Modulation of the Primate Hypothalamo-Pituitary-Gonadal Axis by Gonadotropin-Releasing Hormone-II.

by

Valerie Susanne Densmore

A DISSERTATION

Presented to the Neuroscience Graduate Program

and the Oregon Health and Science University

School of Medicine

in partial fulfillment of

the requirements for the degree of

Doctor of Philosophy

September 2002

School of Medicine
Oregon Health & Sciences University

CERTIFICATE OF APPROVAL

This is to certify that the Ph.D. thesis of

Valerie Susanne Densmore

has been approved

[redacted]

Dr. Henryk F. Urbanski

[redacted]

Dr. Cynthia L. Bethea

[redacted]

Dr. R. Lane Brown

[redacted]

Dr. Oline K. Ronnekleiv

[redacted]

Dr. Harold G. Spies

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

ANOVA	analysis of variance
ArcN	arcuate nucleus of the hypothalamus
DAB	3,3-diaminobenzidine hydrochloride
EB	estradiol benzoate
ELISA	enzyme-linked-immunosorbant assays
ER	estrogen receptor
FSH	follicle-stimulating hormone
GABA	γ-aminobutyric-acid
GAP	GnRH-associated peptide
GnRH	gonadotropin-releasing hormone
GnRH-I	mammalian gonadotropin-releasing hormone
GnRH-II	chicken gonadotropin-releasing hormone-II
H-F	Huynh-Feldt factor
HPLC	high pressure liquid chromatography
Inf	infundibular nucleus
ICV	intracerebroventricular
IHC	immunohistochemistry
IM	intramuscular
ISH	*in situ* hybridization
IV	intravenous
LH	luteinizing hormone
MBH	medial basal hypothalamus
ME	median eminence
NPY	neuropeptide Y
OT	oxytocin
Ovx	ovariectomized animal
Ovx+E	estradiol-treated ovariectomized animal
POA	preoptic area
PVN	paraventricular nucleus of the hypothalamus
RIA	radioimmunoassay
SCN	suprachiasmatic nucleus of the hypothalamus
SON	supraoptic nucleus of the hypothalamus
VHT	ventral hypothalamic tract
VP	vasopressin

ACKNOWLEDGEMENTS

I owe my sincere gratitude to my mentor, Dr. Henryk F. Urbanski, for his gracious support and guidance during my time in his laboratory. He has taught me how to conduct thorough scientific research, how to consider data and scientific issues both creatively and in depth, how to think and write precisely, and how to critically evaluate data and research papers. His ability to synthesize diverse data and the breadth of his approach to understanding scientific concepts have inspired me to pursue integrated concepts. He has prepared me both as an independent scientist and as an individual.

I also want to thank the members of the Urbanski laboratory who have taught me the importance of understanding the logic underlying laboratory techniques and have otherwise been both supportive and enjoyable coworkers. Specifically, I'd like to thank Dr. Steve Kohama, Vasilios Garyfallou, Jodi Downs, Donald Brown, and Laura James. I have also gained support and advice from Dr. David Hess, Dr. Becky Brogan, Dr. Kevin Grove, and many members of the Cooley Building.

Finally, I offer my thanks to Dr. Shane Latimer, for generously providing his scientific opinions on the design of experiments, the choice of statistical methods, and my scientific writing. His mentoring and encouragement have been vital to my success.

PREFACE

In accordance with the guidelines set forth by the Graduate Program of the School of Medicine, Oregon Health & Science University, Portland, Oregon, I have prepared my dissertation, consisting of a general introduction, three chapters of original data, and a general conclusion. The references cited are listed separately in chronological order and follow the format of *The Journal of Clinical Endocrinology and Metabolism*.

Chapter II contains data and figures as they appear in original papers that have been published previously (Urbanski et. al. Endocrinology. 140:1945-1948, 1999; Latimer et. al. Molec. Brain Res. 75:287-292, 2000; Latimer et. al. J. Clin. Endo. Metab. 86:324-329, 2001; Ferro et. al. J. Repro. Immuno. 51:109-129, 2001). Chapter III contains methodology that has been published previously (Gold et. al. J. Pharm. Exp. Therap. 289:1202-1210, 1999) and chapters III and IV contain data and figures that have been prepared and submitted for publication to *The Journal of Clinical Endocrinology and Metabolism* and *Journal of Molecular Endocrinology*, respectively.

ABSTRACT

The hypothalamo-pituitary-gonadal (hpg) axis goversn vertebrate reproduction and consists of a triad of hormones: gonadotropin-releasing hormone (GnRH); the gonadotropins luteinizing hormone (LH) and follicle-stimulating hormone (FSH); and the gonadal sex steroids estrogens, proestins, and androgens. These hormones modulate one anothers' secretion patterns, therefore to influence reproduction, factors will affect on or more onstituents of the hpg axis. However, it remains unclear what mechanism(s) underlie the secretion patterns observed during puberty and how estradiol feedsback onto GnRH and the gonadotropins.

Recently, GnRH-II was discovered in the brains of human and nonhuman primates. This second form of GnRH is the most conserved member of the GnRH decapeptide family, so it may play a crucial role in reproduction. Therefore, this dissertation addresses the following three specific aims in the rhesus macaque (*Macaca mulatta*): 1) to ascertain how GnRH-II may modulate GnRH-I secretion, 2) to ascertain whether GnRH-II stimulates gonadotropin release, and 3) to ascertain if estradiol exhibits negative and/or positive feedback on GnRH-II expression or signaling.

For the first specific aim, I used *in situ* hybridization (ISH) and immunohistochemistry (IHC) to map the hypothalamic distribution of GnRH-II mRNA and peptide, respectively. I found GnRH-II within the supraoptic (SON), suprachiasmatic (SCN), and paraventricular (PVN) nuclei, in addition to the medial basal hypothalamus (MBH). Although the GnRH-I and GnRH-II distributions howed some overlap, the two GnRH forms were not coexpressed in any cells. However, the GnRH-II cell population in the MBH might project to GnRH-I neurons in the preoptic area.

In addition to mapping the hypothalamic distribution of GnRH-II cells, GnRH-II mRNA levels were compared between juveniles and adults and found to

be upregulated developmentally in the MBH, unlike previous findings for GnRH-I. However, whether this increased expression helps mediate the onset of puberty is unlikely because further investigation showed estradiol exerted positive feedback on GnRH-II mRNA in the MBH. Therefore, this increased expression likely resulted from rising estrogen concentrations associated with puberty, and thus was a consequence rather than a cause of puberty.

The ability of GnRH-II to stimulate gonadotropin release was then examined for the second specific aim. Female rhesus macaques were treated with various doses of GnRH-I or GnRH-II both *in vivo* and *in vitro* to determine the relative potency of GnRH-II. The resulting data suggest GnRH-II requires the GnRH-I receptor to stimulate LH release *in vivo* and *in vitro*, and FSH release *in vitro*, however it was as effective as GnRH-I at releasing both LH and FSH.

Finally, IHC, ISH, and radioimmunoassay were used to examine whether estradiol exerts negative and/or positive feedback on GnRH-II expression and signaling to the pituitary. The data suggest that estradiol exerts positive feedback on GnRH-II expression in the MBH by acting directly on GnRH-II cells. In addition, estrogen attenuated GnRH-II stimulating FSH release from the pituitary but had no effect on GnRH-II-stimulating LH release.

Taken together, the current studies suggest that functional subpopulations of GnRH-II occur in the rhesus macaque hypothalamus. Furthermore, GnRH-II may mediate estrogen's positive feedback either to GnRH-I, the gonadotropins, or both.

CHAPTER I

Introduction

Hypothalamo-pituitary-gonadal (hpg) axis

The hypothalamo-pituitary-gonadal (hpg) axis governs reproduction in the majority of vertebrates. It consists of a triad of hormones, beginning with gonadotropin-releasing hormone (GnRH), found in the hypothalamus of the brain. As will be discussed, GnRH stimulates the anterior pituitary to release the second group of hormones, the gonadotropins, luteinizing hormone (LH) and follicle-stimulating hormone (FSH), which are responsible for stimulation of the gonadal sex steroids and gametal development, respectively. The third group in the hpg triad is composed of the gonadal sex steroids, estrogens, progestins, and the androgens; these steroids are able to exert feedback on both GnRH and the gonadotropins, thus forming an integrated system to control reproduction (Fig. 1-1). Although other pituitary and gonadal factors also contribute to the feedback circuitry, the final common pathway to control reproduction is the hpg axis. For this reason, to examine the affect of a peptide or factor on the reproductive system, it is expedient to investigate what influence it may exert on the constituents of the hpg axis. Therefore, I have chosen this approach to determine if gonadotropin-releasing hormone-II (GnRH-II) can modulate the reproductive system of primates.

Mammalian gonadotropin-releasing hormone

Discovery of GnRH-I

In 1971, the neuropeptide mammalian gonadotropin-releasing hormone (GnRH, also called GnRH-I; pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2) was isolated and sequenced following over a decade of fierce competition (1 - 3). Prior to the isolation of hypothalamic neurohormones, it was unorthodox to hypothesize that neurons might act like common glands to secrete peptides, let alone hormones, into the circulation. Despite this skepticism, however, it remained unclear what regulated

the pituitary, a gland suspended ventral to the hypothalamus, to release hormones into the peripheral circulation that controlled a range of physiological processes including reproduction, body growth, and stress. Geoffrey Harris was among the first to seriously propose that the brain might secrete releasing factors into a capillary system, now called the hypothalamic portal blood vessels, which vascularizes the anterior pituitary (4). Supporting this, Harris and Samuel McCann independently demonstrated in 1960 that a factor did exist to stimulate the release of luteinizing hormone (LH) (5). This hypothesis and discovery catalyzed two scientific teams, headed by Roger Guillemin and Andrew Schally, to successfully isolate and sequence the LH-releasing factor from ovine (3) and porcine (1, 2) hypothalami, respectively. This factor, whose sequence revealed it was indeed a hormone (2), demonstrated a high efficacy to stimulate LH release, although bioassays demonstrated it was also capable of stimulating the release of follicle-stimulating hormone (FSH) (5). This discovery that a hypothalamic hormone could stimulate the gonadotropins' release helped spark an ongoing controversy whether this hypothalamic factor was the LH-releasing hormone (LHRH) or the gonadotropin-releasing hormone (GnRH) (5). Adding to this controversy, McCann attempts to isolate bioactive gonadotropin-releasing factors lead to the discovery that a factor which preferentially stimulated FSH release, the FSHRF, might also exist (7, 8). Unfortunately, the methods used to isolate these compounds required thousands of hypothalami, so enough of this FSHRF was never purified to permit sequencing. Consequently, the name predominantly in use is GnRH, and it is still unclear whether a separate FSH-releasing hormone (FSHRH) exists.

Hypothalamic neurohormones

Despite the residual ambiguity, the discovery of GnRH and other neurohormones significantly advanced what is known about neurons and gave birth to the field of neuroendocrinology. True to what Harris proposed, a subpopulation

of hypothalamic neurons terminate on a capillary bed called the hypothalamic portal vessels, which vascularizes the anterior pituitary (4). Hypothalamic neurons are thus able to secrete GnRH into a localized bloodstream, allowing a miniscule amount of hormone to reach pituitary gonadotropes and stimulate LH and FSH release (5). Therefore, GnRH and the other hypothalamic neurohormones, as they came to be called, stimulate anterior pituitary cells to secrete hormones into the peripheral bloodstream. Because of this, measuring the pituitary hormones' release patterns could indicate the hypothalamic neurons' physiology without requiring a terminal experiment. Although the field of neuroendocrinology now had a powerful tool to examine neuronal signaling, the resulting data indicated that the secretion patterns of some anterior pituitary hormones, including the gonadotropins, might be influenced by multiple hypothalamic neurohormones.

In keeping with this, a growing body of evidence suggests that multiple GnRH forms may modulate gonadotropin release. Supporting McCann's evidence that an FSHRH might exist, at least 13 decapeptide forms of GnRH have been isolated and sequenced from various vertebrate species (Fig. 1-2). However, to determine the specific roles of any GnRH form within the hpg axis, it is first necessary to summarize the basic release patterns of LH and FSH.

Gonadotropin release patterns

In 1970, a radioimmunoassay (RIA) to measure rhesus macaque LH was developed, allowing the secretory pattern of LH to be investigated during the normal menstrual cycle (9). What was discovered was that LH was released in pulses occurring roughly once per hour, a circhoral rhythm, during the follicular phase, corresponding to the first 14 days of an approximately 28-day cycle. However, during the luteal phase, or second half of the cycle, LH was secreted once every 2-3 hours (10 - 12). Furthermore, this pulsatile release of LH occurred in the presence and absence of the ovary (11, 12), and this independence of LH pulsatility from gonadal

signals was soon confirmed for male rhesus macaques as well (11). Further investigation demonstrated that the observed LH pulses corresponded to pulsatile secretion of GnRH-I in hypothalamic portal blood (14 - 16). Moreover, additional examinations revealed that maintaining LH secretion required intermittent, rather than continuous, treatment with GnRH (17), and the finding that intravenous (iv) administration of GnRH-I antiserum blocked pulsatile LH release in rhesus macaques (18) provided additional support to the theory that pulsatile LH release was regulated by hypothalamic GnRH-I secretion.

Unlike LH, establishing a clear FSH secretory pattern was more difficult from the start. Using an RIA devised to measure FSH (19, 20), FSH secretion appeared very similar to LH, but the FSH pulses were not as discrete as those seen for LH (21, 22) and appeared to overlay a tonic FSH release (23). As a result, FSH secretion has not been investigated as thoroughly as has LH except in terms of what regulates FSH release. FSH does appear to be primarily under hypothalamic control as ovariectomy did not alter FSH pulse frequency (24, 25), although the molecular size of FSH in the pituitary has been reported to increase following ovariectomy (26). In addition, GnRH-I antiserum decreased plasma FSH concentrations (18). However, these data supporting the theory that GnRH-I controls FSH release are inconsistent with data showing that FSH secretion does not wholly correspond to GnRH-I release patterns.

To address this conflict, a prevalent theory is that the GnRH pulse frequency allows one GnRH form to separately regulate both LH and FSH. Although the pulse frequency varies between species, several studies have demonstrated that the gonadotropes require GnRH pulses within a narrow frequency range to maintain the observed physiological amplitude of LH and FSH release (27, 28). Further work has shown that a slower pulse frequency will more selectively stimulate FSH release (28). In dissention with this, the decreased endogenous frequency observed during the primate luteal phase results in an increased amplitude for both FSH and LH release,

although this may be partly due to increased releasable stores of the gonadotropins (29). Furthermore, lesioning the dorsal paraventricular nucleus attenuated pulsatile FSH release without affecting LH release (30). Regardless, exogenous circhoral pulses of GnRH-I are sufficient to stimulate the natural pattern of gonadotropin release (31); this treatment is also controversially believed sufficient to stimulate an LH and FSH surge (31).

In addition to the tonic pulsatile release, plasma gonadotropin concentrations surge approximately 37 hours prior to ovulation in the rhesus macaque (32). However the mechanisms underlying this pre-ovulatory gonadotropin surge are somewhat unclear. Administration of antiserum to estradiol blocked ovulation in the rat (33), demonstrating that estradiol was necessary, and further investigation revealed that circulating estradiol levels above 200 pg/ml for over 36 hours was necessary to generate a gonadotropin surge in primates (34). However, experiments based on the assumption that an endogenous surge of GnRH-I is necessary for the surge have produced conflicting data.

An initial study was designed to test where estradiol signaled to permit the pre-ovulatory surge and consisted of pituitary stalk section followed by the placement of a Silastic barrier in the portal vessels of rhesus macaques. In this case, estradiol treatment alone was sufficient to generate an LH and FSH surge, leading the investigators to question whether a GnRH surge were necessary (35). Treatment with a GnRH-I antiserum also failed to block an estradiol-induced gonadotropin surge, although pulsatile LH release was abolished (18). In 1980, radiofrequency lesions were used to abolish endogenous GnRH, as measured by the subsequent loss of pulsatile LH and FSH release. The researchers then replaced GnRH using one pulse of GnRH-I per hour; this combined with supplemented estradiol levels generated a gonadotropin surge and lent support to the theory that a GnRH surge was not necessary for ovulation (36). This controversial finding generated renewed

interest in the role of GnRH, as it was rapidly followed by two similar investigations. The first was a study that blocked endogenous GnRH by placing a Silastic barrier in the portal vessels and then replaced exogenous circhoral GnRH pulses in addition to estradiol implants to mimic pre-ovulatory conditions. Although this treatment did result in an LH surge (29), a second investigation that duplicated the study using an impermeable Teflon barrier prevented the generation of a gonadotropin surge (37). In another approach, radiofrequency lesions were again used to remove endogenous GnRH, which was then replaced as before with circhoral GnRH-I. In this case, however, the exogenous GnRH-I was then removed and animals were treated with estradiol benzoate (EB) 24, 48, 72, or 96 hours later. This regimen produced gonadotropin surges at the 24- and 48-hour periods, leading the investigators to conclude both that GnRH was unnecessary for a gonadotropin surge and that EB could function as a gonadotropin-releasing hormone (38). Despite the argument that a GnRH surge was unnecessary, however, several groups observed an increase in portal GnRH concentrations coinciding with the pre-ovulatory surge in several species (14, 16, 39), and both nuclear and cytoplasmic GnRH-I mRNA were shown to increase in rats on the afternoon of proestrus, which is when the LH surge occurs (40). Taken together, these data suggest the mechanisms underlying pulsatile LH and FSH secretion are not necessary to generate the pre-ovulatory gonadotropin surge. However, a hypothalamic factor does seem to be required, and there are increased levels of a GnRH-I in portal blood coinciding with the surge. Therefore, it is possible that a unique pathway, which may involve an alternate form of GnRH, functions to stimulate the pre-ovulatory surge.

