


1998

The central interaction of relaxin with oxytocin

Patricia Ann Heine
Iowa State University

Follow this and additional works at: <https://lib.dr.iastate.edu/rtd>

 Part of the [Animal Sciences Commons](#), [Neuroscience and Neurobiology Commons](#), [Neurosciences Commons](#), [Physiology Commons](#), and the [Veterinary Physiology Commons](#)

Recommended Citation

Heine, Patricia Ann, "The central interaction of relaxin with oxytocin " (1998). *Retrospective Theses and Dissertations*. 11856.
<https://lib.dr.iastate.edu/rtd/11856>

This Dissertation is brought to you for free and open access by the Iowa State University Capstones, Theses and Dissertations at Iowa State University Digital Repository. It has been accepted for inclusion in Retrospective Theses and Dissertations by an authorized administrator of Iowa State University Digital Repository. For more information, please contact digirep@iastate.edu.

INFORMATION TO USERS

This manuscript has been reproduced from the microfilm master. UMI films the text directly from the original or copy submitted. Thus, some thesis and dissertation copies are in typewriter face, while others may be from any type of computer printer.

The quality of this reproduction is dependent upon the quality of the copy submitted. Broken or indistinct print, colored or poor quality illustrations and photographs, print bleedthrough, substandard margins, and improper alignment can adversely affect reproduction.

In the unlikely event that the author did not send UMI a complete manuscript and there are missing pages, these will be noted. Also, if unauthorized copyright material had to be removed, a note will indicate the deletion.

Oversize materials (e.g., maps, drawings, charts) are reproduced by sectioning the original, beginning at the upper left-hand corner and continuing from left to right in equal sections with small overlaps. Each original is also photographed in one exposure and is included in reduced form at the back of the book.

Photographs included in the original manuscript have been reproduced xerographically in this copy. Higher quality 6" x 9" black and white photographic prints are available for any photographs or illustrations appearing in this copy for an additional charge. Contact UMI directly to order.

UMI

A Bell & Howell Information Company
300 North Zeeb Road, Ann Arbor MI 48106-1346 USA
313/761-4700 800/521-0600

NOTE TO USERS

The original manuscript received by UMI contains pages with indistinct and slanted print. Pages were microfilmed as received.

This reproduction is the best copy available

UMI

The central interaction of relaxin with oxytocin

by

Patricia Ann Heine

A dissertation submitted to the graduate faculty
in partial fulfillment of the requirements for the degree of
DOCTOR OF PHILOSOPHY

Co-majors: Neuroscience; Veterinary Anatomy

Major professors: Lloyd L. Anderson and Carol D. Jacobson

Iowa State University

Ames, Iowa

1998

UMI Number: 9841049

UMI Microform 9841049
Copyright 1998, by UMI Company. All rights reserved.

**This microform edition is protected against unauthorized
copying under Title 17, United States Code.**

UMI
300 North Zeeb Road
Ann Arbor, MI 48103

Graduate College
Iowa State University

This is to certify that the Doctoral dissertation of
Patricia Ann Heine
has met the dissertation requirements of Iowa State University

Signature was redacted for privacy.

~~Committee Member~~

Signature was redacted for privacy.

~~Committee Member~~

Signature was redacted for privacy.

~~Committee Member~~

Signature was redacted for privacy.

~~Co-major Professor~~

Signature was redacted for privacy.

~~Co-major Professor~~

Signature was redacted for privacy.

~~For the Co-major Program~~

Signature was redacted for privacy.

~~For the Co-major Program~~

Signature was redacted for privacy.

~~For the Graduate College~~

DEDICATION

This dissertation is dedicated to my dad, who instilled in me the desire to learn, my husband, who changed his life goal to stay in Ames with me following vet school as I pursued my doctorate of philosophy, and my three daughters who have given me inspiration *in utero* and beyond, not only to delve into the field of reproductive neuroendocrinology, but to cherish every moment no matter in which venue of life I am participating. I love you all very dearly.

TABLE OF CONTENTS

CHAPTER 1. GENERAL INTRODUCTION	1
Introduction	1
Dissertation organization	12
CHAPTER 2. THE EFFECT OF RELAXIN ON OXYTOCIN AND PROLACTIN CONCENTRATIONS DURING PREGNANCY IN THE GILT	13
ABSTRACT	13
INTRODUCTION	14
MATERIALS AND METHODS	15
RESULTS	21
DISCUSSION	23
ACKNOWLEDGMENTS	27
REFERENCES	27
CHAPTER 3. RELAXIN-INDUCED EXPRESSION OF FOS IN THE FOREBRAIN OF THE LATE PREGNANT RAT	40
ABSTRACT	40
INTRODUCTION	41
MATERIALS AND METHODS	44
RESULTS	49
DISCUSSION	51
ACKNOWLEDGMENTS	55
REFERENCES	56
CHAPTER 4. EFFECT OF RELAXIN ON THE TRANSCRIPTION OF OXYTOCIN IN THE MAGNOCELLULAR HYPOTHALAMUS OF THE PREGNANT RAT	68
ABSTRACT	68
INTRODUCTION	69
MATERIALS AND METHODS	70
RESULTS	78
DISCUSSION	79
ACKNOWLEDGMENTS	82
REFERENCES	82
CHAPTER 5. GENERAL CONCLUSIONS	99
General summary	99
General discussion	100

REFERENCES	109
ACKNOWLEDGMENTS	120

ABSTRACT

This dissertation is an effort to further define the central relationship of oxytocin (OT) and relaxin. The enclosed experiments have used rats and pigs to study this relationship.

The first study looked at the effect of relaxin on the secretion of oxytocin and prolactin in pregnant gilts. Intravenous administration of relaxin appeared to mildly increase the circulating oxytocin and prolactin in pigs at midgestation, but had no effect in late gestational gilts. However, removal of endogenous relaxin in late gestational gilts did cause a large secretion of oxytocin and a mild release of prolactin, followed by a rapid return to baseline values for both hormones. This may indicate that relaxin acts to prevent the premature release of oxytocin in late gestation.

The second study looked at the effect of relaxin on the activity of oxytocinergic cells in the hypothalamus of late pregnant rats following intravenous administration of relaxin. Cellular activation was visualized using the immunohistochemical labeling of Fos as a marker. In animals which received relaxin, Fos-like immunoreactivity was increased over that in animals which received vehicle. The increased Fos-like immunoreactivity was found in the supraoptic nucleus (SON), paraventricular nucleus (PVN) and subfornical organ. The Fos-like immunoreactivity in the SON and PVN was co-localized in cells exhibiting oxytocin-like immunoreactivity. This suggests that relaxin is changing the cellular activity within oxytocinergic cells.

The third study goes on to determine if the activity seen following relaxin administration within oxytocinergic cells of late pregnant rats, is an increase in the synthesis of oxytocin. *In situ* hybridization was used to compare levels of oxytocin mRNA within the SON and PVN between rats exposed to endogenous or exogenous relaxin and those with no circulating relaxin. No differences were seen in the amount of oxytocin mRNA present as a result of the presence or absence of relaxin in the circulation.

Collectively, the results from these studies indicate that relaxin affects the release of oxytocin during pregnancy. Relaxin probably does this by changing activity at the level of the cell body, but the rate of synthesis of oxytocin is unchanged.

CHAPTER 1 GENERAL INTRODUCTION

Introduction

The phenomenon of parturition is well studied and yet is not fully understood. The interaction of hormones culminating in birth has become less of a mystery over the last half century; however, the search goes on for a complete explanation to this awesome event.

It is well known that oxytocin (OT) plays an important role in the expulsion of the fetus. Its release during parturition is self-controlled through a positive feed back loop (Falke, 1989). But, what prevents it from being released prematurely? Endogenous opioids appear to inhibit the release of OT (Lindow et al., 1996; Douglas et al., 1993), but there may be another player. Relaxin, a polypeptide hormone whose primary role in parturition is to prepare the reproductive tract for fetal expulsion by remodeling its collagen, has been found to interact centrally with oxytocin. This dissertation focuses on gaining a more complete understanding of the interrelationship between OT and relaxin.

Oxytocin is a small peptide made primarily in the brain. It is also made in the placenta and uterus (Lefebvre et al., 1992) of the rat close to term, and in the cow, OT is found in the corpora lutea (CL) during pregnancy (Guldenaar et al., 1984); however, this discussion is limited to its location in the brain. Oxytocin is translated with a co-expressed protein, neurophysin (Morris, 1976), in magnocellular neurons of the supraoptic (SON) and paraventricular nuclei (PVN) within the hypothalamus. Following post-translational processing, oxytocin is comprised of 9 amino acid residues and a disulfide bond. In the

neuron cell body, it is then packaged in neurosecretory vesicles for axonal transport to the neurohypophysis (for review see Gainer and Wray, 1994). From there it can be released into the circulation following neuronal depolarization.

Oxytocin moderates several body functions, the most notable of which are involved in reproductive physiology. The putative oxytocin was named for its ability to hasten childbirth (Ott and Scott, 1909); oxy meaning quick and toco denoting birth. It is the most powerful uterotonic agent known. However, like all hormones, it acts through its receptor, and if its receptor is not expressed in the uterus, it cannot act to increase myometrial contractions. Oxytocin is also responsible for milk letdown. Oxytocin receptors are upregulated in the uterus on the day of parturition (Alexandrova and Soloff, 1980) and in the mammary gland immediately following parturition (Lundin-Schiller et al., 1996). Oxytocin has also been implicated as an osmoregulator, due to its response to slight variations in the plasma osmolality of rats (LeMoine and Young, 1992). It has also been found to enhance natriuresis (Bourque, et al., 1994). These responses are thought to be mediated by the circumventricular organs which have neurons sending axons to synapse on magnocellular neurons of the SON and PVN.

Oxytocin has been implicated as a releasing factor for prolactin (PRL). Prolactin is mammotrophic, and in some species is also luteotrophic. Prolactin acts at the mammary gland to increase the production of casein and α -lactalbumin. Interestingly, relaxin has been shown to increase the release of PRL *in vitro* (Sortino et al., 1989) from cultured cycling-female rat adenohypophyses and *in vivo* following intracerebroventricular (i.c.v.) administration to pseudopregnant pigs (Li et al., 1993). Because OT is a hypothetical PRL

releasing factor, it is possible that relaxin's effect on the release of PRL is mediated through a release of oxytocin. But, Porter et al. (1992), demonstrated that relaxin did not have an effect on the release of OT from cultured cycling-sow neurohypophyses, or from lactating sows administered relaxin intravenously. And, relaxin suppressed the release of OT from cultured bovine luteal cells (Musah, et al., 1990). However, no *in vivo* studies have been completed to look at the effect of relaxin on the release or synthesis of oxytocin in pregnant pigs, in which PRL levels have been shown to be elevated following relaxin administration (Li et al., 1993).

The release of OT is controlled electrophysiologically, but is modulated by stimulatory and inhibitory factors. Electrophysiologically, the release of OT depends on the physiologic state of the animal. Oxytocin is released in increased levels during parturition and lactation, and also in response to dehydration. During times of background activity, OT neurons have a slow irregular or fast continuous pattern and average 1 spike/second (Poulain and Wakerley, 1982). The electrophysiology of OTergic neurons during suckling is well studied. Spinal afferents from the nipples synapse on OTergic cells (Poulain, 1983) producing a characteristic high frequency discharge of action potentials (Poulain and Wakerley, 1982) leading to the release of a bolus of oxytocin from the neurohypophysis (Gainer and Wray, 1994). Oxytocin then acts on the myoepithelial cells in the mammary gland to cause an ejection of milk. During parturition, the firing rate of OTergic cells is increased and undergoes bursts of high frequency firing which are correlated to uterine contractions (Poulain and Wakerley, 1982), and OT is released in boluses during fetal expulsion (Summerlee, 1981). Dehydration causes the mean firing rate

of OTergic neurons to triple, but the pattern continues in the slow irregular or fast continuous fashion typical of these neurons.

The release of OT is also controlled by the presence of inhibitory and stimulatory factors. Oxytocin has a positive effect on its own release both in the hypothalamus (Theodosis, 1985) and at the level of the neurohypophysis (Falke, 1989). During parturition, the Ferguson reflex (increased release of OT following vaginal distention) is probably acting to enhance this positive feedback system. It is also postulated that estrogen facilitates the release of OT during mating (Hadley, 1992). Inhibitory control over the release of OT seems to be a function of opioids. Administration of naloxone, an opioid antagonist, to neural lobes increased the electrically stimulated release of OT; however, this was more pronounced in nonpregnant rats than in late pregnant rats (Douglas et al., 1993). Dynorphin, which is synthesized by vasopressinergic cells (Meister et al., 1990; Bondy et al., 1989) inhibits the electrically stimulated release of OT (Douglas et al., 1993). This effect is also seen on the release of OT from its dendrites in the hypothalamus (Ingram et al., 1996). There is also a reciprocal relationship between OT and β -endorphin release in late pregnant humans (Lindow et al., 1996). Interestingly, it appears that relaxin may be playing a role in developing increased opioid tone in late pregnancy (Way et al., 1993).

Control of the synthesis of oxytocin has not been as thoroughly studied as control over its release. Estrogen has been shown to modulate OT mRNA (Richard and Zingg, 1990) through an estrogen response element located in the oxytocin gene promoter. And, like the regulation of the release of OT during pregnancy, its synthesis may also be

controlled by opioids (Douglas et al., 1994). The role of relaxin as a potential player is being investigated.

Relaxin is a polypeptide hormone which is primarily made in reproductive organs. The primary site of relaxin production varies with species. It is a member of the family of insulin-like growth factors, weighs 6000 daltons, is structurally similar to insulin, and is found in species ranging from the sand shark to the human; however, there is some question regarding its significance in ruminants (Bathgate et al., 1996; Hartung et al., 1995, Roche et al., 1993). Relaxin has been found in males of various species, usually within the accessory sex glands such as the prostate and seminal vesicles (Sherwood, 1994, Juang et al., 1996, Weiss, 1995). More commonly, however, relaxin is studied in females. The most common site of production in females is the CL; however, in some species relaxin is found predominantly in the placenta or the endometrium.

Rats and pigs are the most commonly studied species because their levels of relaxin are easily detected in the CL as well as in the circulation during gestation. Relaxin accumulates in electron dense granules in the CL during the latter half of pregnancy (Anderson et al., 1973a, Anderson et al., 1973b, Belt et al., 1971). Both species have moderate to low levels of relaxin circulating until the antepartum period. At this time the relaxin containing granules in the CL are quickly reduced in number (Anderson et al., 1973a; Anderson et al., 1973b) and there is a peak in the amount of relaxin found in the circulation (Felder et al., 1986, Sherwood et al., 1980). Following parturition, and during the estrous cycle, relaxin is not detectable in the circulation, and is minutely detectable in the CL.

Relaxin is involved in restructuring the collagen in the pelvic ligaments, as well as the cervix, to accommodate parturition. In 1926, Hisaw published a paper indicating that serum from pregnant guinea pigs resulted in a noticeable widening of the pubic symphysis when administered shortly after estrus to virgin guinea pigs (Hisaw, 1926); the active factor in the serum was later identified as relaxin (Fevold et al., 1930) and named for its relaxative characteristics. In addition to the changes seen in the cervix and pelvic ligaments, relaxin also plays a role in the growth of the mammary glands and nipples.

Relaxin not only alters the histologic structure of tissues, but also has a physiologic action. It has a quiescent effect on the uterus during gestation. In animals treated with a monoclonal antibody to relaxin, the beginning of parturition is delayed, it lasts much longer, and there is a higher percentage of fetal death (Lao Guico-Lamm and Sherwood, 1988).

Relaxin has been studied in this reproductive context since the mid 1920's. It was not until nearly sixty years later that relaxin was also found to play a role at the level of the brain. Since that time the rat has been used as a model to show that: 1) there are relaxin binding sites in many discrete regions of the brain (Osheroff and Phillips, 1991); 2) preprorelaxin mRNA is present in regions of the brain in addition to those in which it has binding sites (Osheroff and Ho, 1993); 3) relaxin can elicit, as well as inhibit, the release of oxytocin and vasopressin (AVP) from the neurohypophysis (Dayanithi et al., 1987; Summerlee et al., 1984, Jones and Summerlee, 1986).

Although much is known about relaxin's target tissues, its receptor has yet to be characterized. Numerous autoradiographic binding studies have been carried out that

demonstrate binding of ^{32}P -labeled relaxin in its target tissues (Sherwood, 1994). Studies in the mid 1980's, implicating relaxin's role in the brain, led to the discovery of relaxin binding sites in the rat brain (Osheroff et al., 1990). A subsequent study by Osheroff and Phillips (1991) detailed the exact location of binding sites in the male and female rat brain. Areas where relaxin binding was seen include the: subfornical organ (SFO), organum vasculosum of the lamina terminalis (OVLT), the PVN and SON of the hypothalamus, as well as the neocortex, hippocampus, thalamus, amygdala, midbrain and medulla. Particular interest was given to the binding sites in the PVN and SON because previous data had implicated relaxin in the control of OT and AVP release, both of which are made in these nuclei. Binding sites in the SFO and OVLT were also considered significant because of their incomplete blood brain barrier and therefore potential action as mediators of relaxin's effects on the release of OT and AVP from the supraoptic and paraventricular nuclei.

The SFO is a circumventricular organ, which by definition means that it is exposed to the ventricles, and thereby is also exposed to the cerebral spinal fluid (CSF).

Histologically, many of the capillaries within the SFO are fenestrated (Dellmann and Simpson, 1979), which allows substances from the blood to pass into the tissue and vice versa. This incomplete blood brain barrier is also found in other circumventricular sites, such as the OVLT and the area postrema (Weindel and Sofroniew, 1981).

Circumventricular organs maintain an intact blood-CSF barrier, which prevents substances (which have passed from the blood into the brain through fenestrations in the capillaries) from passively diffusing into the cerebral spinal fluid. This is accomplished

through tight junctions between the ependymal cells lining the ventricular surface of the SFO. Circulating peptides can, however, selectively pass from the blood into the CSF through circumventricular sites (Pardridge et al., 1981). The SFO sends efferents to both the SON and PVN, as well as to areas in the anteroventral third ventricular area of the preoptic region (Miselis, 1981). Summerlee et al. (1987), have shown that lesions of the SFO completely block the inhibitory effects that relaxin has on the reflex milk ejection. This information, along with that demonstrating the location of relaxin binding sites in the SON and PVN, suggests two possible routes for the action of relaxin on the release of OT and AVP: 1) relaxin is selectively transported from the circulation into the CSF through the SFO, and from there reaches its binding sites in the SON and PVN or 2) relaxin is selectively transported from the circulation into the SFO, and is then transported from the SFO to the SON and PVN along the efferent projections from the SFO.

Because relaxin specifically affects magnocellular neurons of the SON and PVN, and no information is available concerning the selective transport of relaxin into the CSF, researchers have decided to look for relaxin mRNA within the brain of rats (Gunnensen et al., 1985; Osheroff and Ho, 1993). Both groups demonstrated a 1kb transcript in rat brain using Northern blots, and Gunnensen and colleagues also found this transcript in uterus, prostate gland, pancreas and kidney. The weight of this isolate correlates well with that of preprorelaxin. Osheroff and Ho (1993) then went on to localize relaxin mRNA to several distinct regions within the brain: the anterior olfactory nucleus, tenia tecta, pyriform- and neo-cortices, and areas CA1-3 and the dentate gyrus of the hippocampus. No

conclusions were drawn by the authors as to the significance of these sites. Relaxin has a low-moderate concentration of binding sites in all of these regions with the exception of the tenia tecta and the piriform cortex, where no binding sites exist. In addition to the mRNA studies, Gunnarsen et al. (1995) also found the relaxin protein, using immunohistochemistry, in the arcuate nucleus of the rat brain. Relaxin has also been identified immunohistochemically in the neurohypophysis of the pig (Bagnell, 1990).

Relaxin has also been discovered in the CSF. Following administration of relaxin into the systemic circulation of rats, relaxin was detected in the CSF from 1-20 minutes post injection (Parry et al., 1991). Interestingly, when anti-relaxin is administered intracerebroventricularly, the length of gestation is shortened as well as the duration of the onset of straining prior to expulsion (Summerlee et al., 1998). This indicates that endogenous relaxin found in the CSF, either from the preprorelaxin mRNA in the brain or through its ability to enter the CSF from the systemic circulation, is important in the timing of birth.

Numerous studies have been published regarding the effect that relaxin has on AVP, blood pressure and heart rate. Intravenous (i.v.) and i.c.v. relaxin administered to rats has been shown to increase circulating AVP as well as to raise blood pressure (Mumford et al., 1989; Parry et al., 1990; Yang et al., 1995). Relaxin's effects on the heart are mediated partly by the central release of AVP, but also by direct interaction with the heart (Kakouris et al., 1992). Relaxin has binding sites in the atria (Osheroff and Ho, 1993). It has been shown *in vitro* to be a very potent inotrope and chronotrope (Kakouris et al., 1992), although, at low doses it decreases heart rate (Yang, et al., 1995). Relaxin's

central actions on the release of AVP seem to be mediated by the SFO and angiotensin II. Lesions of the SFO (Mumford et al. 1989) and i.c.v. administration of angiotensin II antagonists both (Parry and Summerlee, 1991) block the pressor effects of relaxin, and angiotensin II antagonists also decrease circulating levels of vasopressin (Geddes et al., 1994).

The relationship between OT and relaxin has been studied in rats and pigs both *in vitro* and *in vivo*. An *in vitro* study on the rat neurohypophysis by Dayanithi et al. (1987) showed that under basal conditions, relaxin inhibits the release of oxytocin; however, when the nerve endings were depolarized, oxytocin secretion was potentiated. *In vivo* studies in rats have shown contradictory results. In 1984, Summerlee and colleagues found that i.v. and i.c.v. administration of relaxin in rats had an inhibitory effect on the reflex milk ejection, which is mediated through the release of oxytocin. In contrast, Way and Leng (1992) showed that OT levels are elevated following i.v. administration of relaxin to ovariectomized rats. They also demonstrated that the firing rate of OTergic neurons in the SON is increased following i.v. relaxin administration. They hypothesized that the contradiction between these two studies was due to a discrepancy in interpretation. In addition to the aforementioned findings, Way and Leng (1992), in agreement with Summerlee et al. (1984), also reported that i.v. relaxin inhibited the reflex milk ejection. Reflex milk ejections, however, are due to brief episodes of synchronized high-frequency discharges of OTergic cells, which are the consequence of the activation of spinal afferents from the nipples. These bursts, therefore, are not due to pharmacological (or physiological) activation of OTergic cells. Therefore, inhibition of the milk-ejection

pathway and activation of OTergic cells are independent consequences of relaxin administration. Summerlee's research group (Geddes et al., 1994; Parry et al., 1994) went on to confirm the results of Way and Leng (1992) and, in addition, reported that relaxin's effect to increase the release of OT and AVP was attenuated in late pregnancy in the rat. Interestingly, a study in goats (Peaker et al., 1995) showed that the responsiveness of the myoepithelial cells to oxytocin is varyingly dependent upon intramammary relaxin. Relaxin decreases the mammary glands sensitivity to oxytocin during pregnancy and immediately following parturition, but not during the remainder of lactation.

A study in rats showed that the i.c.v. administration of relaxin (McKinley et al., 1997), increases the expression of Fos in OTergic cells of the hypothalamus. Fos is the protein product of an immediate early gene called *c-fos*, which is activated by numerous proteins, one of which is relaxin. Fos acts as a transcription factor by binding to the AP-1 site on genes to alter their transcription. It binds as a heterodimer with Jun, the protein product of another immediate early gene, and can either suppress or enhance the transcription of the gene to which it binds. The expression of Fos in OTergic cells following the administration of relaxin suggests that relaxin is acting to change the transcriptional activity within those cells.

Research has also been conducted in pigs on the relationship between oxytocin and relaxin. *In vitro* studies on sow neurohypophyses showed that relaxin has no effect on the release of oxytocin (Porter et al., 1992). The same study indicated that relaxin does not alter the circulating concentration of OT when administered to lactating sows. The effect of relaxin on circulating OT in pregnant pigs is being investigated.

In summary, OT and relaxin are hormones involved during pregnancy and parturition. Oxytocin binds to receptors in the myometrium at term, to cause uterine contractions, resulting in fetal expulsion. Relaxin has a quiescent effect on the myometrium prior to term, and appears to prevent the premature release of OT during the antepartum period. The present dissertation further explores the relationship of the two hormones in pregnant rats and pregnant pigs.

Dissertation organization

The present dissertation consists of three papers, comprising Chapters 2-4. The paper found in Chapter 2 has been submitted to *Biology of Reproduction* for publication; the paper found in Chapter 3 has been published in *Neuroendocrinology* (1997); and the paper in Chapter 4 will be submitted to *Neuroendocrinology* for publication. The papers were preceded by a general introduction and are followed by a composite summary and general discussion. The literature cited in the introductory and conclusionary chapters is referenced following the general discussion. All of the animal and laboratory work found within this paper was carried out by the author in the laboratory of Carol D. Jacobson, and, with Karen Langner's help on the radioimmunoassays described in Chapter 2 in the laboratory of Lloyd L. Anderson.

