is possible that prolonged activation with RLX might have distinct effects on the VLP or DP subregions, as in the case of prolonged water-deprivation (86). However, while experiments using Fos expression as an index of neuronal activation are useful for short-term responses, they do not reveal long-term activation. Thus, Fos-IR is not an ideal tool to answer this question. This methodological concern is discussed below.

**AVP-IR & Spinal Neurons**
One unexpected result was the low percentage of AVP-Spinal colabeled neurons in the caudal areas of the PVN. This is in contrast to a study by Hallbeck & Blomqvist, where as many as 42% of spinally projecting PVN neurons expressed AVP mRNA (32). One possible reason for this discrepancy is that the former study utilized *in situ* hybridization to localize mRNA for AVP, while our study applied immunohistochemistry to localize neurons that contain AVP peptide. Thus the difference in our findings might partially be
a result of the differences in translational and transcriptional events in the neurons, which
do not always act in parallel (30). Another study by Kc et al. (40) using
immunohistochemistry, found that a substantial percentage of RVLM-projecting pre-
sympathetic neurons (15%) co-labeled with AVP. Since AVP mRNA is located in a
higher percentage of IML projecting neurons (32), and 14% (86) to 30% (79) of IML
projecting neurons in the PVN send collateral projections to the RVLM, we expected to
find significant co-labeling of AVP with IML-projecting neurons in the current study. In
addition to different projection sites (RVLM vs. IML), one possible reason for the
abundance of identified AVP-IR parvocellular projecting neurons in the Kc study (40)
compared to the low incidence of AVP-IR projecting neurons in our study could be
related to the AVP antibody used. Kc et al. (40) used a rabbit anti-vasopressin polyclonal
antibody which is conjugated to thyroglobulin (Chemicon International), while we used a
guinea pig anti-vasopressin polyclonal antibody. Although both antibodies have
demonstrated specificity for AVP containing cells (19, 61), because both are polyclonal
antibodies comprised of many monoclonal antibodies, the surfaces might differ slightly in
how AVP binds to them, thus creating different staining patterns for the same peptide. It
is also possible that the thyroglobulin-conjugated antibody’s greater size might be able to
bind more secondary antibody therefore enhancing its visualization. Finally, differences
in the immunohistochemical protocol could account for different immunofluorescent
findings. Kc et al. used a 4°C incubation, whereas all of the current incubations were
performed at room temperature. Moreover, their tissue was exposed to primary antibodies
sequentially, whereas tissue in the current study was exposed to all primary antibodies
simultaneously. Given the differences in immunohistochemistry protocols between Kc et
al. (40) and the current experiments, it is not possible to determine from these data if there might be a difference in relative AVP colabeling between RVLM-projecting and IML-projecting cells in the PVN.

**Un-phenotyped activated cells in the PVN**

It is important to note that the majority of Fos-IR cells in NP RLX treated rats in our study were otherwise unphenotyped. These unidentified neurons were located medial to the PM subregion, primarily in Level 1 (bregma-1.4mm). We noted that the activated regions are consistent with the distribution of corticotropin-releasing hormone (CRH) mRNA(49) and protein (immunofluorescent) distribution in the PVN (63). Although no work has been performed to evaluate the relationship between RLX and CRH in the PVN, several studies have explored the interaction between RLX and oxytocin and also between oxytocin and CRH. RLX activates oxytocin containing cells (52) and increases release of oxytocin (68). Oxytocin has been shown to potentiate the effects of central CRH (74) and ICV CRH has dose-dependent effects to increase oxytocin release (4).

Thus, it could be expected that RLX might activate central CRH secretion through an oxytocin-mediated mechanism and a portion of the unphenotyped Fos-IR cells seen in the current experiments are likely oxytocin and/or CRH containing cells. Anatomically, the oxytocinergic neurons are located around the edges of the PM region of the PVN anterior to the vasopressinergic neurons and dorsal to AVP neurons in the SON (92). A third possibility is that some of the unphenotyped Fos-IR cells were RVLM-projecting neurons. While some IML-projecting neurons in the PVN would have sent projections to the RVLM (14% (86) to 30% (78)), studies have shown that there are close to the same number(86) or as many as 2 (39) times as many RVLM projecting neurons as PVN-IML projecting neurons. Given the similar location of RVLM-projecting neurons and the
RLX-activated neurons that were otherwise unphenotyped, we might expect to see activated RVLM-projecting neurons if we were to perform the retrograde labeling from the RVLM. This possibility is further discussed in “Future directions”.

**Reduced effects of exogenous RLX in pregnant rats**

Studies examining the CNS effects of RLX in intact term pregnant rats are limited. Ward et al. (96) examined MAP, HR, and plasma AVP levels after an IV bolus injection of high doses of human RLX (0.1 and 2 mg/kg) on day 19 of pregnancy in conscious rats and found no differences between treatment and saline controls. Another study by Parry et al. (68) in anesthetized rats found that pressor responses to pRLX (5 µg, IV) were greatly attenuated by day 14 of pregnancy and remained low until after delivery. Although RLX induces AVP secretion throughout pregnancy, the RLX evoked AVP release was less in near-term pregnant rats (d19). Both of these studies in intact pregnant rats showed that the effects of exogenously administered RLX decreased as parturition approached. Following delivery however, when endogenous RLX levels plummet, the effect of exogenous RLX was restored (68). Consistent with these data, near term pregnant rats in our study did not show the small transient pressor effect or the tachycardia that was seen in NP rats during exogenous RLX infusion.

Regarding immunohistochemical results in the pregnant group of the current experiments, the overall distribution of AVP and spinally projecting cells in P rats was similar to NP rats. However, in response to RLX there was less activation in pregnant rats than in nonpregnant rats. RLX treatment did not activate spinally projecting cells in the PVN, and there was a nonsignificant trend for RLX to activate AVP magnocellular neurons in the PVN and SON of P rats. These data are consistent with the lack of
significant immediate (9 minute peak) changes in MAP seen in P rats, as there was likely little release of AVP or activation of cardiac pre-sympathetic neurons in the IML. Also, although there were fewer Fos-IR cells identified in P rats, similar to NP rats, the majority of Fos-IR cells in the PVN were unphenotyped.