Estrogen feedback

Although several estrogens and progestins exist in the female, the majority of studies have been performed using 17 β-estradiol (estradiol) or estradiol benzoate

(EB) and progesterone. Aside from whether a GnRH surge is necessary for ovulation, positive feedback from high circulating levels of estradiol are required (34); this is in direct contrast to estradiol's tonic signal, which results in negative feedback on gonadotropin release (41). In rhesus macaques, LH and FSH levels increase approximately 10-fold within three weeks of estrogen removal by ovariectomy (11), and replacing physiological estradiol concentrations has been shown to depress plasma gonadotropins back to intact levels within minutes of intravenous (iv) administration (41). That this negative feedback results from the estrogens, rather than another ovarian signal, is further supported by data demonstrating that progesterone treatment did not affect tonic gonadotropin secretion (41), although it was capable of selectively blocking both the spontaneous and estradiol-stimulated pre-ovulatory surge (42, 43).

Estradiol appears to signal negative feedback at the pituitary and the hypothalamus using multiple mechanisms. Pituitary sensitivity to iv GnRH treatment decreased following estradiol treatment in women (44), and estradiol treatment in rhesus macaques decreased LH sensitivity to both intrapituitary and iv GnRH following radiofrequency lesions of the hypothalamus (31, 45). However, estradiol may also affect GnRH expression because gonadectomy has been demonstrated to stimulate increased expression of GnRH-I mRNA. This effect is reversed by steroid replacement in rats (46) but not in ferrets (47). Further investigations demonstrated that ovariectomy both attenuated GnRH neurons' responsiveness to prostaglandins in rats (48) and increased glial ensheathment of GnRH neurons in primates (49). In addition, a population of neurons in the arcuate nucleus (ArcN) of guinea pigs was shown to contain GnRH immunoreactivity and rapidly hyperpolarize after treatment with estradiol (50).

Taken together, the latter data suggest that estrogens can directly affect GnRH neurons in terms of gene expression, morphology, and electrophysiology.

This would indicate a direct negative influence of estrogen on GnRH-I neurons, but until recently, estrogen receptors were either not found on GnRH neurons (51-56), or the techniques used to find receptors were of questionable stringency (57, 58). However, a second estrogen receptor, ERβ, was recently discovered (59) and found colocalized with GnRH-I neurons in some species (60 - 62). Preliminary data has since suggested that ERβ may help control the pre-ovulatory surge due to its distribution and because an ERβ antagonist significantly attenuated the incidence of ovulation in rats (63). However, ERα is necessary for estradiol negative feedback (64). In addition, an ER antagonist, ZM 182,780, blocked the pre-ovulatory surge but did not affect pulsatile LH release, further suggesting that the positive and negative feedback of estradiol may be controlled by different receptors (65). However, further investigation is warranted to clarify the respective roles for both ERα and ERβ and the mechanisms underlying estradiol positive and negative feedback.

Hypothalamic pulse and surge generators

The release patterns of LH and FSH have implied the existence of hypothalamic pulse and surge generators, and lesion studies to map their location suggest these generators are separately housed. An initial study surgically disconnected the caudal hypothalamus, including the medial basal hypothalamus (MBH) and ArcN, and found that both the pulsatile and surge patterns of gonadotropin release remained intact (66). This suggests the caudal hypothalamus is sufficient for both the pulse and surge patterns, however, additional data indicated that after being severed, fiber tracts from the rostral hypothalamus were able to re-terminate on the blood vessels near the scar (67). This corresponded to data that lesioning the rostral hypothalamus around the supraoptic (SON) and suprachiasmatic nuclei (SCN) blocked the spontaneous and estradiol-induced LH surge without altering pulsatile LH release (68). Further support that the surge generator was

located in the rostral hypothalamus came from data showing that estradiol treatment facilitated LH release following electrical stimulation of the rostral hypothalamus, but the response was attenuated when the MBH was stimulated after estradiol treatment (69). In contrast, radiofrequency lesions that destroyed the ArcN blocked both pulsatile and surge release patterns of LH (70), although a later study demonstrated that an estradiol-induced LH surge was possible in rhesus macaques bearing ArcN lesions (31). These inconsistencies in the data may be due to the imprecise nature of lesions; typically the lesioned areas vary in size and exact location. Furthermore, lesions are not confined to cell bodies but may include fiber tracts, which might supply crucial input from distantly-located cells.

In addition to lesion studies, localized treatments of either agonists or antagonists have been used to investigate the site of action for the pre-ovulatory surge. Estradiol microimplants were found to stimulate an LH surge when they were implanted into the MBH, but not the medial preoptic area (POA), of sheep (71). In addition, treatment with EB induced Fos expression in the ArcN, although the rostral hypothalamus was not examined for Fos immunoreactivity (72). However, the administration of an opioid receptor agonist to inhibit the surge was effective in both the MBH and the medial POA of sheep (73). The investigators concluded that the surge generator may have cell bodies located in the MBH that send their axonal projections to the rostral hypothalamus. This argument reconciles the aforementioned data, however further investigation is still required to clarify the pulsatile and surge generators' sites-of-action.

Taken together, the data investigating the neuroendocrine control of mammalian reproduction are extensive, however several questions have yet to be answered. Primary among them are (1) whether separate hormones regulate LH and FSH secretion, (2) the mechanisms underlying both the pulsatile and surge patterns of gonadotropin release, and (3) how the positive and negative feedback effects of

estrogen are transmitted to GnRH release. Although the aforementioned studies have investigated these very issues, they have presupposed in most cases that the mammalian hpg axis is controlled by only one form of GnRH. However, it was recently discovered that mammals express a second GnRH form, which may help to address the remaining questions concerning the neuroendocrine control of the reproductive system.

Chicken Gonadotropin Releasing Hormone-II

History of discovery

The variable synchrony between FSH secretion and GnRH-I release prompted several groups to investigate whether additional gonadotropin-releasing factors occurred in the hypothalami of different species. Consequently, the gonadotropin-releasing hormone (GnRH) family now includes at least 13 decapeptide forms identified from various vertebrate classes (Fig. 1-2), and most of these forms are believed to have reproductive functionality. Matsuo's group first published the GnRH-II sequence in 1984 following its isolation and purification from chicken hypothalamus using ion-exchange chromatography and reverse-phase high pressure liquid chromatography (HPLC) (74). This was soon followed by the construction of GnRH-II-specific antiserum in 1986, which allowed GnRH-II to be identified in the amphibian and reptilian (75) brain using HPLC and radioimmunoassay (RIA). HPLC and RIA were again used to identify GnRH-II in the fish classes, Chondrichthyes (76) in 1986 and Osteichthyes (75) in 1988. Then in 1989, GnRH-II was discovered using HPLC and RIA in the metatherian, or marsupial, mammalian brain (77), but it was commonly held that eutherian, or placental, mammals only expressed GnRH-I. However, the discovery of GnRH-II in the musk shrew, an eutherian mammal, in 1993 (78) both disproved this theory and lead to the eventual finding in 1997 and 1998, respectively, that GnRH-II also occurs in the non-human (79) and human (80)

primate. Because GnRH-II has now been isolated from members of every extant vertebrate class except the fish class, Agnatha, GnRH-II is currently considered the most primitive and conserved form in the GnRH decapeptide family.

Phylogenetic distribution pattern

Fish

GnRH-II was originally isolated from the chicken brain, but most GnRH-II studies since have been carried out using the fish as an animal model. Of the three extant classes of fish, Agnatha, Chondrichthyes, and Osteichthyes, GnRH-II has been identified in several species of the latter two classes.

Chondrichthyes

The cartilaginous fish, Chondrichthyes, lack the hypothalamic portal vessels, a capillary system that connects the median eminence to the pituitary in most vertebrates. However, the gonadotrope-containing pituitary lobe is anatomically distinct from the median eminence in Elasmobranchii, a subclass of Chondrichthyes, therefore it is hypothesized that GnRH may reach the gonadotropes via the general circulation, rather than by hypothalamic portal vessels. As a result, the GnRH-II distribution in Elasmobranchii could suggest whether GnRH-II functions as a gonadotropin-releasing factor in these fish. Initially, the two laboratories that nearly simultaneously reported finding GnRH-II used HPLC and RIA on whole-brain extracts of Elasmobranchii (81, 82). Two years later, detailed immunohistochemistry (IHC) localized GnRH-II to cell bodies in the midbrain tegmentum but with very little immunoreactivity in the pituitary (83), suggesting that GnRH-II likely did not reach pituitary gonadotropes either directly or through the general circulation. This finding that GnRH-II immunoreactivity occurred predominantly in the midbrain rather than the hypothalamus or pituitary would be reiterated for many vertebrate species.

Osteichthyes

Class Osteichthyes, which consists of the bony fish, contains approximately 30,000 species, making it the most diversely populated of all the extant vertebrate classes. Many studies have examined Osteichthyes species for GnRH-II using HPLC and RIA (75, 84-88); although HPLC and RIA proved to be a sensitive measure of GnRH-II occurrence, these methods do not allow the specific distribution of GnRH-II to be mapped with high resolution. Accordingly, several IHC studies were published describing GnRH-II immunoreactivity in the brains of various Osteichthyes species. In the masu salmon (*Oncorhynchus masou*) (89), dwarf gourami (*Colisa lalia*) (90), goldfish (*Carassius auratus*) (91), and medaka (*Oryzias latipes*) (92), for example, GnRH-II cell bodies occurred in the midbrain tegmentum, but very little immunoreactivity was found in the preoptic or terminal nerve areas, which agreed with findings from other vertebrate classes. In the goldfish, a few GnRH-II cells also occurred in the ventrolateral hypothalamus and in parvicellular neurons in the POA, although GnRH-II fibers were widely distributed throughout the brain and had a robust population in the pituitary (91). However, most studies found sparse GnRH-II-immunoreactivity in the pituitary, unlike the other GnRH form present in each case, so the investigators frequently concluded GnRH-II does not primarily function to stimulate gonadotropin release. The medaka study also extended its findings to trace GnRH immunoreactivity throughout pre- and post-natal development and demonstrated that GnRH-II-immunoreactive cells originated from ependymal cells of the third ventricle. Then, in 1995, GnRH-II mRNA was cloned from the catfish (*Clarias gariepinus*) (93) and later from the goldfish in 1997 (94), which demonstrated that GnRH-II is encoded by a distinct gene. Examining the distribution, however, confirmed GnRH-II is restricted to the midbrain in catfish (86) and olfactory bulb and midbrain tegmentum in the goldfish (95). Overall, therefore, the predominance of GnRH-II immunoreactivity in midbrain rather than hypothalamic or pituitary

tissue led most investigators to conclude that GnRH-II did not primarily function to stimulate gonadotropin release but that it might act to influence behavior or as a neurotransmitter.

Amphibians

Although GnRH-II was identified in amphibians using HPLC and RIA on crude brain extracts (75), site-specific localization was delayed a further four years until Muske and Moore performed IHC using antibodies specific for GnRH-II (96). Examining three amphibian species (genus *Rana*) over the course of development, the investigators found the forebrain-spinal cord system had cell bodies and fibers immunoreactive for GnRH-II, but the hypothalamic-pituitary pathway had sparse GnRH-II immunoreactivity. Then, in 1994, Licht et al microdissected the amphibian brain and returned to using HPLC and RIA. They found GnRH-II concentration was equally distributed throughout the brain, resulting in it being the predominant GnRH form in amphibians; however, the average concentration of GnRH-II in the hypothalamus was significantly lower than GnRH-I. The investigators concluded that GnRH-II expression was more consistent with a neurotransmitter function, however they also discovered GnRH-II in amphibian portal blood. Therefore, the investigators extended the potential functions to include pituitary control (97). Furthermore, later studies demonstrated GnRH-II fibers occurred in the infundibular region and posterior pituitary (98). In addition, GnRH-II fibers innervated the spinal cord to terminate in the vicinity of motorneurons (99), suggesting GnRH-II might also influence locomotor activity in amphibians.

Reptiles

Fewer distribution studies have been performed for GnRH-II in the reptilian brain. However, Tsai and Licht mapped GnRH-II distribution using HPLC and

RIA on microdissected brains from the turtle (*Trachemys scripta*). Similar to the amphibian, GnRH-II occurred in equal concentrations across much of the reptilian brain, although GnRH-II concentrations were four times higher in the cerebellum and eight times higher in the medulla compared to the median eminence and optic tectum (100). In 1997, in contrast to previous findings, HPLC and RIA revealed that the green anole (*Anolis carolinensis*) only expressed the GnRH-II form (101). This was in contrast to current thinking that GnRH-II did not primarily have a reproductive function in vertebrates, however, studies to further characterize GnRH-II function in the anole have not been performed. Otherwise, these findings corresponded to those in amphibians, suggesting that GnRH-II primarily functioned as a neurotransmitter/neuromodulator and/or influenced motor activity.

Aves

As mentioned previously, GnRH-II was first identified and sequenced from the chicken brain (*Gallus domesticus*) (74), however the GnRH-II distribution was not mapped using IHC until four years later. Both the caudal hypothalamus and the rostromedial midbrain had GnRH-II-immunoreactive perikarya, but no GnRH-II fibers were found in the rostral hypothalamus or median eminence (102). In contrast, Katz et. al. used microdissected brains and HPLC and RIA to later demonstrate that the hypothalamus and median eminence did possess GnRH-II-immunoreactivity, but the midbrain, cerebellum, and medulla contained higher concentrations of GnRH-II (103). Three years later, another study used radioimmunoassay on hypothalamic and posterior pituitary extracts to demonstrate that GnRH-II occurred in both regions. However, although GnRH-II concentrations in the hypothalamus were comparable to GnRH-I concentrations, the posterior pituitary had significantly more GnRH-I than GnRH-II (104).

Mammals

Not surprisingly, HPLC and RIA were used to first identify GnRH-II in crude hypothalamic extracts from metatherian mammals, the pouch or marsupial mammal (77, 105). However, eutherian, or placental, mammals were thought to express only GnRH-I until 1993, when GnRH-II was described in the musk shrew (*Suncus murinus*) brain (78), and again in 1994 in the musk shrew, mole (*Chrysochloris asiatica*), and bat (*Miniopterus schreibersii*) following HPLC and RIA (106). Although these techniques did not allow much resolution to determine the GnRH-II distribution, they did reveal that 55% of GnRH in the musk shrew is the GnRH-II form (106). Further studies employing IHC revealed that neither GnRH-II cells nor projection sites appeared to overlap with GnRH-I; the major terminal field for GnRH-II in the musk shrew was the medial habenula where immunoreactive cells made axodendritic contacts that were symmetric, which are often inhibitory (107). In addition, males were found to have approximately twice as many GnRH-II cells as intact, but not ovariectomized, females (108). Despite this apparent sex-steroid regulation and although GnRH-II did occur at low levels in the hypothalamus, the investigators concluded that GnRH-II likely acted as a neurotransmitter or neuromodulator. Closely following this study, the cDNA encoding GnRH-II was cloned from the tree shrew (*Tupaia glis belangeri*), which is not only an eutherian mammal, but also considered a presumptive primate (109). However, the potential relevance of a second GnRH form being present was strengthened when GnRH-II was found in rhesus (*Macaca mulatta*) and stumptail (*Macaca speciosa*) macaques in 1997 (79). GnRH-II gained further clinical relevance when it was cloned from the human a year later (80) and then cloned from the rhesus macaque in 1999 (110). In addition, the peptide distribution of GnRH-II was examined in the mouse using IHC (111, 112) and again found in the midbrain close to the 4th ventricle in addition to a caudal hypothalamic population. Furthermore, GnRH-II immunoreactivity was

found in the hypogonadal mouse, which lacks functional GnRH-I (111). However, murine GnRH-II has not yet been cloned, which prevents a GnRH-II-knockout mouse from being generated. Despite this setback, cloning rhesus GnRH-II allowed its mRNA distribution to be mapped, and initial ISH demonstrated that GnRH-II occurred in the dorsal midbrain, as hypothesized, but also had a robust expression in the rostral and caudal hypothalamus (110). Because very little GnRH-II ISH had been carried out in any vertebrate species, these findings re-opened the question if GnRH-II helps modulate gonadotropin-signaling.

Potential functions

Despite GnRH-II being highly conserved across the vertebrate classes and its close similarity to GnRH-I, whose function has been well characterized, a clear physiological function has not emerged from studies on GnRH-II. What primarily indicates potential roles, however, are its distribution pattern and its sequence homology with the GnRH peptide family. Based on these known attributes, several functions have been hypothesized including acting as a neurotransmitter, regulating behavior, stimulating gonadotropin release, and/or mediating sex-steroid signaling, respectively. Although the evidence supporting any of these hypotheses is sparse, it warrants closer examination.

Neurotransmitter

Initial studies examining whether GnRH-II functioned as a neurotransmitter were based on an earlier finding that a GnRH analog was able to mediate the late slow excitatory post-synaptic potential in amphibians. Three years following the discovery of GnRH-II, Jones used whole-cell patch clamp to demonstrate that GnRH-II could potently inhibit the M current in bullfrog sympathetic ganglia (113). This finding prompted a study that found GnRH-II injection caused a significant dose-dependent increase in plasma catecholamines, both adrenaline and

noradrenaline, without affecting heartrate or blood pressure in bullfrogs (*Rana catesbeiana*), leading the investigators to conclude GnRH-II might act as a sympathetic neurotransmitter (114). These studies were further supported by the finding that GnRH-II immunoreactive fibers innervated both the granular layer of the cerebellum and the spinal cord near motorneurons in amphibians (115). Similar to the distribution pattern observed in amphibians, the goldfish and dwarf gourami were found to have GnRH-II cells in the midbrain tegmentum as well as spinal cord (90, 91), although the midbrain tegmentum is implicated to send descending projections into the spinal cord (116). These observations that GnRH-II occurred in the hindbrain around the midbrain tegmentum were verified in several other vertebrate species, as discussed above, however direct demonstrations are less prevalent that GnRH-II acts as a neurotransmitter in vertebrates other than amphibians.