CHAPTER 2
EFFECT OF CIRCULATING RELAXIN ON OXYTOCIN AND PROLACTIN
CONCENTRATIONS DURING PREGNANCY IN THE GILT

A paper submitted to *Biology of Reproduction*

Patricia A Heine, Carol D Jacobson, Ted B Bailey, John Klindt, Lloyd L Anderson

ABSTRACT

In gilts, circulating concentrations of relaxin reach an antepartum peak within 2 d of parturition. During this time, many endocrine changes are occurring in preparation for birth. The present study was designed to determine the effect of intravenously administered relaxin on the circulating concentrations of oxytocin and prolactin in pregnant gilts. Six midgestational and eight late gestational gilts were utilized in this study. Gilts from each gestational period were assigned to one of two treatment groups: relaxin or isotonic saline. Gilts received one of the two treatments and were subsequently bled at sequential time points for a 3-h duration. Prior to treatment, late gestational gilts were ovariectomized to remove the primary source of endogenous relaxin (followed by progesterone administration to maintain pregnancy). Acute exposure to relaxin had no significant effect on the circulating levels of either prolactin or oxytocin at the late gestational age. In mid gestational gilts, however, a significant difference was noted in the circulating concentrations of oxytocin. In the late gestational group, removal of endogenous relaxin did unexpectedly result in a substantial transient increase in the circulating levels of oxytocin and a lesser, yet significant increase in the circulating levels of

prolactin. These results indicate that intravenously administered relaxin is capable of increasing the circulating concentration of oxytocin during midgestation, and suggests that removal of endogenous relaxin causes a release of oxytocin into the circulation during late gestation.

INTRODUCTION

Pregnancy, especially near term, is a time when the hormonal milieu is considerably altered from its normal state. Present in the circulation during pregnancy are hormones that are not detectable in the non-pregnant state, as well as altered levels of hormones regularly found in the circulation. The source of these hormones can be tissues that are present only during pregnancy (placenta) or other endocrine tissues such as the ovaries or pituitary gland. How these hormones interact and the result of their interactions is of ongoing interest.

Gestational length in pigs is 114 d. Many studies have been performed measuring the levels of various hormones throughout pregnancy and during parturition in pigs [1-3]. One hormone of particular interest, relaxin, is made in the corpora lutea (CL) and is therefore detectable in the circulation primarily during pregnancy. Prolactin (PRL) has been shown in recent studies to be released in gilts in response to intracerebroventricularly delivered relaxin[4]. *In vitro* studies suggest that relaxin increases the release of PRL [5, 6]; however, because oxytocin (OT) is a putative releaser of PRL [7], it is plausible that OT is mediating the release of PRL in response to relaxin. It has been shown in rats that OT and vasopressin (VP) are released following injection with relaxin [8, 9]. A study utilizing lactating pigs, however, demonstrated that i.v. administration of relaxin had no effect on

circulating OT concentrations [10]. But, because relaxin is not found in the circulation during lactation, this experimental design did not test a physiologically normal hormonal interaction. The present study utilized late and midgestational gilts to elucidate the effects of relaxin on OT and PRL during a time when gilts are normally exposed to their own circulating relaxin.

We previously determined that levels of PRL and relaxin are equivalent in Yorkshire and Meishan gilts during late pregnancy [11]. The primary objective of this study was to determine if intravenously administered relaxin affected the circulating levels of OT and PRL in pigs during mid- and late gestation. The secondary objective was to determine basal blood concentrations of these hormones in midgestation in the presence of relaxin and in late gestation in the absence of relaxin.

Material and Methods

Animals

All pigs were housed at the Iowa State University Animal Reproduction Farm. They were fed a corn- and soybean meal diet once per day and had free access to water. The room in which they were housed had a controlled ambient temperature of 23-25 °C. All procedures were approved by the Iowa State University Committee on Animal Care.

Six Meishan and eight Yorkshire gilts, weighing approximately 115-180 kg were bred naturally and housed in group pens until 2 d prior to surgery for jugular cannula placement as well as ovariectomy (OVX) in late gestation. At this time, gilts were moved to individual pens, where they were housed for the duration of the study.

Treatments

Following breeding, gilts were placed into groups based on breeding date and blood line. Animals within each group were randomly assigned to receive either relaxin or isotonic saline. To determine the baseline hormone levels, gilts in midgestation (beginning on gestational day 44; g44) were bled every 12 h for 5 d prior to treatment. On the day of treatment, either relaxin or saline was administered intravenously to each gilt according to her group placement (see the Blood Sampling section). Gilts in late gestation underwent surgery between g105 and g107. At that time a jugular cannula was placed and ovaries were removed. From the time of surgery until the end of the experiment, gilts received 200 mg (50 mg/45 kg body weight) of intramuscular progesterone (dissolved in 10 ml sesame oil) every 12 h to maintain pregnancy. Relaxin or saline was administered on g109-g111. Blood was sampled in the same manner as it was in the midgestational gilts.

Surgery

Anesthetic induction of gilts was achieved by intramuscular injection of ketamine (10 mg/kg). Anesthesia was then maintained with halothane, delivered through a mask connected to a semiclosed circle anesthetic machine (Ohio Medical Products, Madison, WI). Oxygen levels were set at 2 l of oxygen/min with delivery of 1.5-2% halothane.

A cannula (i.d., 1.27 mm; o.d., 2.29 mm; Tygon® microbore tubing, Fisher Scientific, Pittsburgh, PA) was placed in the external jugular vein and passed caudally to the level of the right atrium. At the point of entry through the skin of the neck, the cannula was secured to the surrounding tissues using silk suture. The free end of the tubing was tunneled under the skin and exteriorized at the nape of the neck and secured to the

skin using prolene (#1; Ethicon, Somersville, NJ). The cannula was filled with heparinized saline (15units/ml) to maintain patency.

In addition to cannula placement, late gestational gilts underwent ovariectomy (OVX). Both procedures were conducted during the same anesthetic episode to reduce the chance of fetal complications due to multiple anesthetic exposures. Ovariectomy was performed through a midventral laparotomy. To enter the peritoneal cavity, a 20-cm incision was made on the ventral midline extending from the level of the second-from-the-last teat, cranially. Ovaries were elevated from the abdomen, and the proper ligament between the ovary and uterus and the mesovarium were crushed. Chromic cat gut (#3; Braun, Jorgensen Laboratories, Inc., Loveland, CO) was used to ligate (by transfixation) the ovarian pedicle allowing for excision. The remaining ovarian stump then was cauterized. Penicillin G (Solvay, Mendota Heights, MN) was administered intraperitoneally as well as intramuscularly (7 million IU). The abdominal incision was closed in three layers. Chromic cat gut (#3; Braun) was used to close the two deep layers, utilizing a vest-over-pants pattern on the dorsal rectus sheath and a simple continuous pattern on the fat layer. A Ford interlocking pattern utilizing 1/8" cotton umbilical tape (Ethicon) was used to close the skin. Following surgery, the gilts inhaled 100% oxygen until they regained consciousness and were then wheeled back into their individual pens with heated floors for full recovery.

Blood Sampling

The blood sampling protocol was the same for mid- and late gestational gilts. One 10-ml blood sample was obtained every 12 h from each gilt's i.v. cannula. To maintain

patency, cannulas were flushed with heparinized saline following blood withdrawal. The collected blood samples were transferred to heparinized vacutainer tubes (Becton Dickinson, Rutherford, NJ) and centrifuged for the recovery of plasma, which was stored in duplicate in plastic tubes. One tube contained 20 μ l protinin A (ICN Pharmaceuticals, Inc., Costa Mesa, CA) to prevent the proteolysis of small peptides such as oxytocin. Tubes were snap frozen at -70°C and then stored at -20°C until radioimmunoassays (RIAs) were run for relaxin, OT, PRL, and progesterone. All four hormones were assayed in both gestational ages. Relaxin was assayed to determine baseline levels in the midgestational gilts and to affirm that the treatment did enter the circulation, and reached levels similar to those seen after degranulation from the CL in late pregnancy. Progesterone was assayed to determine baseline values in the midgestational gilts and to verify that an adequate level of the hormone was being exogenously maintained in the late gestational group.

Days on which the gilts received their treatments, blood samples were drawn 20 and 10 min prior to treatment administration. Treatments were given through the same cannula from which samples were drawn. At time zero, gilts received either 1 mg [12] purified porcine relaxin (C. Schwabe, Medical University of South Carolina, Charleston, SC) dissolved in 2 ml of isotonic saline, or just the vehicle. Blood samples then were obtained at 2.5, 5, 10, 15, and 20 min following the challenge, and every 20 min thereafter for a total of 180 min. The procedure for blood sample collection was the same as previously discussed.

/

Radioimmunoassay of progesterone, relaxin, prolactin and oxytocin

Plasma progesterone was extracted with a benzene-hexane mixture (1:2 vol/vol) using a modified version of the procedures described by Louis et al.[13]. One hundred microliters of plasma samples were extracted in duplicate. A third 100- μ l replicate received 5,000 cpm [1,2,6,7-N- 3 H]progesterone (97 Ci/mmol; NEN Research Products, Boston, MA). With modification of the RIA procedure described by Anderson et al.[14], plasma extracts were assayed for progesterone using the fully characterized antibody (GDN antiprogestrone-11-BSA 337;[15, 16]). The limit of detection of the progesterone assay was 230 pg/tube. The average intraassay coefficient of variation (CV) was 6.85% and the interassay CV was 4.03%. The mean recovery of P4 after benzene-hexane extraction was 90.0%.

Relaxin levels in the plasma were quantified in duplicate in aliquots of 100 μ l. Monotyrosylated relaxin, prepared by C. Schwabe, was radioiodinated by the chloramine T method, and served as the tracer. The primary antibody, R6, was provided by B.G. Steinetz (New York University Medical Center, Tuxedo, NY), and the secondary antibody was goat anti-rabbit γ -globulin. The procedures were followed as have been previously described [17, 18]. The assay sensitivity was 40 pg/tube. The average intra- and interassay CVs were 3.85% and 7.3%, respectively.

Prolactin plasma concentrations were quantitated using a double-antibody RIA [19]. Prolactin (USDA-pPRL-I-1) was radioiodinated by the chloramine -T method to a specific activity of approximately 90 μ Ci/ μ g. Primary antibody was purchased from

Research Products International Corp. (Mt. Prospect, IL, Lot 10115), and 100 μ l at a dilution of 1:37,500 was added to each tube. Reference preparation was USDA-pPRL-B-1. Each tube contained 200 μ l of plasma. The limit of detection was 0.15 ng/tube, intraassay CV was $13.1 \pm 10.3\%$ (mean \pm SD), and interassay CV was $15.1 \pm 7.6\%$ (mean \pm SD).

Concentrations of oxytocin in the plasma were determined by use of a double antibody procedure and ^{125}I oxytocin (NEN Research Products) as the tracer. The primary antibody, made in rabbit, was a gift from J. Kotwica (Division of Reproductive Endocrinology and Pathophysiology, Center for Agrotech and Veterinary Science, Polish Academy of Sciences, Olsztyn, Poland). The secondary antibody was a goat anti-rabbit γ -globulin (Organnon Teknika, Cappel Research Products, Durham, NC). Plasma oxytocin was extracted using acetone (2 ml, cold)/ 0.5 ml duplicate. The extracts were resuspended in 0.4 ml of assay buffer. To this volume, 100 μ l of 1:11,500 of the primary antibody was added and this was incubated at 4 ° C for 24 h. The tracer was then added at approximately 5,500 cpm/100 μ l. This was incubated at 4 ° C for 24 h. Then 200 μ l of the secondary antibody at 1:15 was added, and this was incubated at 4 ° C for 48 h. Then 200 μ l of polyethylene glycol (Sigma, St. Louis, MO) and 3 ml of 0.01 M phosphate buffered saline were added, and this was centrifuged for 1 h at 3,000 x g. The supernatant was decanted and the pellets were counted in the γ spectrometer for 1 min. The sensitivity of the assay was 0.97 pg/tube. The average intraassay CV was 3.7%, and the interassay CV was 8.1%. The mean recovery of OT after acetone extraction was 75.5%.

Statistical Analyses

The experimental units were the individual gilts. Analyses of midgestational and late gestational stages were completed independently. Because each gilt was measured several times our initial analysis was a univariate ANOVA corresponding to a split-plot design, with time as the split-plot factor [19]. Treatment (saline or relaxin) means were compared via the ANOVA F-test. Tests for time and time by treatment interaction effects were completed using reduced numerator and denominator degrees of freedom to account for the correlation of measurements at the different times. Finally, tests for linear and non-linear trends over time were carried out for the relaxin treatment [20]. All tests were conducted using a five percent-level of significance.

RESULTS

Midgestational Gilts

Baseline

Baseline hormonal values for gilts in midgestation are shown in Fig. 1. Midgestational values for all four hormones are similar to those measured previously [3, 11]. Circulating concentrations of OT, which previously had not been documented during midgestation, averaged 3 pg/ml of plasma.

Following treatment

After receiving exogenous relaxin, the detected levels of circulating relaxin were similar to those previously determined during the antepartum peak [3, 22]. Following relaxin administration, the circulating PRL concentration gradually increased over the 3-h trial (Fig. 2A), however, the linear component of the line graph (Fig. 2A) was not

significantly different from a line with zero slope. Plasma OT levels in the midgestational gilts underwent a transient rise followed by a rapid return to baseline (Fig. 2B). The linear component of the relaxin treatment group in Fig. 2B indicates a significant difference from a line of zero slope ($p < 0.002$). As was expected, circulating progesterone showed no response to the administration of relaxin (Fig. 2C).

Late Gestational Gilts

Baseline

Ovariectomy affects circulating progesterone and relaxin concentrations because the CL are the main source for both hormones. Therefore, following removal of the ovaries, there should be no detectable relaxin or progesterone in the circulation. As was expected, the concentration of plasma relaxin (Fig. 3D) was not measurable in gilts post-OVX. But, P4 was detectable due to its exogenous replacement. Progesterone values plateaued at 4 d post-OVX (Fig. 3C). Sufficient circulating P4 was present in all gilts, validated by the lack of spontaneous abortions, and also the comparative data from intact gilts. The circulating concentrations of PRL and OT both decreased significantly in the first 2 d following ovariectomy (Fig. 2A and 2B). The OT concentration in the circulation reached an average of 12 pg/ml immediately following OVX, and by 3 d post-OVX had plummeted to 2.5 pg/ml, where it remained for the duration of the study. A regression analysis showed that the linear component of this line was significantly different from a line of zero slope ($p > 0.0001$). Plasma PRL levels were 3.5 ng/ml following OVX, and by 3 days post-OVX had decreased to 1.5 ng/ml, where they remained throughout the duration of the experiment. Regression analysis of the linear component of this line

showed a significant difference from a line a zero slope ($p > 0.0002$). Data from intact gilts at similar gestational points are shown for comparison.

Following hormone or vehicle treatment

Following administration of exogenous relaxin, detectable levels of relaxin in the circulation were similar to that seen in the antepartum peak [3, 22]. The response of circulating OT and PRL concentrations to i.v. administration of relaxin was insignificant in the late gestational gilts. Plasma PRL incurred only a transient rise (Fig. 4A), which did not occur until 20 min after relaxin had been administered and slowly returned to baseline values over the remainder of the study. The circulating concentrations of OT did not show any response to i.v. relaxin administration in the late gestational group (Fig. 4B).

DISCUSSION

The intravenous administration of relaxin results in a significant increase in the circulating concentration of OT in midgestational gilts. Surprisingly, changes were noted in the circulating concentrations of OT and PRL in response to removal of the ovaries in late gestation. Because progesterone was replaced, the changes seen likely resulted from the acute drop in circulating relaxin concentrations. This unexpected change is similar to one documented in late pregnant rats, in which ovariectomy decreased the endogenous opioid inhibition of OT release [23].

Midgestational gilts

The nonstatistically significant increase in circulating PRL following treatment with relaxin may be explained by similar findings seen in a previous study [4], in which relaxin was administered intracerebroventricularly to pseudopregnant gilts. This caused a

rapid transient rise in peripheral prolactin. The increases in circulating PRL are much less pronounced in the present study; however, it can be assumed that relaxin administered intravenously reached the adenohipophysis in smaller amounts than it did using the intracerebroventricular method of administration.

The significance of the increase in circulating OT in the midgestational gilts suggests that relaxin's effect on the release of OT in pigs is similar to that in rats [8]. In the current study, the increase in circulating OT, in response to relaxin, peaks at 30 min following relaxin administration and has returned to baseline by 60 min post treatment. The rise in PRL doesn't begin until 60 min post treatment and peaks at 100 min, followed by a return toward baseline. Circulating OT, a putative releaser of PRL, may be responsible for this late increase in PRL, however, the half life of OT in the circulation is only 3 min, which would indicate that the cellular mechanism behind the OT induced release of PRL must be slow. From another point of view, although the increase in circulating OT was significant following the administration of relaxin, the absolute values of the OT in the circulation are still within the limits of the baseline values (Fig. 1B). This suggests that the changes seen may only be variations within the normal range of circulating oxytocin.

Late gestational gilts

The loss of relaxin as seen in the present study results in a response of the circulating concentrations of both PRL and oxytocin. The circulating levels of PRL immediately following OVX are greater than baseline values; however, there is a rapid return to the baseline values. This reduction in circulating PRL could be a direct or

indirect result of the loss of relaxin. Prolactin has been shown to respond to changes in relaxin [4-6], but it also appears to be regulated by oxytocin[7]. Oxytocin's diurnal rhythm mirrors that of PRL [24], suggesting that fluctuations in OT release result in changes in peripheral PRL concentrations. It is likely that the marked decrease in circulating PRL following the loss of relaxin was due to prolactin's response to the decrease in oxytocin.

Following the removal of relaxin, the circulating concentration of PRL is much lower than that seen in the intact gilts. This suggests that the presence of relaxin may be necessary for the release of PRL. In support of this, previous work has shown that relaxin has a much more potent effect in females on the release of PRL from cultured cells of the adenohypophysis than it does in males [6].

General discussion

The physiology of the hormonal milieu during the antepartum period in swine is such that relaxin peaks within 24 h of parturition. Oxytocin does not increase in the circulation until after parturition begins [25]. Therefore, prepartum circulating relaxin may prevent a premature release of oxytocin. Oxytocin is produced in the supraoptic and paraventricular nuclei of the hypothalamus and is released into the circulation from the neurohypophysis. In the rat, relaxin, which has been shown in the cerebral spinal fluid [26], binds to the supraoptic and paraventricular nuclei [27], making it feasible that relaxin alters some facet of OT production [28]. Additionally, relaxin is found in the neurohypophysis of the pig [29], indicating that relaxin may play a role in monitoring the release of OT into the circulation. *In vitro* studies have shown that relaxin does increase the release of OT from rat neurohypophysis [30].

Studies on the effect of relaxin administration on plasma OT levels have had contradictory results. Results from early studies on rats indicated that relaxin inhibited the release of oxytocin [31]. Contrary to these results, further studies indicated that in the rat, OT is released into circulation following the administration of relaxin [8]. The current study indicates that in gilts, a loss of relaxin in late gestation results in the release of oxytocin. The circulating concentrations of OT are elevated above the baseline values immediately following OVX and then rapidly decline to levels at or below baseline. Likewise, a study in rats indicated that removal of relaxin decreased the inhibitory effect of opioids on the release of oxytocin during late pregnancy [23]. This is likely the same mechanism that was seen in the current study. The opioid dynorphin is co-released with VP from the neurohypophysis and binds locally to kappa opioid receptors inhibiting the release of oxytocin [32].

Why does circulating OT in the late gestational group show no response to treatment with relaxin? A previous study [10] indicated that relaxin had no effect on circulating OT in lactating pigs. This would indicate that after parturition and during lactation, oxytocinergic neurons are unable to respond to relaxin. Normally, pigs are exposed to an antepartum peak followed by a rapid decrease in circulating relaxin concentrations [2, 3]. Oxytocin is then seen to increase after an hour or more of active labor [25]. In the current study, the loss of circulating relaxin post-OVX may activate the same hormonal response as is seen in the antepartum relaxin decrease. The large release of OT following OVX could be one of the normal hormonal responses to a loss in circulating relaxin. Interestingly, the effect of opioids on OT is only seen during

pregnancy. Therefore, during other reproductive stages, relaxin would be unable to interact with the opioids to alter the release of oxytocin. This may explain why, in the current study, there was no change in circulating OT following an acute dose of relaxin in the ovariectomized late pregnant gilts, nor was there a response of lactating gilts to relaxin in the previous study [10].

Physiologically, the relationship between relaxin, PRL, and OT appears to be complex. From previous studies it is clear that there is an interrelationship between the three hormones. During late pregnancy, prolactin is luteotropic in the pig [33], suggesting that the increase in circulating relaxin during pregnancy may act to release PRL for this purpose. Relaxin in the rat acts to release OT, which can then enhance the release of prolactin. This experiment shows that relaxin is also responsible for increasing the release of OT in midgestational pigs. In addition, findings herein support results from work previously completed in the rat and implicate relaxin as a control mechanism preventing the premature release of OT in the late pregnant pig.

ACKNOWLEDGMENTS

We thank Karen Langner, Eric Mathias, S-Joon Cho, John Silbernagel, Dan Isaacson, and Mahlon Shell for excellent technical assistance. Purified porcine relaxin was generously provided by Dr. Christian Schwabe, Department of Biochemistry and Molecular Biology, Medical University of South Carolina, Charleston, SC. Purified porcine relaxin (CM-B) and relaxin antiserum were generously provided by Dr. Bernard G. Steinetz, New York University Medical Center, Tuxedo, NY. Progesterone antiserum (GDN 337) was generously supplied by Dr. Gordon D. Niswender, Department of

Physiology and Biophysics, Colorado State University, Fort Collins, CO. Oxytocin antibody was a gift from Dr. K. Kotwica, Polish Academy of Sciences, Olsytn, Poland. This paper was presented in part at the 79th Annual Meeting of the Endocrine Society, 1997, Minneapolis, MN (abstract P2-360).

REFERENCES

1. Adair V, Stromer MH, Anderson LL. Progesterone secretion and mitochondrial size of aging porcine corpora lutea. *Anat Rec* 1989;223:252-256.
2. Sherwood OD, Nara BS, Welk FA, First NL, Rutherford JE. Relaxin levels in the maternal plasma of pigs before, during and after parturition and before, during and after suckling. *Biol Reprod* 1981;25:65-71.
3. Dlamini BJ, Li Y, Klindt J, Anderson LL. Acute shifts in relaxin, progesterone, prolactin, and growth hormone secretion in Chinese Meishan gilts during late pregnancy and after hysterectomy. *J Anim Sci* 1995;73:3732-3742.
4. Li Y, Huang C, Klindt J, Anderson LL. Stimulation of prolactin secretion in the pig: Central effects of relaxin and the antiprogestosterone RU 486. *Endocrinology* 1993;133:1205-1212.
5. Sortino MA, Cronin MJ, Wise PM. Relaxin stimulates prolactin secretion from anterior pituitary cells. *Endocrinology* 1989;124:2013-2015.
6. Cronin MJ, Malaska T. Characterization of relaxin-stimulated cyclic AMP in cultured rat anterior pituitary cells: influence of dopamine, somatostatin and gender. *J Mol Endocrinol* 1989;3:175-182.

7. Lumpkin MD, Samson WK, McCann SM. Hypothalamic and pituitary sites of action of oxytocin to alter prolactin secretion in the rat. *Endocrinology* 1983;112:1711-1717.
8. Way SA, Leng G. Relaxin increases the firing rate of supraoptic neurons and increases oxytocin secretion in the rat. *J Endocrinol* 1989;122:747-755.
9. Yang RH, Bunting A, Wyss M, Bereck KH, Zhang L, Jin H. Pressor and bradycardic effects of centrally administered relaxin in conscious rats. *Am J Hypertens* 1995;8:375-381.
10. Porter DG, Ryan PL, Norman L. Lack of effect of relaxin on oxytocin output from the porcine neural lobe *in vitro* or in lactating sows *in vivo*. *J Reprod Fertil* 1992;96:251-260.
11. Cho S-J, Dlamini B, Klindt J, Jacobson CD, Anderson LL. Comparative adenohipophyseal and ovarian hormone secretion in pregnant and hysterectomized Chinese Meishan and Yorkshire gilts. *J Anim Sci* 1996;74 (suppl 1):226 (abstract 475).
12. Cho S-J, Jacobson CD, Anderson LL. Acute effects of antiporcine relaxin on progesterone and relaxin secretion, and parturition in gilts. *J Anim Sci* 1996;74 (suppl 1):73 (abstract 198).
13. Louis TM, Hafs HD, Seguin BE. Progesterone, LH, estrus and ovulation after prostaglandin F_{2α} in heifers. *Proc Soc Exp Biol Med* 1973;143:152.
14. Anderson LL, Hard DL, Kertiles LP. Progesterone secretion and fetal development during prolonged starvation in the pig. *Am J Physiol* 1979;236:E335.
15. Niswender GD. Influence of the site of conjugation on the specificity of antibodies to progesterone. *Steroids* 1973;22:413-424.