Different from NP rats, in near-term P rats, RLX did not result in an increase in Fos-IR in the SFO. Thus, it is likely that the reduced activation of AVP neurons in the PVN and SON and the lack of activation of spinally projecting neurons in the PVN are related to the lack of activation of the SFO during acute infusions of RLX in near-term P rats. The current experiments did not directly test the mechanisms for this observation, but we considered several possibilities. Conrad and colleagues (11, 12, 51) demonstrated that the cellular pathways for peripheral vasodilation differ between chronic and acute exposure to RLX. It is possible that CNS pathways/mechanisms for effects of acute and chronic RLX also differ. Another possibility is that RXFP1 receptor expression in the SFO declines during gestation, as it does in myometrial tissue (94). If this were the case the effects of RLX would decrease as pregnancy progresses due to decreased receptor expression.

Although responses to exogenous RLX are attenuated by day 14 of pregnancy (68), if endogenous RLX is immunoneutralized during pregnancy, the normal cardiovascular adaptations that are present at day 14 of gestation are prevented (23). Thus, endogenous RLX is clearly contributing to cardiovascular changes at the same time that responses to exogenous RLX are attenuated. In regard to CNS adaptations, pregnant rats that have been ovariectomized at mid-term (to reduce endogenous RLX) and treated with estrogen and progesterone to maintain pregnancy, respond to acute RLX administration at near-
term (d19) with activation of neurons in the SFO, PVN, and SON (36). Taken together these studies indicate that endogenous RLX contributes to the adaptations of pregnancy and that pregnant animals respond to exogenous RLX if endogenous levels are low. Therefore, another possibility we considered is that in intact pregnant rats, RLX receptors at the SFO might be maximally occupied by endogenous RLX and that long-term activation of the SFO is mediating on-going excitation of AVP and spinally projecting neurons. In this case exogenous administration of RLX would have no further effect. Since Fos expression does not reveal long term activation, our experimental approach would not address this possibility.

**Technical Considerations**

One methodological consideration is the use of Fos-IR to evaluate the effects of RLX. While Fos is a well-established tool for examining neuronal activation, it is not without limitations (56). Fos is the protein product of the immediate early-gene, \( c-fos \), which is sensitive to anesthesia and environmental stimuli (56). Thus experiments need to be performed in conscious rats and conditions around the rat must be well controlled in order to avoid nonspecific \( c-fos \) expression. Regarding minimal effects of RLX in near-term pregnant rats, one possible explanation for the lack of Fos-IR colabeling in AVP and spinally projecting cells is that RLX receptors may be chronically activated by elevated endogenous plasma RLX levels during pregnancy. Expression of the early gene \( c-fos \) is a tool for examining responses to an acute stimulus and it would not reveal baseline differences between NP and P rats due to chronic neuronal activation by endogenous RLX. Since Fos expression is low in the absence of an acute stimulus and is expressed during neuronal excitation (56, 66, 71, 75), another consideration is that the use of \( c-fos \)
expression will not reveal if neuronal inhibition is occurring in response to a stimulus. Thus, for example, if the sympathoexcitatory actions of RLX were dependent on inhibition of neurons participating in a sympathoinhibitory pathway from the PVN, we would not be able to identify these neurons with the current experimental design.

An important feature of the current experiments is the use of a low sub-pressor dose of RLX administered intracarotid artery, to access the forebrain circulation. The dose of RLX used in this study was lower than typically used by others to study acute CNS effects of RLX. We infused RLX in conscious rats at a rate 1 µg/ hr for a total of 1.5 µg over 90 minutes. For their immunohistochemistry studies, also using conscious rats, McKinley and colleagues infused RLX (IV) at a rate of 25 µg/ hr for 1-1.5 hours (89, 90). As noted above, the amount of RLX given during our infusion was 1.5µg over 90 minutes, which would produce plasma levels well within the physiological range. Other studies using bolus IV injections (versus infusions) commonly used from 2µg (16) to 10µg (89). In fact, doses exceeding 10ug often have been used in exploring the central and cardiovascular effects of RLX (36, 90, 96, 98, 100). Although these doses rapidly induce tachycardia, AVP secretion, Fos-IR, a pressor response, and water drinking, the resulting plasma RLX levels may not be representative of physiological states. Therefore, Fos-IR in the current study was more likely the result of the direct action of the ica RLX, rather than the product of dramatic increases in MAP or other cardiovascular responses.

The route of RLX administration is another important contrast between previous CNS examinations of RLX and our own. Rather than administering RLX into the cerebral ventricular system (ICV) or the peripheral circulation (IV), we utilized a catheter in the carotid artery directed towards the forebrain circulation. Intracarotid administration
permitted the use of a lower dose given our proximity to the forebrain circulation and reduced possible effects in the periphery. In addition, ica administration provides a vascular route to the sensory circumventricular organs, to more closely approximate physiological exposure to the peptide. One consideration for ica administration is that our catheter was seated below the carotid sinus region and therefore infusion of RLX could have effects on carotid sinus baroreceptor s and/or chemoreceptors. It is unlikely that our low infusion rate (~16 ul/min) would produce a stimulus to carotid sinus baroreceptors. This is evidenced by the rise in HR, where a decrease in HR would be expected during baroreflex stimulation. In addition, both the RLX and saline infusions were given at the same rate, so that any minimal pressure or mechanical stimulation would have been identical between the control (SAL) and experimental (RLX) groups. In regard to possible activation of carotid body chemoreceptors, no RLX receptors have been identified in the carotid sinus or carotid body regions, so it is unlikely that carotid body chemoreceptors would have been activated by our infusions.