Regulate behavior

The GnRH-II distribution patterns in multiple species also suggest it could function to regulate behavior because of the theorized functions of these regions where GnRH-II occurs. For example, in addition to sending projections to the spinal cord, the midbrain tegmentum has been implicated to help modulate behaviors (119). Furthermore, a major terminal field for GnRH-II fibers in musk shrews was observed in the medial habenula (107), an area that may help regulate female sexual receptivity (118). Altering catecholaminergic activity has also been shown to influence reproductive behavior (119), which correlates to the finding discussed above that GnRH-II treatment elevates adrenaline and noradrenaline in amphibians (114).

In addition to circumstantial data that GnRH-II might modulate reproductive behavior, a few direct measurements have been performed. In red-sided garter snakes (*Thamnophis sirtalis parietalis*), intracerebroventricular (icv) injection of GnRH-II did not potentiate or attenuate the courtship behavior provoked by either

visual or olfactory cues (120). In contrast, icv injection of GnRH-II, but not chicken GnRH-I, in female white-crowned sparrows (*Zonotrichia leucophrys gambelii*) did maintain copulatory behavior for a significantly longer time after the birds were exposed to an auditory stimulus (121). In agreement with this study, icv injections of GnRH increased the number of spawning acts performed by female goldfish, however, this effect was seen with both GnRH-II and salmon GnRH, another form present in these fish (122). Therefore it is difficult to draw a clear conclusion whether GnRH-II functions physiologically to modulate reproductive behavior because although the GnRH-II distribution pattern would allow it to regulate behavior, its *in vivo* effects vary between species.

Gonadotropin-releasing factor

The potential functions theorized for GnRH-II have frequently been drawn from its distribution pattern in addition to its sequence homology with known gonadotropin-releasing hormones. In addition to these, GnRH-II is highly conserved, providing enough evidence to justify addressing a primary question: does GnRH-II stimulate LH and/or FSH release and could it function as the putative FSHRH? As previously mentioned, the existence of an FSHRH has long been hypothesized (7, 8), but no potential candidates are unequivocally supported (23). However, several studies have examined whether GnRH-II can act as a gonadotropin-releasing factor, and particularly an FSHRH both *in vitro* and *in vivo*.

The comparative ability of GnRH-II to stimulate LH and FSH release has been examined in several species, but, understandably, the preliminary work frequently focused on birds, where GnRH-II was originally discovered. In static 2-hour incubations on primary dispersed chicken pituitary cells, GnRH-II demonstrated a significantly ($P<0.01$) greater potency to stimulate both LH (ED_{50} 0.055) and FSH release (ED_{50} 0.034) compared to chicken GnRH-I (ED_{50} 0.28, ED_{50} 0.37, respectively) and mammalian GnRH-I (ED_{50} 0.27 for LH) (132, 133). Similarly,

GnRH-II showed significantly more potency to release LH from dispersed quail pituitary cells than chicken GnRH-I or mammalian GnRH-I (ED_{50} 0.12 for GnRH-II and 0.96 for both chicken and mammalian GnRH-I) (132). One receptor subtype appeared to mediate the effects of all the GnRH forms, as combining the maximal doses of both forms in the chicken produced neither an additive nor subtractive effect on LH release (131). These relative potencies were later confirmed in sexually-immature cockerels (133) and in turkeys (*Meleagris gallopavo*), both in an *in vitro* perifusion system and *in vivo* (134).

The ability of GnRH-II to stimulate gonadotropin release was also examined repeatedly in fish species. In catfish, GnRH-II was significantly more effective ($P<0.05$) than catfish GnRH to stimulate LH release both *in vitro* from perifused pituitary fragments and *in vivo* (135). Furthermore, GnRH-II demonstrated a ten-fold higher potency than catfish GnRH to release LH *in vivo* consistently throughout the pubertal development of male catfish (136). In addition, GnRH-II was found significantly more effective to stimulate ovulation than salmon GnRH in catfish (137) and goldfish (88) and was implicated to be primarily responsible to stimulate ovulation in goldfish, as blocking salmon GnRH, the other form known to be present, did not impair the goldfishes' ability to ovulate (138). In addition, GnRH-II was found to be between 2 and 8 times more potent to stimulate LH release than seabream or salmon GnRH in the gilthead seabream (139).

Studies in reptiles (140) and amphibians (97, 141) also demonstrated that GnRH-II more effectively stimulated LH release *in vivo* than the other GnRH forms present in each respective species. However, the clearance rate of GnRH-II from plasma was found to be slower than either chicken GnRH-I (26% versus 17% not degraded after more than 7 hours) or mammalian GnRH-I, respectively, and the investigators concluded in both cases that the higher observed potency might be due to a longer exposure to GnRH-II than the other forms.

A similar observation was later made in the human, where GnRH-II remained in circulation longer than GnRH-I (142). This might be caused by GnRH-II resisting degradation due to the histidine at position 5, which hinders proteolytic degradation (140, 143). In addition, plasma proteins that selectively bound GnRH-II were identified in both goldfish (144) and spotted ratfish (*Hydrolagus colliei*) (145), which might further allow GnRH-II to resist proteolytic degradation or reuptake and therefore remain in the circulation longer than other GnRH forms. Despite this argument that the increased potency of GnRH-II might result from resisting removal from the serum, the final conclusion remains that GnRH-II was more effective at stimulating LH release *in vivo* than other naturally occurring GnRH forms in both reptiles and amphibians.

In contrast to the relative potency of GnRH-II in other vertebrates, GnRH-II initially appeared less effective to stimulate LH release in mammals. Early studies that examined the relative ability of GnRH-II to release LH and bind pituitary gonadotropes *in vitro* found GnRH-II to be approximately ten-fold less effective in sheep and rats, respectively (130). A later study comparing the abilities of GnRH-II and GnRH-I to release both LH and FSH from sheep pituitary cells revealed GnRH-II to be four- and ten-fold less potent, respectively, and further investigation demonstrated that the mammalian GnRH receptor required an arginine at position 8 for optimal receptor binding (146). Although GnRH-II has a tyrosine in this position, the combined effect of the histidine at position 5 and the tryptophan at position 7 compensated, in part, for the arginine to tyrosine substitution (146). Following these *in vitro* studies, an *in vivo* examination demonstrated that GnRH-I treatment resulted in significantly more musk shrews ovulating than GnRH-II treatment ($P<0.04$) (107). However, with the discovery that GnRH-II occurred in the primate, the ability of GnRH-II to stimulate LH release was reported to be similar to that for GnRH-I, although the LH-releasing abilities of the two GnRH

forms were not directly compared (81). In 2001, a GnRH-II receptor was identified in primates by two separate laboratories (147, 148). Because the lesser potency of GnRH-II to release LH in mammals had been attributed to its decreased effectiveness to bind the mammalian GnRH receptor, finding a second, GnRH-II-selective, receptor strengthened the argument that GnRH-II might function physiologically to release either or both gonadotropins.

Mediate estradiol feedback

Besides the other functions mentioned, the GnRH-II distribution pattern also suggests that GnRH-II may help mediate sex-steroid feedback onto the reproductive axis. Therefore, in addition to mapping GnRH-II distribution, several investigators investigated whether GnRH-II content or expression was altered in concert with the changing sex-steroid milieu due to exogenous manipulation or the stage of the breeding cycle.

Studies using the cichlid (*Oreochromis mossambicus*) and the European silver eel (*Anguilla anguilla*) failed to demonstrate that midbrain GnRH-II expression was altered by progesterone (123), estradiol (124, 125), or triiodothyronine, a thyroid hormone analogue (125). However, both testosterone and androstenedion, a nonaromatizable androgen, caused a decrease in midbrain GnRH-II immunoreactivity (124). Considering that GnRH-II expression and immunoreactivity were not observed to change developmentally or in response to changing estrogen and progesterone milieu, however, made the androgen data difficult to interpret. Worth noting is that the androgen treatment was given to female eels, which might suggest the observed negative feedback was not physiologically relevant, but studies examining relative GnRH-II concentrations between male and female fish also did not demonstrate a significant difference (126). In addition to the effects of exogenous steroids on GnRH-II content, the peptide levels were measured by enzyme-linked-immunosorbant assays (ELISA) in gilthead

seabream (*Sparus aurata*) during the breeding and non-breeding seasons. Under these conditions when the sex-steroid milieu changes naturally, GnRH-II content was observed to remain constant, although the levels of seabream GnRH, another form present, increased during the breeding, compared to the non-breeding, season (126). Furthermore, another study used IHC to find that GnRH-II peptide content does not change developmentally in the rainbow trout (*Salmo gairdneri*) (127). In summary, existing data in fish suggest that changing sex-steroid milieu exogenously, during development, or during the breeding season does not affect GnRH-II levels.

In cockerels, castration resulted in higher chicken GnRH-I but not GnRH-II content (128), although injecting progesterone intramuscularly (im) to stimulate an LH surge in hens did decrease GnRH-II hypothalamic immunoreactivity (129). In addition, photostimulated turkey hens had increased GnRH-II peptide concentrations in the hypothalamus, but ovariectomy decreased GnRH-II levels (104).

In reptiles, HPLC demonstrated that adult female turtles did show increased GnRH-II compared to hatchling females (100). The existing data for mammals also correspond to the reptile data, as ovariectomy increased GnRH-II cell number in musk shrews (108). To date, experiments examining the effect of sex-steroid milieu on GnRH-II in amphibians have not been published.

Although these studies are consistent within each vertebrate class, the overall outcome seems mixed. Birds, reptiles, and mammals show a change in GnRH-II immunoreactivity in response to exogenous sex-steroid manipulation, but fish do not demonstrate a similar pattern. Furthermore, when GnRH-II concentrations are examined during conditions that naturally change sex-steroid milieu, the levels remain constant. It is therefore possible that either any GnRH-II changes observed following steroid manipulation occur artifactually, or the observed GnRH-II

sensitivity to changing milieu evolved after the appearance of fish. Therefore, whether GnRH-II functions to mediate sex-steroid feedback remains unclear.

All in all, there is considerable variability between vertebrate classes of how GnRH-II functions. As a result, it effects on the hpg axis have not been clarified. However, this same variability raises the question whether GnRH-II has a conserved function, its physiological role has evolved, or whether GnRH-II is a vestigial neuropeptide. These questions aside, one factor potentially compounding the inconsistency is that the data are most frequently collected from a variety of species and then compiled, rather than one species being examined systematically.

The rhesus macaque as an animal model

The confluence of multiple factors make the rhesus macaque (*Macaca mulatta*) an excellent model to examine whether GnRH-II modulates the hpg axis. Similar to the human reproductive system, female rhesus macaques undergo a spontaneous menstrual cycle that lasts approximately 28 days and contains both a follicular and luteal phase and menstruation, which is not present in all primates. In addition, GnRH-I exhibits a circhoral release pattern during the follicular phase, and it is secreted in pulses every 2-3 hours during the luteal phase; this secretion is under estradiol negative feedback, as is also true of humans. Furthermore, the preovulatory surge appears almost identical between rhesus macaques and humans. Therefore, the neuroendocrine system of rhesus macaques appears to regulate reproduction nearly identically to what is seen for humans. In addition to the reproductive similarities, however, the GnRH-II gene has been cloned from rhesus macaques, which allows additional techniques, like *in situ* hybridization, to be used to examine the GnRH-II distribution and expression patterns with high resolution. This combination of a similar hpg axis coupled with the sequence of GnRH-II mRNA as a tool has not been duplicated in any other animal to date. Finally, the Oregon National Primate

Research Center can be utilized to provide a source of animal subjects under exceptional care that will allow the interactions of GnRH-II with the primate hpg axis to be investigated in depth.

Aims of the thesis

To determine if GnRH-II can modulate the hpg axis, the following three specific aims are proposed:

Specific Aim #1: To ascertain whether GnRH-II may modulate GnRH-I by determining whether GnRH-II is histologically capable of signaling to GnRH-I and if GnRH-II expression varies according to development or sex.

Specific Aim #2: To ascertain whether GnRH-II may modulate gonadotropin release by determining if GnRH-II can stimulate FSH and/or LH release *in vivo*, how GnRH-II compares in potency to GnRH-I, if GnRH-I and GnRH-II compete for a common receptor or signaling pathway, and if endogenous GnRH-II reaches the pituitary gonadotropes *in vivo*.

Specific Aim #3: To ascertain whether GnRH-II might mediate some of estradiol's effects on the reproductive axis by determining if estradiol exhibits negative and/or positive feedback on GnRH-II expression or release *in vivo* and if these effects are mediated via estrogen receptors expressed by GnRH-II cells.

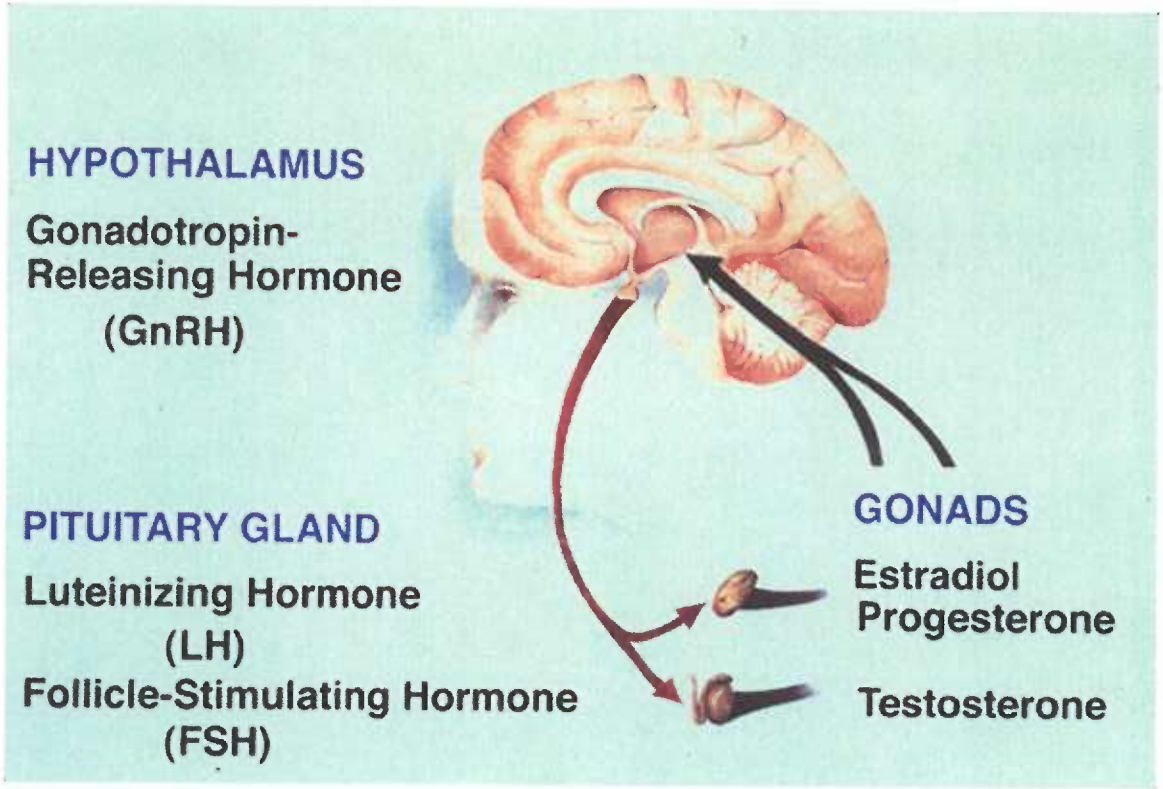

Figure 1-1. Representation of the hypothalamo-pituitary-gonadal (hpg) axis. The axis is composed of a triad of hormones (1) gonadotropin-releasing hormone(GnRH), (2) the gonadotropins, luteinizing hormone (LH) and follicle-stimulatinghormone (FSH), and (3) the sex steroids, estrogen, progesterone, and testosterone. The sex steroids exert feedback on both the hypothalmus andpituitary to modulate the release of GnRH and the gonadotropins. This axis formsthe backbone of the reproductive system in most vertebrates.

	1	2	3	4	5	6	7	8	9	10	
Mammalian	pGlu	- His	- Trp	- Ser	- Tyr	- Gly	- Leu	- Arg	- Pro	- Gly	- NH2
Chicken-II	pGlu	- His	- Trp	- Ser	- His	- Gly	- Trp	- Tyr	- Pro	- Gly	- NH2
Salmon	pGlu	- His	- Trp	- Ser	- Tyr	- Gly	- Trp	- Leu	- Pro	- Gly	- NH2
Seabream	pGlu	- His	- Trp	- Ser	- Tyr	- Gly	- Leu	- Ser	- Pro	- Gly	- NH2
Chicken-I	pGlu	- His	- Trp	- Ser	- Tyr	- Gly	- Leu	- Gln	- Pro	- Gly	- NH2
Catfish	pGlu	- His	- Trp	- Ser	- His	- Gly	- Leu	- Asn	- Pro	- Gly	- NH2
Dogfish	pGlu	- His	- Trp	- Ser	- His	- Gly	- Trp	- Leu	- Pro	- Gly	- NH2
Lamprey-I	pGlu	- His	- Tyr	- Ser	- Leu	- Glu	- Trp	- Lys	- Pro	- Gly	- NH2
Lamprey-III	pGlu	- His	- Trp	- Ser	- His	- Asp	- Trp	- Lys	- Pro	- Gly	- NH2
Tunicate-I	pGlu	- His	- Trp	- Ser	- Asp	- Tyr	- Phe	- Lys	- Pro	- Gly	- NH2
Tunicate-II	pGlu	- His	- Trp	- Ser	- Leu	- Cys	- His	- Ala	- Pro	- Gly	- NH2
Guinea Pig	pGlu	- Tyr	- Trp	- Ser	- Tyr	- Gly	- Val	- Arg	- Pro	- Gly	- NH2
Herring	pGlu	- His	- Trp	- Ser	- His	- Gly	- Leu	- Ser	- Pro	- Gly	- NH2

Figure 1-2. Amino acid sequences for thirteen known forms of gonadotropin-releasing hormone. Each form is typically named after the species from which it was first isolated and sequenced, and all the forms are decapeptides.