16. Gibori GE, Pintezak E, Rothchild I. The role of estrogen in the regulation of luteal progesterone secretion in the rat after day 12 of pregnancy. *Endocrinology* 1978;100:1483-1495.
17. O'Byrne EM, Steinetz BG. Radioimmunoassay (RIA) of relaxin in sera of various species using an antiserum to porcine relaxin. *Proc Soc Exp Biol Med* 1976;152:272.
18. Felder KJ, Molina JR, Benoit AM, Anderson LL. Precise timing for peak relaxin and decreased progesterone secretion after hysterectomy in the pig. *Endocrinology* 1986;119:1502-1509.
19. Klindt J, Stone RT. Porcine growth hormone and prolactin: Concentrations in the fetus and secretory patterns in the growing pig. *Growth* 1984;48:1-15.
20. Spector PC, Goodnight JH, Sall JP, Sarle WS. The GLM procedure. In: *SAS User's Guide: Statistics* (5th ed.). Cary, NC: Statistical Analysis System Institute, Inc.; 1985:433-506.
21. Snedecor GW, Cochran WG. *Statistical Methods* (6th ed.). Ames, IA: Iowa State University Press; 1980.
22. Felder KJ, Molina JR, Benoit AM, Anderson LL. Precise timing for peak relaxin and decreased progesterone secretion after hysterectomy in the pig. *Endocrinology* 1986;119:1502-1509.
23. Way SA, Douglas AJ, Dye S, Bicknell RJ, Leng G, Russell JA. Endogenous opioid regulation of oxytocin release during parturition is reduced in ovariectomised rats. *J Endocrinol* 1993;138:13-22.

24. Arey BJ, Freeman ME. Activity of oxytocinergic neurons in the paraventricular nucleus mirrors the periodicity of the endogenous stimulatory rhythm regulating prolactin secretion. *Endocrinology* 1992;130:126-132.
25. Forsling ML, Taverne MAM, Parvizi N, Elsaesser F, Smidt D, Ellendorf F. Plasma oxytocin and steroid concentrations during late pregnancy, parturition and lactation in the miniature pig. *J Endocrinol* 1979;82:61-69.
26. Parry LJ, Poterski RS, Wasnidge C, Summerlee AJS. Relaxin in the cerebrospinal fluid of female rats. *Soc Neurosci Abstr* 1991;17:1196 (abstract 474.28).
27. Osheroff PL, Phillips HS. Autoradiographic localization of relaxin binding sites in rat brain. *Proc Natl Acad Sci USA* 1991;88:6413-6417.
28. Heine PA, Di S, Ross LR, Anderson LL, Jacobson CD. Relaxin-induced expression of fos in the forebrain of the late pregnant rat. *Neuroendocrinology* 1997;66:38-46.
29. Bagnell, CA. Identification of immunoreactive relaxin in the neurohypophysis of the pig. *Biol Reprod* 1990; 42(suppl 1):102 (abstract 184) .
30. Dayanithi G, Cazalis M, Nordmann JJ. Relaxin affects the release of oxytocin and vasopressin from the neurohypophysis. *Nature* 1987;325:813-816.
31. Summerlee AJS, O'Byrne KT, Paisley AC, Breeze MF, Porter DG. Relaxin affects the central control of oxytocin release. *Nature* 1984;309:372-374.
32. Bondy CA, Whitnall MH, Brady LS, Gainer H. Coexisting peptides in hypothalamic neuroendocrine systems: Some functional implications. *Cell and Mol Neurobiol* 1989;9:427-446.

33. Li Y, Molina JR, Klindt J, Bolt DJ, Anderson LL. Prolactin maintains relaxin and progesterone secretion by aging corpora lutea after hypophysial stalk transection or hypophysectomy in the pig. *Endocrinology* 1989;124:1294-1304.

Figure 1. Midgestational baseline hormonal values of prolactin (A), oxytocin (B), progesterone (C) and relaxin (D). Gilts were bled every 12 h for 5 d beginning on g44. Values are the mean \pm SE (n = 6).

Midgestational baseline plasma hormone concentrations

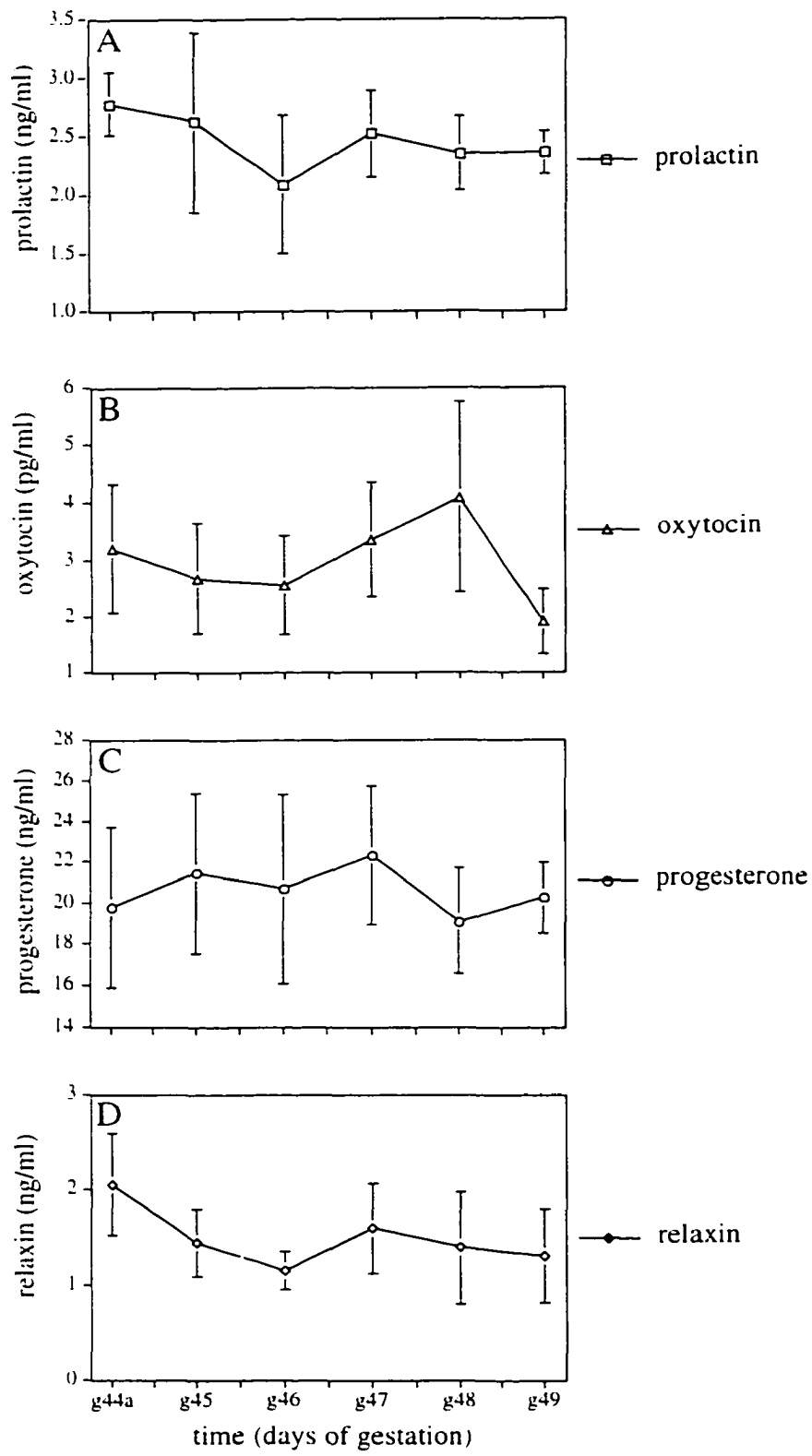


Figure 2. Midgestational hormonal values for gilts treated with relaxin (closed shapes; n = 3) or saline (open shapes; n = 3). Treatment (2 ml saline with or without 1 mg dissolved relaxin) was administered intravenously at time zero. Blood was drawn prior to treatment administration, as well as over a 180 min period following treatment. Hormone profiles are present for prolactin (A), oxytocin (B), progesterone (C) and relaxin (D). Data points are the mean \pm SE.

Midgestational plasma hormone concentrations following relaxin or saline treatments

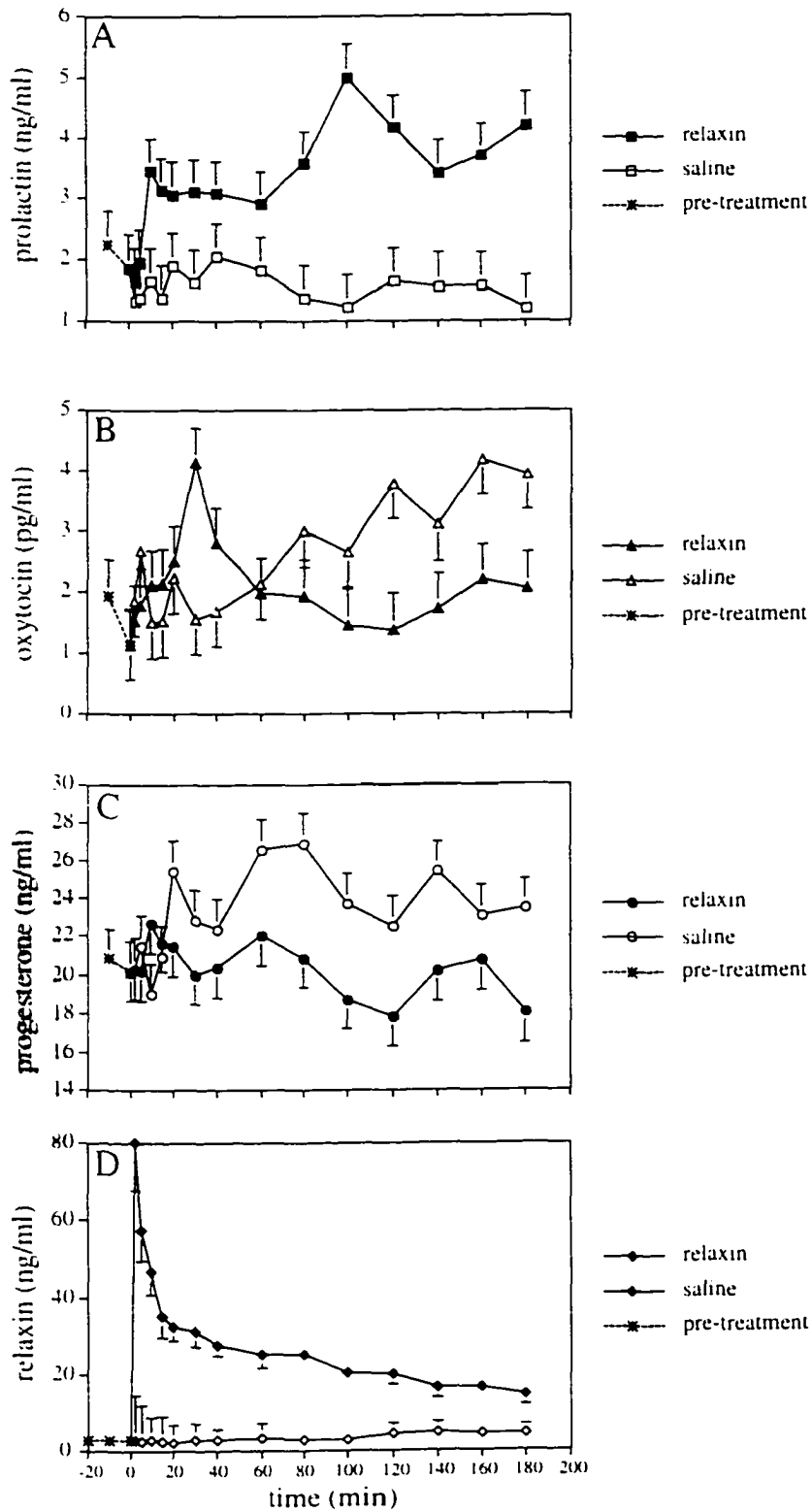


Figure 3. Gilts were ovariectomized at the beginning of the study, and were bled to obtain baseline hormonal values for 8 d following surgery (designated as day 0). Pregnancy was maintained using IM progesterone b.i.d. Hormone profiles are shown for prolactin (A), oxytocin (B), progesterone (C) and relaxin (D). Data from intact gilts at the same gestational time points is included for comparison (comparative data is not shown for the oxytocin values). Data points are the means \pm SE (n = 8).

Late gestational gilt baseline plasma hormone concentrations: ovariectomized and intact

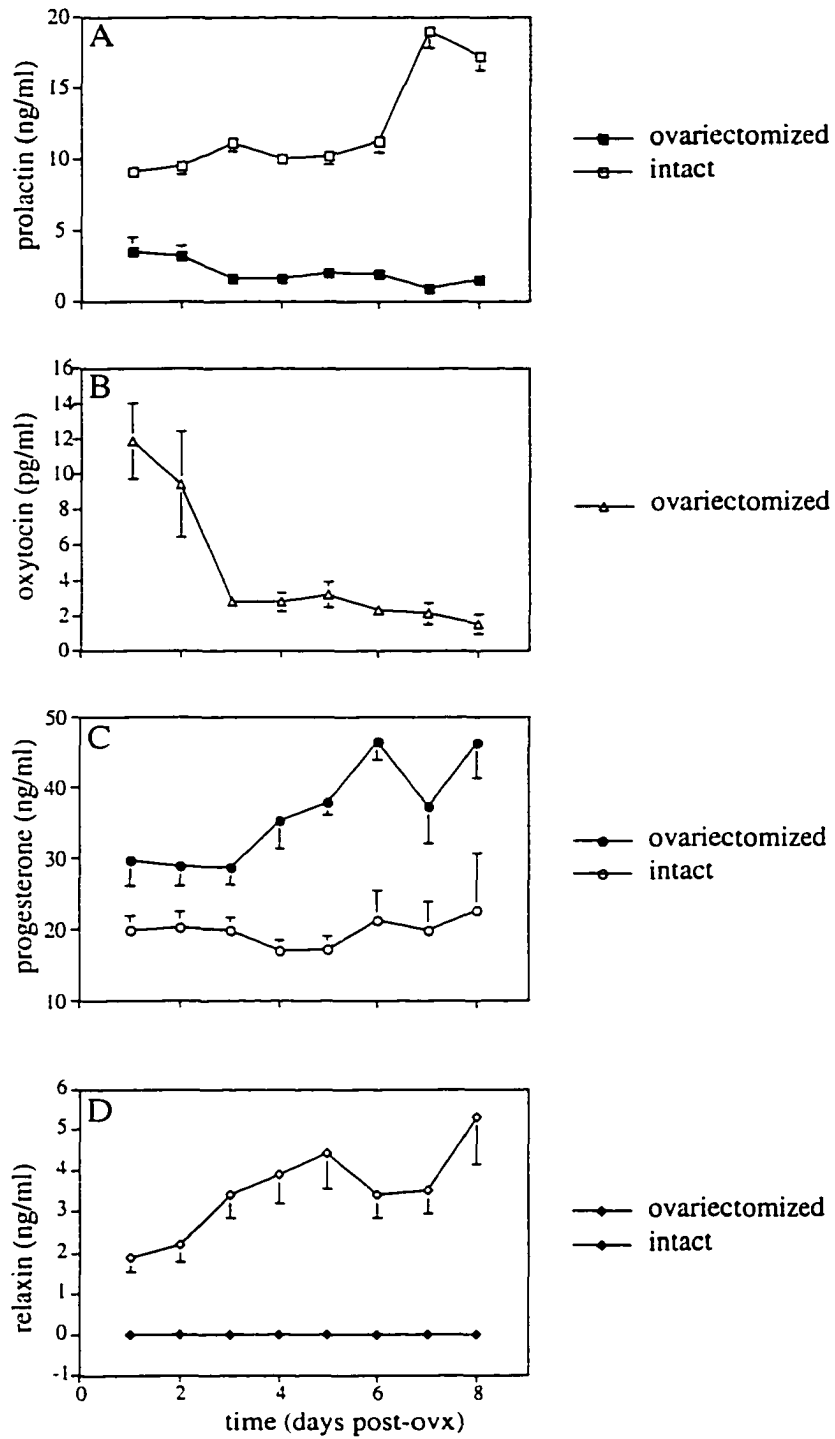
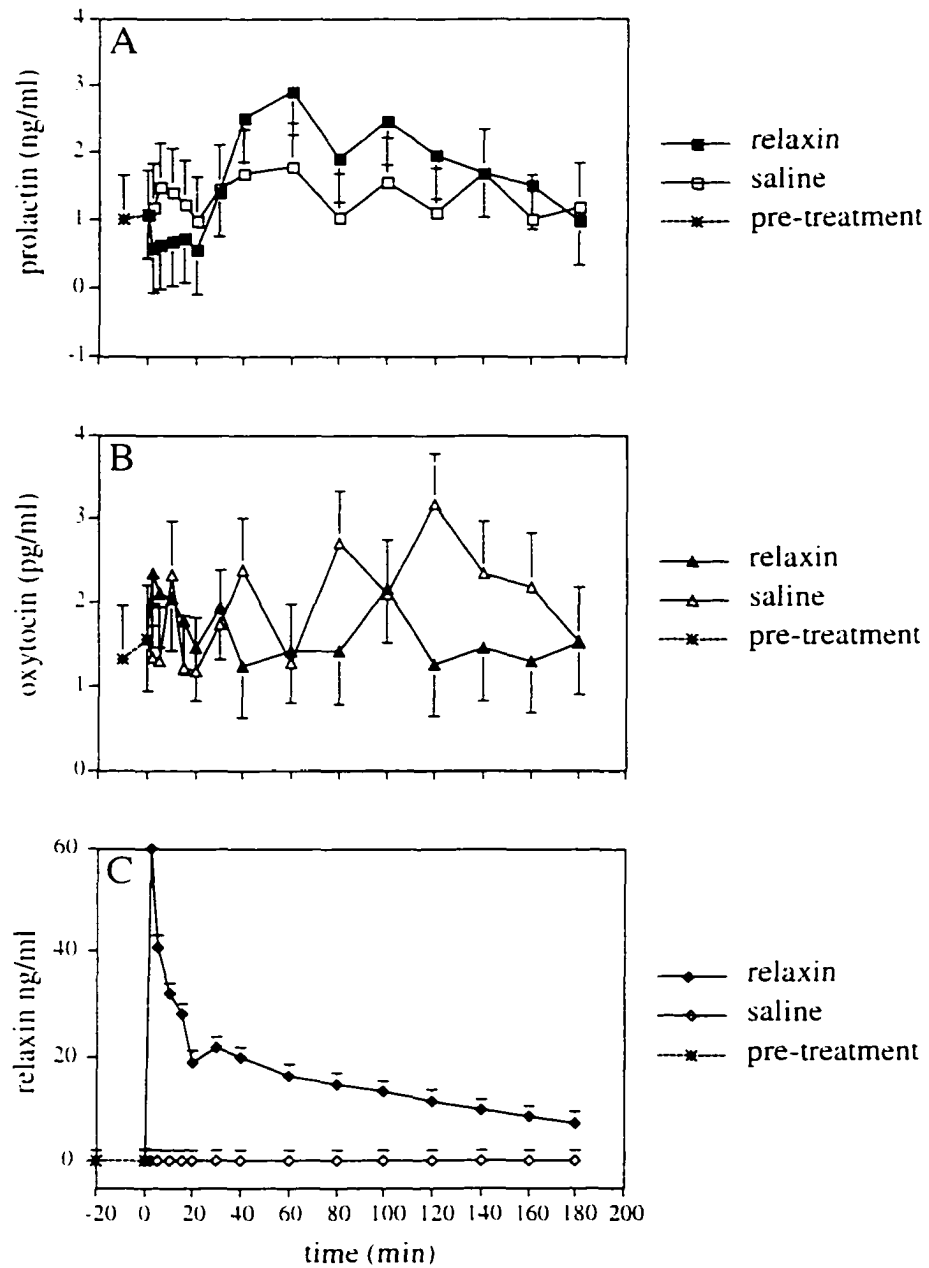


Figure 4. Late gestational gilts were treated with either relaxin (closed shapes; n = 4) or saline (open shapes; n = 4) following ovariectomy. Treatments (2 ml saline with or without 1 mg dissolved relaxin) were administered at time zero. Blood was drawn prior to treatment and for a period of 180 min following treatment. Hormone profiles are shown for prolactin (A), oxytocin (B) and relaxin (C). Data points are the mean \pm SE.

Late gestational plasma hormone concentrations post-ovx following relaxin or saline treatments



CHAPTER 3
RELAXIN-INDUCED EXPRESSION OF FOS IN THE FOREBRAIN OF THE
LATE PREGNANT RAT

A paper published in *Neuroendocrinology*

Patricia A Heine, Shi Di, Lynne R Ross, Lloyd L Anderson,
Carol D Jacobson

ABSTRACT

Relaxin, administered parenterally, has been shown to increase the release of oxytocin (OT) into the circulation and increase the firing rate of OTergic neurons. The objective of the present study was to determine if relaxin administration can result in the expression of a transcription factor, which would suggest that it alters transcriptional activity within OTergic neurons at the level of the hypothalamus. Primigravid rats were ovariectomized and a jugular cannula was inserted on day 11 of gestation (g11). Pregnancy was maintained by implanting 17 β -estradiol and progesterone caplets subcutaneously at the time of ovariectomy. At g19, rats were challenged with intravenous relaxin or isotonic saline and the brains were removed for study. Immunohistochemistry was performed on coronal brain sections, utilizing Fos as a marker of cellular activation. In the group receiving relaxin, Fos-like immunoreactivity (Fos-IR) was abundant only in the supraoptic (SON) and paraventricular nuclei (PVN) of the hypothalamus, as well as in the subfornical organ (SFO). In contrast, Fos-IR in the group given isotonic saline was lacking in these three brain regions. A double label study using antibodies against Fos and OT, demonstrated that a majority of the Fos labeled cells in the hypothalamus were OTergic. Because Fos can act as

a transcription factor, we interpret these data to indicate that transcription within OTergic cells is altered following relaxin administration, with abundant Fos-IR being limited to the SON and PVN of the hypothalamus and the SFO during late pregnancy in the rat.

INTRODUCTION

Relaxin is a polypeptide hormone found in elevated concentrations during pregnancy, in a variety of vertebrates [1]. It is involved in many of the anatomical changes seen during the gestation of the rat, in part through its remodeling of collagen. Changes are seen in the cervix [2,3], vagina [3], and mammary apparatus [4]. Relaxin weighs 6000 daltons and is a member of the family of insulin-like growth factors [1]. In female rats, relaxin is made in the corpora lutea, where it is found in elevated concentrations in the latter part of pregnancy [5,6]. Relaxin concentrations in the rat first become detectable in the blood on gestational day 10 (g10), and plateau between 40-70 ng/ml by g14 [1,7]. Serum relaxin levels during the antepartum period rise in two phases. The first rise occurs 60 h prior to parturition and is maintained until the second phase occurs about 24 h prior to parturition [1]. At these times, levels reach between 80 and 200 ng of relaxin per milliliter of serum [1,7].

In addition to the changes seen in the collagen of the reproductive tract, relaxin also has an influence at the level of the central nervous system (CNS). Although a receptor for relaxin has not yet been characterized, relaxin binding sites have been localized autoradiographically in the rat brain [8]. Areas that bind the ^{32}P -labeled relaxin include the subfornical organ, paraventricular, arcuate and supraoptic nuclei, amygdala, hippocampus

and neocortex. In addition, relaxin has been found immunohistochemically in the arcuate nucleus of the rat brain [9]. Because relaxin is a large molecule, and not likely to pass the blood brain barrier (BBB), these findings led to a search for relaxin messenger RNA in the rat brain. The relaxin message was found in the brain, as well as in other tissues such as the placenta, endometrium, and prostate [9,10]. In the brain, relaxin mRNA was located in the hippocampus, taenia tecta, anterior olfactory nucleus, piriform cortex and neocortex [10]. Additionally, relaxin has access to the CNS through circumventricular organs (CVOs). One CVO, commonly implicated in water balance homeostasis, is the subfornical organ (SFO). The SFO, like other CVOs, does not have an intact BBB, and therefore, allows substances to pass from the circulation into the neural tissue [11]. Because of its location surrounded by the dorsal third ventricle, peptides can be transported into the cerebral spinal fluid (CSF) through tight junctions between ependymal cells lining the ventricular surface [12]. In support of this theory, relaxin has been detected in the CSF following intravenous (i.v.) administration to rats [13]. And, in addition, relaxin's effects on the reflex milk ejection and pressor response are abolished following lesioning of the subfornical organ [14,15].

It seems that relaxin can modulate the concentration of circulating hormones through its actions in the central nervous system. Oxytocin (OT) [16], vasopressin [17,18], and prolactin [19] plasma levels are changed following relaxin administration. Previous work has shown that relaxin administered intravenously to rats increased the firing rate of neurons in the supraoptic nucleus (SON) and also increased circulating concentrations of OT in the periphery [16]. But, no studies have yet demonstrated an alteration in the

transcriptional activity within hormone producing neurons of the hypothalamus as a result of relaxin administration.