One concern regarding RLX administration in pregnant rats is the “biphasic” dose dependent effect of RLX described by Conrad et al., where the vascular beds that are affected may differ depending on the concentrations of RLX. In these cases, low doses of RLX may preferentially act in certain vascular beds (e.g., renal vasodilation), where at higher doses, effects on other regions could predominate (16). Since pregnant rats had high levels of endogenous circulating RLX prior to exogenous RLX infusion, the total concentration of RLX would be expected to be higher in pregnant compared to nonpregnant rats following exogenous RLX administration. If CNS effects of RLX are
also biphasic, then higher total plasma concentrations of RLX in pregnant rats might contribute to different effects compared to nonpregnant rats.

Another consideration also discussed above is that the mechanism for the effects of RLX might differ between acute and chronic exposure. For example, peripheral vasodilatory effects of RLX are mediated by different cellular pathways following chronic versus acute RLX administration (Fig 2) (12, 51). It is possible that CNS effects of RLX might also be mediated by different pathways after acute versus chronic exposure, as occurs in pregnancy. Because the duration of the infusion and recording in this study were 1.5 hours (also in part a limitation of Fos immunostaining), it is difficult to predict if the activity of RLX persists long term. Under the chronically elevated levels a pregnant animal experiences, the central actions of RLX exposure might be different, as demonstrated by Parry et al. with regard to the pressor effect and oxytocin/AVP release (68), or Vodstrcil et al. (94) with regard to decreased myometrial RXFP1 expression during gestation. A more thorough cross-sectional study examining potential changes in CNS RLX receptor expression is necessary to predict the contributions of CNS RLX to the adaptations of pregnancy.

**Remaining Questions and Future directions**

AngII has been known to play an important role in the central actions of RLX. Parry and Summerlee (69) demonstrated that ICV pretreatment with the angiotensin II peptide blocker, saralasin, eliminated pressor responses to ICV RLX and attenuated the pressor response to IV injections of RLX. In this same study, the combination of ICV angiotensin II blockade and an IV V₁ antagonist completely blocked the pressor response to IV RLX. These results support a role for AVP and AngII in the actions of central RLX and its
pressor effect. Both RLX and AngII rely, in part, on the release of AVP for their pressor effects (62, 67). There is ample evidence that AngII increases sympathetic nerve activity through a CNS mechanism (62), and indirect evidence that RLX may also result in sympathoexcitation (67). Preliminary experiments from our laboratory in which lumbar sympathetic nerve activity was recorded suggest that RLX, through an action at the SFO, increases sympathetic outflow (3). Moreover, electrophysiological studies have located single neurons in the SFO that are excited by both AngII and RLX (89). Additionally, the pressor response to ICV RLX is blocked in the presence of CNS angiotensin II antagonism (69). Yang et.al (100) also showed in conscious rats that both IV and ICV vasopressin V1 receptor blockade inhibited the pressor responses to ICV RLX in rats. Therefore, there is evidence that central effects of RLX are dependent on CNS AngII and possibly also CNS AVP.

Questions remain in exploring the actions of RLX in the central nervous system. For example, does increased Spinal-Fos colabeling translate to an increase in sympathetic nerve activity? Preliminary physiological data from our laboratory (3) show that microinjection of RLX into the SFO of anesthetized, female NP sinoaortic denervated rats increased lumbar sympathetic nerve activity by 10±1%. As above, we showed that the pressor effect of RLX in the SFO was completely reversed by intravenous administration of either an AVP V1 receptor antagonist (Manning Compound), or the Angiotensin II AT1 receptor blocker, L-158,809. However, in the presence of RLX, lumbar sympathetic nerve activity in our experiments was not decreased after blockade of either AngII or V1 receptors. Based on previous literature (28, 69), we expected that the effect of L-158,809 to block the pressor response to SFO RLX is due to blockade of CNS
AT₁ receptors. It is possible that the pressor response was mediated through a regional sympathetic nerve activity, other than the lumbar sympathetic nerve. AT₁ blockade therefore would block the pressor responses and other regional nerve activity, but not the lumbar sympathetic nerve activity. Alternately, CNS actions of RLX to increase sympathetic nerve activity may not be dependent on AVP or AngII alone and other CNS mechanisms could be involved.

Future experiments are necessary to evaluate a possible interdependence between CNS actions of angiotensin II, AVP and RLX. One such experiment might be a subpressor ICV dose of AngII in P and NP rats, and a subsequent ICA infusion of RLX. We would then measure lumbar sympathetic nerve activity, and changes in arterial pressure. We might expect that the CNS actions of RLX (increase in LSNA, MAP) would be elevated in the presence of AngII, and further potentiated in pregnant rats. This is in part because the CNS effects of AngII are potentiated in pregnancy (34), a state with elevated RLX. Moreover, CNS effects of RLX are partially mediated by AngII, since AngII antagonism partially blocks RLX induced secretion of AVP and oxytocin (28). This experiment might further help us understand the nature of the dependence of RLX on the central AngII system.

How might the central effects of RLX change over the course of pregnancy? As mentioned above, preliminary data in term-pregnant rats suggests that the pressor and sympathoexcitatory responses to AngII microinjection into the SFO are potentiated (34). It is possible this is due to altered AT₁ receptor expression in the SFO. A preliminary observation in one rat reported by Summerlee et al.(87) showed that AT₁ receptor expression increases from days 12-16 of pregnancy, and then decreases. Although it
appeared that AT$_1$ receptor expression declined between days 16-22 of pregnancy in the one rat studied, the possible contribution of changes in AT$_1$ receptor expression in the SFO to adaptations of pregnancy merits further study. Importantly, they also showed that when RLX is immunoneutralized in pregnant rats, AT$_1$ receptor expression in the SFO is nearly undetectable by Western blot. This suggests that RLX might be increasing the expression of AT$_1$ receptors in the SFO.

It is also important to consider the involvement of other brain regions involved in control of sympathetic outflow, namely the rostral ventrolateral medulla. The PVN has direct efferent projections to the IML which were evaluated in the current experiments. However, parvocellular neurons in the PVN also project to the RVLM to impact sympathetic outflow (15). The RVLM is under greater GABAergic inhibition in pregnancy (44), and yet, the RVLM appears to be a site mediating potentiated active sympathoexcitation from the SFO in pregnant rats (45). Thus, evaluating the effects of RLX on activation of RVLM projecting PVN neurons would be of great interest.