CHAPTER II

Distribution of GnRH-II in the Rhesus Macaque Brain: Potential to Modulate GnRH-I secretion.

INTRODUCTION

To date, the GnRH family includes at least 13 decapeptide forms identified from various vertebrate classes. Most of these forms are believed to have reproductive functionality, based on their ability to exogenously either stimulate gonadotropin release, modulate reproductive behaviors, or a combination of both. In addition, it has become apparent that many vertebrate species express two or more of the multiple forms of GnRH, supporting the hypothesis that neuroendocrine control of the reproductive system is a conjunctive process between two or more GnRH forms.

To classify them, the forms are often subdivided into three groups based on their distribution and specific postulated functions, and it rapidly becomes apparent that many GnRH forms are specific to a particular vertebrate class or family (Fig. 2-1) (151). What is unknown, however, is whether these forms arise post-transcriptionally due to alternative splicing. GnRH-II is an exception in this case because it is known that a unique gene sequence encodes GnRH-II, thus it is not derived from GnRH-I post-transcriptionally (80, 109, 110). In addition, the GnRH-II gene occurs very early on the phylogenetic tree, therefore current theory holds that it is the most primitive GnRH form found in vertebrates (Fig 2-1) (149).

Although GnRH-II has been cloned from the human, rhesus macaque, and tree shrew, little is known about its regional expression in the mammalian brain. GnRH-II immunoreactivity has been detected in the midbrain of the tree and musk shrews (106, 109) and in the midbrain and hypothalamus of the rhesus macaque, but it is unclear whether these cells represent a source of GnRH-II synthesis or uptake (79). In addition, if GnRH-II is constituitively released or occurs at low levels, immunohistochemical methods may give an incomplete representation of its regional expression.

The importance of mapping GnRH-II distribution is multi-fold. Although many GnRH forms are believed to modulate the hpg axis, the biological function of GnRH-II is presently unknown, and it is possible that GnRH-II acts in a neuroendocrine cascade modulating GnRH-I. Current theory holds that the majority of neuroendocrine signals that influence the hpg axis act either directly on GnRH-I release or on GnRH-I signaling to the gonadotropes. As a result, it is expedient to determine whether GnRH-II is histologically capable of influencing GnRH-I.

In humans and rhesus macaques, two distinct genes encode GnRH-I and GnRH-II (80, 110); this suggests that different neuroendocrine pathways control the synthesis and release of GnRH-I and GnRH-II in primates. What is unknown is whether the same neurons synthesize both GnRH-I and GnRH-II. This possibility certainly exists in primates because preliminary studies have demonstrated that GnRH-II is highly expressed in hypothalamic areas where GnRH-I has also been found, notably around the ventral hypothalamic tract and in the medial basal hypothalamus (79, 110). Thus it is unknown whether two populations of GnRH-releasing neurons exist and if the two GnRH forms play different physiological roles. As a result, a primary question to be addressed is whether GnRH-II occurs in a distinct cell population from GnRH-I, and to resolve this issue, it is necessary to determine the extent to which GnRH-I neurons express mRNA encoding GnRH-II.

In addition, knowing where GnRH-II occurs in the hypothalamus may also elucidate potential functionality. In particular, it may help to formulate hypotheses about the function of GnRH-II if it is located within any well-characterized hypothalamic regions, because this will indicate what neurotransmitters, neuropeptides, or axonal terminal fields might modulate GnRH-II and also suggest where GnRH-II cells themselves project. In addition, studying its projection sites may serve as an indication whether GnRH-II signals to GnRH-I or other specific

cell types within a known neuroendocrine system. Examining the GnRH-II terminal fields will require immunological methods, however, using *in situ* hybridization to initially study its distribution will also allow an investigation into whether GnRH-II mRNA expression is altered due to sexual dimorphisms or to different developmental states. Such a determination will further what can be hypothesized about GnRH-II's function(s).

GnRH-I mRNA levels have not been demonstrated to differ between male and female or juvenile and adult primates. However, castrated male primates will undergo a gonadotropin surge if primed with estradiol (150), suggesting that their reproductive neuroendocrine systems are not sexually dimorphic. Similarly, if juvenile primates are treated with circhoral GnRH-I their LH profiles will correspond to those of an adult (151), and this suggests that their neuroendocrine systems controlling reproduction do not differ completely. Unlike the first case, however, where the sex-steroid makeup provides the definition of "male" or "female" to the neuroendocrine system, the signal that triggers the hpg axis to undergo puberty has not been defined. As a result, it was hypothesized that increasing GnRH expression might signal puberty to begin (155). Contrary to this, GnRH-I hypothalamic expression does not increase during development unless the animals are gonadectomized as juveniles (156 - 159). In addition, GnRH-I mRNA also does not decrease at puberty, which might be expected due to the negative feedback that might be expected with rising estradiol levels. Taken together, this suggests that a change in GnRH-I expression does not trigger the onset of puberty. However, it has since been discovered that GnRH-II also occurs in the primate hypothalamus (110). Developmentally, GnRH-II expression has been observed to both increase (130) and decrease (127), as well as not change (125, 126), depending on the species, however many of these studies examined midbrain, rather than hypothalamic populations of

GnRH-II. Taken together, this provides the impetus to examine whether GnRH-II expression changes during development.

GnRH-II cDNA was recently cloned from the rhesus macaque, which for aforementioned reasons is an excellent model to examine GnRH-II mRNA distribution and expression. In particular, this is due to the striking similarities between the rhesus macaque and human hpg axes and the placement of the rhesus macaque in the phylogenetic tree. If GnRH-II is primitive and has a highly conserved function, studying the peptide near the end of its functional evolution may elucidate what, if any, essential roles GnRH-II plays in reproduction.

This chapter, therefore, addresses where GnRH-II occurs in the rhesus hypothalamus, and specifically, the ability of GnRH-II cells to signal to GnRH-I cells is examined as well as whether GnRH-II expression differs between males and female and juveniles and adults.

MATERIALS &METHODS AND RESULTS

Technical approach

Although the regional distribution of GnRH-II has been examined in several species, the approach taken has depended heavily on HPLC and IHC. HPLC permits high sensitivity but has less resolution when mapping distribution. The second method, IHC, supplies both sensitivity and resolution to mapping distribution, but it does not answer the question whether immunoreactive cells represent a source of peptide synthesis or uptake. Furthermore, the GnRH-II peptide has 70% sequence homology with the GnRH-I peptide (Fig. 1-2), therefore antibody specificity becomes a pertinent concern. Alternatively, the GnRH-associated peptide for GnRH-II is unique from GnRH-I, which allows for a highly specific GnRH-II riboprobe to be constructed suitable for *in situ* hybridization (ISH). As a result, I chose to initially map the GnRH-II distribution using ISH,

followed by IHC to determine both if the mRNA was translated into peptide and where GnRH-II nerve terminals projected. Having examined the mRNA and peptide distributions, the degree of overlap between the GnRH-II and GnRH-I populations was investigated as was whether GnRH-II expression varied between males and females or juveniles and adults.

mRNA distribution

Animals

Tissues from adult male and female rhesus macaques (*Macaca mulatta*) were obtained from the Oregon Regional Primate Research Center's (ORPRC, now the Oregon National Primate Research Center or ONPRC) Tissue Distribution Program. These animals had been maintained in accordance with the NIH *Guide for the Care and Use of Laboratory Animals*. They had been housed under controlled lighting (12 hours of light and 12 hours of darkness per day) and temperature (23 ± 2°C), and had been provided a diet consisting of Purina monkey chow and fresh fruit, with unlimited access to drinking water.

Tissue preparation

The animals were deeply anesthetized using ketamine/pentobarbital according to procedures established by the Panel on Euthanasia of the American Veterinary Society. Their brains were fixed by perfusing 1 L 0.9% saline through the ascending aorta followed by 6.5 L ice-cold 4% paraformaldehyde in 0.1 mol/L phosphate-buffered (pH 7.6) saline (0.9%, wt/vol). Hypothalami were blocked just rostral to the optic chiasm and rostral to the mammillary bodies. Midbrains were blocked just rostral to the mammillary bodies and rostral to the pons. Hippocampal tissue included the entire fundus to the rostral edge of central sulcus. They were then immersed in fresh fixative for an additional 3 h (at 4°C) and cryoprotected. This involved their immersion in 0.02 mol/L phosphate buffer (pH 7.4) containing

glycerol (10%, vol/vol) and dimethylsulfoxide (2% vol/vol) for 24 h, followed by immersion in a more concentrated glycerol (20%) phosphate/dimethylsulfoxide solution for an additional 72 h. The tissue blocks were rapidly frozen in 2-methyl butane (precooled in an ethanol/dry-ice bath) and stored at -85°C. Subsequently, a freezing, sliding microtome was used to cut coronal sections (25 μm) that were mounted on glass microscope slides (Fisherbrand SuperFrost/Plus; Fisher Scientific, Auburn, WA). After being air-dried for 30 min and vacuum-dried overnight, the mounted sections were stored at -85°C for later use.

cDNA isolation and sequencing

The cDNA used to produce the GnRH-II riboprobe was generously supplied by Drs. Richard White and Russell Fernald at Stanford University. To obtain the cDNA, rhesus macaque midbrain tissue was homogenized in a guanidine thiocyanate-phenol-chloroform reagent and total RNA extracted (UltraSpec-II RNA, Biotecx Laboratories, Houston, TX). SuperScript II RT was then used to transcribe 5 μg of total RNA into cDNA, following the manufacturer's protocol (Life Technologies, Gaithersburg, MD) except that the reaction was performed at 45°C and primed with $dT_{22}V$. A portion (0.01%) of the cDNA synthesis reaction was amplified in glass microcapillary tubes using a Rapidcycler (Idaho Technologies, Idaho Falls, ID) and the following cycling protocol: a 15-sec denaturation at 94°C followed by 35 cycles of a 0-sec hold at 94°C, a 0-sec primer annealing step at 60°C and a 15-sec extension step at 72°C. The primers were based on the known human GnRH-II cDNA (113): exon 1, 5'-CTG CAG CTG CCT GAA GGA G-3' and exon 4, 5'-CGG AGA ACC TCA CAC TTT ATT GG-3'. The amplified products were sequenced directly to confirm their identity (GenBank Accession Number AF097356).

In situ hybridization (ISH)

ISH involved the use of ^{35}S-labeled antisense riboprobes to rhesus macaque GnRH-II mRNA. The riboprobe was about 430-base-pairs long, and spanned the complete decapeptide coding region as well as most of the GnRH-associated peptide (GAP) coding region.

ISH was performed on a series of coronal hippocampal, midbrain, and hypothalamic sections from each animal, collected at approximately 200-µm intervals. First, the sections were postfixed in 4% paraformaldehyde in 0.1 mol/L phosphate buffer (pH 7.4) for 15 min, rinsed in Tris-ethylenediamine tetraacetate (Tris-EDTA), and then digested with proteinase K (10 µg/mL) in Tris-EDTA buffer (pH 8.0, 100 mmol/L Tris and 50 mmol/L EDTA) for 30 min. Next, they were acetylated, dehydrated with ascending concentrations of ethanol, and dried under vacuum for 2 h. They were then hybridized for 18 h at 65°C with 100 µL ^{35}S-labeled antisense riboprobe diluted to 1 X 10^7 cpm/mL hybridization buffer [50 mmol/L dithiothreitol (DTT), 250 µg/ml transfer RNA, 50% formamide, 0.3 mol/L sodium chloride, 1 X Denhardt's solution, 20 mmol/L Tris (pH 8.0), 1 mmol/L EDTA, and 10% dextran sulfate]. For the hybridization, glass coverslips were affixed to the slides using DPX mounting medium (BDH Laboratory Supplies, Poole, UK). The posthybridization procedure involved removing the coverslips, after two 30-min soakings in 4X SSC (saline-sodium citrate buffer; the 20X stock SSC solution comprised 175.3 g sodium chloride and 88.2 g sodium citrate/L, pH 7.0) containing 20 mmol/L DTT. The sections were then incubated in Tris-EDTA buffer (pH 8.0; 10 mmol/L Tris, 1 mmol/L EDTA, and 0.5 mol/L sodium chloride) containing ribonuclease A (10 mg/mL) for 30 min at 37°C, followed by two 30-min washes at room temperature with 2X SSC containing 1 mmol/L DTT. After a final 30-min wash at 70°C with 0.1X SSC containing 1 mmol/L DTT, they were dehydrated

through ascending concentrations of ethanol containing 0.3 mol/L ammonium acetate and then air-dried for 30 min. To visualize the hybridization pattern the sections were apposed to Hyperfilm β-max (Amersham Pharmacia Biotech, Piscataway, NJ) for 6 days (i.e., an exposure period that maintained the hybridization signal in the linear response range of the film). As a negative control, ISH was also performed on a few sections using a ^{35}S-labeled sense riboprobe.

The autoradiographs were uniformly transilluminated (Northern Light, Imaging Research, Inc., St. Catherines, Canada) and images captured using a Sony XC-77 CCD camera (Sony Corp. of America, Cypress, CA) equipped with a 50-mm macrolens. They were then digitized using a DT-2255 frame grabber (Data Translation, Marlboro, MA) connected to a Macintosh Power-PC computer (Apple Computer Inc., Cupertino, CA). To examine regional mRNA distribution at the cellular level, sections were subsequently dehydrated using increasing concentrations of ethanol, defatted in xylenes for 1 h, and dipped in photographic emulsion (NTB-2, Eastman Kodak Co., Rochester, NY). They were exposed at 4°C in a light-tight box for 12 days then processed with Kodak developer (D-19) and fixer. To determine whether mRNA occurred in magnocellular or parvicellular cells, sections were then washed for 5 min in distilled water and dipped in toluidine blue (160) for 10 min followed by two 5-min washes in water. Sections were then dehydrated with ethanol, cleared with xylenes, and finally coverslipped using DPX mounting medium.

Results

Both midbrain and hippocampal sections highly expressed GnRH-II mRNA in discrete clusters. Midbrain expression was confined to the central region, ventro-medial to the fourth ventricle (Fig. 2-2). This distribution appeared to coincide approximately with the dorsal raphe, however colocalization studies with tryptophan hydroxylase, a marker for serotonin, are necessary to confirm if GnRH-II occurs in

the dorsal raphe. Hippocampal GnRH-II expression appeared to coincide with the dentate gyrus (Fig. 2-2) although, the high cellular density of this region precluded individual cells to be identified.

The hypothalamus also highly expressed GnRH-II mRNA in discrete areas. Autoradiographs revealed a clustered hybridization pattern in the supraoptic (SON) and paraventricular (PVN) nuclei and the medial basal hypothalamus (MBH) (Fig. 2-3); in some sections, moderate hybridization also occurred in the suprachiasmatic nucleus (Fig. 2-3). Control sections hybridized with a ^{35}S-labeled sense probe, showed a uniform low level of hybridization (Fig. 2-4). In addition, when sections were exposed to increasing temperature stringency during post-hybridization, a nonlinear decrease in expression occurred, which further supports that the hybridization of the GnRH-II probe was specific.

To determine whether the magnocellular or parvicellular cells in the SON and PVN expressed GnRH-II mRNA, hypothalamic sections were counterstained with toluidine blue, which gives parvicellular nuclei a dark, granulated appearance while magnocellular nuclei stain a more uniform light color. As shown in Fig. 2-3, silver grain deposition coincided entirely with magnocellular nuclei, indicating that the magnocellular cell population in the SON and PVN express GnRH-II mRNA.

Distribution of GnRH-II versus GnRH-I

Animals

This study was approved by the Institutional Animal Care and Use Committee at the ONPRC and used six male rhesus macaques (*Macaca mulatta*), aged 0.6-15 years. They were cared for by the ORPRC in accordance with the NIH *Guide for the Care and Use of Laboratory Animals* and eventually were painlessly killed to provide a source of brain tissue both for this and other related studies. Tissue was perfusion-fixed and processed as described above.

Immunohistochemistry

IHC was performed on a series of 8-12 hypothalamic sections from each animal, collected at approximately 200-μm intervals. All solutions made for this procedure used RNase-free, diethyl pyrocarbonate-treated water. Sections were washed three times, 5 min each, with Tris buffer (0.05 M Tris, pH 7.6, containing 0.15 M sodium chloride). They were then incubated for 48 h at 4°C with a GnRH-I monoclonal antibody (HU4H) (159) at a 1:1000 dilution. They were then washed three times, 5 min each, incubated in biotinylated horse anti-mouse IgG (Vector Laboratories; Burlingame, CA) at 1:1000 dilution in Tris buffer for 1 h at room temperature, and again washed. To detect the signal, the sections were exposed to an avidin/biotin complex (Standard ABC kit; Vector Laboratories) for 1 h, washed three times in Tris buffer, 5 min each, and exposed to 3,3'-diaminobenzidine tetrachloride (1 mg/ml Tris buffer; Sigma, St. Louis, MO) for 10 min, concluding with 3 more washes. The sections were then mounted on glass microscope slides (Fisherbrand; Fisher Scientific), fan dried for 10 min, dehydrated in ascending concentrations of ethanol, dipped in xylenes, and coverslipped using DPX mounting medium.

In situ hybridization

ISH was performed as described above using a ^{35}S-labeled antisense riboprobe against either macaque GnRH-II precursor cDNA or macaque GnRH-I precursor cDNA, which was a riboprobe approximately 224-nucleotides in length, for a positive control.

Results

Representative autoradiographs depicting the general distribution pattern of GnRH-I and GnRH-II mRNAs in the hypothalamus are shown (Fig. 2-5). Cells expressing GnRH-I mRNA were scattered widely throughout the hypothalamus, especially in the ventral regions, whereas cells expressing GnRH-II mRNA were

found to be concentrated mainly in the SON and PVN nuclei, as well as in the MBH. Despite the marked difference in the general distribution pattern of GnRH-I and GnRH-II mRNAs, some overlap was also evident, especially around the ventral hypothalamic tract and in the MBH.