C-fos is a proto-oncogene that is activated immediately following certain cellular stimuli but is only active transiently [20,21]. Fos, the protein translated by *c-fos*, has been widely used as a marker of cellular activation. After Fos has heterodimerized with Jun (the product of a similar immediate-early gene), and under certain conditions as a homodimer [21], it can act as a transcription factor by binding to the AP-1 site on DNA [22,23]. Stimuli, such as osmotic stress [24,25] and copulation [26], have been shown to activate *c-fos* in the rat hypothalamus. In addition, Fos is present in nuclei following administration of peptides such as interleukin 1b [27] and angiotensin II [28]. A preliminary study in our laboratory indicated that the peptide relaxin is also capable of activating *c-fos*. Because relaxin has been shown to influence the release of OT, the present study has been designed to examine the possibility that relaxin also alters the transcriptional activity within OTergic nuclei. The aim of this experiment is to locate Fos immunohistochemically to indicate areas of cellular activation and possible transcriptional activity. Utilizing Fos to functionally map the hypothalamus in response to relaxin has allowed us to explore our hypothesis that: i.v. administration of relaxin alters the transcriptional activity within the nuclei of OTergic cells of late pregnant rats.

MATERIALS AND METHODS

Animals

Sprague-Dawley virgin female rats, 2 months of age, were obtained from Harlan Sprague-Dawley (Indianapolis, IN). Rats were housed in plastic cages at 23°C, on a 14.10 h light-dark cycle, with food (Teklad Rodent Diet: Harlan Sprague-Dawley) and water available *ad libitum*. The animals and procedures used were in accordance with the guidelines and approval of the Iowa State University Committee on Animal Care.

Animal Preparation

Eight virgin cycling rats were housed with males and checked daily by vaginal smear at 08:00 for the presence of sperm. Females were returned to individual housing units upon positive sperm checks. The day on which sperm were found was designated g1. Rats were ovariectomized (OVX) on g11, to remove the primary source of relaxin, and rats in the experimental group were then given relaxin on g19, to mimic the first spike that is seen in intact animals. The pregnancies were maintained by subcutaneously placed timed-release pellets of progesterone and 17 β -estradiol.

On g11, rats were anesthetized with ether, bilaterally ovariectomized (OVX) and fitted with a jugular vein cannula [29]. Cannulas were made of Silastic tubing (0.020 in. ID, 0.037 in. OD) and sheeting (Dow Corning, Midland, MI). Each cannula was filled with heparinized saline (10 units/ml) and flushed every 24–48 h until the animals were sacrificed. During surgery, timed-release progesterone (63 mg) and 17 β -estradiol (4.2 mg) pellets (Innovative Research of America, Sarasota, FL) were placed subcutaneously, at the level of

the interscapular space, to maintain pregnancy. This method of steroid replacement has previously been shown to sustain blood levels of progesterone and estradiol [30,31], which have been shown necessary to maintain pregnancy [32]. Blood levels of these hormones were not checked in the present experiment to determine if optimal levels were maintained. An average of six fetuses were retained per dam using this method of steroid replacement (data not shown). This number of fetuses is lower than one might expect, suggesting that circulating progesterone might not have been present in optimal levels. But, at this time, a definitive explanation is not apparent.

Tissue Preparation

All animals remained in the colony until g19. This point in gestation coincides with the initial antepartum rise in the circulating levels of relaxin in intact rats [1]. Relaxin was administered to rats in the experimental group on g19, to mimic this natural peak. Rats in this group (n=4) were given 10 µg of relaxin [16] dissolved in 0.2 ml of 0.9% sodium chloride solution (isotonic saline), and control animals (n=4) received an equivalent volume of vehicle (n=4). All treatments were given intravenously. This dosage of relaxin and route of administration has been shown to increase circulating concentrations of OT in rats [16]. Relaxin for this experiment was extracted from the ovaries of pregnant pigs and purified according to procedures described previously [33]. Injections were given through the indwelling cannula at 12:00. Two hours postinjection, rats were deeply anesthetized with ether, and transcardially perfused with 500 ml isotonic saline, followed by 500 ml chilled 4% paraformaldehyde (PF; pH 7.3). All rats were checked for viable fetuses, and only those

with at least four live fetuses were used in the study. The dams' brains were then removed and blocked using identifiable landmarks and postfixed in 4% PF for 24 h at room temperature. Brains were then sunk in a sodium phosphate buffered saline 30% sucrose solution at 4°C, followed by sectioning on a cryostat (Reichert-Jung 2800N) into 40 µm serial coronal sections. Sections were stored in tissue culture wells (one brain/six wells) containing cryoprotectant [34] at -20°C until processed for immunohistochemistry (IHC).

Immunohistochemistry

The protocol utilized for IHC was a modification of what we have previously reported [35,36] with some changes due to the nature of the tissue preparation. Free floating sections, 240-310 µm apart, were rinsed with 50 mM potassium phosphate buffered saline (KPBS). To rid sections of endogenous peroxidase, they were placed in 0.1% H₂O₂ for 20 min at room temperature. Tissue was then incubated in blocking solution composed of KPBS, 1% bovine serum albumin (BSA; Sigma, St. Louis, MO), 0.4% Triton-X 100 (TrX; Sigma) and 1.5% Normal Goat Serum (NGS; Vector, Burlingame, CA), for 2 h. Tissue was subsequently incubated for 36 h at 4°C in rabbit polyclonal Fos antibody (Ab-2, Oncogene, Cambridge, MA) diluted to 1:1000 in KPBS, 1% BSA, 0.4% TrX and 1% NGS. Sections were then washed in KPBS with 0.02% Triton-X 100 (KPBS/TrX), followed by a 2-h incubation in biotinylated goat anti-rabbit secondary antibody (2°; Vector), composed of diluent: KPBS/TrX, 1% BSA, 1.5% NGS plus 0.5% 2° antibody. This same diluent was used to make the Avidin Biotin Complex (ABC; Vector Elite Kit), using 0.2% ABC. Sections were incubated in ABC for 1 h at room temperature. Following additional washes,

the tissue was reacted with diaminobenzidine (DAB; Sigma) in a solution consisting of 0.1 M sodium acetate, 2.5% NiSO₄, 0.04% DAB and 0.01% H₂O₂, for 8 min. The reaction was terminated with isotonic saline. The tissue sections were then mounted onto gelatin coated slides, dried overnight, counterstained using a 1% neutral red solution (Fisher Scientific, Pittsburgh, PA), dehydrated in graded alcohols and coverslipped. Negative controls, used in each run, were generated by omitting the incubation in primary antibody and substituting blocking solution during the incubation period. *Double label study*

The double label IHC study was performed in the same manner as the single label study for the first 2 days. Following termination of the DAB reaction, the tissue was washed four times in KPBS, followed by a 15-min incubation in 0.1% H₂O₂. Tissue was then put in blocking solution, made as above, for 2 h. Following this, tissue was incubated in the second primary antibody, a rabbit polyclonal OT antibody (1:3000; Peninsula, Belmont, CA), overnight at room temperature. The protocol for the third day was identical to that for the second day, with the exception of the ABC concentration, which was 0.05% for the cytoplasmic antigen, and the DAB solution, which did not include Ni₂SO₄, and resulted in a brown immunoreactive product.

Analysis

The brain sections were analyzed by using a Zeiss Axiophot microscope and an IBAS-20 image analysis system. Thirty equally spaced sections from the mid- and fore-brain were analyzed for all rats in the control and experimental groups. Sections were viewed, without knowledge of treatment group, under the light microscope for areas of Fos-like

immunoreactivity (Fos-IR). The areas of the brain expressing Fos-IR were delineated by using anatomical landmarks in the coronal plane and a stereotaxic atlas of the rat brain [37]. The Fos-IR was quantified by using a protocol similar to that used previously to quantify the number of Fos-IR estrogen receptor containing cells in the brain [35,38], by counting the total number of immunoreactive nuclei/specified area. The brain sections were viewed on a Zeiss Axiophot microscope at 12.5X, and the nuclei containing Fos-IR were discriminated from those not exhibiting Fos-IR by using an IBAS-20 image analysis system. The regions examined were the SON [37; SO plates 21-25], PVN [37; Pa plates 23-26], SFO [37; plates 21-24] and the retrochiasmatic supraoptic nucleus (SOR) [37; plates 26-30]. The total unilateral field area of each region, per individual 40 μm section, was determined as well as the number of immunoreactive nuclei within each unilateral field area. The data obtained were then statistically analyzed with a Student's *t*-test. Values were deemed statistically significant if $p < 0.05$.

The double label study looked for localization of Fos- and OT-IR in the same cells. Coronal brain sections mounted serially onto slides were analyzed at 10X and 40X on the light microscope. Because oxytocin's primary site of synthesis is in the SON and PVN, these were the only brain regions examined for double labeled cells.

RESULTS

Localization of Fos-IR

Prominent Fos-IR was apparent within the SON, PVN and SFO of the relaxin-treated animals. In addition to these three regions, occasional cellular nuclei exhibiting Fos-

IR were seen in the following brain regions: cortex, periventricular hypothalamic nucleus, paraventricular nucleus of the thalamus, arcuate and retrochiasmatic supraoptic nuclei, supramammary decussation, lateral and medial geniculate nuclei, central gray, and various regions of the amygdala. Fos immunoreactivity was comparable in these regions between the two treatment groups. Although a significant difference was not obvious in the SOR, the amount of immunoreactivity within the nucleus was determined; there was not a statistically significant difference between the two groups (data not shown).

Quantification of Fos-IR

Fos immunoreactivity was abundant in the SON, PVN and SFO (fig. 1B,D,F) 2 h following relaxin treatment, whereas Fos-IR was virtually absent in the saline-treated controls (fig. 1A,C,E). Because the SFO varies significantly in size, from rostral to caudal, it was divided into rostral and caudal parts for statistical analysis. Both parts of the SFO, as well as the SON and PVN displayed immunoreactive nuclei evenly distributed throughout the respective region. Results of the quantification of the Fos-IR demonstrated a significant difference between the two treatment groups in the SON ($t=4.7$, $p < 0.01$), PVN ($t=4.1$, $p < 0.01$) and both parts of the SFO (rostral: $t=19.1$, $p < 0.0001$; caudal: $t=10.6$, $p < 0.0002$), but not in the SOR ($t=1.9$, $p < 0.1$). In order to demonstrate that the areas under study were not significantly different in the two groups, the average total unilateral field area of each region was determined. There was not a significant difference between groups in the averages of the field areas that were evaluated for any of the 4 regions shown in fig. 2. Therefore, it was not necessary to take differences in total field area into

consideration when calculating the average number of immunoreactive nuclei per field area.

Fos and OT double label study

Oxytocinergic cells were found throughout the SON and in the magnocellular PVN. Brain sections from each animal were carefully examined under the light microscope, to approximate the number of cells in which both OT- and Fos-IR were located. Because of the lack of a technique for quantifying the exact number of cells in which two non-fluorescing dyes are located in thick tissue sections, no statistical analysis was performed on these observations. Within the SON, there was a high degree of cellular coexistence of Fos- and OT-IR in the relaxin-treated rats. A majority of the magnocellular cells in the rostral and caudal PVN also showed coexistence of OT- and Fos-IR (fig. 3A and B, respectively); however, many of the parvocellular nuclei in the caudal portion of the PVN were positive for Fos and not for OT (fig. 3B). As in the single-label study for Fos, very little Fos-IR was seen in the control animals (fig. 3C). In one of the animals receiving isotonic saline, several of the nuclei within the caudal PVN were Fos immunoreactive, suggesting that additional factors may have also induced *c-fos* in animals under study.

DISCUSSION

This study demonstrates that Fos-IR is visualized in the magnocellular hypothalamic system, as well as in the SFO, following i.v. administration of relaxin. Significant differences in the amounts of Fos-IR seen between the experimental and control groups, were found in the SFO, PVN, and SON. Cells of the SON and PVN produce and secrete OT, and relaxin increases the release of OT, as well as increasing the electrical activity within OTergic cells

of the SON [16]. Furthermore, results from the double label IHC study demonstrate that many of the OTergic cells within the SON and PVN express Fos after relaxin administration, suggesting that i.v. relaxin alters transcriptional activity within OTergic cells.

The use of Fos as a marker of transcriptionally or synaptically activated neurons has been previously reviewed [23]. However, it is imperative to acknowledge the limitations of interpreting the immunohistochemical localization of Fos as a marker of transcriptional activity within OTergic neurons. The AP-1 site must be bound by a dimer to be activated, and Fos is only stable as a heterodimer with Jun. Both of these proteins are present in high levels following cellular stimulation [22], and although Jun wasn't directly localized in this study, the antibody utilized precipitates not only the 62 kD Fos protein, but the Fos/Jun complex [39]. However, due to significant post-translational modification [23] resulting in proteins ranging from 55 to 72 kD, it is unclear in which form Fos is found in high concentrations following relaxin stimulation, and if that form is adequately recognized by the antibody. The ability of the Fos/Jun complex to bind to the OT gene is uncertain. There is a sequence in the OT gene which differs by one base from the AP-1 site [40,41]. If Fos is unable to act as a transcription factor at this site, Chan et al. [42] have previously discussed Fos' ability to interact with other cellular components, and in that way alter cellular output. It should also be pointed out that Fos has been identified as a transcription factor for proenkephalin [43], which is also a product of OTergic cells.

Fos is generally regarded as a transcription factor, induced by environmental signals. The signal studied in the present experiment was relaxin; previously shown to not only bind to cells of the SON and PVN [8], but to affect the electrophysiology of these neurons [16]. As demonstrated in this experiment, relaxin results in an expression of Fos in the hypothalamus. Due to the acute release of OT following relaxin administration [16], it is possible that Fos is responding to the decreased amount of stored OT, by activating the transcription of oxytocin messenger.

Fos is found in activated cells from approximately 30 min postactivation to about 8 h. It reaches its peak concentration at about 90-120 min [23,24]. For this reason, Fos is a useful indicator of early, yet transient, cellular activation. It has been previously used to locate OTergic neurons activated in response to suckling pups [44,45]. Other neurons have shown Fos-IR following stimuli such as osmotic stress [24,25], sexual activity [26], and peptide administration [27,28,45]. Our unpublished data indicate that intracerebroventricular injection of relaxin in rats activates *c-fos*, resulting in the expression of Fos in nuclear regions, including the SON and PVN. The study described here demonstrates that i.v. administration of relaxin is also a stimulus for the activation of *c-fos*.

The amount of Fos-IR in the PVN varied among animals receiving isotonic saline; however, the average number cells exhibiting Fos-IR for all control animals was significantly smaller than that in animals receiving relaxin. The limited variability in Fos expression seen within the control group can be explained by differences in animal response to the stress of being handled, because stress has been shown to induce Fos-IR in the

hypothalamus [46]. In the relaxin group, the parvocellular PVN has numerous non-O₂Tergic cells expressing Fos (fig. 3B), which might also be activated because of individual differences in the response to stress. However, cells in the PVN are also responsible for producing vasopressin, which is released in response to relaxin [17,18]. Therefore, the non-O₂Tergic Fos immunoreactive cells in the parvocellular PVN of relaxin-treated animals may be responding to stress, may be vasopressinergic cells responding to relaxin, or may be cells responsible for synthesizing other peptides not yet shown to be activated by relaxin.

Fos immunoreactivity in the SFO was almost exclusively seen in the group of animals receiving relaxin. The SFO is a circumventricular organ involved in maintaining water balance, and is therefore sensitive to osmotic changes. As mentioned previously, osmotic stress has been shown to activate Fos; however, testing with an osmometer showed only a minute difference between the osmolarity of isotonic saline and relaxin (data not shown). Therefore, Fos should not have been induced in the SFO of relaxin-treated animals in this study in response to a change in the osmolarity of the blood. Previous studies have shown that lesions of the SFO negate the effects that relaxin has on blood pressure [15], which demonstrates that relaxin can act at the level of the brain to exert effects on the periphery. Autoradiographic binding studies have also shown that the SFO has a high concentration of binding sites for relaxin [8]. Interestingly, connections between the SFO and SON have previously been shown to exist [46], and relaxin preferentially activates these connections over connections between the SFO and other areas of the brain [47]. Because the SFO has an incomplete BBB, and relaxin is too large to pass through the BBB, it is

possible that relaxin's actions are mediated through the SFO. But, there are also relaxin binding sites in other areas of the brain, including the PVN and SON [8], and therefore a question as to how it reaches these sites. Relaxin could be acting directly on the SON and PVN as a neurotransmitter from the SFO following endocytosis by the cell bodies of the SFO and transport down the axons. On the contrary, relaxin could be acting indirectly on the SON and PVN by eliciting an action potential in the cells of the SFO that project to the SON and PVN. Or, relaxin transported into the CSF from the SFO, may reach the SON and PVN. Relaxin mRNA has been found in discrete regions of the rat brain, however, such as the anterior olfactory nucleus, taenia tecta, and the hippocampus [10], which supports the idea that relaxin is synthesized within the brain and from there transported to its areas of action in other regions of the brain.

Previous studies have shown that estrogen priming is necessary to obtain the optimal action of relaxin in relaxing the pubic symphysis in guinea pigs and mice [1]. It is possible that the action of relaxin in the brain is also facilitated by estradiol. Estrogen receptors are found in the brain of rats, including areas within the hypothalamus. The rats in the present study received timed-release pellets containing 4.2mg of 17 β -estradiol. This has been shown, when used in conjunction with progesterone replacement [30,31], to sustain adequate blood levels of these ovarian steroids to maintain pregnancy following ovariectomy [32]. Producing pseudopregnant rats and ovariectomizing them before the challenge with relaxin might produce an answer to the necessity of estrogen in facilitating the effects of relaxin in the brain of pregnant rats.

In summary, we have demonstrated that i.v. relaxin administration results in abundant Fos-IR within the SON, PVN and SFO. Because Fos can be a marker of transcriptional activation, these findings begin to define a role for relaxin as a signal for cells to alter their transcriptional activity. It has already been shown that relaxin increases DNA synthesis in porcine granulosa cells [49]. The present study is the first presentation of evidence that relaxin may have the ability to alter activity of OTergic neurons at the DNA level. In addition to the response that OTergic cells show to relaxin by altering their release of OT, as has been previously shown, this study indicates that relaxin could be involved in the synthesis of OT or other products of OTergic cells.

ACKNOWLEDGMENTS

We thank Cathy Kuehl-Kovarik, Tracey Pepper, Jay Swanson, and Joel Elmquist for technical support and Margie Carter, Iowa State University Image Analysis Facility and Li Wu for analysis of data. Purified porcine relaxin was generously provided by Christian Schwabe, Department of Biochemistry and Molecular Biology, Medical University of South Carolina, S.C. This paper was presented in part at the 25th Annual Meeting of the Society of Neuroscience, 1995, San Diego (abstract 351.15). This work was supported by the US Department of Agriculture, ARS, CSRS and OGPS Competitive Grant 93-37203-8965. This is Journal Paper No. J-17054 of the Iowa Agriculture and Home Economics Experiment Station, Ames, Iowa, Projects No. 3316 and 3223, and supported by the Hatch Act and State of Iowa funds.

REFERENCES

1. Sherwood OD: Relaxin; in: Knobil E, Neill JD (eds): *The Physiology of Reproduction*, ed 2. New York, Raven Press, 1994, vol 1, pp 861-1009.
2. Downing SJ, Sherwood, OD: The physiological role of relaxin in the pregnant rat. III. The influence of relaxin on cervical extensibility. *Endocrinology* 1985;116:1215-1220.
3. Burger LL, Sherwood OD: Evidence that cellular proliferation contributes to relaxin-induced growth of both the vagina and cervix in the pregnant rat. *Endocrinology* 1995;136:4820-4826.
4. Hwang JJ, Lee AB, Fields PA, Haab LM, Mojonier LE, Sherwood OD: Monoclonal antibodies specific for rat relaxin. V. Passive immunization with monoclonal antibodies throughout the second half of pregnancy disrupts development of the mammary apparatus and, hence, lactational performance in rats. *Endocrinology* 1991;129:3034-3042.
5. Anderson LL, Bast JD, Melampy RM: Relaxin in ovarian tissue during different reproductive stages in the rat. *J Endocrinol* 1973;59:371-372.
6. Fields PA: Intracellular localization of relaxin in membrane-bound granules in the pregnant rat luteal cell. *Biol Reprod* 1984;30:753-762.
7. Sherwood OD, Crnekovic VE, Gordon WL, Rutherford JE: Radioimmunoassay of plasma relaxin throughout pregnancy and during parturition in the rat. *Endocrinology* 1980;107:691-698.
8. Osheroff PL, Phillips HS: Autoradiographic localization of relaxin binding sites in rat brain. *Proc Natl Acad Sci USA* 1991;88:6413-6417.

9. Gunnarsen JM, Crawford RJ, Tregar GW: Expression of the relaxin gene in rat tissue. *Mol Cell Endocrinol* 1995;110:55-64.
10. Osheroff P, Ho WH: Expression of relaxin mRNA and relaxin receptors in postnatal and adult rat brains and hearts. *J Biol Chem* 1993;268:15193-15199.
11. Dellmann HD, Simpson JB: The subfornical organ. *Internatl Rev Cytol* 1979;58:333-421.
12. Pardridge WM, Frank HJL, Conford EM, Braun LD, Crane PD, Oldendorf WH: Neuropeptides and the blood-brain barrier; in: Martin JB, Reichlin S, Blick KL (eds): *Neurosecretion and Brain Peptides*. New York, Raven Press, 1981, pp 321-328.
13. Parry LJ, Poterski RS, Wasnidge C, Summerlee AJS: Relaxin in the cerebrospinal fluid of female rats. *Soc Neurosci Abstr* 1991;17:1196 (474.28).
14. Summerlee AJS, O'Byrne KT, Jones SA, Eltringham L: The subfornical organ and relaxin-induced inhibition of reflex milk ejection in lactating rats. *J Endocrinol* 1987;115:347-353.
15. Mumford D, Parry LJ, Summerlee AJS: Lesion of the subfornical organ affects the hemotensive response to centrally administered relaxin in anaesthetized rats. *J Endocrinol* 1989;122:747-755.
16. Way SA, Leng G: Relaxin increases the firing rate of supraoptic neurones and increases oxytocin secretion in the rat. *J Endocrinol* 1992;132:149-158.
17. Yang RH, Bunting A, Wyss M, Bereck KH, Zhang L, Jin H: Pressor and bradycardic effects of centrally administered relaxin in conscious rats. *Am J Hyperten* 1995;8:375-381.