One experiment that would explore the involvement of the RVLM would include retrogradely labeling the RVLM-PVN projecting neurons instead of, or in addition to the IML-PVN projecting neurons in P and NP rats and reproducing the current studies. Given the role of the RVLM in modulating SNA, we might see an increase in Fos-IR through the PVN-RVLM pathway in the current study. CNS RLX activation of RVLM neurons is consistent with the current study’s evidence of activation of spinally-projecting neurons, as well as our previous physiological data showing that RLX in the SFO increases lumbar sympathetic nerve activity (3). Additional staining for phenylethanolamine- N-methyl -transferase (PNMT)—the enzyme responsible for converting norepinephrine to
epinephrine—would identify C1 catecholaminergic neurons in the RVLM, which project to the IML of the spinal cord. This particular immunostaining would further inform the nature of relaxin’s excitatory pathway, as the Parry et al. study demonstrated that blockade of α1 receptors with phentolamine partially blocked the pressor response (67). Consistent with these data, we would expect to see greater Fos-IR in PNMT neurons in the RVLM after CNS RLX treatment. This study could also then perform staining for RVLM-projecting neurons in PVN tissue and fill in some of the gaps in the knowledge of the unphenotyped neurons in the PVN that were activated in the current study.

Conclusions
Intracarotid RLX increased Fos-IR in the lateral margins of the SFO, consistent with activation of PVN projecting neurons in the SFO. The role of RLX in AVP secretion is well accepted. The current experiments provide quantitative data demonstrating that magnocellular AVP neurons in both the PVN and SON are activated by centrally directed RLX. Specifically, the percent of activated AVP neurons in each region was 21% and 25%, respectively, and therefore it appears that both PVN and SON magnocellular neurons contribute to increased AVP secretion due to RLX.

Less is known about the role of RLX on the control of sympathetic outflow. Our data demonstrate that 19% of IML projecting neurons in the PVN are activated by low dose ica RLX. These data support our hypothesis that non-pressor doses of blood-borne RLX activate spinally projecting neurons in the PVN, which would result in increased sympathetic outflow. In contrast to our hypothesis however, and different from responses to angiotensin II in the SFO, the response to centrally directed RLX was suppressed, rather than potentiated, in term-pregnant rats.
RLX receptor expression in the forebrain during pregnancy has not been examined, although further study of this and the interaction with the brain AngII system will be important. It is possible that the elevated endogenous RLX in term-pregnant rats might maximally activate RLX receptors in the SFO. Thus, there would be no further activation by exogenously administered RLX. Since c-fos expression is restricted to acute stimuli, our experimental approach did not allow us to evaluate baseline differences between NP and P rats. Future experiments using more long term indicators of activation such as Fos Related Antigen (20), or Fos-B (71) would allow us to evaluate the effects of chronic elevations of RLX in P rats over the course of pregnancy.

In summary, pregnancy is characterized by resetting of vasopressin secretion and increased baseline sympathetic nerve activity and it has been proposed that activation of the PVN might contribute to these adaptations. Although our experimental approach did not allow us to evaluate chronic neuronal activation in pregnant rats, we did demonstrate that acute administration of RLX to NP rats increased activation of both AVP containing and spinally projecting neurons in the PVN. Since relaxin secretion is elevated in pregnancy, our data are consistent with a role for endogenous RLX in the resetting of vasopressin secretion and increased baseline sympathetic nerve activity seen in pregnant animals.
FIGURES
Fig. 1: COMPARATIVE distribution of RLX family receptors (A, B), $[^{35}P]$-RLX binding sites (B, B'), RXFP3 mRNA (C, D, E), and $[^{125}I]$-R3/I5 binding sites (C', D'). RXFP1 mRNA has been shown in magnocellular neurons in the PVN and SON (SO), along with layers of the cerebral cortex (1, 5, 6b), the SFO, and the amygdala (BLA, BMA, CEA). RXFP3 mRNA has been found in the bed nucleus of the stria terminalis (BNST), lateral preoptic nucleus (LPO) and dentate gyrus (DG). Many of these regions also contained radiolabeled RLX3 binding sites (C', D'). Abbreviations: Motor cortex (MO), Fields of the hippocampus (CA1-3), paraventricular thalamic nucleus (PV, PVA), Reuniens thalamic nucleus (Rem), Centromedial thalamic nucleus (CM), Peripeduncular nucleus (PP), periaqueductal gray (PAG), superior colliculus (SC), Lateral septum (LS).