To examine whether the GnRH-I neurons in these regions also express GnRH-II mRNA, some of the brain sections were first processed for IHC using a monoclonal antibody to GnRH-I and then for ISH using a riboprobe to monkey GnRH-II mRNA. The sections were then coated with photographic emulsion to allow microscopic examination for double labeling. Overall, three times as many GnRH-II as GnRH-I cells were detected. More importantly, in no instance was GnRH-II mRNA found to be coexpressed with GnRH-I peptide (Fig. 2-6).

For control purposes, some of the GnRH-I immunolabeled cells were processed for ISH using a riboprobe to monkey GnRH-I mRNA. As expected, numerous GnRH-I immunolabeled cells were found scattered around the ventral hypothalamic tract and in the MBH, and each one showed a high level of GnRH-I mRNA expression (Fig. 2-6). This is in marked contrast to the results obtained when using the GnRH-II riboprobe; although several cells in the vicinity of the GnRH-I neurons showed a high level of hybridization to the GnRH-II riboprobe, the GnRH-I neurons themselves did not (Fig. 2-6).

Peptide distribution

Tissue preparation

The animals were deeply anesthetized and brains were fixed as described above. Hypothalami were blocked, cryoprotected, and sectioned as described. Coronal sections (25 µm) were stored free-floating at -20°C in a cryoprotectant solution comprised of 0.05 M sodium phosphate buffer (pH 7.3) with ethylene glycol

(30% vol/vol) and glycerol (20% vol/vol) until use. Pituitaries were cryoprotected and axial or sagittal sections were stored in cryoprotectant as described.

Immunohistochemistry (IHC)

IHC was performed as described above except the antibodies used were a previously characterized (162) GnRH-II polyclonal antibody (Ferro 1) at a 1:500 dilution in Tris buffer, and a GnRH-I monoclonal antibody (HU4H) (159) at a 1:1000 dilution, a previously characterized FSH monoclonal antibody at 1:50 (Fisher Scientific) (163) and LH polyclonal antibody at 1:100 (Chemicon International; Temecula, CA) (164). Furthermore, the secondary antibodies used were a biotinylated goat anti-rat and a biotinylated horse anti-mouse IgG, respectively (Vector Laboratories).

Results

mRNA expression, while providing an indication of GnRH-II distribution, does not reveal whether the message is translated into the peptide, where GnRH-II cells might project, or whether the GnRH-II peptide may be constituitively released. Therefore it became necessary to also map hypothalamic GnRH-II distribution using immunohistochemical methods. A previously characterized (161) polyclonal rat antibody specific for GnRH-II was used to map peptide distribution. In the hypothalamus, immunoreactive fibers occurred predominately along the ventral hypothalamic tract, presumably coming from the SON population (Fig. 2-7), or originated ventral to the PVN and ran parallel to the third ventricle, however immunoreactive cell bodies were not detected in either nucleus.

Approximately 90% of magnocellular cells in the SON and PVN project to the pars nervosa, therefore IHC was also performed on a series of pituitary sections to determine whether GnRH-II cells projected to the pituitary. As hypothesized, both the pituitary stalk and the pars nervosa had fibers immunoreactive for GnRH-

II (Fig. 2-9). In the latter case, GnRH-II fibers occurred predominately at the base of the stalk and near the border of the pars nervosa with the pars distalis (Fig. 2-7). In the pars distalis, GnRH-II immunoreactivity was not observed, although GnRH-I immunoreactivity was present in cells that corresponded to both LH- and FSH-producing gonadotropes (Fig. 2-8).

Although the GnRH-II antibody used has been characterized using ELISA, it was also preabsorbed with synthesized GnRH-II to confirm that the immunoreactivity seen was specific. Results, shown in Fig. 2-7, indicated that nonspecific binding was low to nonexistent using this IHC protocol.

GnRH-II secretion into peripheral plasma

Animals

Blood samples (0.5 ml) were obtained from six gonad-intact adult females during their follicular and luteal phases, as determined by detailed menstruation records and radioimmunoassay for circulating estradiol and progesterone concentrations.

Animal care was provided as described above by the ONPRC in accordance with the NIH *Guide for the Care and Use of Laboratory Animals.*

Radioimmunoassay

GnRH-II standards were prepared by diluting 5 µg purified GnRH-II (Bachem Laboratories) in 245 µg 0.1 N acetic acid. This was then further diluted 50 µl into 9.95 ml of assay buffer (0.01 M phosphosaline-EDTA, 0.1% gelatin, pH 7.4) to make a 10 ng/100 µl stock solution that was used to make the seven GnRH-II standards: 25 µg, 50 µg, 100 µg, 200 µg, 400 µg, 800 µg, and 1600 µg/100 µl assay buffer. The choice of standards to optimize the calibration curve was determined empirically.

All samples, including the GnRH-II standards, were prepared in triplicate for the GnRH-II assay. Standards (100 µl) were added to borosilicate glass culture tubes (12mm x 75, Fisherbrand; Fisher Scientific) containing 50 µl of a rabbit polyclonal GnRH-II antibody generously provided by Dr. Els d'Hondt diluted 1:50,000 in assay buffer, and 150 µl assay buffer to bring the final volume to 300 µl. Experimental tubes contained a dilution curve of 10, 25, 50, or 100 µl of sample, and the assay buffer volume was adjusted accordingly to bring the final volume to 300 µl. Tubes were vortexed briefly and incubated at 4°C overnight. Next, 50 µl of radiolabeled GnRH-II (Peninsula Laboratories; Belmont, CA), diluted in assay buffer to a final concentration of 8,000 to 10,000 cpm, was added. The tubes were again vortexed and incubated at 4°C overnight. Following this, 100 µl of goat anti-rabbit antibody diluted 1:10 in assay buffer was added and the tubes were vortexed and incubated as before. The following day, 1 ml of assay buffer was added and the tubes were centrifuged at 3000 rpm for 30 minutes. The supernatant was gently aspirated off and the remaining pellet counted using a Packard Cobra gamma counter (Packard; Meridan, CT) for 1 min.

Results

The SON and PVN populations of GnRH-II cells appeared to send projections to the posterior pituitary, where they might release GnRH-II into the general circulation. This would allow GnRH-II release to be measured in peripheral, rather than portal, blood samples. Single time-point blood samples were obtained from follicular- and luteal-phase intact rhesus macaques, and the plasma was assayed for GnRH-II concentrations. However, GnRH-II was below the detectable limits of the assay for intact animals during either the follicular or luteal phase (Fig. 2-9).

Comparison between males and females and juveniles and adults

Animals

Tissues from six male and six female rhesus macaques (*Macaca mulatta*) were obtained from the ONPRC Tissue Distribution Program to provide tissue for this and other studies. Half of the animals from each sex were sexually immature (0.6 yr old), and half were adults (10-15 yr old). Based on detailed menstruation records, one of the three adult females was in the early follicular phase of the menstrual cycle, one was in the late follicular phase, and one was in the luteal phase. The immature animals had all been weaned several months before use. Animal care was provided by the ONPRC in accordance with the NIH *Guide for the Care and Use of Laboratory Animals*. They had been housed under controlled lighting (12 hours of light and 12 hours of darkness per day) and temperature (23 ± 2°C), and had been provided a diet consisting of Purina monkey chow and fresh fruit, with unlimited access to drinking water.

Tissue preparation

The animals were deeply anesthetized using ketamine/pentobarbital according to procedures established by the Panel on Euthanasia of the American Veterinary Society. Their brains were fixed by perfusion as described above. Hypothalami were blocked just rostral to the optic chiasm and rostral to the mammillary bodies. The tissue blocks were frozen, sectioned, and stored as described above.

In situ hybridization (ISH)

ISH involved the use of ^{35}S-labeled antisense riboprobes to macaque GnRH-II mRNA. The riboprobe was 430-base-pairs long and spanned the complete decapeptide coding region as well as most of the GnRH-associated peptide (GAP)

coding region. Because the GAP region is unique for each GnRH precursor form, this probe specifically identified only those cells that express GnRH-II mRNA.

ISH was performed as described above on a series of eight coronal hypothalamic sections from each animal, collected at approximately 200-μm intervals. As a negative control, ISH was also performed on a few sections using a ^{35}S-labeled sense riboprobe. For quantitation, the autoradiographs were uniformly transilluminated, and the images captured and digitized as described above. Images were analyzed using the NIH Image program (version 1.59). Specific hypothalamic areas were defined using the freehand outlining tool, and the mean optical density was measured after correcting for background noise. For each animal, the mean optical densities from different autoradiographs were averaged and then combined according to age and sex to give an overall group mean (± SEM; n=3). Two-way ANOVA was used to assess statistical differences in regional GnRH-II mRNA expression between the sexes and also between the immature and adult animals. Because no sex differences were detected, the data from the males and females were pooled (i.e., n=6/age group) and further analyzed by one-way ANOVA.

To further quantitate the expression of GnRH-II mRNA in the different groups, the hypothalamic sections were subsequently dehydrated, defatted, and dipped in photographic emulsion as described above. Silver grain density was examined under a microscope using a X40 objective lens. Only cells that had an obvious round or fusiform silver grain deposition pattern were counted and analyzed. The images were digitized, as described above, and the NIH Image program was then used to determine the silver grain density per cells (expressed as pixels per cell) and to determine the total number of positive cells per section. Again, for each animal the mean silver grain densities were averaged and then combined according to age and sex to give an overall group mean (± SEM; n=3). Two-way ANOVA was used

to assess statistical differences in regional GnRH-II mRNA expression between the sexes and also between the immature and adult animals. Because no sex differences were detected, the data from the males and females were pooled (i.e., n=6/age group) and further analyzed by one-way ANOVA.

Results

Autoradiographic analysis

To analyze sex and age differences in GnRH-II gene expression in these hypothalamic regions the autoradiographic images were digitized, and mean optical densities were determined for the SON, PVN, and MBH. In all of the hypothalamic regions examined the level of GnRH-II mRNA expression was similar in the males and females (Fig. 2-9), which was confirmed by two-way ANOVA, so data from the two sexes were pooled for the developmental analysis. Comparing the distribution between adult and immature animals, GnRH-II mRNA appeared equivalent in the SON and PVN, suggesting that the rostral hypothalamus does not undergo a significant change in GnRH-II expression developmentally (Fig. 2-10). However, in the caudal hypothalamus, GnRH-II mRNA was expressed in the adult MBH but this hybridization was either markedly attenuated or missing in the juvenile MBH (Fig. 2-11). This observation was confirmed using ANOVA to be a statistically significant difference between adults and juveniles, regardless of sex ($P<0.05$).

Silver grain analysis

Based on the autoradiographs alone it was unclear whether the intense hybridization in the MBH of adults reflected an increase in GnRH-II mRNA expression per cell or the cell population itself increased developmentally. To resolve this issue, the same hybridized sections were dipped in photographic emulsion, and the resulting silver grain deposition patterns were analyzed microscopically (Fig. 2-11). A total of 2604 GnRH-II cells were identified in hypothalamic sections from the

12 males and females, however the number of silver grains per cell was found to be similar regardless of the animal's age ($P<0.05$; Fig. 2-11). In contrast, the number of MBH cells expressing detectable quantities of GnRH-II mRNA was significantly ($P<0.001$) greater in the adults than in the immature animals (Fig. 2-11).

Due to the apparent, but not significant, increase seen developmentally in the SON and PVN using autoradiographs, silver grain analysis was also used in the rostral hypothalamus to make a more sensitive measure of GnRH-II mRNA levels. As seen with the autoradiographs, GnRH-II mRNA expression appeared greater in the adult than immature animals in the rostral and caudal hypothalamus (Fig. 2-12), however, whereas the juvenile SON and PVN expressed a moderate level of GnRH-II, the MBH population of GnRH-II appeared largely absent in the juvenile. Upon analysis, both the quantity of silver grains per cell and the total number of cells expressing GnRH-II were not significantly greater in the adult SON or PVN compared to the juvenile SON and PVN, respectively (Fig. 2-12).

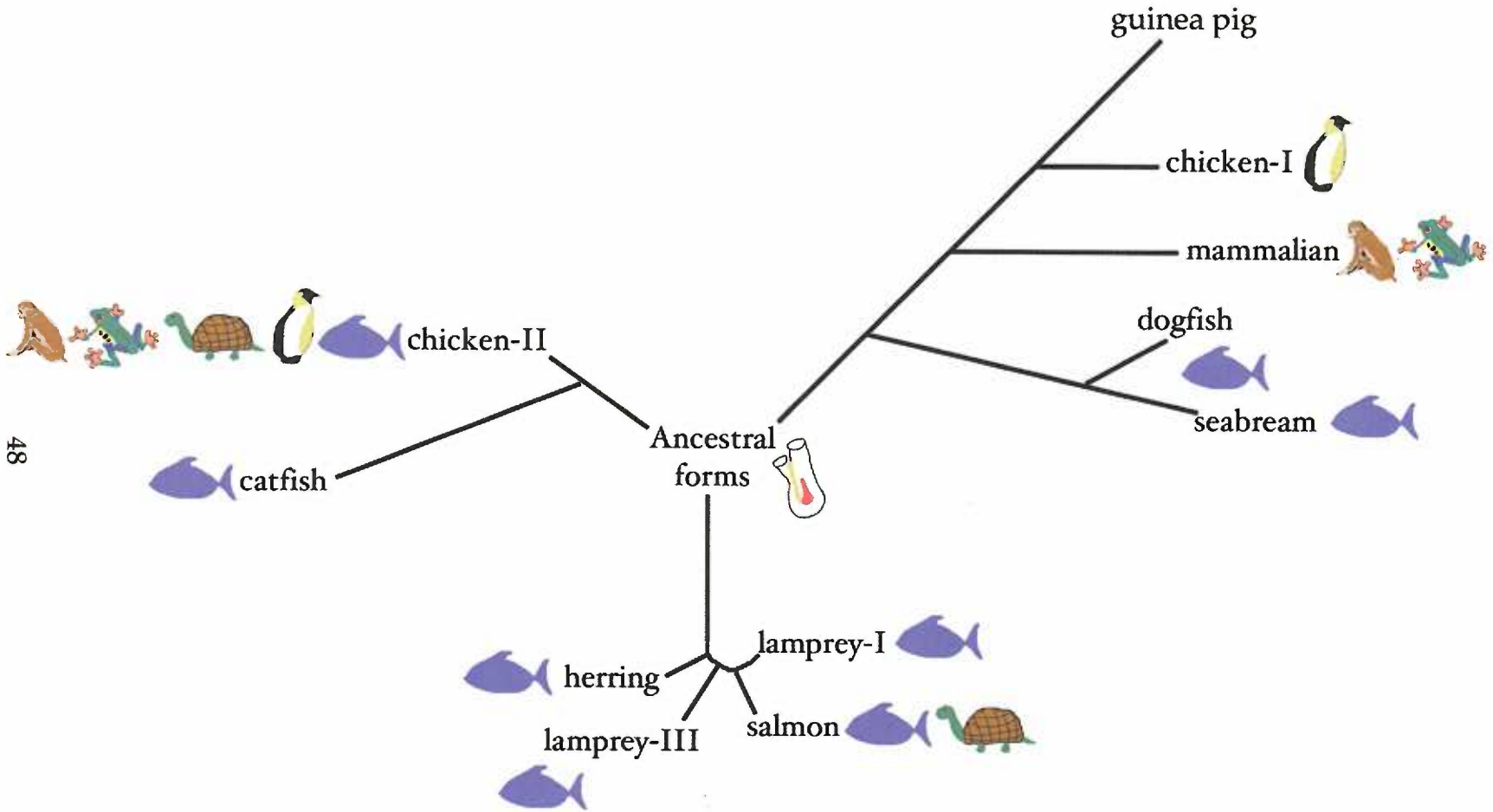

Figure 2-1. Phylogenetic tree for the GnRH gene family. To date, 13 forms have been identified. Of these, chicken-II (GnRH-II) is the most highly conserved across the vertebrate classes.

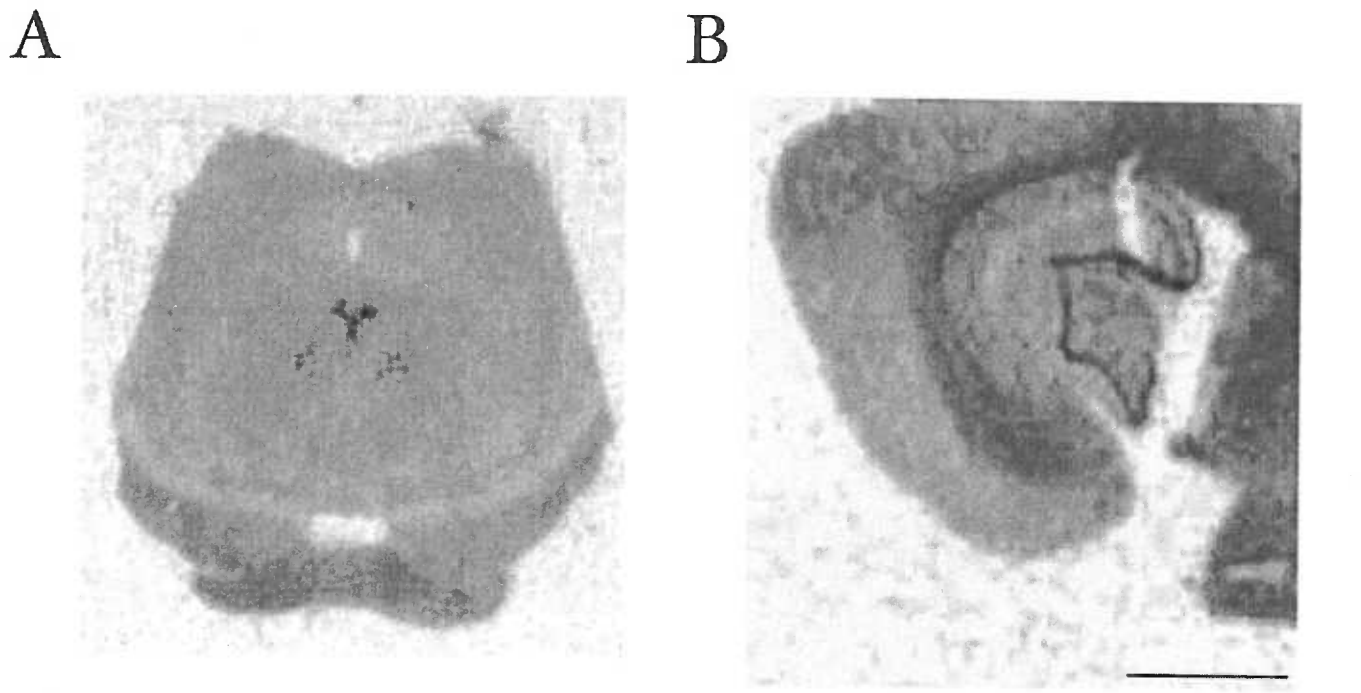

Figure 2-2. Regional expression of GnRH-II mRNA in the rhesus macaque brain as revealed by radioisotopic *in situ* hybridization. Autoradiographs of coronal brain sections represent hybridization of an antisense GnRH-II riboprobe in the central region of the midbrain (A) and hippocampus (B). *Scale bar*, 5 mm.