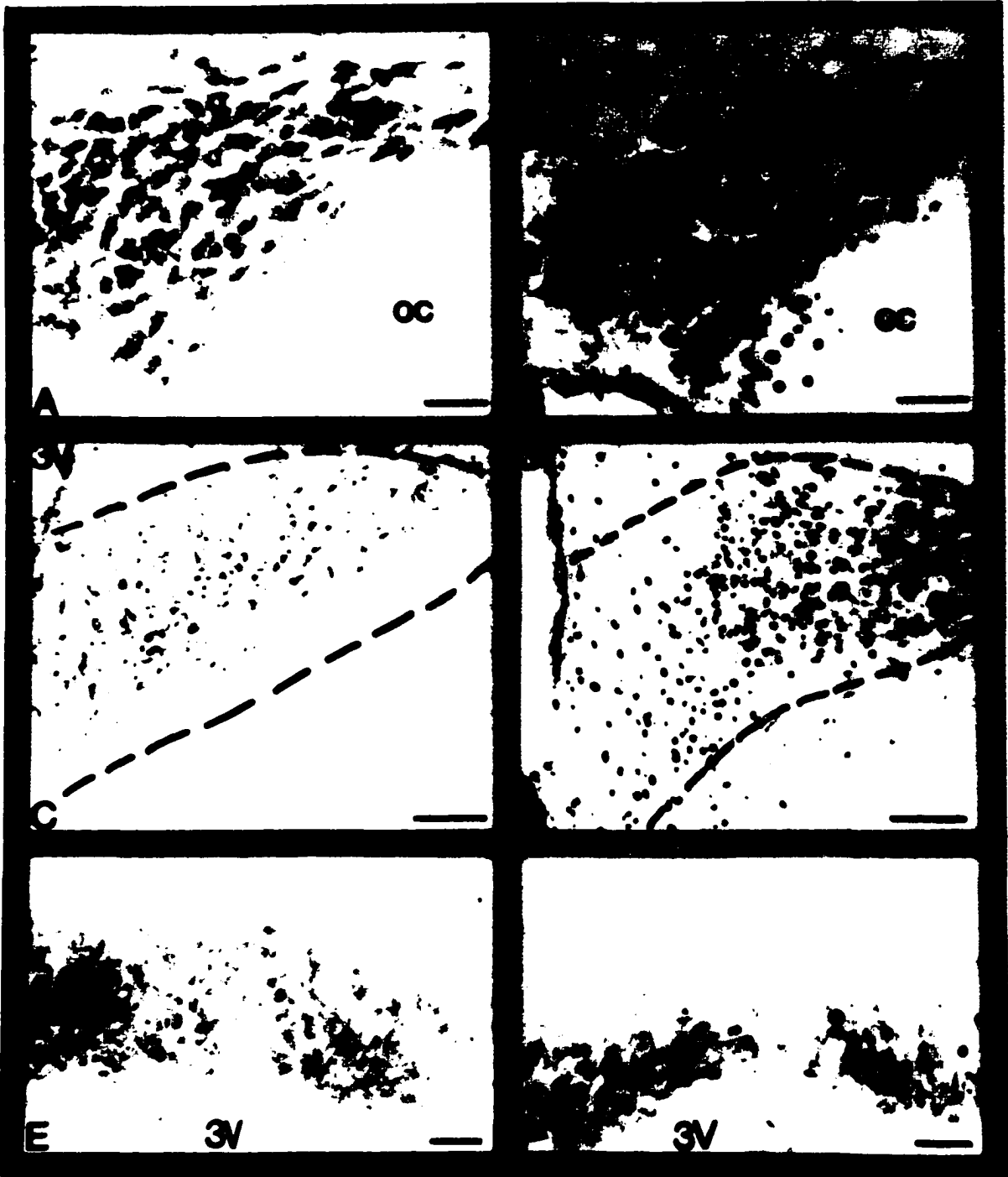
18. Jones SA, Summerlee AJS: Relaxin increases blood pressure and vasopressin levels in anaesthetized rats. *J Physiol* 1986;381:37P.
19. Li Y, Huang C, Klindt J, Anderson LL: Stimulation of prolactin secretion in the pig: Central effects of relaxin and the antiprogestone RU 486. *Endocrinology* 1993;133:1205-1212.
20. Sagar SM, Sharp FR, Curran T: Expression of *c-fos* protein in brain: metabolic mapping at the cellular level. *Science* 1988;240:1328-1330.
21. Hesketh R: FOS; in: *The Oncogene Handbook*. San Diego, Academic Press, pp 178-199.
22. Curran T, Franza BR: Fos and Jun: The AP-1 connection. *Cell* 1988;55:395-397.
23. Morgan JI, Curran T: Stimulus-transcription coupling in the nervous system: involvement of the proto-oncogenes *fos* and *jun*. *Annu Rev Neurosci* 1991;14:421-451.
24. Sharp FR, Sagar SM, Hicks K, Lowenstein D, Hisanaga: C-fos mRNA, Fos, and Fos-related antigen induction by hypertonic saline and stress. *J Neurosci* 1991;11:2321-2331.
25. Giovannelli L, Shiromani PJ, Jirikowski GF, Bloom FE: Oxytocin neurons in the rat hypothalamus exhibit *c-fos* immunoreactivity upon osmotic stress. *Brain Res* 1990;531:299-303.
26. Flanagan LM, Pfaus JG, Pfaff DW, McEwen BS: Induction of FOS immunoreactivity in oxytocin neurons after sexual activity in female rats. *Neuroendocrinology* 1993;58:352-358.
27. Rivest S, Torres G, Rivier C: Differential effects of central and peripheral injection of interleukin 1b on brain *c-fos* expression and neuroendocrine function. *Brain Res* 1992;587:13-23.

28. Xu Z, Herbert J: Regional suppression by lesions in anterior third ventricle of *c-fos* expression induced by either angiotensin II or hypertonic saline. *Neuroscience* 1995;67:135-147.
29. Terkel J, Rosenblatt JS: Humoral factors underlying maternal behavior at parturition: cross transfusion between freely moving rats. *J Comp Physiol Psychol* 1972;80:365-371.
30. Barron WM, Schreiber J, Linheimer MD: Effect of ovarian sex steroids on osmoregulation and vasopressin secretion in the rat. *Am J Physiol* 1986;250:E352-E361.
31. Katovich MJ, O'Meara J: Effect of chronic estrogen on the skin temperature response to naloxone in morphine-dependent rats. *Can J Physiol Pharm* 1987;65:563-567.
32. Arkaravichien W, Kendle KE: Critical progesterone requirement for maintenance of pregnancy in ovariectomized rats. *J Reprod Fertil* 1990;90:63-70.
33. Büllsbach EE, Schwabe C: Naturally occurring porcine relaxin and large scale preparation of the B29 hormone. *Biochemistry* 1985;24:7717-7722.
34. Watson RE, Wiegand SJ, Clough RW, Hoffman GE: Use of cryoprotectant to maintain long-term peptide immunoreactivity and tissue morphology. *Peptides* 1986;7:49-58.
35. Elmquist JK, Ackerman MR, Rimler RB, Ross LR, Jacobson CD: Induction of Fos-like immunoreactivity in rat brain following *Pasteurella multocida* endotoxin administration. *Endocrinology* 1993;133:3054-3057.
36. Elmquist JK, Fox CA, Ross LR, Jacobson CD: Galanin-like immunoreactivity in the adult and developing Brazilian opossum brain. *Dev Brain Res* 1992;67:161-179.

37. Paxinos G, Watson C: The rat brain in stereotaxic coordinates. 1986. Academic Press, San Diego.
38. Pearson PL, Ross LR, Jacobson CD: Differential down regulation of estrogen receptor-like immunoreactivity by estradiol in the female Brazilian opossum brain. *Brain Res* 1993;617:171-175.
39. Chan RKW, Sawchenko PE: Spatially and temporally differentiated patterns of *c-fos* expression in brainstem catecholaminergic cell groups induced by cardiovascular challenges in the rat. *J Comp Neurol* 1994;348:433-460.
40. Fenelon VS, Poulain DA, Theodosis DT: Oxytocin neuron activation and fos expression: a quantitative immunocytochemical analysis of the effect of lactation, parturition, osmotic and cardiovascular stimulation. *Neuroscience* 1993;53:77-89.
41. Ivell R, Richter D: Structure and comparison of the oxytocin and vasopressin genes from rat. *Proc Natl Acad Sci USA* 1984;81:2006-2010.
42. Chan RWK, Brown ER, Ericsson A, Kovacs KJ, Sawchenko PE: A comparison of two immediate-early genes, *c-fos* and NGFI-B, as markers for functional activation in stress-related neuroendocrine circuitry. *J Neurosci* 1993;13:5126-5138.
43. Sonnenberg JL, Rauscher FJ, Morgan JI, Curran T: Regulation of proenkephalin by fos and jun. *Science* 1989;246:1622-1625.
44. Eriksson M, Ceccatelli S, Uvnas-Moberg K, Iadarola M, Hökfelt T: Expression of Fos-related antigens, oxytocin, dynorphin and galanin in the paraventricular and supraoptic nuclei of lactating rats. *Neuroendocrinology* 1996;63:356-367.

45. Verbalis JG, Stricker EM, Robinson AG, Hoffman GE: Cholecystokinin activated *c-fos* expression in hypothalamic oxytocin and corticotropin-releasing hormone neurons. *J Neuroendocrinol* 1991;3:205-213.
46. Pezzone MA, Lee WS, Hoffman GE, Rabin BS: Induction of *c-fos* immunoreactivity in the rat forebrain by conditioned and unconditioned aversive stimuli. *Brain Res* 1992;597:41-50.
47. Sgro S, Ferguson AV, Renaud LP: Subfornical organ-supraoptic nucleus connections: An electrophysiologic study in the rat. *Brain Res* 1984;303:7-13.
48. Wilson BC, Summerlee AJS: Relaxin excites subfornical organ neurones antidromically identified as projecting to the supraoptic nucleus but not those projecting to medial septum. *Soc. Neurosci Abstr* 1992;18:822(346.2).
49. Ohleth KM, and Bagnell CA: Relaxin-induced deoxyribonucleic acid synthesis in porcine granulosa cells is mediated by insulin-like growth factor-I. *Biol Reprod* 1995;53:1286-1292.

Figure 1. Photomicrographs of coronal sections of rat brain. Fos-like immunoreactivity is represented by black stained nuclei in the isotonic saline (A,C,E) and relaxin (B,D,F) treated animals in the SON (A,B), PVN (C,D), and caudal SFO (E,F). Abbreviations: oc=optic chiasm, 3V=third ventricle. Scale bars represent 20 μ m.



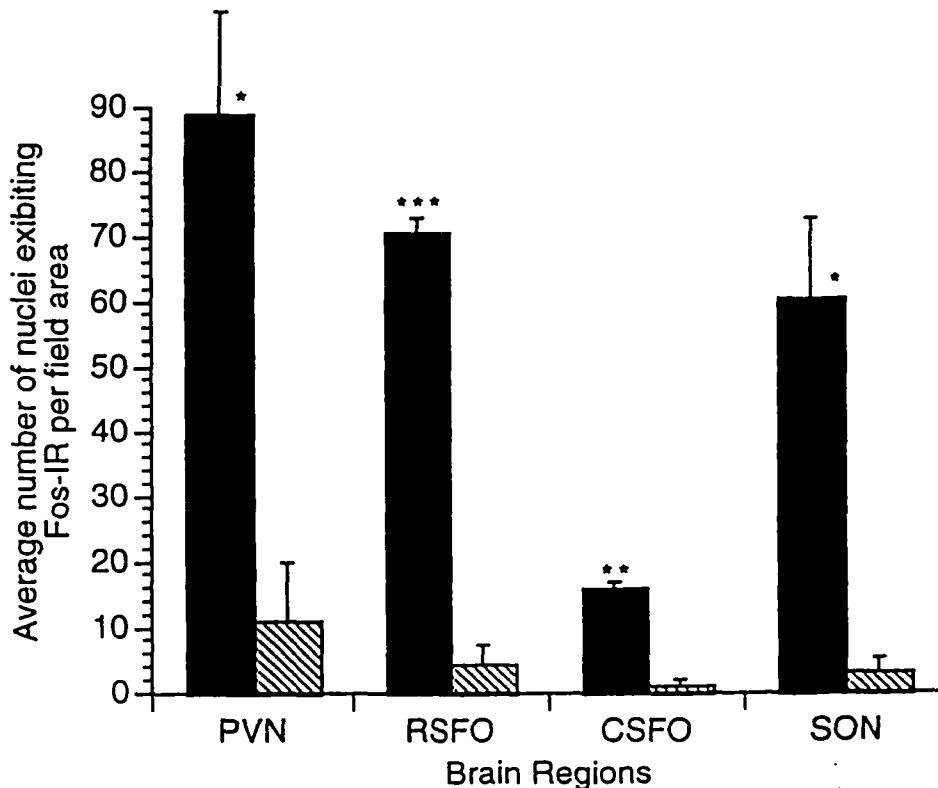
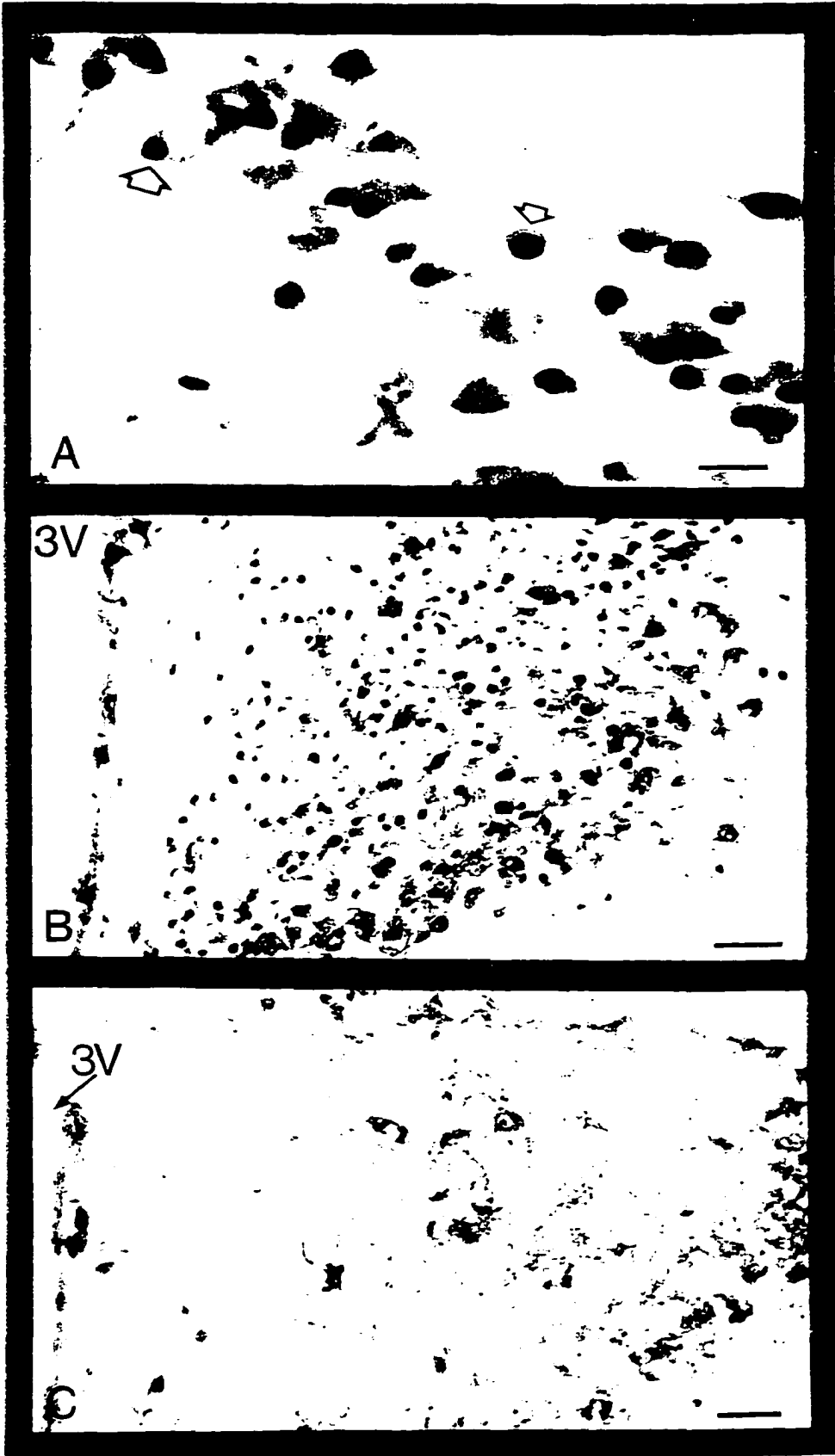


Figure 2. The effects of intravenous relaxin administration on the average number of nuclei displaying Fos-IR in each of the four regions: rostral subfornical organ (RSFO), caudal SFO (CSFO), supraoptic nucleus (SON), and paraventricular nucleus (PVN). Numbers represent averages from counts taken unilaterally (in paired regions). On average, ten field areas in the PVN were examined per animal, fourteen field areas per animal in the SON, two field areas per animal in the RSFO, and three field area per animal in the CSFO. The average number of Fos immunoreactive nuclei per field area, in animals receiving relaxin, is represented by the solid bars. The hatched bars indicate the average number of nuclei exhibiting Fos-IR per field area in the isotonic saline treatment group. In relaxin treated animals, the number of Fos-like immunoreactive cells is significantly greater than in the relaxin-treated animals in all four of the brain regions presented here: * - $p < 0.01$; ** - $p < 0.0002$; *** - $p < 0.0001$.

Figure 3. Double label photomicrographs depicting the dark Fos-like immunoreactive nuclei and brownish gold colored OT-like immunoreactive cytoplasm. **A**: rostral paraventricular nucleus (PVN) demonstrating colocalization of Fos- and OT-IR; **B** and **C** represent the caudal PVN: **B** from a relaxin-treated animal, illustrating colocalization of Fos- and OT-IR, and **C** from a control animal, illustrating the lack of Fos-IR within OT immunoreactive cells. Abbreviation: 3V = third ventricle. Open arrows point to examples of cells with coexistence of Fos- and OT-IR. Scale bar = 20 μm (**A**), 50 μm (**B,C**).



CHAPTER 4
EFFECT OF RELAXIN ON THE TRANSCRIPTION OF OXYTOCIN IN
THE MAGNOCELLULAR HYPOTHALAMUS OF THE PREGNANT RAT

A paper to be submitted to Neuroendocrinology

Patricia A Heine, Carol D Jacobson and Lloyd L Anderson

ABSTRACT

Relaxin administered to late pregnant rats induces the expression of Fos in oxytocin producing cells of the hypothalamus, indicating a change of cellular activity within these neurons. The objective of the present study was to determine if the change in cellular activity was due to an alteration in the amount of oxytocin mRNA present in these cells following relaxin administration. Primigravid rats were ovariectomized on gestational day 9 (g9). Pregnancy was maintained using progesterone and 17 β -estradiol implants. There were three control groups of rats; one which had no surgery, one which had a sham surgery, and one which was ovariectomized and received relaxin replacement. Rats were killed either on g19, g21 or g 23. Their brains were removed and prepared for *in situ* hybridization. OT mRNA levels were visualized using a ³⁵S labeled oligonucleotide probe. Slides with hybridized brain sections were exposed to radiographic film. Images on the film were quantitatively analyzed for differences in optical density. The amounts of OT mRNA were not different between any of the treatment groups or any of the gestational time points analyzed.

INTRODUCTION

Control of the synthesis and release of oxytocin (OT) during pregnancy is not fully understood. Its release depends not only on a self regulating positive feedback loop, but also upon local opioids [1, 2]. The factors controlling the synthesis of OT may also involve opioid control [3].

Relaxin and OT are both hormones which play integral roles in pregnancy and parturition. In rats, relaxin is found nearly exclusively in the corpora lutea during the latter half of pregnancy [4, 5]. Pregnancy in rats lasts 23 days, and relaxin is first detectable in the serum at 10 days of gestation (g10) [6]. It plateaus from g14 to g19 and then rises in two peaks 60 and 24 h prior to parturition [7]. It is necessary for remodeling the collagen in the reproductive tract which allows for passage of the fetuses [6], and also for developing the mammary apparatus [6]. Oxytocin is released in a diurnal rhythm [8] except during physiological states which demand its release such as parturition, when it is released in boluses to promote uterine contraction [9].

The interaction between OT and relaxin has been studied in both pigs and rats. Although not completely understood, several studies have examined the effect of relaxin on the release of oxytocin. In pigs, relaxin has been shown to have no effect on circulating OT concentrations during lactation [10]. In ovariectomized nonpregnant rats, relaxin appears to increase the release of OT into the circulation [11]. A study using pregnant rats showed that this effect was attenuated in late pregnancy [12], the findings of which were supported by a study demonstrating that the absence of relaxin decreases the opioid inhibition of OT release [13]. Several studies have looked at the relationship between

relaxin, OT and opioids [14-19]. The relationship of relaxin to the synthesis of OT, however, has not been thoroughly studied.

The relationship between relaxin and OT is best studied at the end of pregnancy when both hormones are found in the circulation in high concentrations and their relationship is dynamic. In a previous study conducted in our laboratory on late pregnant rats [19], indirect evidence pointed toward relaxin's ability to alter OT synthesis. Cellular activity within OTergic cells following relaxin administration was visualized using Fos, a transcription factor [20], as a marker. Fos binds to the AP-1 site of genes to activate or repress transcription [20, 21]. The localization of Fos in OTergic neurons in the previous study, therefore, demonstrated that a transcriptional change was occurring, but was not conclusive as to the nature of the transcriptional alteration nor the gene being transcribed. A further study needed to be designed to directly examine the levels of OT mRNA to determine if they were modulated by the presence or absence of relaxin.

The purpose of this study was to determine if relaxin is directly involved in regulating the synthesis of OT in the magnocellular hypothalamic nuclei of the late pregnant rat. *In situ* hybridization for OT mRNA in the brains of late pregnant rats was utilized to answer this question.

MATERIALS AND METHODS

Animals

Rats were housed in plastic cages, one per cage, under a 12:12 h light:dark cycle. They were fed a rodent diet (Teklad, 6% fat; Madison, WI) and provided water *ad libitum*. All procedures were approved by the Iowa State University Committee on Animal Care.

Treatment groups

This study utilized four different hormone treatments and three different gestational stages (fig. 1). The gestational days studied were g19, 21 and 23. The hormone treatments were ovariectomy with progesterone and estradiol replacement (OPE); ovariectomy with progesterone, estradiol, and relaxin replacement (OPER); sham ovariectomy, in which the animals underwent the same surgical procedure as the OPE group, except they maintained their ovaries (S); and a control group which underwent no surgery (C). Each of the twelve time by treatment groups had three rats, for a total of 36 animals in the study.

Surgery

Rats were obtained from Harlan Sprague Dawley (Indianapolis, IN). Primigravid females (2-4 months old) were housed with males for breeding. Pregnancy was determined by observing sperm in the vaginal smear. The day sperm were found was designated g1. On g9 rats in groups S, OPE and OPER underwent surgery. Anesthesia was induced and maintained using isoflurane (Solvay; Mendota Heights, MN) delivered by an anesthetic machine (Fraser Harlake; Orchard Park, NY) through a nonrebreathing system. Rats were placed in an enclosed chamber for induction using 3% isoflurane, and were maintained for the duration of surgery using 1.5% isoflurane. All anesthetic was delivered with oxygen at 1L/min.

Rats underwent bilateral laparotomy for ovariectomy. Incisions were 1 cm long, and were closed in two layers using 3-0 Ethilon (Ethicon; Somersville, NJ) in a simple

continuous pattern on the muscle layers and staples (Autoclip, 9 mm; Becton Dickenson; Sparks, MD) to close the skin. Only rats with at least 6 fetuses were retained in the study.

During the same surgical procedure animals received hormone replacement, which has been detailed elsewhere [22]. Briefly, 4 silastic tubes (Sani-Tech, Inc., LaFayette, NJ; id 1.5 mm, od 1.9 mm; 52 mm in length) filled with crystalline progesterone (Sigma, St. Louis, MO) were placed subcutaneously along the flank through one 2 cm incision over the thoracolumbar spinal region, which was closed using staples (Autoclip, 9 mm; Becton Dickenson). One silastic tube (Sani-Tech, Inc.; id 1.6 mm, od 3.2 mm, 1.6 cm in length) of 17 β -estradiol (2 μ g; Sigma) was placed subcutaneously at the nape of the neck.

The estradiol implant was replaced with an implant containing 12 μ g 17 β -estradiol on g18 to mimic the increase in estrogen levels that occur during late pregnancy [22, 23]. Progesterone implants were removed early on the morning of g22 to mimic the decline in progesterone levels that occurs at luteolysis [22-24]. Rats in the sham group received empty implants (progesterone controls) and implants containing sesame seed oil (17- β estradiol controls). All implants were sealed using Silastic glue (Dow Corning, Midland, MI). The OPER group received an Alzet osmotic pump (Alza Corp., Palo Alto, CA) subcutaneously caudal to the scapulae for the delivery of porcine relaxin (0.5 μ l/h for 14 days = pump rate). Relaxin was put into solution using 0.9% NaCl. Porcine relaxin has been proven to be biologically active in rats [6].

Tissue Preparation

Brains from the dams were removed on g19, 21 or 23 at 0600 h. Rats were lightly anesthetized using isoflurane (Solvay) followed by decapitation. Brains were carefully

removed from the calvaria, divided into fore- and hindbrain blocks, and quick-frozen in isopentane (Fisher Scientific, Pittsburgh, PA). Brains were then stored at -70°C in air tight plastic bags until sectioned. Brains were sectioned on a cryostat (Reichert-Jung 2800N) into 10- μ m coronal sections. Sections were thaw mounted onto poly-L-lysine coated slides. Approximately every other section throughout the hypothalamic region was mounted. A total of 96 sections was kept from each hypothalamus, divided into 48 rostral and 48 caudal sections. There were 8 brain sections on each slide and 6 slides in each of the two groups (twelve slides/rat). Brain sections were fixed in 4% paraformaldehyde for 5 min, rinsed in 0.1 M phosphate buffered saline, and then stored in 95% ethanol (EtOH) at -70 ° C until used for *in situ* hybridization (ISH).

In situ hybridization

Prehybridization

Slides on which brain sections were mounted, were removed from their -70 ° C storage in EtOH and allowed to dry in a dust free environment at room temperature. One rostral and one caudal slide from one of the three rats in each of the twelve time by treatment groups were run together as a hybridization group. The 36 rats were randomly assigned to one of three hybridization groups, such that each of the three groups contained one rat from each of the twelve time by treatment groups. After the slides were dry, a PAP pen (Newcomer Supply, Oak Park, IL) was used to form a hydrophobic ring around the periphery of the tissue sections to keep the labeled hybridization buffer on the tissue.

Probe preparation

Deoxyribonucleic acid (DNA) oligomers (probes) were synthesized at the Iowa State University DNA Facility. Three antisense probes, each 45 bases in length, were synthesized for three different regions of the OT DNA. All were from exons, and none showed more than a 62% homology with the same area of the vasopressin gene. These were designed to compare the labeling patterns produced by each one to ensure the probe was labeling OT mRNA. The probe which was used in all of the analyzed work was: 5'TTGCGCATATCCAGGTCTAGCGCAGCCCTCTTGCCGCCCA3'. This was antisense to the OT DNA sequence starting at base 341 [25].