Figures adapted from Ma et al., 2006, 2007.
Fig. 2: TIMELINE of pressor and vasodilatory responses to exogenous RLX. CNS actions of RLX might persist during chronic exposure, increasing baseline SNA, and higher than expected AVP. These effects are not reflected in MAP because of the profound vasodilation. Adapted from Conrad, 2010; Callander, 2010.
Fig. 3: SCHEMATIC outlining basic forebrain connectivity and projections to the hindbrain and spinal cord that might be involved in CNS actions of RLX.
Fig. 4: BASELINE body weight, MAP, and HR. Compared to nonpregnant rats (NP, n=14), pregnant rats (P, n=10) had a higher baseline weight, lower MAP, and higher HR (* = p ≤ 0.05; Student’s t-test).
**Fig. 5:** PHOTOMICROGRAPHS showing distribution of AVP-IR and spinally projecting cells in the PVN: Schematics are adapted from Paxinos and Watson 6th ed. **A:** Level 1 is approximately bregma -1.4mm and contains a cluster of AVP-IR cells (pseudocolored green) in the posterior magnocellular region (PM). **B:** Level 2 is approximately bregma -1.6mm and has a characteristic PM cluster, with spinally projecting neurons (pseudocolored white) in the dorsal (DP) and ventrolateral parvocellular (VLP) subregions. **C:** Level 3 is approximately bregma -1.8mm and consists primarily of spinally projecting neurons, DP and VLP were often indistinguishable at this level. **D:** Level 4 is approximately bregma -2.0mm and contains spinally projecting neurons (Spinal) extending lateral and dorsal to the third ventricle (3V), with a small cluster of AVP-IR cells often found dorsal to the fornix (f). (Scale bar = 500μm)
Fig. 6: RLX activates AVP-IR and spinally projecting cells in the PVN of NP rat. A. Panel i: Level 2 (bregma – 1.6mm) of a RLX treated rat shows a high number of Fos-IR cells within and medial to the PM group. (Scale bar = 500µm; 3V = third ventricle) Panel ii: Level 2 of a SAL treated rat shows fewer Fos-IR cells and less colabelling. B,C: Pseudocolored images of AVP-IR, Fos-IR, and spinally projecting cells from RLX treated rats. B: Level 2--Panel i: AVP-IR cells (green). ii: Fos-IR cells with characteristic dark nucleolar region (red). iii: Merged image. Arrows indicate AVP-Fos colabelling. C. Level 3--Panel i: Shows spinally projecting cells with cytoplasmic staining (white) outside of the nuclear region. ii: Fos-IR cells. iii: Merged image showing Spinal and Fos cells. Arrows indicate examples of colabelled cells. (Scale bar = 100µm)
Fig. 7: ROSTRAL-caudal distribution of activated vasopressin (AVP-Fos) and spinally projecting (Spinal-Fos) cells in the PVN of nonpregnant rats: 2-way repeated measures ANOVA found effects of treatment and level for both phenotypes. 

A. AVP-Fos cells. Level 2 was higher than levels 1, 3, and 4; RLX > SAL. 

B. Spinal-Fos cells. Level 3 is the highest, while levels 2 and 4 are both higher than level 1; RLX > SAL. § = main effect of Treatment; † = main effect of Level. (p ≤ 0.05)
Fig. 8: RELAXIN activates spinally projecting cells in DP and VLP subregions of the PVN in NP rats: A. Absolute counts of Spinal-Fos cells in DP and VLP regions were higher in RLX treated rats. B. RLX activated more cells in both regions as a percentage of total spinally projecting cells. There was no effect of Subregion (DP or VLP). § = effect of Treatment. (p ≤ 0.05)
**Fig. 9: RLX activates magnocellular neurons in the SON of nonpregnant rats:**

**A:** Photomicrograph of the SON of a RLX treated NP rat. AVP-IR cells are pseudocolored green, and Fos-IR cells are pseudocolored red. Arrows indicate AVP-Fos colabeled cells. Opt = optic tract. Scale bar = 200um.

**B:** Activation of AVP cells in the SON of NP and P rats: 2 way ANOVA revealed main effects of treatment and group, such that RLX treated rats had a higher percentage of activated AVP-IR cells compared to SAL, and the NP group had a higher percentage of activated AVP-IR cells than the P group. Student’s t-tests in the SON comparing RLX to SAL within Group revealed an increase in the % activated AVP cells in the nonpregnant group. † = main effect of Group, § = main effect of Treatment.
Fig. 10: PERCENTAGE of activated AVP-IR and spinally projecting cells in the PVN of nonpregnant and pregnant rats. **A:** Percentage of total AVP-IR cells that were activated (AVP-Fos/AVP * 100). An interaction was present between group and treatment such that NP-RLX treated rats had a higher percentage of activated AVP cells compared to NP-SAL, P-SAL, and P-RLX rats. **B:** Percentage of activated spinally projecting cells (Spinal-Fos/Spinal * 100). There was an interaction such that NP-RLX treated rats had a higher percentage of activated cells compared to NP-SAL, P-SAL and P-RLX rats. ‡ interaction; * = different from SAL within NP group; # = different from P within RLX treatment (p ≤ 0.05).
Fig. 11: SUBREGIONAL activation of spinally projecting cells in DP and VLP subregions of pregnant rats: A. Absolute counts of Spinal-Fos cells in DP and VLP subregions. Counts were higher in VLP subregion. B. Counts shown in A expressed as a percentage. Percent activation was higher in VLP subregion, but counts were overall low. † = main effect of Region (VLP or DP).
Fig. 12: ACTIVATION of AVP cells in the SON of NP and P rats: 2 way ANOVA revealed main effects of treatment and group, such that RLX treated rats had a higher percentage of activated AVP-IR cells compared to SAL, and the NP group had a higher percentage of activated AVP-IR cells than the P group. Student’s t-tests in the SON comparing RLX to SAL within Group revealed an increase in the % activated AVP cells in the nonpregnant group. † = main effect of Group, § = main effect of Treatment.
Fig. 13: RELAXIN activates magnocellular AVP cells in the hypothalamus. A. Percent of vasopressin containing cells also expressing Fos in the PM region of the PVN (A) and the SON (B) in nonpregnant and pregnant rats. In the PVN magnocellular neurons, there was a main effect of treatment (RLX > SAL). B. In the SON there were main effects of Group (NP > P) and Treatment (RLX > SAL).
Fig. 14: RELAXIN activates cells in the SFO of NP but not P rats. Representative photomicrographs of SFO from RLX and SAL treated NP and P rats, **A.** NP SAL treated rat. **B.** NP RLX treated rat with characteristic activation of the peripheral edges of the SFO lateral and dorsal to the third ventricle **C.** Pregnant SAL treated rat. **D.** Pregnant RLX treated rat. Fos-IR in SFO’s from SAL treated P and NP and RLX treated P rats was minimal. D3V: Dorsal 3\textsuperscript{rd} Ventricle. Scale bar = 200um. (Approximately -1.3 to -1.4mm caudal to bregma).
Table 1: RLX family peptides in the rat CNS. In humans and higher primates, the equivalent gene is RLN2; in humans and higher primates, the equivalent protein is relaxin-2. While RLX peptide and receptors have been located within the brain parenchyma, the focus of the current studies is on the effects of blood-borne RLX on the CNS. Adapted from Callander & Bathgate 2010; Ma et al., 2006, 2007.