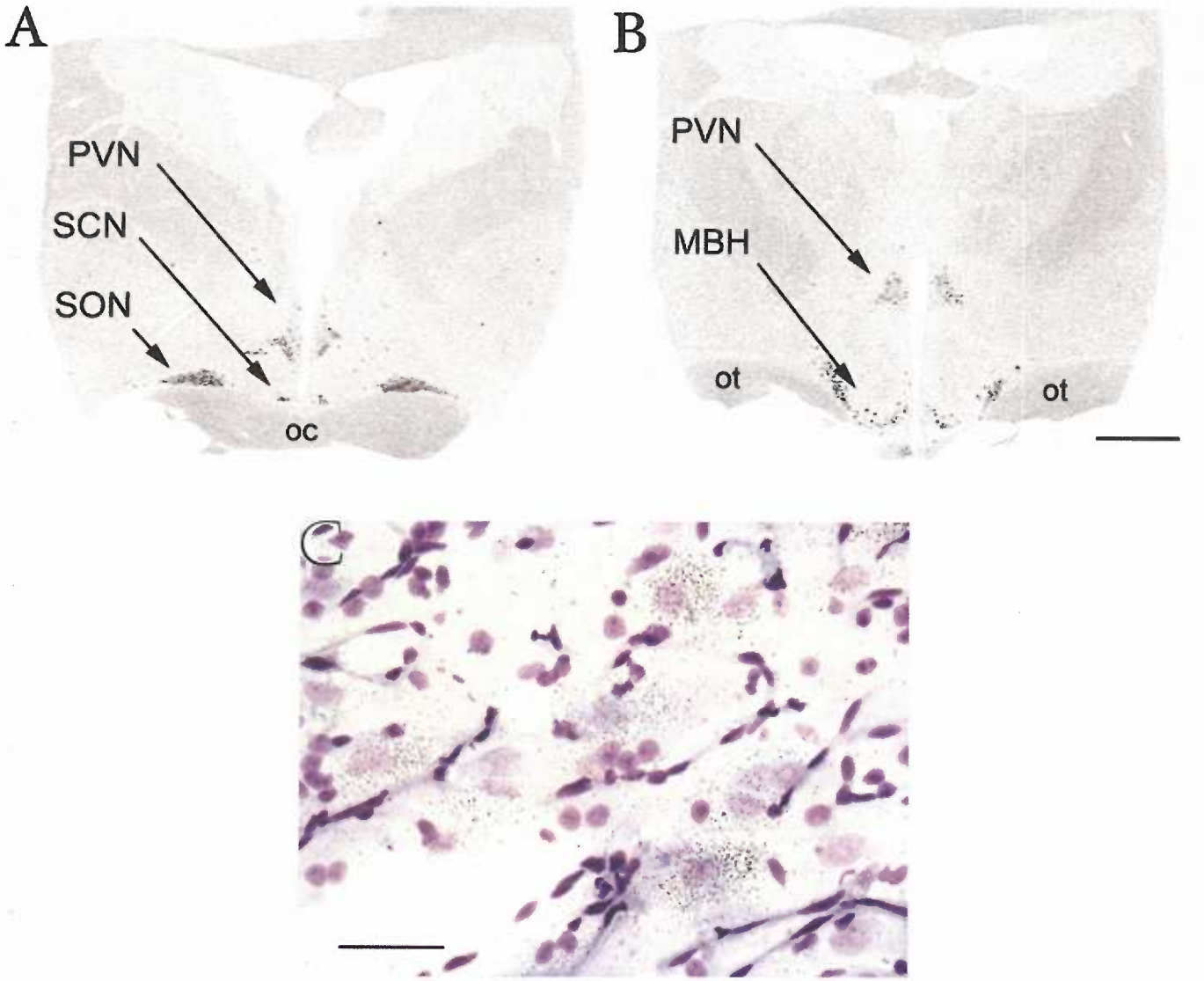

Figure 2-3. Regional expression of GnRH-II mRNA in the rhesus macaque brain as revealed by radioisotopic *in situ* hybridization.Autoradiographs of coronal brain sections represent hybridization of an antisense GnRH-II riboprobe in the central region of the supraoptic, paraventricular, and suprachiasmaticnuclei of the hypothalamus (A) and in the mediobasal hypothalamus (B). Silver grains representing mRNA for GnRH-II are combined with toluidine blue staining to distinguish magnocellular and parvocellular nuclei in the SON and PVN (C). Notice the silver grains occur predominately over the magnocellular nuclei. *Scale bars*, 5 mm (A, B), 50 µm (C). oc=optic chiasm, ot = optic tract.

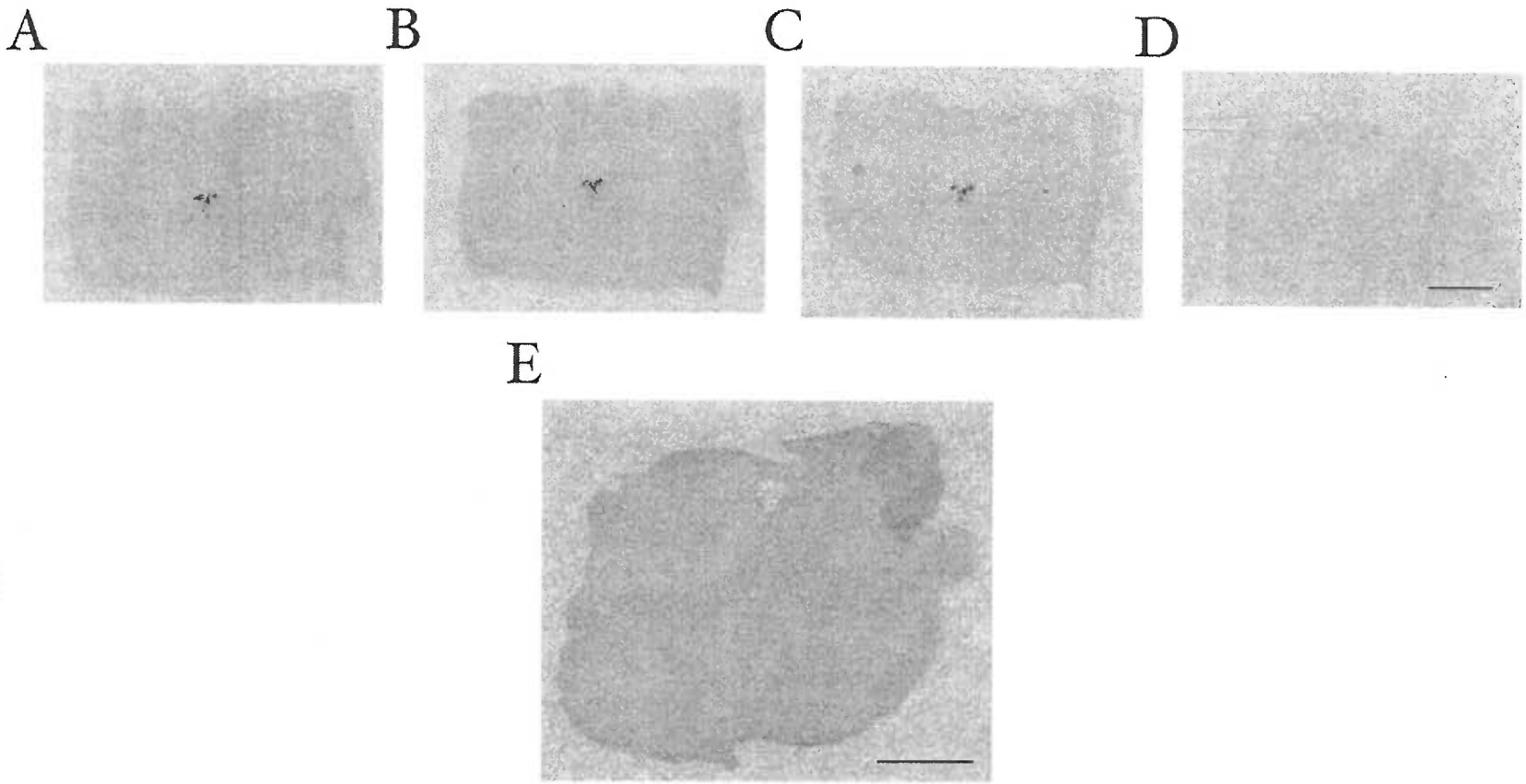

Figure 2-4. Specificity of the antisense GnRH-II riboprobe used for *in situ* hybridization. Autoradiographs of coronal midbrain sections hybridized with an antisense GnRH-II riboprobe followed by exposure to increasingly stringent temperatures (A-D). A, Usual post-hybridization temperature of 70 C. B, Post-hybridization temperature of 80 C. C, Post-hybridization temperature of 90 C. D, Post-hybridization temperature of 100 C. Note that the degree of hybridization does not decrease gradually, but disappears suddenly at 100 C. E, Autoradiograph of coronal midbrain section hybridized with a sense GnRH-II riboprobe to test the probe specificity. No binding was observed with the sense probe. *Scale bar*, 5 mm.

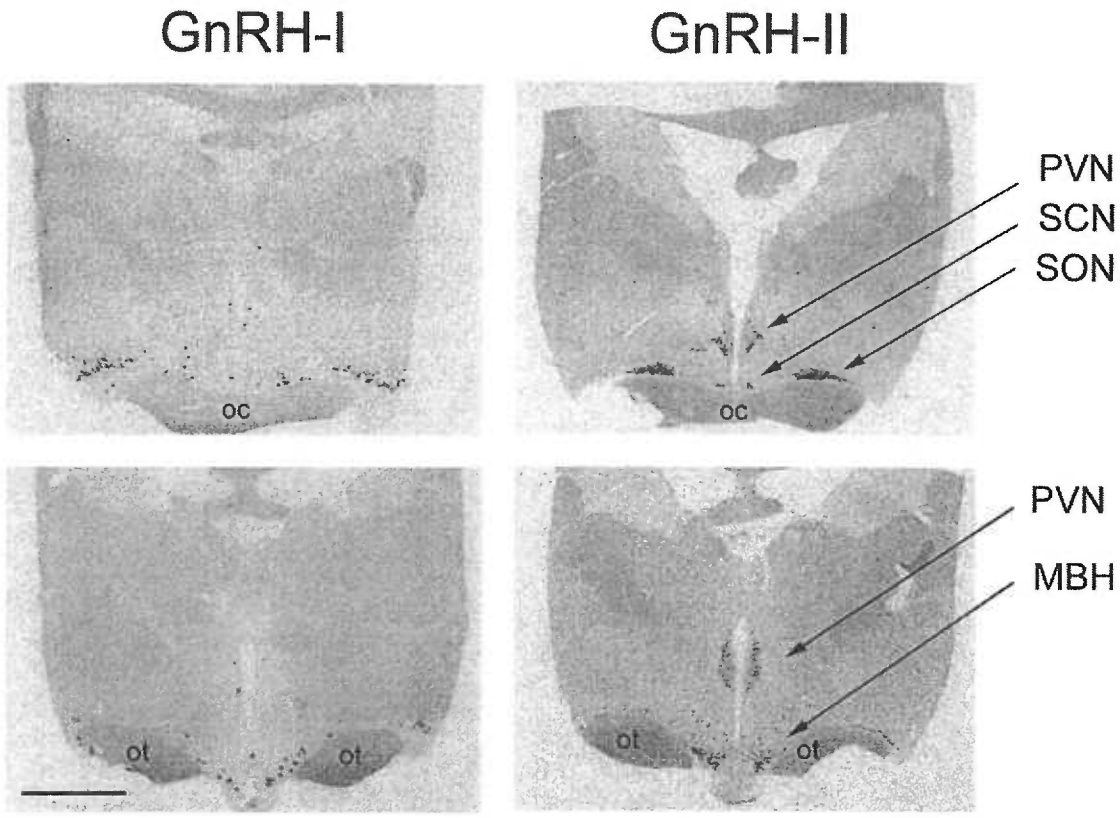

Figure 2-5. Regional distribution of GnRH mRNA in the rostral (*upper panels*) and caudal (*lower panels*) hypothalamus of male rhesus macaques, as revealed by *in situ* hybridization. Representative autoradiographs depicted in the *left panels* show a scattered pattern of GnRH-I mRNA expression, especially in ventral regions of the hypothalamus. Representative autoradiographs depicted in the *right panels* show a concentrated pattern of GnRH-II mRNA expression, especially in the suproptic (SON), paraventricular (PVN), and suprachiasmatic nuclei (SCN), and also in the medial basal hypothalamus (MBH). oc = optic chiasm; ot=optic tract. *Scale bar*, 5 mm.

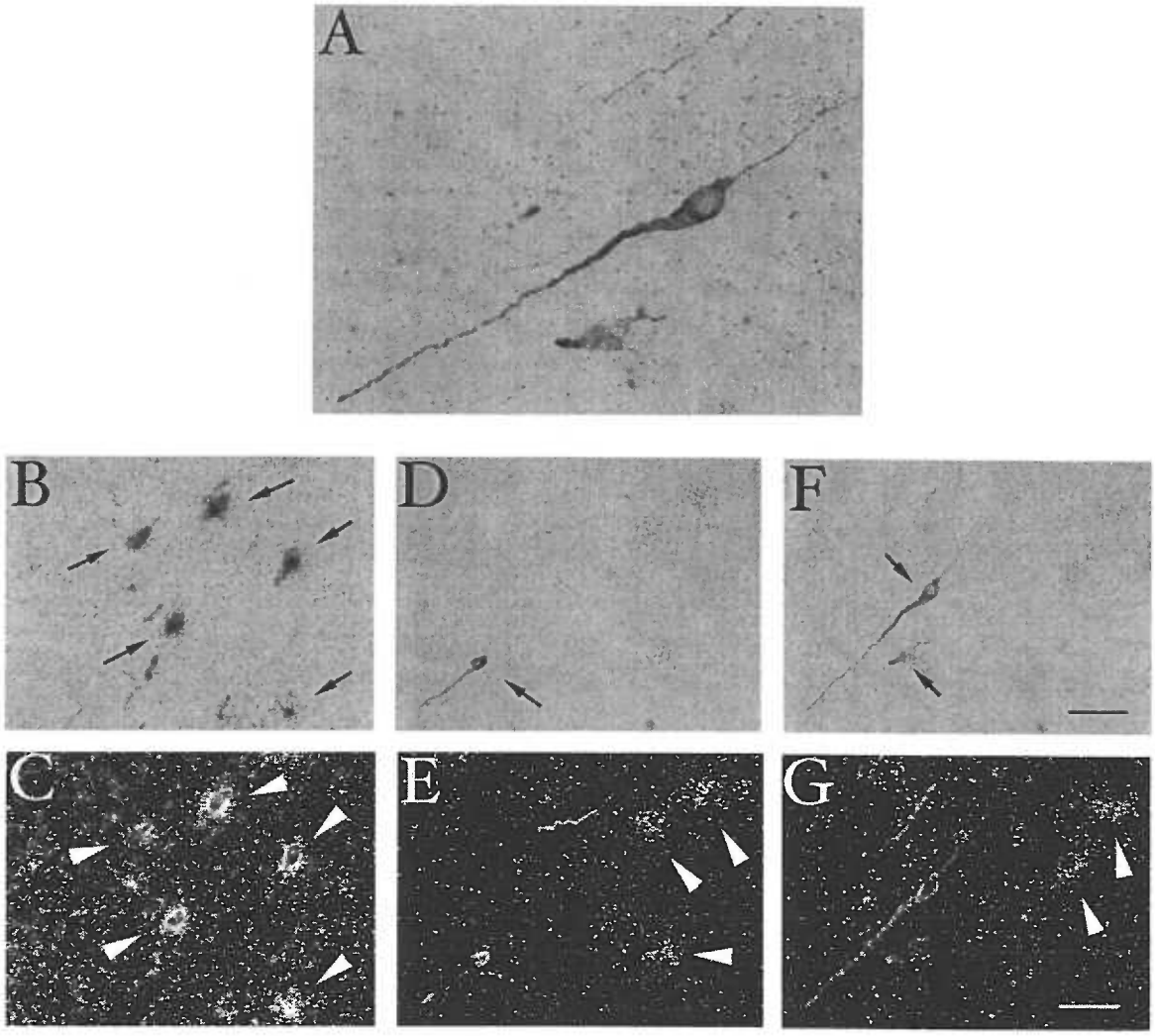

Figure 2-6. Representative photomicrographs of hypothalamic sections from male rhesus macaques, double-labelled using a procedure that combined immunohistochemistry (IHC) with *in situ* hybridization (ISH). The sections were initially processed for GnRH-I IHC and then for ISH, using a riboprobe to either GnRH-I mRNA (B, C) or to GnRH-II mRNA (A, D-G). In the bright-field photomicrographs (*upper panels*), GnRH-I immunopositive cells are indicated by *black arrows*. In the corresponding dark-field photomicrographs (*lower panels*), cells expressing GnRH-I or GnRH-II mRNA are identified by regions of high silver grain density (indicated by *white arrow heads*). Note the colocalization of GnRH-I mRNA, but not GnRH-II mRNA in the GnRH-I immunopositive cells. *Scale bars*=50 μm.