Radioactive labeling of the oligonucleotide was utilized. The terminal deoxynucleotidyl transferase (TdT) end-labeling protocol was a modification of that previously published [26]. One microliter of oligonucleotide at a concentration of 0.3 pmol/ μ l was added to 2 μ l 5x reaction buffer (Boeringer Mannheim, Indianapolis, IN), 0.6 μ l CoCl₂ (Boeringer Mannheim), 6 μ l ³⁵SdATP (NEN Research Products, Boston, MA), 5 μ l sterile H₂O and 1 μ l TdT (Boeringer Mannheim). The labeling reaction was carried out in a 37 ° C waterbath for 15 min. The reaction was stopped when 35 μ l of sterile water was added to the mixture. The probe was added to prepared spin columns (Biospin 6 chromatography columns, BioRad; Hercules, CA) and centrifuged as per product specifications. Two microliters of the eluate were evaluated using liquid scintillation counting. Probes were only retained if counts were between 50,000-100,000 dpm/ μ l. If probes (labeled oligonucleotides) were not used immediately, they were stored at -20 ° C for later use.

Hybridization

The protocol utilized for the hybridization was a modification of that previously published [26]. Hybridization buffer was composed of 50% formamide (Fluka, Ronkonkoma, NY)/4 x SSC/10% dextran sulphate (Sigma). One microliter of probe was added to 100 μ l of hybridization buffer. Each slide was dipped in hybridization buffer prior to the addition of the probe/buffer mixture. This provided a fluid contact allowing the probe/buffer to more easily cover the sections on the slide. Approximately 300 μ l of the probe/buffer mixture was added to each slide. A much cleaner signal was obtained without the use of coverslips, so this step, which is a part of many ISH protocols, was omitted in this study. The slides were then placed in a humid chamber (humidified with 1:1 mixture of water and formamide) and incubated at 44 ° C overnight.

Post-hybridization

Slides were removed from the humid chamber and washed in 1 x SSC. Probes which may have hybridized to the wrong sequence were removed through an incubation in 1x SSC at 60°C for 30 min, followed by rinsing in 1 x SSC and 0.1 x SSC. Slides were then dehydrated and allowed to dry. Slides were kept in their hybridization groups (one slide from an animal in each of the twelve treatment groups) for image production which was accomplished by taping the slides to paper and exposing them to X-ray film (XAR-5, Eastman Kodak Co., New Haven, CT). In addition to the 12 experimental slides, each film had a set of brain paste standards for densitometric calculations, and two controls. The protocol for making the brain paste standards was followed exactly as previously

published [26]. Optimal exposure time for the slides on the X-ray film was 2 h. The film was developed using an automatic processor (Eastman Kodak).

Following exposure to film, the slides were dipped in emulsion. NBT-2 emulsion (Kodak Eastman) was brought to 42°C and diluted 1:1 with ddH₂O in a dark room. The liquified emulsion was transferred to a slide dipping chamber and all of the slides were dipped in the emulsion and allowed to dry in a dark box for 5 h. They were then placed in a light proof slide box at 4°C for 24 h. The slides were developed for 2 min in Dektol (Kodak Eastman) at 17°C, rinsed in ddH₂O and fixed for 20 min. Following fixation, slides were rinsed in running water for 1 h, during which the emulsion was removed with a razor blade from the back of the slides. Slides were then counter stained with a 1 % neutral red solution (Fisher Scientific), dehydrated through graded EtOH, cleared with xylene (Fisher Scientific) and coverslipped with Permount synthetic mounting media (Fisher Scientific). Figure 3 demonstrates the labeling obtained through this technique.

Image analysis

The SON [27, SO plates 21-25] and PVN [27, Pa plates 23-26] were the brain areas studied. To analyze areas on the radiographic film which corresponded to the OT producing SON and PVN, images were acquired by the Iowa State University Image Analysis Facility using an Agfa Arcus II flatbed scanner (Wilmington, MA) attached to an Apple 8500 Power PC (Cupertino, CA). The brain paste standards (dpm/mg) on each film were used with the internal scaling features of the software to accurately calibrate the densitometric data. The film images were interactively thresholded and then edited to isolate the SON and PVN for measurement. These regions were then measured to obtain

the densitometric reading. A mean value for all eight coronal brain sections on each slide was determined for both the SON and PVN. To avoid researcher bias, the treatment by time group to which each slide belonged remained coded until after the image analysis was completed.

For the purpose of illustration, a color table was created for each set of images using Adobe Photoshop (San Jose, CA) in which the grey values, corresponding to the various density levels of the brain paste standards, were assigned a specific color (Fig. 4). The same color table was applied to each brain section on the film.

Controls

Two controls were used in this experiment. The first control was a competitive control to assess for non-specific binding. Twice as much unlabeled as labeled oligonucleotide was added to the probe/buffer mixture to compete with the labeled oligonucleotide for binding sites. The second control was checking to determine if the OT gene actually was what was being labeled. This was accomplished by using all three probes synthesized against different areas of the exons of the OT gene to observe if the resultant labeling patterns were similar. The first control was exposed to film along with each of the hybridization groups. The second control was run initially to determine whether or not the probe was labeling OT mRNA.

Statistical analysis

The mean dpm/mg value for each slide on the film was uncoded and placed into its proper time by treatment group. There were two values from each animal (one from the more rostral brain sections and one from the more caudal brain sections) and three

animals per group. There was one exception in which there were only two animals because one rat managed to remove her estradiol implant, resulting in fetal resorption, so her brain was not used in the study. A mean dpm/mg value was calculated from the six individual mean values for each of the twelve treatment groups. This was conducted separately for the two regions (SON and PVN). An ANOVA was used to determine the statistical significance, which was set at $p < 0.05$.

RESULTS

This probe appeared to be very specific, as there was very little binding to any areas of the brain other than the hypothalamus. Because of this, the outline of the brain was not at all apparent in the figures, but was artificially drawn in for the purpose of orientation.

The average fetal survival for the rats which were exposed to relaxin was 13.2 pups \pm 2.86 for g19, 10.7 pups \pm 2.41 for g21 and 10.6 pups \pm 4.77 for g23. Rats which were not exposed to relaxin had a fetal survival rate of 16.3 pups \pm 2.14 for g19, 12.3 pups \pm 2.89 for g21, and 12.0 pups \pm 4.36 for g23. As was previously noted [22], rats in the OPER group had the lowest fetal survival rate of the four treatment groups.

Controls

The binding of the ^{35}S labeled oligonucleotide probe to the OT mRNA was greatly decreased when it was incubated with the unlabeled oligonucleotide, as compared to a brain section which did not have the unlabeled probe added to the incubation. Figure 2 demonstrates the similarity in the labeling from the three different probes, indicating that the probes are specific for OT mRNA.

Treatment groups

The dpm/mg values did not show a statistically significant difference in either the SON ($p > 0.05$) or the PVN ($p > 0.05$) between the treatment groups (Fig. 5) or the gestational time points (Fig. 6).

DISCUSSION

The present study showed that relaxin does not play a role in promoting the synthesis of OT in the brain of the late pregnant rat. Results of ISH for OT mRNA indicated that there was no significant difference in the dpm/mg value in either the SON or PVN when comparing either treatment groups or gestational time points. The same mean dpm/mg value was present in rats exposed to relaxin as those not exposed to relaxin. Likewise, the mean dpm/mg value was not altered in response to the proximity of parturition.

The hypothesis of this study was that relaxin plays a role in the synthesis of OT during pregnancy. It was expected that the three control groups (C, S, OPER), which were all exposed to relaxin, would all show a higher dpm/mg value than the OPE group which did not have any circulating relaxin. To closely define any differences seen between C and OPE, the sham groups was included to control for changes caused by anesthetic or the stress of handling. In addition, the OPER group was included to control for the possibility that some ovarian factor other than relaxin was responsible for observed differences. As it turned out, all three controls had nearly identical dpm/mg values. Surprisingly, the OPE group also had mean dpm/mg values which were not significantly different from the controls. Analysis of one of the six hybridization groups alone did

show that the OPE group had a lower mean dpm/mg than the controls, but this finding was not repeated in the other five hybridization groups.

Previous experiments indicated that OT mRNA concentrations did not increase significantly in the antepartum period [28, 29]. This finding was supported in the current study. Figure 5 shows that rats from group C from each of the three gestational stages did not show significantly different dpm/mg values. This is somewhat surprising in light of the imminent demand on the cells to provide sufficient OT to stimulate uterine contraction during parturition. Therefore, because neurohypophysial OT is necessary for parturition to occur, it is likely that the release of OT is inhibited since its synthesis is not increased. Relaxin has been shown to aid opioids in their inhibition of OT release [13], so perhaps that is the mechanism for increasing stores of oxytocin.

The lack of effect of relaxin on the presence of OT mRNA concentrations begs the question of what the purpose of the Fos is, observed in OTergic cells following relaxin administration [19]. The initial assumption would be that Fos is responsible for altering the transcription of another gene in the OTergic cells. One candidate is preproenkephalin. This has previously been shown to have an AP-1 site [30] and its protein product is colocalized in granules with OT [31]. However, little proenkephalin is expressed in magnocellular neurons [32]. Another gene that Fos could be regulating is the one which encodes for the μ -opioid receptor. This receptor appears to be responsible for the inhibition of OT release in late pregnancy [33], as the κ -opioid restraint seen in early pregnancy is reduced [34]. Another possibility is that Fos is coincidentally present due to a change in the firing characteristics [35] of the OTergic cells in response to the relaxin

[11] and is not actively involved in any transcriptional regulation. However, relaxin's ability to release OT is attenuated in late pregnancy [12] and therefore presumably the firing rate of the OTergic cells is also less sensitive to relaxin during this time. This indicates that Fos' expression as a result of relaxin administration may not be due to a change in the firing pattern. Additionally, it has been shown that in magnocellular neurons of the hypothalamus, synaptic activation is necessary for the expression of Fos, not a change in spike activity [36].

The OT gene does have an estrogen response element [37], indicating that its transcription may depend on the presence of estrogen. Interestingly, many of the relaxin induced changes seen during pregnancy are enhanced by the presence of estrogen [6]. Perhaps this is also the case in regard to the production of OT, but estrogen likely plays a more important role than relaxin. However, estrogen's role will be difficult to sort out due to the inability to maintain pregnancy without its presence. Interestingly, intracerebroventricular administration of relaxin to male rats also results in Fos expression in the SON and PVN [38], indicating that Fos' actions are not necessarily codependent on circulating female hormones.

In summary, relaxin does not appear to modulate the presence of OT mRNA within the SON or PVN during late pregnancy. Exactly why relaxin stimulates the presence of Fos in these cells remains unknown; several hypotheses have been discussed. A final determination will depend on future studies, perhaps looking at the role of opioids and their receptors or estrogen and its receptors.

ACKNOWLEDGMENTS

We thank Margie Carter of the Iowa State University Image Analysis Facility which is supported by the ISU Biotechnology Council, and David Primm for their assistance with data analysis. Purified porcine relaxin was generously provided by Christian Schwabe, Department of Biochemistry and Molecular Biology, Medical University of South Carolina, Charleston, SC.

REFERENCES

1. Lindow SW, Newham A, Hendricks MS, Thompson JW, van der Spuy ZM: The 24-hour rhythm of oxytocin and β -endorphin secretion in human pregnancy. *Clin Endocrinol* 1996;45:443-446.
2. Douglas AJ, Dye S, Leng G, Russell JA and Bicknell RJ: Endogenous opioid regulation of oxytocin secretion through pregnancy in the rat. *J Neuroendocrinol* 1993;5:307-314.
3. Douglas AJ, Johnstone LE, Neumann I, Leng G, Russell JA: Oxytocin neurones in the supraoptic nucleus (SON) are inhibited by endogenous opioids in late pregnant rats. *Gene Therapy* 1994;1,Suppl.1:S84.
4. Anderson LL, Bast JD, Melampy RM: Relaxin in ovarian tissue during different reproductive stages in the rat. *J Endocrinol* 1973;59:371-372.
5. Fields PA: Intracellular localization of relaxin in membrane-bound granules in the pregnant rat luteal cell. *Biol Reprod* 1984;30:753-762.
6. Sherwood OD: Relaxin; in Knobil E, Neill JD (eds): *The Physiology of Reproduction*, ed 2. New York, Raven Press, 1994, vol 1, pp 861-1009.

7. Sherwood OD, Crnedovic VE, Gordon WL, Rutherford JE: Radioimmunoassay of plasma relaxin throughout pregnancy and during parturition in the rat. *Endocrinology* 1980;107:691-698.
 8. Arey BJ, Freeman ME: Activity of oxytocinergic neurons in the paraventricular nucleus mirrors the periodicity of the endogenous stimulatory rhythm regulating prolactin secretion. *Endocrinology* 1992;130:126-132.
 9. Summerlee AJS: Extracellular recordings from oxytocin neurones during the expulsive phase of birth in unanaesthetized rats. *J Physiol (London)* 1981;321:1-9.
 10. Porter DG, Ryan PL, Norman LL: Lack of effect of relaxin on oxytocin output from the porcine neural lobe *in vitro* or in lactating sows *in vivo*. *J Reprod Fertil* 1992;96:251-260.
 11. Way SA, Leng G: Relaxin increases the firing rate of supraoptic neurones and increases oxytocin secretion in the rat. *J Endocrinol* 1992;132:149-158.
 12. Parry LJ, Poterski RS, Summerlee AJS: Effects of relaxin on blood pressure and the release of vasopressin and oxytocin in anesthetized rats during pregnancy and lactation. *Biol Reprod* 1994;50:622-628.
 13. Way SA, Douglas AJ, Dye S, Bicknell RJ, Leng G, Russell JA. Endogenous opioid regulation of oxytocin release during parturition is reduced in ovariectomised rats. *J Endocrinol* 1993;138:13-22.
 14. O'Byrne KT, Eltringham L, Clarke G, Summerlee AJS: Effects of porcine relaxin on oxytocin release from the neurohypophysis in the anaesthetized lactating rat. *J*
-

- Endocrinol 1986;109:393-397.
15. Jones SA, Summerlee AJS: Relaxin acts centrally to inhibit oxytocin release during parturition: an effect that is reversed by naloxone. J Endocrinol 1986;111:99-102.
 16. Eltringham L, O'Byrne KT, Summerlee AJS: Opioids, from the adrenal glands, may mediate relaxin inhibition of oxytocin release in lactating rats. J Physiol (London) 1986;371:183P
 17. O'Byrne KT, Eltringham KT, Summerlee AJS: Central inhibitory effects of relaxin on the milk ejection of the rat depends upon the site of injection into the cerebroventricular system. Brain Res 1987;405:80-83.
 18. Jones SA, Summerlee AJS: Effects of chronic infusion of porcine relaxin on oxytocin release in lactating rats. J Endocrinol 1987;114:241-246.
 19. Heine PA, Di S, Ross LR, Anderson LL, Jacobson CD: Relaxin-induced expression of fos in the forebrain of the late pregnant rat. Neuroendocrinology 1997;66:38-46.
 20. Curran T, Franza BR Jr: Fos and jun: The AP-1 connection. Cell 1988;55:395-397.
 21. Sassone-Corsi P, Sisson J, Verma IM: Transcriptional autoregulation of the proto-oncogene *fos*. Nature 1988;334:314-319.
 22. Burger LL, Sherwood OD: Evidence that cellular proliferation contributes to relaxin-induced growth of both the vagina and the cervix in the pregnant rat. Endocrinology 1995;136:4820-4826.
 23. 23. Sherwood OD, Downing SJ, Golos TG, Gordon WL, Tarbell MK. Influence of light-dark cycle on antepartum serum relaxin and progesterone immunoactivity levels on birth in the rat. Endocrinology 1983;113:997-1003.

24. Taua K, Greenwald GS. *In vivo* and *in vitro* ovarian steroidogenesis in the pregnant rat. *Biol Reprod* 1981;25:683-691.
25. Ivell R, Richter D: Structure and comparison of the oxytocin and vasopressin genes from rat. *Proc Natl Acad Sci USA* 1984;81:2006-2010.
26. Wisden W and Morris BJ. *In situ* hybridization with synthetic oligonucleotide probes; in Wisden W, Morris BJ (eds): *In Situ Hybridization Protocols for the Brain*. Academic Press Harcourt Brace and Co, London, pp 9-34.
27. Paxinos G, Watson C: *The Rat Brain in Stereotaxic Coordinates*. San Diego, Academic Press, 1986.
28. Miller FD, Ozimek G, Milner RJ, Bloom FE: Regulation of neuronal oxytocin mRNA by ovarian steroids in the mature and developing hypothalamus. *Proc Natl Acad Sci USA* 1989;86:2468-2472.
29. Van Tol HMM, Bolwerk ELM, Liu B, Burbach JPH: Oxytocin and vasopressin gene expression in the hypothalamo-neurohypophysial system of the rat during the estrous cycle, pregnancy and lactation. *Endocrinology* 1988;122:945-951.
30. Sonnenberg JL, Rauscher FJ, Morgan JI, Curran T: Regulation of proenkephalin by Fos and Jun. *Science* 1989;246:1622-1625.
31. Adachi T, Hisano S, Daikoku S: Intragranular colocalization of immunoreactive methionine-enkephalin and oxytocin within the nerve terminals in the posterior pituitary. *J Histochem Cytochem* 1985;33:891-899.
32. Lightman SL, Young WS: Vasopressin, oxytocin, dynorphin, enkephalin and

- corticotrophin-releasing factor mRNA stimulation in the rat *J Physiol (London)* 1987;394:23-39.
33. Douglas AJ, Neumann I, Meeren HKM, Leng G, Johnstone LE, Munro G, Russell JA: Central endogenous opioid inhibition of supraoptic oxytocin neurons in pregnant rats. *J Neurosci* 1995;15:5049-5057.
34. Leng G, Dye S, Bicknell RJ: Kappa-opioid restraint of oxytocin secretion: plasticity through pregnancy. *Neuroendocrinology* 1997;66:378-383.
35. Curran T. The fos oncogene; in Reddy EP and Skalka AM (eds): *The Oncogene Handbook*. Elsevier, Amsterdam, pp 307-325.
36. Luckman SM, Dyball RE, Leng G.: Induction of *c-fos* expression in hypothalamic magnocellular neurons requires synaptic activation and not simply increased spike activity. *J Neurosci* 1994;14:4825-4830.
37. Richard S, Zingg HH: The human oxytocin gene promoter is regulated by estrogens. *J Biol Chem* 1990;265:6098-6103.
38. McKinley MJ, Burns P, Colvill LM, Oldfield BJ, Wade JD, Weisinger RS, Tregear GW. Distribution of Fos immunoreactivity in the lamina terminalis and hypothalamus induced by centrally administered relaxin in conscious rats. *J Neuroendocrinol* 1997;9:431-437.

Figure 1. Diagram of the treatment design. The length of the bars indicates the gestational date on which rats were sacrificed. For details on hormone replacement, see *Materials and Methods*.

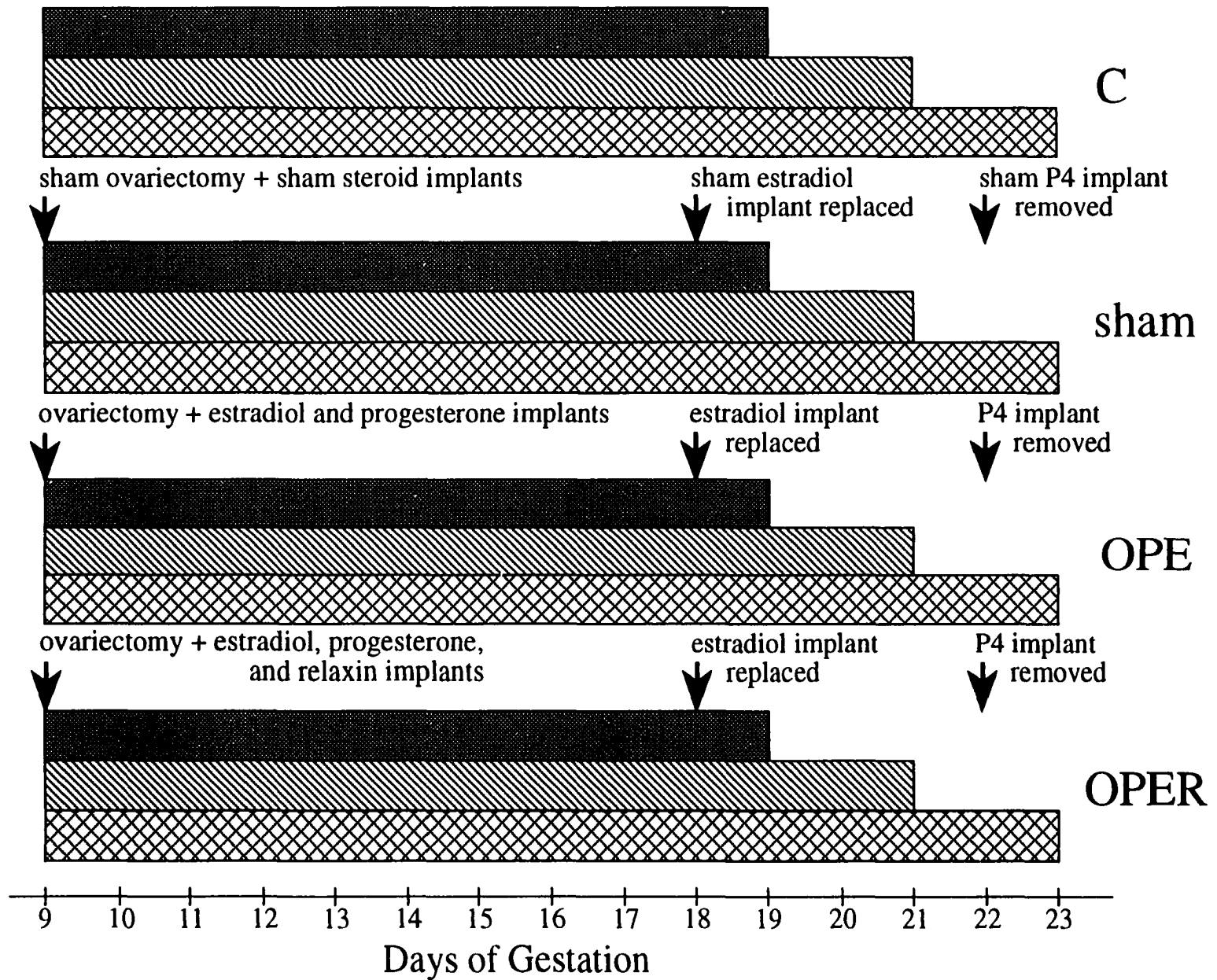


Figure 2. Scanned images from x-ray film exhibiting coronal brain sections showing the SON and PVN. A, B and C are examples of the three different oligo probes made against different areas of the OT gene. Notice the similarity in labeling pattern. SON = supraoptic nucleus, PVN = paraventricular nucleus

A PVN
SON

B PVN
SON

C PVN
SON

Figure 3. Photomicrographs of coronal brain sections exhibiting the SON (A) and the PVN (A and B). The tissue was exposed to ³⁵S-labeled oligonucleotides designed to bind to OT mRNA. The slides were then dipped in emulsion and developed to demonstrate the labeled area, which is represented by the black areas. Neutral red was used as a background stain. Notice the specificity of the labeling to the OT producing areas of the hypothalamus. 3V= third ventricle, SON = supraoptic nucleus, PVN = paraventricular nucleus

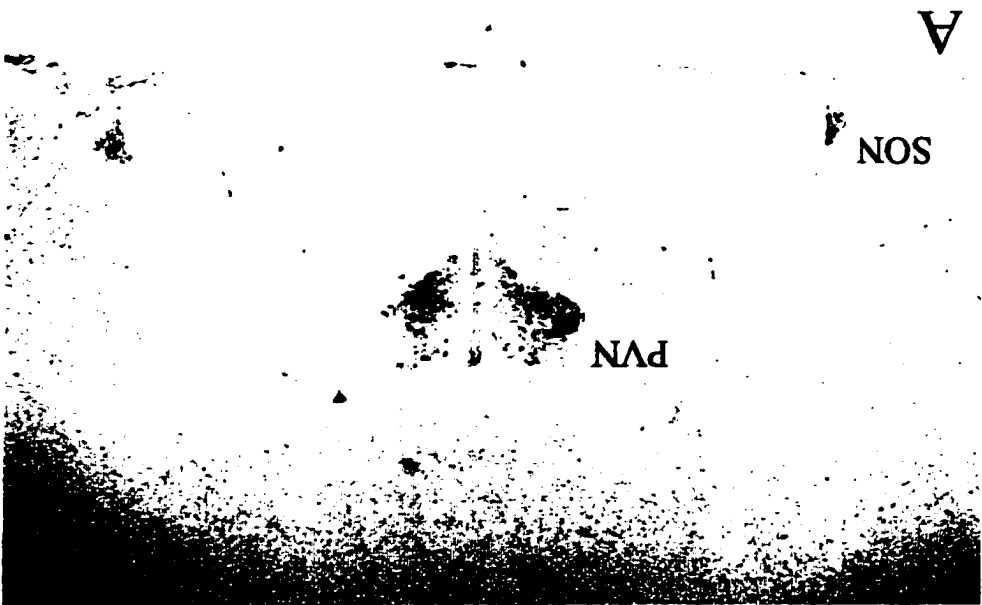
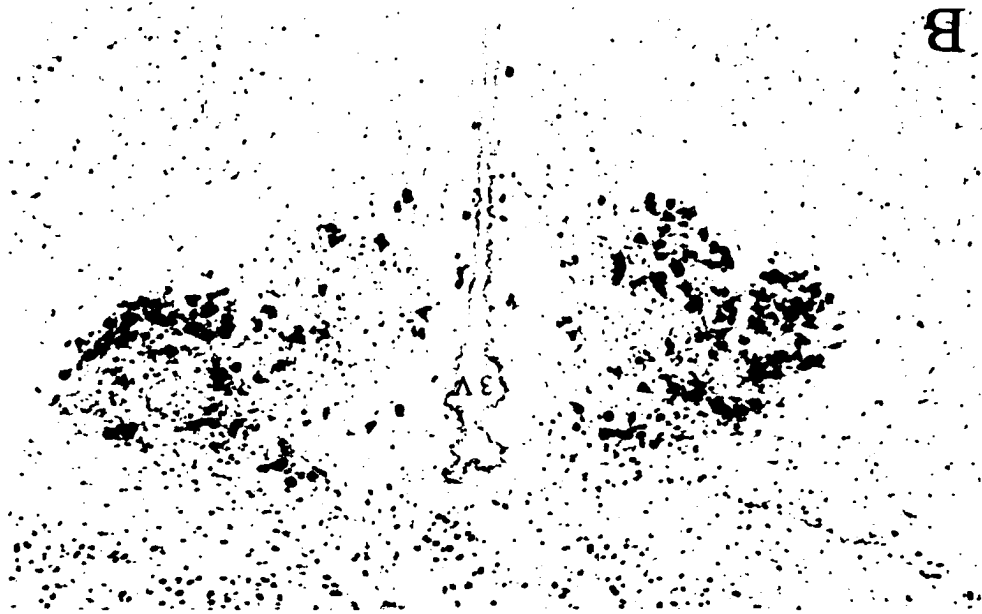


Figure 4. Pseudocolored brain paste standards. Red = 101,500 dpm; orange = 62,000 dpm; yellow = 58,000 dpm; green = 38,500 dpm; blue = 24,000 dpm; purple = 13,000 dpm; pink = 5,500 dpm.