<table>
<thead>
<tr>
<th>PEPTIDE</th>
<th>Gene</th>
<th>Protein</th>
<th>Example brain regions (mRNA, mRNA)</th>
<th>Expression in brain regions (mRNA)</th>
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<tr>
<td>RLN1</td>
<td>Relaxin-1, (Relaxin, or “RLX”)</td>
<td>Amygdala, SFO, SON, PVT, Cerebral cortex (L5, L6), pineal gland</td>
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<td>Relaxin-2</td>
<td>Cerebral cortex (L2, 3, 5, 6), piriform cortex, arcuate nucleus</td>
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<tr>
<td>RLN3</td>
<td>Relaxin-3</td>
<td>Lateral preoptic area, posterior hypothalamic area, lateral hypothalamic area, periaqueductal grey, dentate gyrus, nucleus incertus</td>
<td>Yes</td>
<td></td>
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Table 2: CHANGES in MAP and HR due to ICA SAL and RLX infusions. Changes ± SEM in MAP and HR are indicated for peak (~9 minutes) and 90 minute average. In NP rats only, peak increases in MAP were greater in RLX compared to SAL treated rats. Tachycardia, but not the pressor effect was maintained over the 90 min RLX infusion. RLX had no significant effects on MAP or HR in P rats. * Student’s T-test, ∆ RLX > ∆ SAL; (p≤0.05).

<table>
<thead>
<tr>
<th></th>
<th>Peak ∆ MAP (mmHg)</th>
<th>90 min ∆ MAP (mmHg)</th>
<th>Peak ∆ HR (beats/min)</th>
<th>90 min ∆ HR (beats/min)</th>
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<tr>
<td>SAL</td>
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<td>4 ± 2</td>
<td>16 ± 4</td>
<td>12 ± 13</td>
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<td></td>
<td>n=8</td>
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</tr>
<tr>
<td>RLX</td>
<td>13 ± 1*</td>
<td>7 ± 2</td>
<td>42 ± 12*</td>
<td>50 ± 5*</td>
</tr>
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<td>SAL</td>
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<td>-7 ± 2</td>
<td>6 ± 8</td>
<td>9 ± 10</td>
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<td>RLX</td>
<td>2 ± 4</td>
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Table 3: MAP and HR before and after ICA SAL and RLX infusions: Results of 2 Way ANOVA comparisons within the NP and P Groups are indicated. Baseline values prior to the treatment infusion (before) were compared to peak values (~ 9 minutes with RLX treatment) or to the average value over the course of the 90 min infusion (after). Changes from baseline ± SEM are shown.