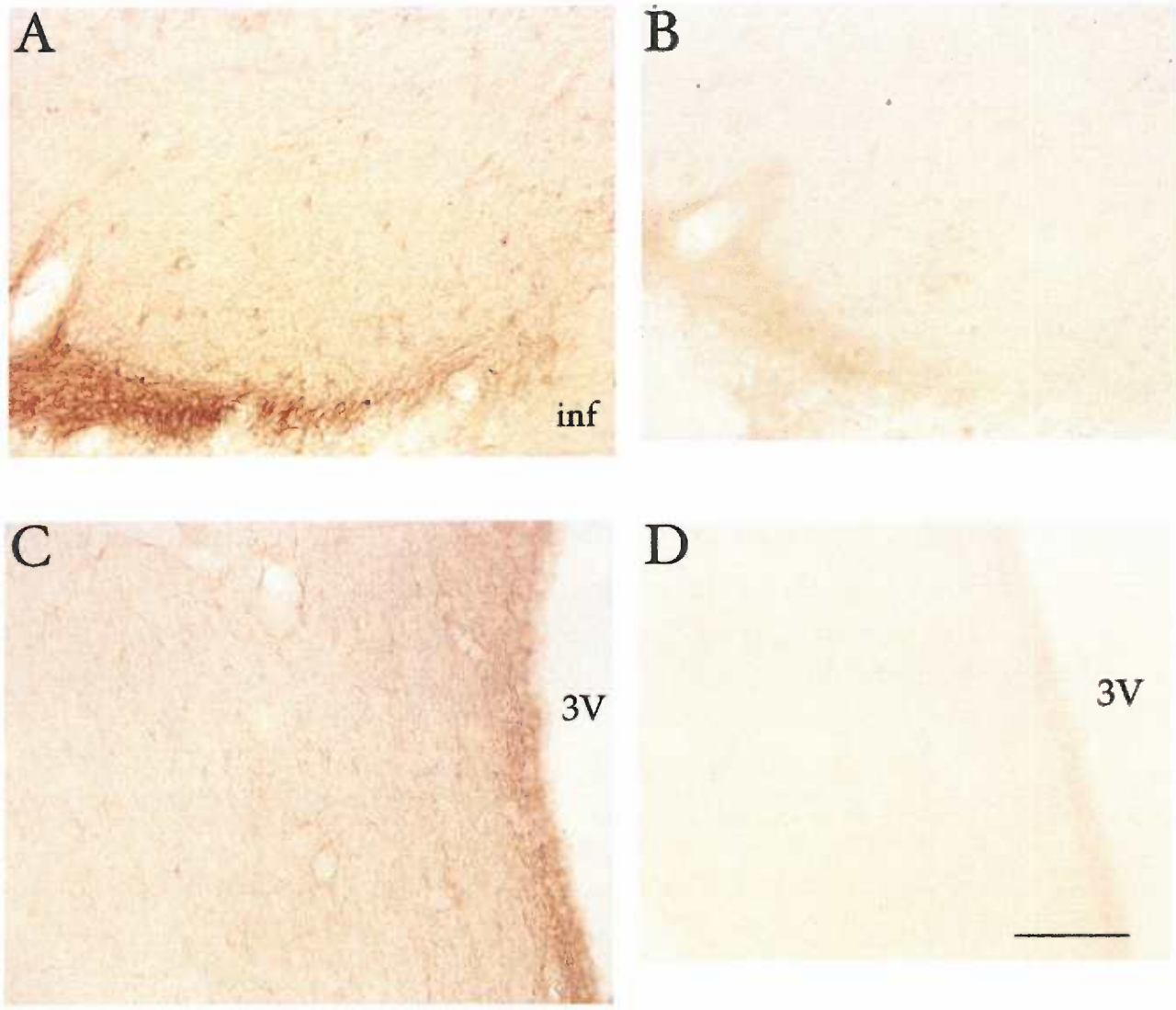

Figure 2-7. Representative photomicrographs showing GnRH-II immunoreactivity in the rhesus macaque hypothalamus. Coronal sections (25 μm) were processed for immunohistochemistry using an antibody to GnRH-II (A, C). Immunostained fibers in the ventromedial hypothalamus (A) presumably originate from the supraoptic nucleus while fibers extending parallel to the third ventricle presumably originate from the paraventricular nucleus. Immunohistochemistry on adjacent sections used the GnRH-II antibody after it had been preabsorbed with purified GnRH-II (B, D). *Scale bars,* 50 μm, 3V=third ventricle, inf=infundibulam.

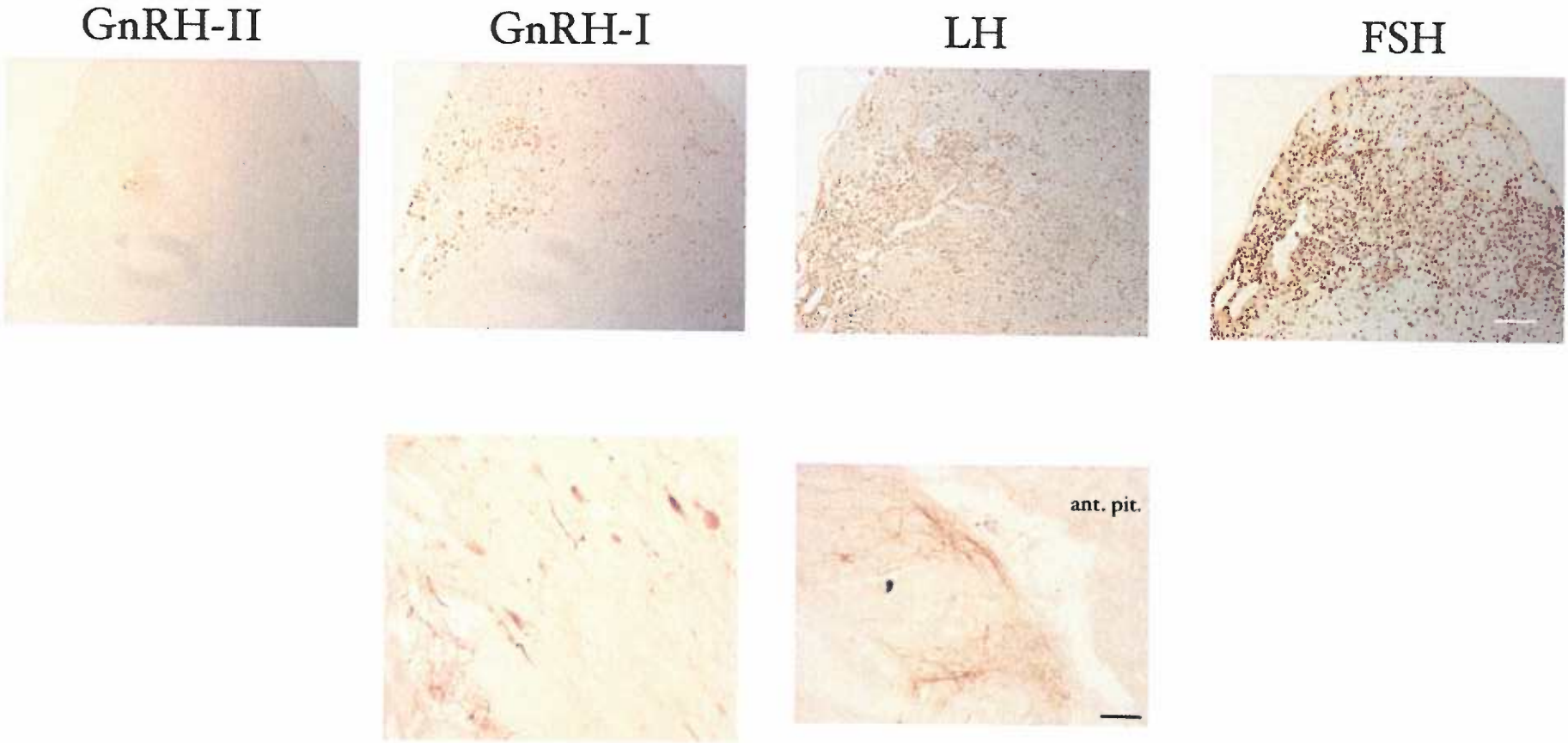

Figure 2-8. Representative photomicrographs showing GnRH-II immunoreactivity in the rhesus macaque pituitary. Axial sections (*upper panels*) were processed for immunohistochemistry using an antibody to GnRH-II, GnRH-I, LH, or FSH. Immunostained cells in the anterior pituitary were observed for each hormone except GnRH-II. Sagittal sections including the posterior pituitary (*lower panels*) revealed fibers immunoreactive for GnRH-II in the pituitary stalk (*left panel*) and near the border between the posterior and anterior pituitary (*right panel*). *Scale bars*, 100 µm. ant. pit.=anterior pituitary.

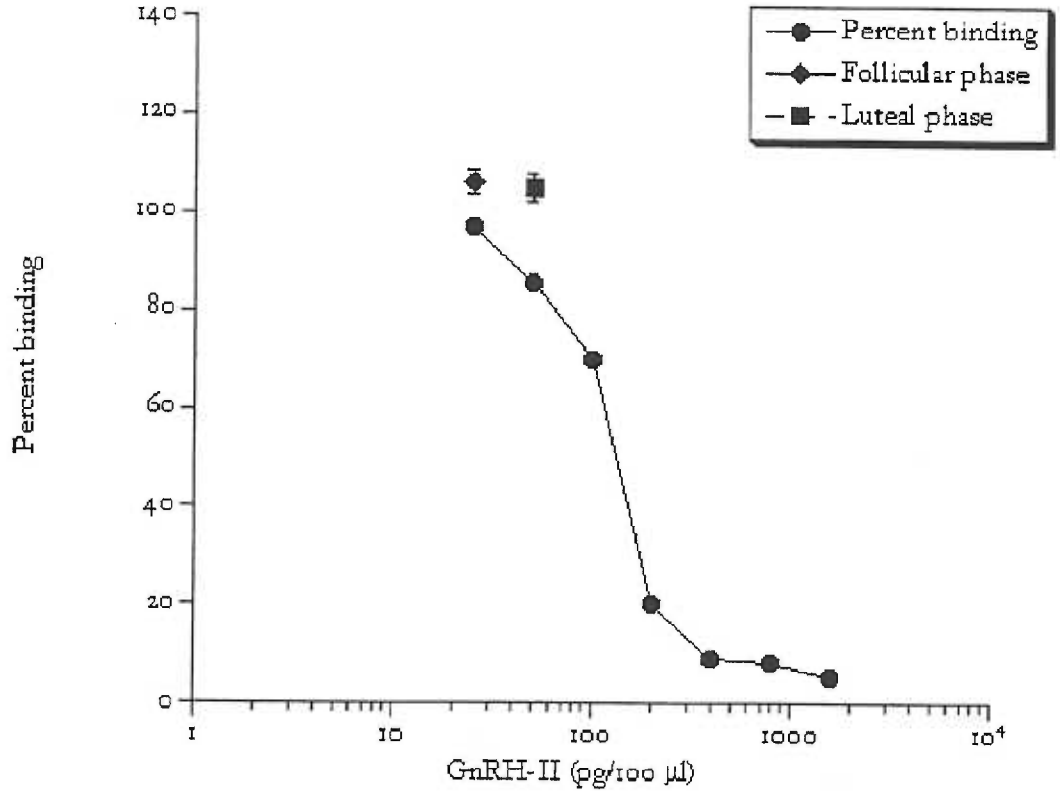

Figure 2-9. Representative graph demonstrating GnRH-II plasma concentrations as determined using radioimmunoassay. Calibration curve shows the ability of cold GnRH-II standards to compete for 125-labeled GnRH-II. GnRH-II levels were not detectable in peripheral plasma from follicular (*diamond*) or luteal (*square*) phase rhesus macaques. Values represent the mean and SEM from 4 animals for each group.

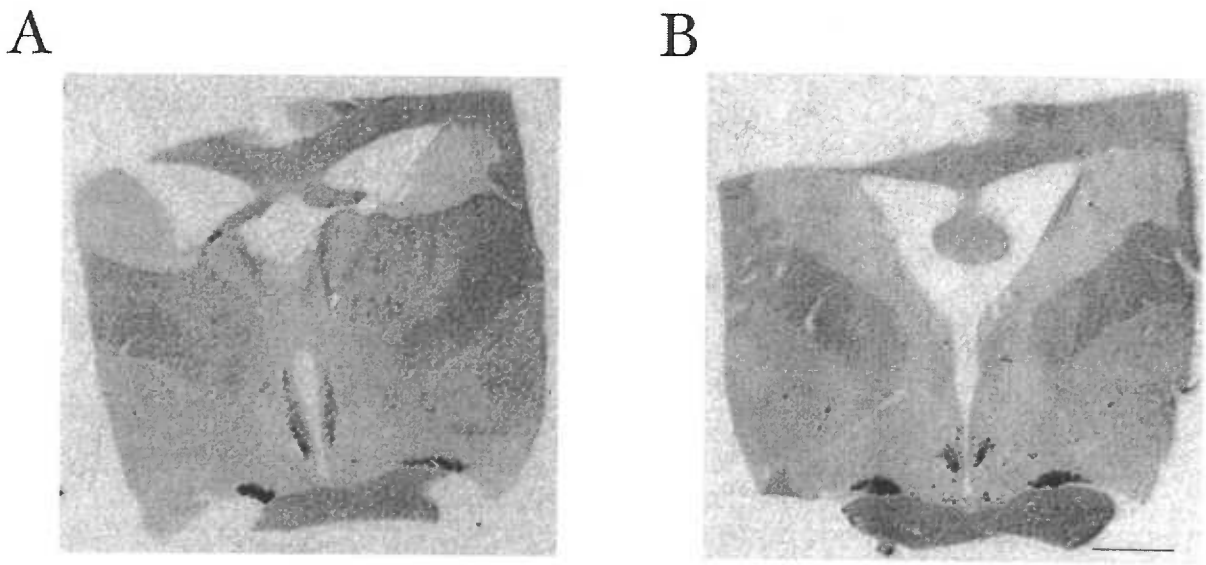

Figure 2-10. Regional expression of GnRH-II mRNA in the rhesus macaque hypothalamus, as revealed by radioisotopic *in situ* hybridization. Autoradiographs depict representative female (A) and male (B) rostral hypothalamic sections. Note that the hybridization is equally intense between males and females. *Scale bar*, 2.5 mm. oc=optic chiasm.

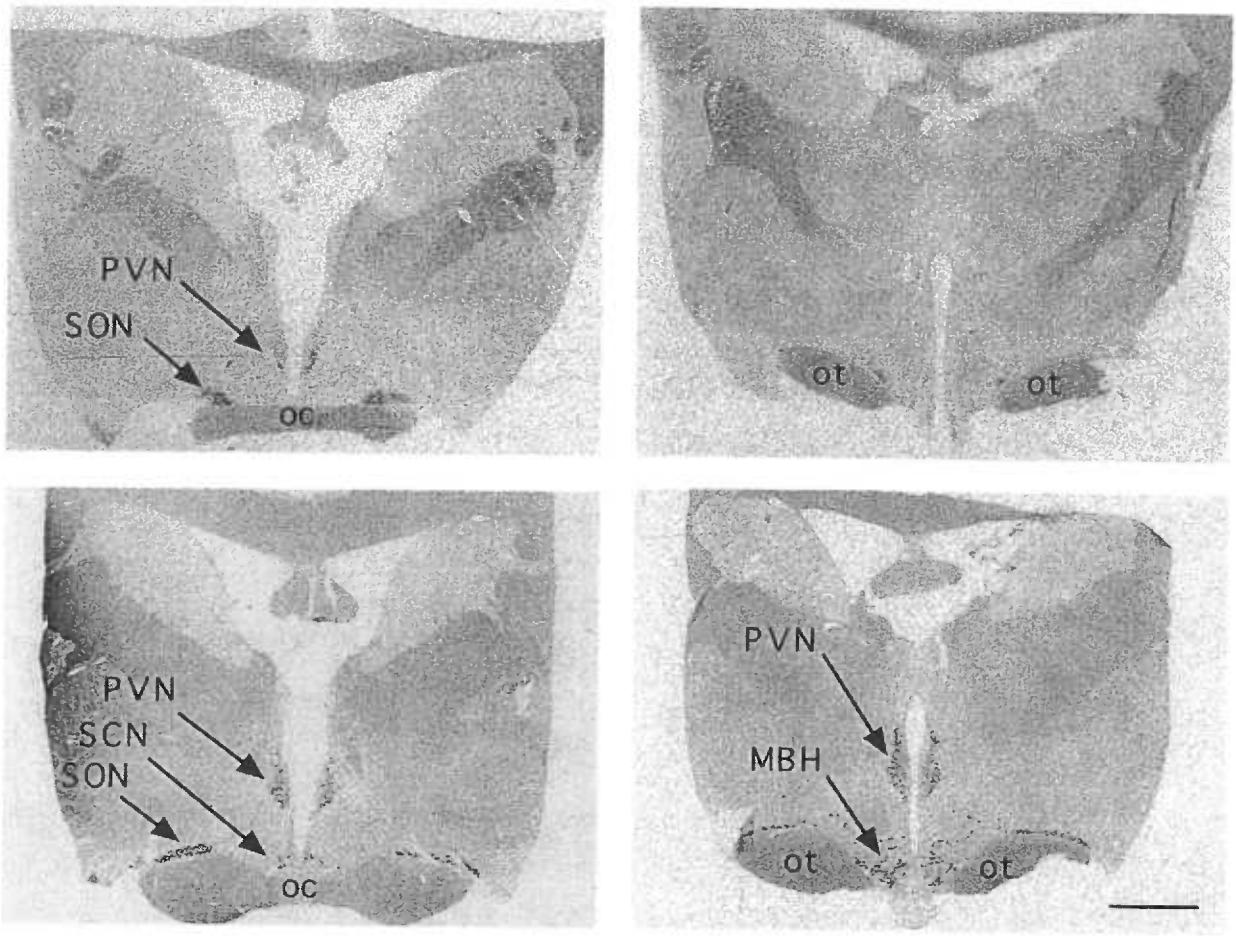

Figure 2-11. Regional expression of GnRH-II mRNA in the rhesus macaque hypothalamus, as revealed by radioisotopic *in situ* hybridization. Representative autoradiographs of rostral and caudal regions of the hypothalamus are depicted in the *left* and *right columns*, respectively. Note the less intense hybridization in the prepubertal animals (*upper panels*) compared with the adults (*lower panels*). *Scale bar*, 3.5 mm. oc=optic chiasm, ot=optic tract.