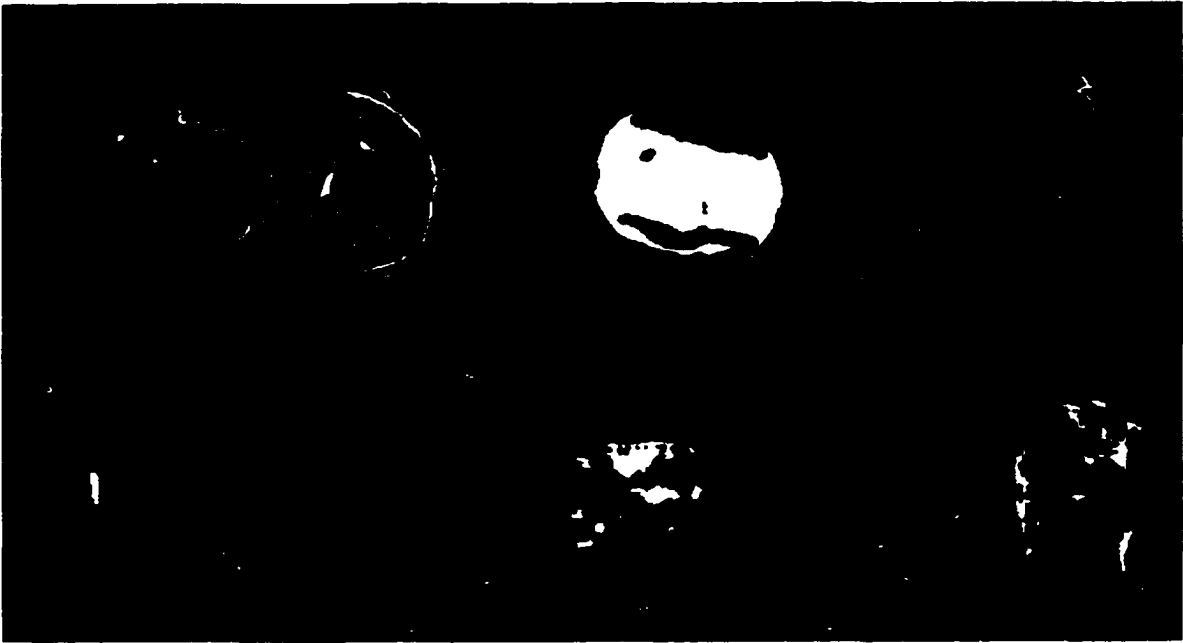


Figure 5. Pseudocolored prints of representative brain sections from the four different treatment groups. A = an ovariectomized animal that received relaxin replacement, B = an ovariectomized animal that received only progesterone and estradiol replacement, C = a sham ovariectomized animal, D = a control animal which underwent no surgery. Note that the color, which represents the optical density (a measure of the density of the OT mRNA), is essentially the same in all of the treatments. An approximate outline of the brain was drawn for the purpose of orientation. SON = supraoptic nucleus, PVN = paraventricular nucleus

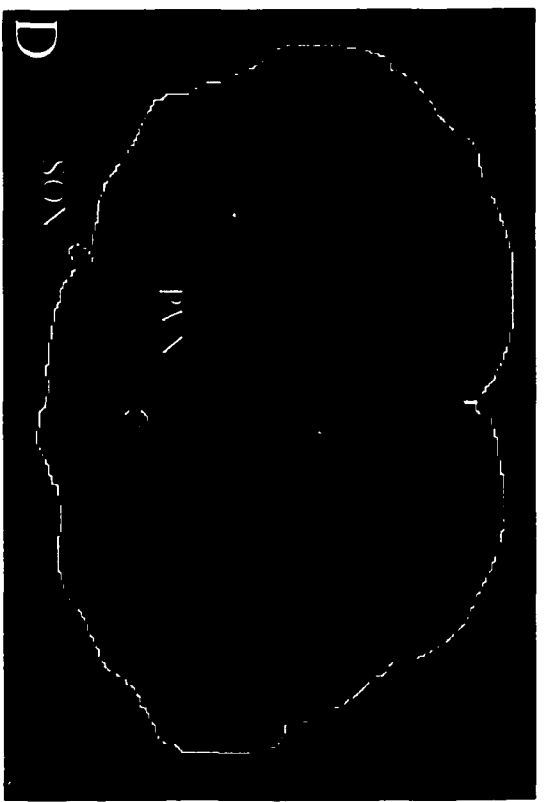
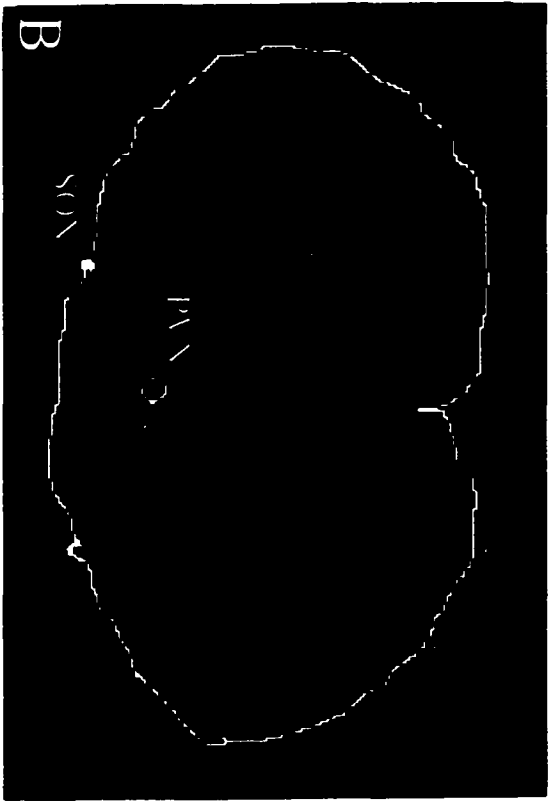
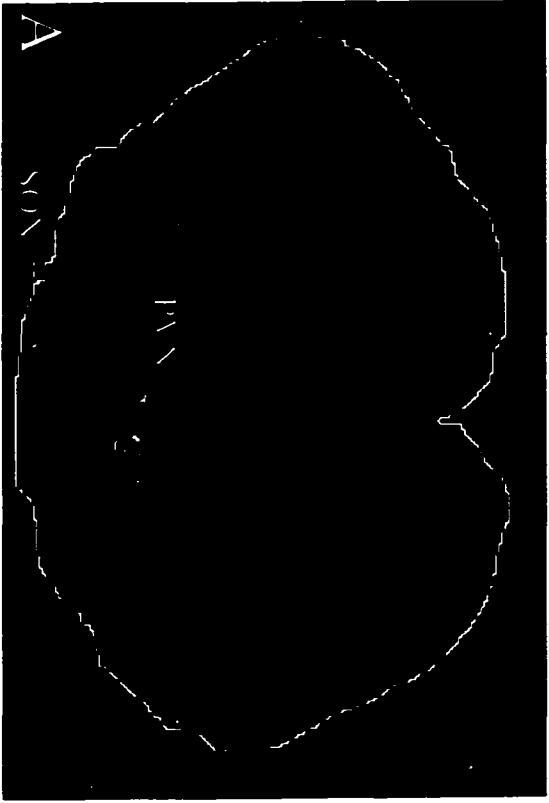
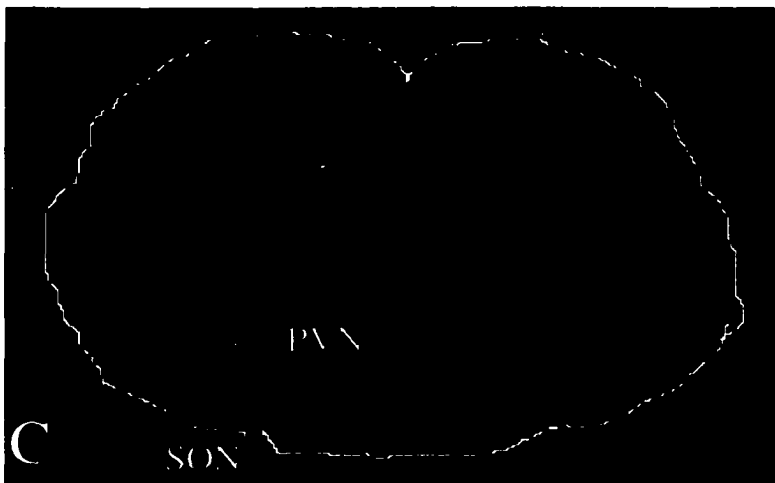
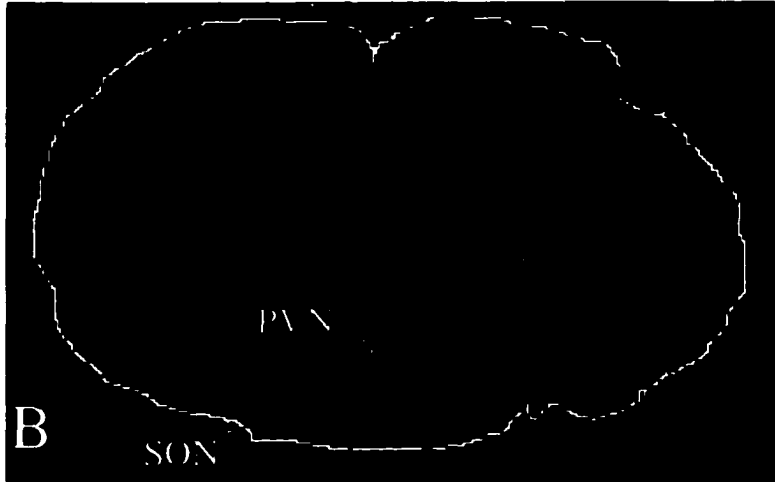
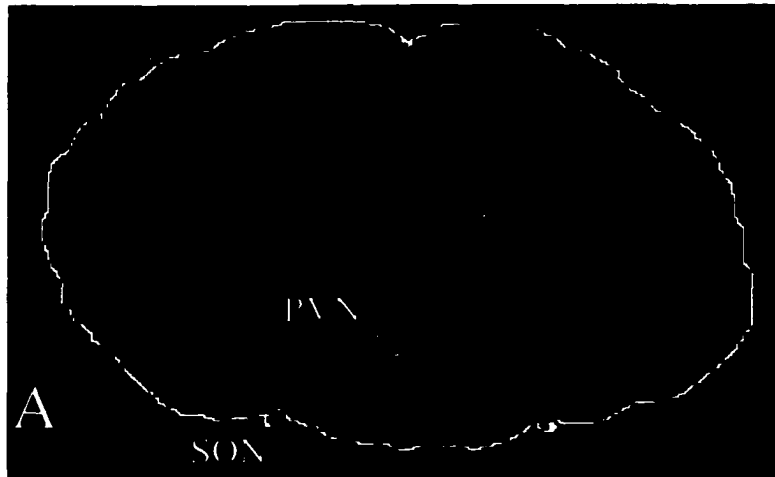


Figure 6. Pseudocolored prints of representative brain sections from animals representing the three different time points. A = g19, B = g21, C = g23. Note the similarity in color between the three groups. An approximate outline of the brain was drawn for the purpose of orientation. SON = supraoptic nucleus, PVN = paraventricular nucleus



CHAPTER 5 GENERAL CONCLUSIONS

General Summary

The first study investigated the relationship between OT, PRL and relaxin in the pregnant pig. In midgestational gilts, relaxin appeared to mildly increase the circulating levels of prolactin and significantly increase the levels of oxytocin. In late gestational gilts, however, there was no apparent effect of exogenous relaxin on the circulating concentrations of OT or prolactin. The most interesting and prominent result arising from this study was an unexpected large transient rise in the circulating levels of both OT and PRL following the removal of the gilts' ovaries. The rise in both hormones was statistically significant, however the rise in OT was much more remarkable. The circulating levels of both PRL and OT remained elevated for only a short period, followed by a return to baseline levels. The study was designed to determine the effects of the iv administration of relaxin on OT and PRL, and the most prominent finding was incidental. Its unplanned nature, however, makes this discovery no less important. The lack of relaxin on circulating concentrations of OT and PRL fortuitously led us to a new hypothesis, that relaxin in the antepartum period is acting to inhibit the premature release of OT from its stores in the neurohypophysis. A study in pregnant rats indicated that the inhibitory effect of opioids on the release of OT from its nerve terminals in the neurohypophysis is decreased in rats after ovariectomy (Way et al., 1993).

The second study turned to rats to look at the effect of relaxin on the somata of OTergic neurons in the hypothalamus. The iv administration of relaxin to late pregnant

rats increased the expression of Fos, used as a marker of cellular activation, in the magnocellular OTergic neurons of the SON and PVN as well as within the SFO. These results indicated that relaxin had an effect on the cellular activity within OTergic neurons as well as within the subfornical organ.

The third study further looked at the effect of iv relaxin administered to late pregnant rats on the synthesis of oxytocin. On three different days during late pregnancy, rats were sacrificed to determine the levels of OT mRNA expressed in the SON and PVN of the hypothalamus in response to the presence or absence of endogenous relaxin or the addition of exogenous relaxin. Findings from this study indicated that relaxin does not have an effect on the transcription of OT mRNA in late pregnancy.

Collectively, the findings from these studies show that during the antepartum period, relaxin appears to be inhibiting a premature release of OT from the neurohypophysis (most likely through synergistic activity with opioids which are known inhibitors of OT release), as well as promoting activity within the OTergic neurons of the hypothalamus. However, the activity seen in the SON and PVN does not appear to be an increase in the transcription the OT gene.

General discussion

The present studies were designed to further our understanding of the relationship between OT and relaxin in late pregnancy. We determined the effects of relaxin and the lack thereof on the circulating concentrations of OT and prolactin in pregnant pigs. We

have also investigated the effect of relaxin on OTergic cellular activity and the synthesis of oxytocin in late pregnant rats.

Centrally, the physiological and anatomical relationships of OT and relaxin have been studied in the rat, and the physiological relationship has been investigated in the pig. In rats, relaxin enhances the release of OT (Dayanithi et al., 1987; Way and Leng, 1992) except during pregnancy when this relationship is attenuated (Parry et al., 1994), and relaxin binding sites are also found in the SON and PVN of rats (Osheroff and Phillips, 1991). In the pig, relaxin has been shown to have no effect on the circulating levels of OT during lactation (Porter et al., 1992). These relationships in addition to facts and queries concerning each hormone individually led us to design the studies within this dissertation.

Oxytocin is the most powerful uterotonic agent known. However, its potency is regulated by the concentration of myometrial OT receptors. Oxytocin receptors in the uterus are upregulated by an abrupt decrease in circulating progesterone concentration just prior to the onset of labor (Alexandrova and Soloff, 1980), and circulating OT does not increase until after labor has started (Giraldi et al., 1990, Forsling et al., 1979). Premature release of OT, therefore, would not initiate labor unless the uterus was primed, and would reduce the amount of OT available for fetal expulsion during parturition. The regulation of the prevention of this premature release of OT is of interest.

Although control over the release of OT during parturition has been well documented, control over its release in late pregnancy is not as well understood. There appears to be some inhibition of OT release through endogenous opioids. Dynorphin is stored and released with vasopressin from the neurohypophysis. It acts on κ -opioid

receptors in the neurohypophysis to inhibit the release of oxytocin (Bondy et al., 1989). Beta-endorphins exhibit a diurnal rhythm opposite that of OT in late pregnant women, suggesting that an increased level of circulating β -endorphins prevents the release of oxytocin (Lindow, et al., 1996). Work presented in Chapter 2 on pregnant pigs shows that removal of relaxin results in a mass release of OT, followed by its return to baseline levels. This suggests that relaxin may act as a tonic inhibitor of OT release in late pregnant pigs. In support of this, studies in the rat indicate that relaxin's ability to cause a release of OT is attenuated in late pregnancy (Parry et al., 1994). Additionally, the opioid restraint of OT is decreased in ovariectomized rats (Way et al., 1993). This implicates relaxin as a modulator of the opioid restraint seen during pregnancy.

Activity within OTergic perikarya can be documented using the expression of proto-oncogenes as markers of cellular activity. One such gene is *c-fos*, which has been shown to be activated in OTergic cells by various stimuli (Flanagan et al, 1993; Verbalis et al, 1991; Giovannelli et al, 1990). *C-fos* expresses its protein Fos in response to depolarization and calcium influx (Morgan and Curran, 1986). This depolarization can result from numerous stimuli. Expression of Fos in the magnocellular hypothalamus is not activated by changes in spike activity, but rather by changes in synaptic activity (Luckman et al., 1994). In light of the findings from Chapter 3, one such stimulus could be the binding of relaxin to receptors on the cell membrane. Although the relaxin receptor has not been characterized, Osheroff and Phillips (1991) have shown that the SON and PVN do have binding sites for relaxin. In this way, relaxin could be activating *c-fos* within the OTergic neurons.

What role is Fos playing within the OTergic neurons? Fos is a transcription factor. It can act either to stimulate or repress transcription (Sassone-Corsi et al., 1988). It alters transcription by binding as a heterodimer with Jun (the protein product of the related proto-oncogene *c-jun*) to the AP-1 site in DNA (Morgan and Curran, 1991). Many genes have an AP-1 site. The OT gene has a site which differs from the AP-1 site by only one base (Fenelon et al., 1993). Also found within OTergic neurons is the gene for proenkephalin (Lightman et al., 1987), which has an AP-1 sequence (Sonnenberg et al., 1989). Because relaxin does not appear to be altering the transcription of OT, as defined in Chapter 4, it may be acting on proenkephalin, which binds to μ -opioid receptors. These receptors are believed to be involved in OT restraint in late pregnancy (Douglas et al., 1995). Alternately, relaxin could be acting through Fos to upregulate the expression of the μ -opioid receptors themselves.

The primary site in which the OT gene is found is within the magnocellular nuclei of the hypothalamus. It is also found in the uterus of the rat during late pregnancy (Lefebvre et al., 1992) and in the ovary of the cow during pregnancy (Musah et al., 1990). Using ISH, OT mRNA levels in the SON and PVN have been shown to be increased in late pregnant rats over those found in virgin rats (Miller et al., 1989). And, although OT is present in the uterus, neurohypophysial OT is necessary for parturition to occur (Luckman et al., 1993).

Interaction of relaxin and oxytocin

Circulating relaxin acts in several ways in the prepartum female. It keeps the uterus quiescent and remodels the collagen of the pelvic ligaments and reproductive tract to

accommodate for passage of the fetus. In the rat and pig, relaxin's concentration in the circulation is steady until the antepartum period at which time it peaks and then returns to its baseline level (for review see Sherwood, 1994). It is unclear what role this peak release of relaxin plays. It is tempting to postulate that the interhormonal relationship between relaxin and OT is temporally active around this antepartum period.

The mass release of relaxin at the end of pregnancy may be acting as a final restraint mechanism to prevent the release of oxytocin. Results from Chapter 2 suggest that this may be a mechanism in pigs, and a study from another laboratory showed similar findings in ovariectomized rats (Way et al., 1993). No studies have been done to determine whether blocking peripheral relaxin exclusively during the antepartum period affects circulating OT concentrations or the birthing process. Blocking peripheral relaxin throughout pregnancy prolongs gestation as well as lengthening the time of parturition and the amount of straining. This is primarily due to the lack of cervical ripening. However, a recent study by Summerlee (1998), demonstrated that intracerebroventricular (icv) administration of a relaxin monoclonal antibody to late pregnant rats allowed parturition to occur earlier than it otherwise would, as well as proceeding more quickly. The author's explanation was that central and peripheral relaxin systems must be separate. I postulate that rather than separate relaxin systems, the large increase in circulating relaxin during the antepartum period was blocked by the monoclonal antibody when it entered the CSF. Although it is possible that the source of relaxin in the brain is central (Osheroff and Ho, 1993; Gunnensen et al., 1985), relaxin can gain access to the CSF (Parry et al., 1991). With the higher circulating amounts of relaxin during this antepartum period, it would seem

likely that relaxin is also entering the CSF in higher concentrations. Likewise, it is unlikely that the antepartum release of relaxin has no physiological effect. I therefore postulate that the antepartum elevation in relaxin is acting to prevent the premature release of OT in late pregnancy.

How exactly is relaxin preventing the release of OT? Indeed, the relaxin receptor has not yet been characterized, so we do not know if the nerve terminals in the neurohypophysis have relaxin receptors to enable relaxin to act in this way. We do know, however, that relaxin has binding sites in the SON and PVN (Osheroff and Phillips, 1991) of non-pregnant rats, but we do not know what happens to these binding sites during pregnancy. The neurohypophysis has not been studied for relaxin binding sites, however *in vitro* experiments do show that relaxin alters the release of oxytocin (Dayanithi et al., 1987). This indicates that neurohypophysial relaxin receptors modulate these changes.

We also know that the opioid restraint of OT appears to switch from κ -opioid receptor restraint in the neurohypophysis during early and mid pregnancy to μ -opioid receptor restraint on the OTergic cell bodies at the end of pregnancy (Leng et al., 1997). Dynorphin, co-released with vasopressin from nerve terminals in the neurohypophysis, is a κ -opioid receptor agonist. It is active in the restraint of OT release during pregnancy. This restraint becomes weaker in late pregnancy, at which time μ -opioid receptors are thought to take over the control of OT release (Douglas et al., 1995). These receptors, however, are located on the cell bodies, not on the nerve terminals like κ -opioid receptors (Leng et al., 1997). Likewise, as mentioned previously, relaxin binding sites are found in the SON and PVN, where the OTergic cell bodies are located. Therefore, relaxin may be

modulating the μ -opioid receptors found on these cells; perhaps through the expression of Fos. Interestingly, in rats the OT receptors in the uterus are not upregulated until approximately 8 h prior to parturition, and the circulating peak of relaxin is on a decline 12 h prior to parturition. This opens a window for the circulating OT to start increasing. However, there are relaxin surges during parturition, which causes a problem with this hypothesis. There are two possible explanations for this diskumboomeration (Merlin, *The Sword and the Stone*, Walt Disney Productions). First, this could be explained if the circulating OT, which binds to OTergic cells (Neumann et al., 1996), changes the expression of either the relaxin or the μ -opioid receptors on these cells. This would prevent relaxin or proenkephalin from binding to the cells in high enough quantity to prevent the release of oxytocin. A second explanation is theorized by the *in vitro* finding that relaxin potentiates the release of OT from depolarized nerve terminals (Dayanithi et al., 1987). This suggests that during times of physiological excitation, such as parturition, the neurohypophysial nerve terminal release of OT would be enhanced by the presence of relaxin.