<table>
<thead>
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<th></th>
<th>Peak MAP Response (mmHg)</th>
<th>90 min MAP Response (mmHg)</th>
<th>Peak HR Response (beats/min)</th>
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<tr>
<td><strong>Treatment</strong></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>RLX &gt; SAL</td>
<td></td>
<td></td>
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</tr>
<tr>
<td><strong>Time</strong></td>
<td></td>
<td>After ~ 9 minutes</td>
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<td></td>
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<tr>
<td>After &gt; Before</td>
<td></td>
<td></td>
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<tr>
<td><strong>SAL</strong></td>
<td>Before 110 ± 2.1</td>
<td>After 111 ± 3</td>
<td>Before 392 ± 12</td>
<td>After 404 ± 11</td>
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<tr>
<td><strong>RLX</strong></td>
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<td>Before 368 ± 6</td>
<td>After 437 ± 18</td>
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<td><strong>Treatment</strong></td>
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<tr>
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<tr>
<td><strong>SAL</strong></td>
<td>Before 90 ± 4.8</td>
<td>After 85 ± 8</td>
<td>Before 420±13</td>
<td>After 429±5</td>
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<tr>
<td><strong>RLX</strong></td>
<td>Before 81±3</td>
<td>After 83±4</td>
<td>Before 415±15</td>
<td>After 423±9</td>
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Table 4: EFFECTS of relaxin on PVN cell counts in nonpregnant rats: Mean cell counts ± SEM at four levels of the PVN are provided. Results for 2-way ANOVA for repeated measures (Level) are indicated at top (Treatment and Level comparisons). SAL (n=9); RLX (n=8).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>AVP</th>
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<th>Fos</th>
<th>AVP-Fos</th>
<th>Spinal-Fos</th>
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<tbody>
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<tr>
<td>2 &gt; 1 &gt; 3, 4</td>
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<td>2 &gt; 3 &gt; 4</td>
<td>2 &gt; 1, 3, 4</td>
<td>3 &gt; 4, 2 &gt; 1</td>
<td>2, 1 &gt; 3 &gt; 4</td>
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</tr>
<tr>
<td>Level 1</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>SAL</td>
<td>42 ± 8</td>
<td>13 ± 2</td>
<td>52 ± 10</td>
<td>1 ± 1</td>
<td>0.7 ± 0.4</td>
<td>50 ± 10</td>
</tr>
<tr>
<td>RLX</td>
<td>27 ± 7</td>
<td>15 ± 2</td>
<td>98 ± 29</td>
<td>4 ± 1</td>
<td>3 ± 0</td>
<td>91 ± 29</td>
</tr>
<tr>
<td>Level 2</td>
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<td></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SAL</td>
<td>103 ± 7</td>
<td>32 ± 5</td>
<td>45 ± 12</td>
<td>2 ± 1</td>
<td>2 ± 0</td>
<td>41 ± 12</td>
</tr>
<tr>
<td>RLX</td>
<td>98 ± 10</td>
<td>37 ± 7</td>
<td>180 ± 53</td>
<td>26 ± 8</td>
<td>7 ± 1</td>
<td>147 ± 46</td>
</tr>
<tr>
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<td></td>
<td></td>
</tr>
<tr>
<td>SAL</td>
<td>16 ± 5</td>
<td>77 ± 8</td>
<td>38 ± 6</td>
<td>0.2 ± 0.1</td>
<td>6 ± 2</td>
<td>32 ± 6</td>
</tr>
<tr>
<td>RLX</td>
<td>17 ± 5</td>
<td>89 ± 6</td>
<td>70 ± 14</td>
<td>4 ± 2</td>
<td>18 ± 3</td>
<td>48 ± 13</td>
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<tr>
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<td></td>
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</tr>
<tr>
<td>SAL</td>
<td>8 ± 2</td>
<td>62 ± 10</td>
<td>15 ± 3</td>
<td>0 ± 0</td>
<td>3 ± 1</td>
<td>12 ± 3</td>
</tr>
<tr>
<td>RLX</td>
<td>12 ± 2</td>
<td>67 ± 7</td>
<td>37 ± 4</td>
<td>2 ± 1</td>
<td>11 ± 3</td>
<td>26 ± 3</td>
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</table>
Table 5: PERCENTAGE activated cells in the PVN of nonpregnant rats: Cell counts expressed as percentage of a given phenotype in the PVN of NP rats. Results of 2 Way ANOVA for repeated measures (Level) are indicated. Mean ± SEM are shown. SAL (Saline, n=9); RLX (Relaxin, n=8).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>%AVP- Fos/AVP</th>
<th>%Spinal- Fos/Spinal</th>
<th>%Fos only</th>
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<tbody>
<tr>
<td>Level</td>
<td></td>
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<td></td>
</tr>
<tr>
<td>2&gt; 4 (P=0.055)</td>
<td>RLX &gt; SAL</td>
<td>RLX &gt; SAL</td>
<td>SAL &gt; RLX</td>
</tr>
<tr>
<td>Level 1</td>
<td>SAL</td>
<td>3 ± 2</td>
<td>3 ± 2</td>
</tr>
<tr>
<td></td>
<td>RLX</td>
<td>18 ± 6</td>
<td>20 ± 3</td>
</tr>
<tr>
<td>Level 2</td>
<td>SAL</td>
<td>2 ± 1</td>
<td>7 ± 1</td>
</tr>
<tr>
<td></td>
<td>RLX</td>
<td>24 ± 6</td>
<td>22 ± 4</td>
</tr>
<tr>
<td>Level 3</td>
<td>SAL</td>
<td>2 ± 2</td>
<td>8 ± 2</td>
</tr>
<tr>
<td></td>
<td>RLX</td>
<td>22 ± 6</td>
<td>20 ± 3</td>
</tr>
<tr>
<td>Level 4</td>
<td>SAL</td>
<td>0 ± 0</td>
<td>4 ± 1</td>
</tr>
<tr>
<td></td>
<td>RLX</td>
<td>12 ± 3</td>
<td>15 ± 3</td>
</tr>
</tbody>
</table>
Table 6: EFFECTS of relaxin on PVN cell counts in pregnant rats: Mean cell counts ± SEM at four levels of the PVN are provided. Results for 2-way ANOVA for repeated measures (Level) are indicated at top (Treatment and Level comparisons). SAL (n=6); RLX (n=6).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Level 1</th>
<th>Level 2</th>
<th>Level 3</th>
<th>Level 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>SAL</td>
<td>29 ± 3</td>
<td>36 ± 7</td>
<td>2 ± 1</td>
<td>8 ± 3</td>
</tr>
<tr>
<td>RLX</td>
<td>2 ± 1</td>
<td>1 ± 1</td>
<td>2 ± 1</td>
<td>2 ± 1</td>
</tr>
<tr>
<td>SAL</td>
<td>8 ± 3</td>
<td>1 ± 1</td>
<td>2 ± 1</td>
<td>2 ± 1</td>
</tr>
<tr>
<td>RLX</td>
<td>36 ± 7</td>
<td>2 ± 1</td>
<td>2 ± 1</td>
<td>2 ± 1</td>
</tr>
</tbody>
</table>

Table 6
Table 7: POSTERIOR magnocellular (PM) cell counts in the PVN of NP and P rats: Results of 2 Way ANOVA for PM subregion in NP and P rats are shown. * Different from SAL within NP group; # different from P within RLX treatment (p ≤ 0.05). Mean ± SEM are shown. NP, SAL (n=9); RLX (n=8). P, SAL (n=6); RLX (n=5).

<table>
<thead>
<tr>
<th>Group</th>
<th>AVP</th>
<th>Fos</th>
<th>AVP-Fos</th>
<th>%AVP-Fos/AVP</th>
<th>Fos Only</th>
<th>%Fos Only</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatment</td>
<td>RLX: NP &gt; P</td>
<td>NP &gt; P</td>
<td>NP &gt; P (p=0.139)</td>
<td>RLX: NP &gt; P</td>
<td>---</td>
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<tr>
<td>---</td>
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<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>Nonpregnant</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>SAL</td>
<td>120 ± 11</td>
<td>21 ± 5</td>
<td>2 ± 1</td>
<td>2 ± 1</td>
<td>19 ± 5</td>
<td>91 ± 3</td>
</tr>
<tr>
<td>RLX</td>
<td>109 ± 16</td>
<td>108 ± 34 *#</td>
<td>25 ± 6</td>
<td>22 ± 5</td>
<td>83 ± 28*#</td>
<td>74 ± 5</td>
</tr>
<tr>
<td>Pregnant</td>
<td>---</td>
<td>---</td>
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<td>---</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>SAL</td>
<td>94 ± 8</td>
<td>14 ± 6</td>
<td>1 ± 1</td>
<td>1 ± 1</td>
<td>12 ± 5</td>
<td>87 ± 8</td>
</tr>
<tr>
<td>RLX</td>
<td>92 ± 12</td>
<td>19 ± 10</td>
<td>7 ± 4</td>
<td>7 ± 5</td>
<td>12 ± 6</td>
<td>67 ± 10</td>
</tr>
</tbody>
</table>
Table 8: TOTAL cell counts for PVN in NP and P groups: Mean cell counts ± SEM of the sum of all four levels of the PVN. Results of 2-way ANOVA for repeated measures are indicated at top (Group and Treatment comparisons). * 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; NP-RLX: n=6.