Conclusions

There are obviously many postulations involving the possible interaction of relaxin and oxytocin. Too much data implicate a relationship to ignore the possibility. However, the data available are not conclusive. Specific studies need to be designed to act as the connecting pieces for this unfinished jigsaw puzzle.

There are several future studies I envision to answer the questions raised by this dissertation. Initially, it would be worthwhile to conduct relaxin binding studies on the

brains of pregnant rats at different points during gestation. The data currently available show that relaxin in nonpregnant rats binds moderately to the SON and PVN. How is this binding altered during pregnancy? Is it increased in late pregnancy?

Broader studies using icv and peripheral administration of relaxin antibodies are also necessary. Does peripheral administration of a monoclonal relaxin antibody in late pregnancy result in the same findings as an icv administration? What if pregnant rats are exposed to icv monoclonal relaxin antibody throughout pregnancy instead of just at the end? Answering these questions will help identify the source of the relaxin interacting in the brain with oxytocin.

Further investigation is necessary to understand the role of Fos as a messenger for relaxin in OTergic cells. Utilizing ISH for proenkephalin and the μ -opioid receptor in the SON and PVN of late pregnant rats in relaxin deficient states, may indicate whether or not the genes for these two proteins undergo transcriptional changes in response to relaxin.

Lastly, a study should be done to examine the possibility of estrogen modulating the transcription of oxytocin. The presence of the estrogen response element on the OT gene (Richard and Zingg, 1990) suggests an interaction between these two hormones. Estrogen receptors are found in the hypothalamus (Li et al, 1997). To determine the role of estrogen in the transcription of OT during pregnancy, icv raloxifene (an estrogen antagonist) administration could be used to block the estrogen receptor, and in that way prevent activation of the estrogen response element.

From these studies, one of the most important conclusions is that not only does relaxin act differently in different species, as has been noted before, but it seems to have different actions depending on the gestational status of the animal. Therefore, when studying hormonal interrelationships, it is important to carefully define the model so that it is appropriate for answering the question. Hormonal relationships during pregnancy will not necessarily be the same in the non-pregnant model and vice versa.

REFERENCES

- Alexandrova M, Soloff MS. Oxytocin receptors and parturition. I. Control of oxytocin receptor concentration in the rat myometrium at term. *Endocrinology* 1980;106:730-735.
- Anderson LL, Bast JD, Melampy RM. Relaxin in ovarian tissue during different reproductive stages in the rat. *J Endocrinol* 1973a;59:371-372.
- Anderson LL, Ford JJ, Melampy RM, Cox DF. Relaxin in porcine corpora lutea during pregnancy and after hysterectomy. *Am J Physiol* 1973b;225:1215-1219.
- Bagnell, CA. Identification of immunoreactive relaxin in the neurohypophysis of the pig. *Biol Reprod* 1990; 42(suppl 1):102 (abstract 184) .
- Bathgate R, Balvers M, Hunt N, Ivell R. Relaxin-like factor gene is highly expressed in the bovine ovary of the cycle and pregnancy: sequence and messenger ribonucleic acid analysis. *Biol Reprod* 1996;55:1452-1457.
- Belt WD, Anderson LL, Cavazos LF, Melampy RM. Cytoplasmic granules and relaxin levels in porcine corpora lutea. *Endocrinology* 1971;89:1-10.
- Bondy CA, Whitnall MH, Brady LS, Gainer H. Coexisting peptides in hypothalamic neuroendocrine systems: Some functional implications. *Cell Mol Neurobiol* 1989;9:427-446.
- Bourque CW, Oliet SHR, Richard D. Osmoreceptors, osmoreception and osmoregulation. *Front Neuroendocrinol* 1994;15:231-274.

- Dayanithi G, Cazalis M, Nordmann JJ. Relaxin affects the release of oxytocin and vasopressin from the neurohypophysis. *Nature* 1987;325:813-816.
- Dellmann HD, Simpson JB. The subfornical organ. *Internatl Rev Cytol* 1979;58:333-421.
- Douglas AJ, Dye S, Leng G, Russell JA, Bicknell RJ. Endogenous opioid regulation of oxytocin secretion through pregnancy in the rat. *J Endocrinol* 1993;5:307-315.
- Douglas AJ, Russell JA. Corticotrophin releasing hormone, proenkephalin A and oxytocin mRNA's in the paraventricular nucleus during pregnancy and parturition in the rat. *Gene Therapy* 1994;1 (Suppl 1):S85.
- Douglas AJ, Neumann I, Meeren HKM, Leng G, Johnstone LE, Munro G, Russell JA. Central endogenous opioid inhibition of supraoptic oxytocin neurons in pregnant rats. *J Neurosci* 1995;15:5049-5057.
- Falke N. Oxytocin stimulates oxytocin release from isolated nerve terminals of rat neural lobe. *Neuropeptides* 1989;14:269-274.
- Felder KJ, Molina JR, Benoit AM, Anderson LL. Precise timing for peak relaxin and decreased progesterone secretion after hysterectomy in the pig. *Endocrinology* 1986;119:1502-1509.
- Fenelon VS, Poulain DA, Theodosis DT. Oxytocin neuron activation and fos expression: A quantitative immunocytochemical analysis of the effect of lactation, parturition, osmotic and cardiovascular stimulation. *Neuroscience* 1993;53:77-89.
- Fevold HL, Hisaw FL, Meyer RK. Isolation of the relaxative hormone in the corpus luteum. *Proc Soc Exp Biol Med* 1930;27:604-606.

- Flanagan LM, Pfaus JG, Pfaff DW, McEwen BS. Induction of FOS immunoreactivity in oxytocin neurons after sexual activity in female rats. *Neuroendocrinology* 1993;58:352-358.
- Forsling ML, Taverne MAM, Parvizi N, Elsaesser F, Schmidt D, Ellendorff F. Plasma oxytocin and steroid concentrations during late pregnancy, parturition and lactation in the miniature pig. *J Endocrinol* 1979;82:61-69.
- Gainer H, Wray S. Cellular and molecular biology of oxytocin and vasopressin; in: Knobil E, Neill JD (eds): *The physiology of reproduction*, ed 2. New York, Raven Press, 1994, pp 1099-1129.
- Geddes BJ, Parry LJ, Summerlee AJS. Brain angiotensin-II partially mediates the effects of relaxin on vasopressin and oxytocin release in anesthetized rats. *Endocrinology* 1994;134:1188-1192.
- Giovanelli L, Shiromani PJ, Jirikowski GF, Bloom FE. Oxytocin neurons in the rat hypothalamus exhibit *c-fos* immunoreactivity upon osmotic stress. *Brain Res* 1990;531:299-303.
- Giraldi A, Enevoldsen AS, Wagner G. Oxytocin and the initiation of parturition. A review. *Dan Med Bull* 1990;37:377-383.
- Guldenaar SEF, Wathes DC, Pickering BT. Immunocytochemical evidence for the presence of oxytocin and neurophysin in the large cells of the bovine ovary. *Cell Tissue Res* 1984;237:349-352.
- Gunnarsen JM, Crawford RJ, Tregar GW. Expression of the relaxin gene in rat tissue. *Mol*

Cell Endocrinol 1985;110:55-64.

Hadley ME. Neurohypophysial Hormones; in: Endocrinology. New Jersey, Prentice Hall, 1992, pp153-178.

Hartung S, Kondo S, Abend N, Hunt N, Rust W, Balvers M, Bryant-Greenwood G, Ivell R. The search for ruminant relaxin; in: MacLennan AH, Tregear GW, Bryant-Greenwood GD (eds): Progress in Relaxin Research. Singapore, World Scientific Publishing Co., 1995, pp 439-456.

Hisaw FL. Experimental relaxation of the pubic ligament of the guinea pig. Proc Soc Exp Biol Med 1926;23:661-663.

Ingram CD, Kavadas V, Thomas MRM, Threapleton JD. Endogenous opioid control of somatodendritic oxytocin release from the hypothalamic supraoptic and paraventricular nuclei in vitro. Neuroscience Res 1996;25:17-24.

Jones SA, Summerlee AJS. Relaxin acts centrally to inhibit oxytocin release during parturition: an effect that is reversed by naloxone. J Endocrinol 1986;111:99-102.

Juang HH, Musah AI, Schwabe C, Ford JJ, Anderson LL. Relaxin in peripheral plasma of boars during development, copulation, after administration of hCG and after castration. J Reprod Fertil 1996;107:1-6.

Kakouris H, Eddie LW, Summers RJ. Cardiac effects of relaxin in rats. Lancet 1992;339:1076-1078.

Lao Guico-Lamm M, Sherwood OD. Monoclonal antibodies specific for rat relaxin. II. Passive immunization with monoclonal antibodies throughout the second half of

- pregnancy disrupts birth in intact rats. *Endocrinology* 1988;123:2479-2485.
- Lefebvre DL, Giaid A, Bennett H, Lariviere R, Zingg HH. Oxytocin gene expression in rat uterus. *Science* 1992;256:1553-1555.
- Le Moine C, Young WS III. *RHS2*, a POU domain-containing gene, and its expression accompanying the development and plasticity of cell phenotypes in the neuroendocrine system. *Proc Natl Acad Sci USA* 1992;89:3285-3289.
- Leng G, Dye S, Bicknell RJ. Kappa-opioid restraint of oxytocin secretion: plasticity through pregnancy. *Neuroendocrinology* 1997;66:378-383.
- Li X, Schwartz PE, Risman EF. Distribution of estrogen receptor- β -like immunoreactivity in rat forebrain. *Neuroendocrinology* 1997;66:63-67.
- Li Y, Huang C, Klindt J, Anderson LL. Stimulation of prolactin secretion in the pig: central effects of relaxin and the antiprogesterone RU 486. *Endocrinology* 1993;133:1205-1212.
- Lightman SL, Young WS. Vasopressin, oxytocin, dynorphin, enkephalin and corticotrophin-releasing factor mRNA stimulation in the rat. *J Physiol (London)* 1987; 394:23-39.
- Lindow SW, Newham A, Hendricks MS, Thompson JW, van der Spuy ZM. The 24-hour rhythm of oxytocin and β -endorphin secretion in human pregnancy. *Clin Endocrinol* 1996;45:443-446.
- Luckman SM, Antonijevic I, Leng G, Dye S, Douglas AJ, Russell JA, Bicknell RJ. The maintenance of normal parturition in the rat requires neurohypophysial oxytocin. *J Neuroendocrinol* 1993;5:7-12.

- Luckman SM, Dyball REJ, Leng G. Induction of *c-fos* expression in hypothalamic magnocellular neurons requires synaptic activation and not simply increased spike activity. *J Neurosci* 1994;14:4825-4830.
- Lundin-Schiller S, Kreider DL, Rorie RW, Hardesty D, Mitchell MD, Koike TI. Characterization of porcine endometrial, myometrial and mammary oxytocin binding sites during gestation and labor. *Biol Reprod* 1996;55:575-581.
- McKinley MJ, Burns P, Colvill LM, Oldfield BJ, Wade JD, Weisinger RS, Tregear GW. Distribution of *fos* immunoreactivity in the lamina terminalis and hypothalamus induced by centrally administered relaxin in conscious rats. *J Neuroendocrinol* 1997;9:431-437.
- Meister B, Cortes R, Villar MJ, Schalling M, Hokfelt T. Peptides and transmitter enzymes in hypothalamic magnocellular neurons after administration of hyperosmotic stimuli: comparison between messenger RNA and peptide/protein levels. *Cell Tissue Res* 1990;260:279-297.
- Miller FD, Ozimek G, Milner RJ, Bloom FE. Regulation of neuronal oxytocin mRNA by ovarian steroids in the mature and developing hypothalamus. *Proc. Natl. Acad. Sci. USA* 1989;86:2468-2472.
- Miselis RR. The efferent projections of the subfornical organ of the rat: a circumventricular organ within a neural network subserving water balance. *Brain Res* 1981;230:1-23.
- Mumford D, Parry LJ, Summerlee AJS. Lesion of the subfornical organ affects the hemotensive response to centrally administered relaxin in anaesthetized rats. *J Endocrinol* 1989;122:747-755.

- Morgan JI, Curran T. Role of ion flux in the control of *c-fos* expression. *Nature* 1986;322:552-555.
- Morgan JI, Curran T. Stimulus-transcription coupling in the nervous system: involvement of the inducible proto-oncogenes *fos* and *jun*. *Annu Rev Neurosci* 1991;14:421-451.
- Morris JF. Hormone storage in individual neurosecretory granules of the pituitary gland: A quantitative ultrastructural approach to hormone storage in the neural lobe. *J Endocrinol* 1976;68:209-224.
- Musah A-HI, Scwabe C, Anderson LL. Relaxin and prostaglandin on oxytocin secretion from bovine luteal cells during different stages of gestation. *Acta Endocrinol (Copenh)* 1990;122:369-402.
- Neumann I, Douglas AJ, Pittman QJ, Russell JA, Landgraf R. Oxytocin released within the supraoptic nucleus of the rat brain by positive feedback action is involved in parturition-related events. *J Neuroendocrinol* 1996;8:227-233.
- Osheroff PL, Ling VT, Vandlen RL, Cronin MJ, Lofgren JA. Preparation of biologically active ³²P-labeled human relaxin: displaceable binding to rat cervix, uterus, and brain. *J Biol Chem* 1990;265:9396-9401.
- Osheroff PL, Phillips PS. Autoradiographic localization of relaxin binding sites in rat brain. *Proc Natl Acad Sci USA* 1991;88:6413-6417.
- Osheroff PL, Ho WH. Expression of relaxin mRNA and relaxin receptors in postnatal and adult rat brains and hearts. *J Biol Chem* 1993;268:15193-15199.
- Ott I, Scott JC. The actions of glandular extracts upon the contractions of the uterus. *J*

Exp Med 1909;11:326-330.

Pardridge WM, Frank HJL, Conford EM, Braun LD, Crane PD, Oldendorf WH.

Neuropeptides and the blood-brain barrier; in: Martin JB, Reichlin A, Blick KL, (eds): Neurosecretion and Brain Peptides. New York, Raven Press, 1981, pp 321-328.

Parry LJ, Poterski RS, Summerlee AJS. Mechanisms of the haemotensive action of porcine relaxin in anaesthetized rats. J Neuroendocrinol 1990;2:53-58.

Parry LJ, Poterski RS, Wasnidge C, Summerlee AJS. Relaxin in the cerebrospinal fluid of female rats. Soc Neurosci Abstr 1991;17:1196 (abstr 474.28).

Parry LJ, Summerlee AJS. Central angiotensin partially mediates the pressor action of relaxin in anesthetized rats. Endocrinology 1991;129:47-52.

Parry LJ, Poterski RS, Summerlee AJS. Effects of relaxin on blood pressure and the release of vasopressin and oxytocin in anesthetized rats during pregnancy and lactation. Biol Reprod 1994;50:622-628.

Peaker M, Fleet IR, Davis AJ, Taylor E. The effects of relaxin on the response of the intramammary pressure and mammary blood flow to exogenous oxytocin in the goat. Exp Physiol 1995;80:1047-1052.

Porter DG, Ryan PL, Norman L. Lack of effect of relaxin on oxytocin output from the porcine neural lobe *in vitro* or in lactating sows *in vivo*. J Reprod Fertil 1992;96:251-260.

Poulain DA, Wakerley JB. Electrophysiology of hypothalamic magnocellular neurones

- secreting oxytocin and vasopressin. *Neuroscience* 1982;7:773-808.
- Poulain DA. Electrophysiology of the afferent input to oxytocin- and vasopressin-secreting neurones. Facts and problems; in: Cross BA, Leng G (eds): *The neurohypophysis: Structure, function and control*. Progress in Brain Research, Elsevier Science Publishers, 1983, vol 60, pp 39-52.
- Richard S, Zingg HH. The human oxytocin gene promoter is regulated by estrogens. *J Biol Chem* 1990;265:6098-6013.
- Roche PJ, Crawford RJ, Tregear GW. A single-copy of relaxin-like gene sequence is present in sheep. *Mol Cell Endocrinol* 1993;91:21-28.
- Sassone-Corsi P, Sisson JC, Verma IM. Transcriptional autoregulation of the proto-oncogene *fos*. *Nature* 1988;334:314-319.
- Sherwood OD. Relaxin; in: Knobil E, Neill JD (eds): *The Physiology of Reproduction*, ed.2 New York, Raven Press, 1994, vol 1, pp 861-1010.
- Sherwood OD, Crnekovic VE, Gordon WL, Rutherford JE. Radioimmunoassay of relaxin throughout pregnancy and during parturition in the rat. *Endocrinology* 1980;107:691-698.
- Sonnenberg JL, Rauscher FJ, Morgan JI, Curran T. Regulation of proenkephalin by Fos and Jun. *Science* 1989;246:1622-1625.
- Sortino MA, Cronin MJ, Wise PM. Relaxin stimulates prolactin secretion from anterior pituitary cells. *Endocrinology* 1989;124:2013-2015.
- Summerlee AJS. Extracellular recordings from oxytocin neurones during the expulsive

- phase of birth in unanaesthetized rats. *J Physiol* 1981;321:1-9.
- Summerlee AJS, O'Byrne KT, Paisley AC, Breeze MF, Porter DG. Relaxin affects the central control of oxytocin release. *Nature* 1984;309:372-374.
- Summerlee AJS, O'Byrne KT, Jones SA, Eltringham L. The subfornical organ and relaxin-induced inhibition of reflex milk ejection in lactating rats. *J Endocrinol* 1987;115:347-353.
- Summerlee AJS, Ramsey DG, Poterski RS. Neutralization of relaxin within the brain affects the timing of birth in rats. *Endocrinology* 1998;139:479-484.
- Theodosis DT. Oxytocin-immunoreactive terminals synapse on oxytocin neurones in the supraoptic nucleus. *Nature* 1985;313:682-684.
- Verbalis JG, Stricker EM, Robinson AG, Hoffman GE. Cholecystokinin activates *c-Fos* expression in hypothalamic oxytocin and corticotropin-releasing hormone neurons. *J Neuroendocrinol* 1991;3:205-213.
- Way SA, Leng G. Relaxin increases the firing rate of supraoptic neurones and increases oxytocin secretion in the rat. *J Endocrinol* 1992;132:149-158.
- Way SA, Douglas AJ, Dye S, Bicknell RJ, Leng G, Russell JA. Endogenous opioid regulation of oxytocin release during parturition is reduced in ovariectomized rats. *J Endocrinol* 1993;138:13-22.
- Weindel A, Sofroniew MV. Relation of neuropeptides to mammalian circumventricular organs; in: Martin JB, Reichlin S, Blick KL (eds): *Neurosecretion and Brain Peptides*. New York, Raven Press, 1981, pp 303-319.

Weiss G. Relaxin in the male; in: MacLennan AH, Tregear GW, Bryant-Greenwood GD (eds): Progress in Relaxin Research. Singapore, World Scientific Publishing Co., 1995, pp 569-578.

Yang, RH, Bunting S, Wyss JM, Bereck KH, Zhang L, Jin H. Pressor and bradycardic effects of centrally administered relaxin in conscious rats. *Am J Hypertens* 1995;8:375-381.

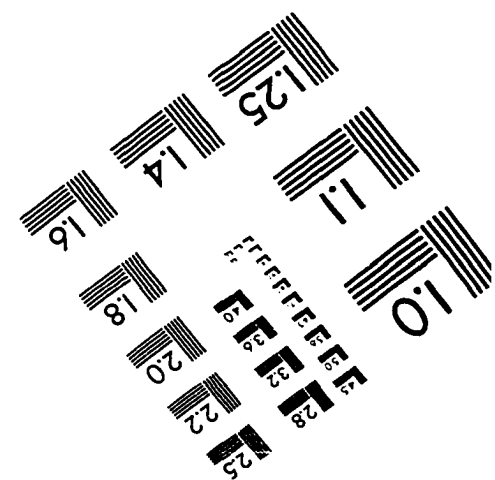
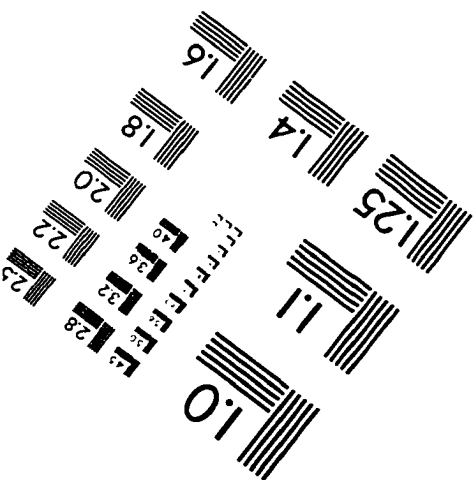
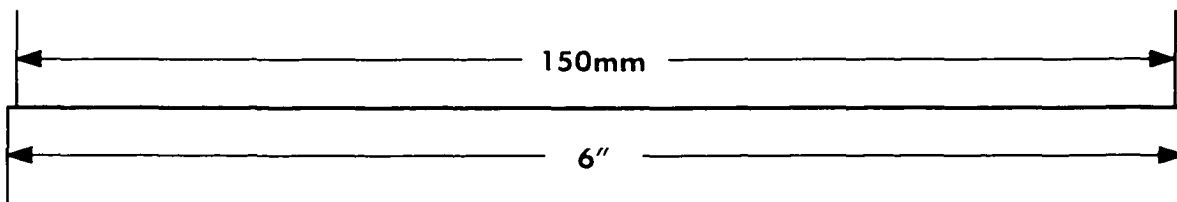
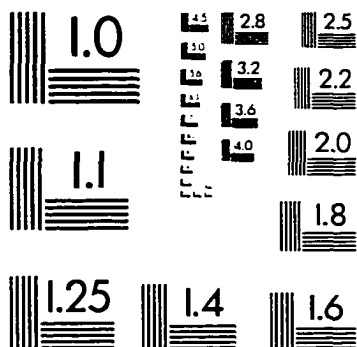
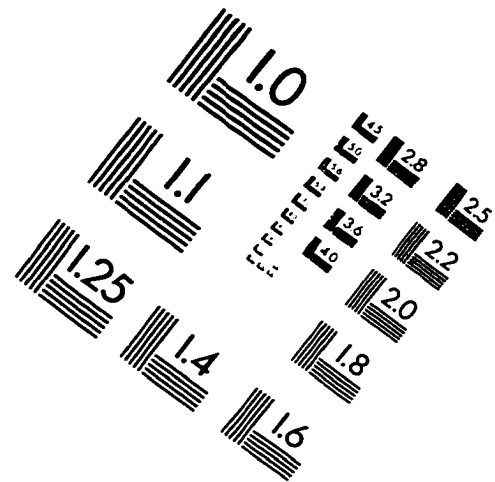
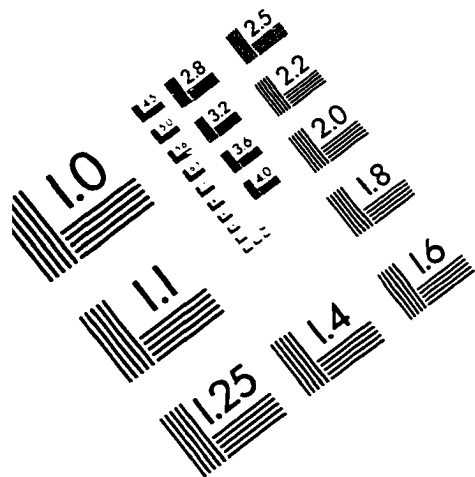
ACKNOWLEDGMENTS

The studies within this dissertation have been completed only with the support of many people. From my undergraduate experience at Occidental College, I thank Dr. Nancy Dess for peaking my interest in the field of Neuroscience and Dr. Stuart Rugg for sharing and teaching his enthusiasm of science. For their ongoing long distance support, I'd like to thank the past members of the Jacobson laboratory, Tracey Pepper, Dr. Jack Swanson, Dr. Paul Pearson, Dr. Cathy Kuehl-Kovarik, Dr. Joel Elmquist, and Dr. Shi Di. Currently in our department, I'd like to thank Dr. Ioana Sonea for her helpful suggestions, Margie Carter, Lynne Ross and Cathy Martens for their technical support, and Dr. Nani Ghoshal for his continued support and interest in my progress toward the completion of my dissertation. For his help with animal handling, I give much gratitude to Mahlon Shell, and for her help in the laboratory, I thank Karen Langner. For her smiling face and assistance with "paperwork", a big thanks to Donna Johnston.

For their guidance and constructive criticism, I thank my committee members, Dr. Srdija Jeftinija and Dr. Eugenia Farrar and particularly Dr. Mary Helen Greer for her assistance with handling atypical circumstances. An especially big thanks to my major professors Dr. Lloyd Anderson and Dr. Carol Jacobson for their patience and support.

Last but not least a very deep and heart felt thank you to my family and friends who have put up with my never ending scholastic goals. Many of you, especially Rita and Deanne, have given loving assistance with the kids, which has afforded me the time I needed to tie things up. Thank you all, I will always be grateful.

IMAGE EVALUATION TEST TARGET (QA-3)



APPLIED IMAGE . Inc
1653 East Main Street
Rochester, NY 14609 USA
Phone: 716/482-0300
Fax: 716/288-5989

© 1993, Applied Image, Inc., All Rights Reserved