<table>
<thead>
<tr>
<th></th>
<th>AVP</th>
<th>Spinal</th>
<th>Fos</th>
<th>AVP-Fos</th>
<th>Spinal-Fos</th>
<th>Fos Only</th>
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</thead>
<tbody>
<tr>
<td><strong>Group</strong></td>
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<td></td>
<td></td>
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</tr>
<tr>
<td><strong>Treatment</strong></td>
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<td></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>---</td>
<td>---</td>
<td>---</td>
<td>NP: RLX &gt; SAL</td>
<td>NP: RLX &gt; SAL</td>
<td>NP: RLX &gt; SAL</td>
<td>NP: RLX &gt; SAL</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Nonpregnant</td>
<td>SAL</td>
<td>169 ± 11</td>
<td>183 ± 20</td>
<td>151 ± 22</td>
<td>4 ± 1</td>
<td>12 ± 2</td>
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<tr>
<td></td>
<td>RLX</td>
<td>154 ± 17</td>
<td>207 ± 6</td>
<td>385 ± 82 *#</td>
<td>36 ± 10 *#</td>
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<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Pregnant</td>
<td>SAL</td>
<td>118 ± 11</td>
<td>175 ± 10</td>
<td>133 ± 8</td>
<td>1 ± 1</td>
<td>11 ± 1</td>
</tr>
<tr>
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<td>RLX</td>
<td>115 ± 14</td>
<td>200 ± 13</td>
<td>118 ± 31</td>
<td>8 ± 4</td>
<td>12 ± 4</td>
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</table>
Table 9: PERCENTAGES of total activated cells of a given phenotype in the PVN of NP & P rats. Results of 2 Way ANOVA comparing the total percentages of PVN counts in NP and P rats are indicated. There was a trend for a main effect of Group in %Fos Only cells where P > NP. * Different from SAL within NP group; # Different from P within RLX treatment (p ≤ 0.05). Changes ± SEM are shown. NP-SAL (n=9); NP-RLX (n=8). P-SAL (n=6); P-RLX (n=5).

<table>
<thead>
<tr>
<th></th>
<th>%AVP-Fos/AVP</th>
<th>%Spinal-Fos/Spinal</th>
<th>%Fos Only</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Group</strong></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>RLX: NP &gt; P</td>
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<td></td>
<td>P &gt; NP (p=0.08)</td>
</tr>
<tr>
<td><strong>Treatment</strong></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>NP: RLX &gt; SAL</td>
<td></td>
<td></td>
<td>SAL &gt; RLX</td>
</tr>
<tr>
<td><strong>Total Nonpregnant</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SAL</td>
<td>2 ± 1</td>
<td>7 ± 1</td>
<td>88 ± 3</td>
</tr>
<tr>
<td>RLX</td>
<td>21 ± 5*#</td>
<td>19 ± 2*#</td>
<td>77 ± 3*</td>
</tr>
<tr>
<td><strong>Total Pregant</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SAL</td>
<td>1 ± 1</td>
<td>6 ± 1</td>
<td>90 ± 1</td>
</tr>
<tr>
<td>RLX</td>
<td>6 ± 4</td>
<td>6 ± 2</td>
<td>84 ± 3</td>
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</table>
Table 10: CELL counts in the SON of NP and P rats. Results of 2 Way ANOVA comparing cell counts in SON neurons of NP and P are indicated. NP, SAL (n=9); RLX (n=8). P, SAL (n=6); RLX (n=5).
Additional Figure 1: CHANGES in MAP and HR in SAL and RLX treated rats: A: Change in MAP in nonpregnant and pregnant rats treated with SAL and RLX after 90 min infusion. Although over time MAP tended to increase in NP rats and decrease in P rats, within each group there was no difference in MAP between SAL and RLX treated rats. B: Change in HR after 90 minute infusion of SAL or RLX in nonpregnant and pregnant rats. Infusion of RLX produced a significant increase in HR in nonpregnant rats, while SAL and treatments in pregnant rats were not significant. # = Different from P within treatment; *= different from SAL within group; ‡ = interaction (p ≤ 0.05).
Additional Figure 2: BAR charts representing cell counts by level from NP and P rats. Data is also available in tables 2 and 3. These graphs highlight the similar overall distribution of AVP and Spinal-IR cells between NP & P rats and the contrast in Fos-IR between SAL and RLX treated rats in the NP group. † = Main effect of Level; § = main effect of Treatment (p ≤ 0.05).
Additional Table 1: PERCENTAGE activated cells in the PVN—pregnant. Results of 2 Way ANOVA for repeated measures (Level) comparing the percentages (by level) of colabeled or unphenotyped cells in pregnant rats are indicated. SAL (Saline, n=6); RLX (Relaxin, n=6).

<table>
<thead>
<tr>
<th>Treatment</th>
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<th>%Spinal-Fos/Spinal</th>
<th>%Fos Only</th>
</tr>
</thead>
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<td>Level</td>
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<td>2, 3 &gt; 1</td>
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</tr>
<tr>
<td>Level 1</td>
<td>Sal</td>
<td>2 ± 2</td>
<td>4 ± 3</td>
</tr>
<tr>
<td></td>
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<td>11 ± 2</td>
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<td></td>
<td>RLX</td>
<td>9 ± 6</td>
<td>7 ± 3</td>
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<tr>
<td>Level 3</td>
<td>Sal</td>
<td>1 ± 1</td>
<td>8 ± 2</td>
</tr>
<tr>
<td></td>
<td>RLX</td>
<td>6 ± 3</td>
<td>8 ± 3</td>
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<tr>
<td>Level 4</td>
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<tr>
<td></td>
<td>RLX</td>
<td>0 ± 0</td>
<td>4 ± 1</td>
</tr>
</tbody>
</table>
WORKS CITED