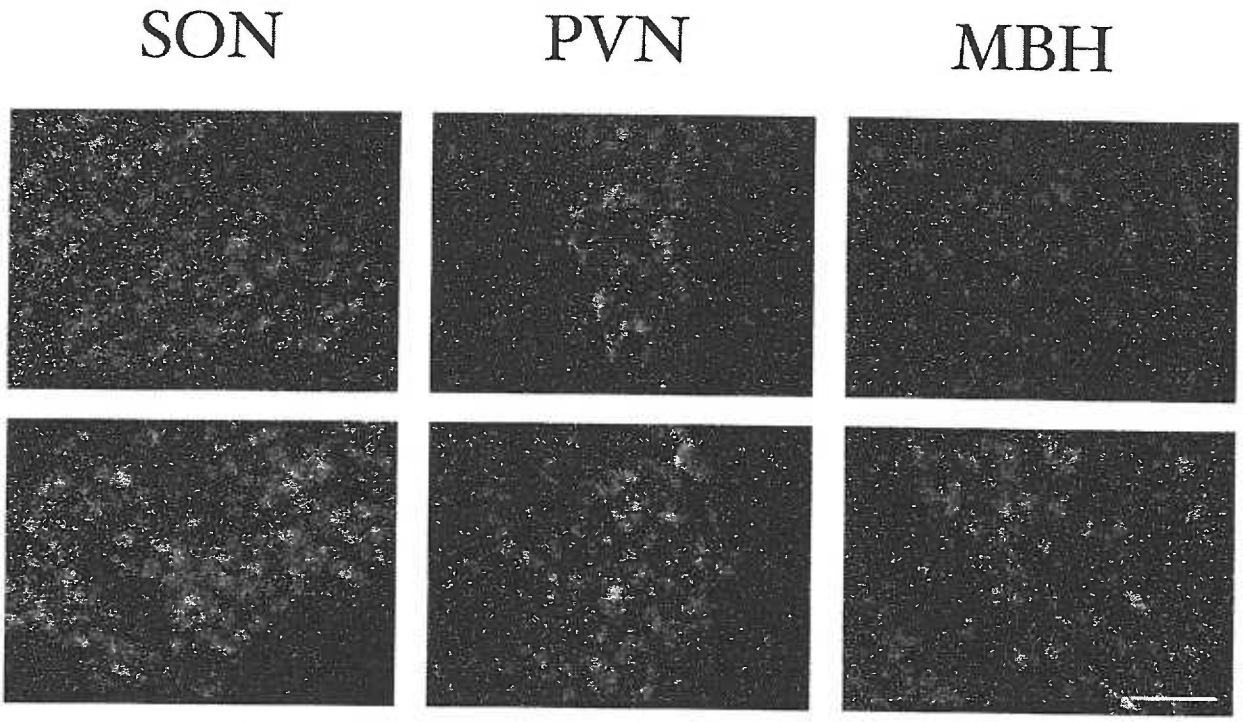

Figure 2-12. Expression of GnRH-II mRNA in the rhesus macaque SON, PVN, and MBH, as revealed by radioisotopic *in situ* hybridization. Representative darkfield photomicrographs from prepubertal and adult animals are shown in the *upper* and *lower panels*, respectively. Note the increased density of silver grains in the adults, especially in the MBH. *Scale bar*, 400 μm.

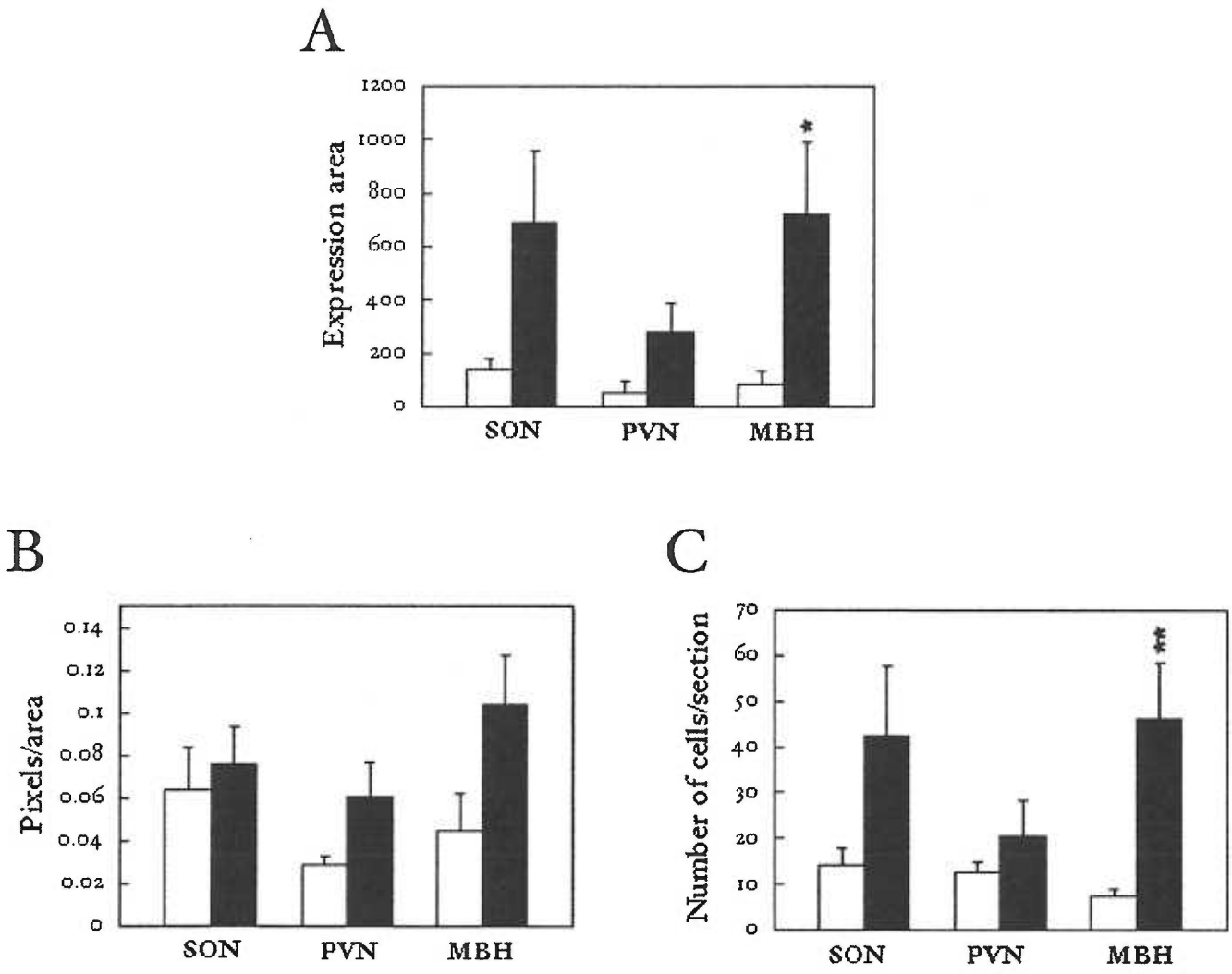

Figure 2-13. Comparison of GnRH-II mRNA expression in the rhesus macaque SON, PVN, and MBH before and after the onset of puberty (*white* and *black bars*, respectively). (A) The total area of expression was determined by optical density analysis of autoradiographs. (B) The mean expression per cell was determined by silver grain density analysis of emulsion-dipped slides. (C) The number of GnRH-II mRNA-expressing cells per section. Each *bar* represents the mean value from six animals (three males and three females), and the SEM is represented by the *vertical lines*. *, $P<0.05$; **, $P<0.001$.

DISCUSSION

For the last 2 decades, it has generally been assumed that only one molecular form of GnRH (i.e., GnRH-I) exists in the primate brain, but recent evidence from HPLC (79), immunohistochemistry (79, 111), and *in situ* hybridization (110, 164) supports the existence of at least one additional form (i.e., GnRH-II). This discovery that a second form of GnRH exists in the primate brain introduces questions concerning the neuroendocrine control of the reproductive system. In particular, the neuroendocrine mechanisms regulating specific events, such as puberty and ovulation, have proven difficult to clearly resolve using current assumptions that only one GnRH form is present. However, little is currently known about the distribution and regulation of GnRH-II in the primate brain.

mRNA distribution

The present results use ISH to corroborate and extend previous findings (79) by showing a high level of GnRH-II mRNA expression in four discrete regions of the primate hypothalamus: the SON, SCN, PVN, and MBH. Counterstaining the sections further revealed that the clustered pattern of GnRH-II expression in the SON and PVN appeared restricted to the magnocellular population. Further caudal, the hybridization observed in the MBH had a more medial to lateral spread, however the overall distribution of the cell bodies appeared continuous. As a result, this expression pattern for GnRH-II was strikingly different from the more diffuse pattern observed for GnRH-I mRNA (165) and suggests the neuroendocrine pathways for the two forms are likely to show some differences. However, there also appeared to be some overlap in ventral hypothalamic regions between GnRH-I and GnRH-II mRNA expression, therefore it is possible that a population of cells uses the two GnRH forms as cofactors.

Distribution of GnRH-II versus GnRH-I

To investigate whether a subpopulation of cells contain both GnRH-I and GnRH-II, over 2000 cells were examined for GnRH-I peptide and GnRH-II mRNA. However, no coexpression was observed, suggesting the individual cells expressed only one of the two molecular forms. It is unlikely that this lack of GnRH-I/GnRH-II double-labeling reflected a limitation of the techniques employed in this study, because the combined IHC/ISH approach that was used was validated by demonstrating that GnRH-I immunolabeled neurons also express GnRH-I mRNA. In addition, GnRH-I-immunolabeled neurons were often observed within the same field of view as GnRH-II-positive cells, but in no instance was colocalization of GnRH-I peptide and GnRH-II mRNA evident. Therefore, because distinct hypothalamic cell populations contain GnRH-I and GnRH-II mRNAs and they show a largely dissimilar distribution pattern, these populations, and thus the GnRH forms, are likely to receive a distinct set of inputs, and to show some differences in their physiological roles.

The pivotal involvement of GnRH-I in the control of the primate reproductive axis is well established, but it is unclear whether GnRH-II can modulate GnRH-I. The pattern of GnRH-II expression observed supports the hypothesis that GnRH-II could function to help regulate GnRH-I secretion or signaling. In particular, it has not been resolved how GnRH-I release is coordinated into either the observed pulses or a preovulatory surge due to the GnRH-I cell bodies being scattered throughout the ventral hypothalamus. However, the distribution of GnRH-II in the MBH or the SON and PVN may allow GnRH-II to coordinate GnRH-I secretion or modulate its signaling.

Peptide distribution

Although GnRH-II occurs in the MBH, it is currently unknown whether GnRH-II neurons project to the median eminence and or even secrete the decapeptide into the hypothalamo-hypophyseal portal blood vessels. Such a humoral route to the pituitary gonadotropes is certainly plausible given the GnRH-II mRNA distribution observed, however the possibility also exists that MBH GnRH-II cells project to the POA in the vicinity of GnRH-I cells (166). Furthermore, IHC analysis demonstrated GnRH-II-immunoreactive fibers in the dorsal region of the caudal hypothalamus, but it is unknown if these fibers originate from the MBH or the SON. Thus, the MBH population potentially allows GnRH-II to be secreted humorally into the hypophysial portal vessels, or to signal to GnRH-I cells in the median eminence or POA.

It is also possible that the posterior lobe of the pituitary gland, or pars nervosa, secretes some GnRH-II directly into the peripheral circulation. Although speculative, this view gains support from the observation that very high levels of GnRH-II mRNA were detected in the SON and PVN, two hypothalamic nuclei that have major projections to the pars nervosa (167, 168). The primary neuropeptides produced by these nuclei include arginine VP and OT, which resemble GnRH-II in many ways. For example, the biosynthesis of each of these peptides involves amidation of the terminal glycine residue at the carboxyl end of the molecule by the donation of an amide group from an adjacent glycine residue. Furthermore, each peptide's precursor contains an associated peptide that is cleaved off at a lysine-arginine processing site (167, 169). Taken together, these peptide similarities emphasize that some of the neurons in the SON and PVN share the same biochemical machinery to produce VP, OT, and GnRH-II. Furthermore, these magnocellular neurons provide the main axonal projections from the SON and PVN to the pars nervosa (168).

The similarities between OT, VP, and GnRH-II peptide processing may also elucidate the unexpected IHC results for GnRH-II. GnRH-II immunoreactivity was seen in nerve fibers and terminals but no somal immunoreactivy was observed in any hypothalamic nucleus. This raised the question whether the IHC produced artifactual results, however, it is also possible that the data reflect how the GnRH-II peptide is processed post-translationally. Both the OT and VP preprohormones encode a second peptide, the neurophysins. Studies originally using the Brattleboro rat, which lacks a functional VP neurophysin, demonstrated that the neurophysins are cleaved off OT or VP during transport down the axon and are furthermore necessary to chaperone the peptides during transport. This became clear because the Brattleboro rat expressed VP mRNA, but the VP peptide was not transported successfully to nerve terminals without the exogenous addition of the VP neurophysin *in vitro*. (170) To construct an antibody specific for GnRH-II likely requires including an epitope located adjacent to the cleavage site between the GnRH-II peptide and the GnRH-associated peptide (GAP). Because post-translational processing has some marked similarities between GnRH-II and OT or VP, it is possible that for the antibody to recognize GnRH-II, the peptide must first be cleaved from its GAP sequence, which may take place during axonal transport, just as occurs for OT and VP. If this were the case, one would expect immunoreactivity would only be detectable within the axons and terminals rather than the cell bodies, which is what was observed following GnRH-II IHC. Moreover, this might explain why IHC detected less hypothalamic GnRH-II than HPLC and RIA in the other vertebrate classes.

However, a population of GnRH-II fibers was observed in the external lamina of the median eminence extending down the pituitary stalk and terminating in the pars nervosa, but it is unknown what the ultimate function could be for circulating GnRH-II. Previous studies have shown that synthetic GnRH-II is a

potent stimulator of gonadotropin release in rhesus macaques *in vivo* (79) Taken together, the findings that GnRH-II mRNA exists in the rhesus hypothalamus and that GnRH-II peptide may reach GnRH-I cell bodies, the portal vessels, and/or the peripheral circulation suggests that more than one molecular form of GnRH may play a physiological role in regulating the release of LH and FSH. Thus, GnRH-I and GnRH-II may coordinately control gonadal steroidogenesis, sperm production, follicular development, ovulation, and/or reproductive development.

GnRH-II secretion into peripheral plasma

Plasma GnRH-II concentrations were not detectable in either follicular- or luteal-phase samples from female rhesus macaques. These data would suggest that the SON and PVN cells that express GnRH-II do not function to help modulate the hpg axis during either the follicular or luteal phase. However, it remains possible that GnRH-II in the SON and PVN may have a reproductive neuroendocrine function during the pre-ovulatory surge at mid-cycle, or GnRH-II may have a specific, rather than constituitive, action on the hpg axis. Alternatively, the SON and PVN populations of GnRH-II may have a non-reproductive neuroendocrine function. In particular, GnRH-II may act as a cofactor with either oxytocin and/or vasopressin, which also occur in magnocellular neurons in the SON and PVN.

Comparison of expression between juvenile and adult

Although the level of GnRH-II mRNA expression in the SON and PVN was similar in immature and adult animals, it showed a marked developmental increase in the MBH. This increase appeared to stem from an increase in the total number of cells expressing GnRH-II mRNA rather than from an increase in the level of expression per cell. Although the literature describing GnRH-II expression during development seems mixed, these results corroborate what has been observed in other vertebrates, namely that hypothalamic GnRH-II increases developmentally

(128). Other existing studies, which find GnRH-II does not show a developmental increase, examine populations of GnRH-II that are all non-hypothalamic but primarily occur in the midbrain (127 - 129). However, these data do not elucidate whether a developmental increase in GnRH-II expression is associated with the central mechanism that triggers the onset of puberty, or whether it simply reflects a consequence of the maturational change in the sex steroid environment.

Possibly the earliest manifestation of pubertal onset in primates is a change in the pulsatile release pattern of LH, which is highlighted by high amplitude nocturnal peaks (173). To date, the underlying cause of this neuroendocrine activation is unclear, although a pubertal change in the pulsatile release pattern of GnRH almost certainly plays a pivotal role (174). In addition, there is evidence to indicate that the pubertal activation of LH secretion following an extended juvenile hiatus is associated with increased glutamatergic stimulation (171, 173) and/or reduced γ-aminobutyric acidergic inhibition (174 - 176) of the GnRH neuronal circuitry. It is possible that developmental changes in the expression of neuropeptide Y within the MBH are also involved (157, 177 - 179). However, because enhanced GnRH release is maintained after puberty, one might expect an underlying increase in GnRH gene expression to also become prominent during prepubertal development. Surprisingly, there is little evidence to support a developmental increase in the expression of GnRH-I mRNA (156, 158) or GnRH-I peptide (159) in the hypothalamus of primates, although a developmental increase in GnRH-I mRNA expression has been observed recently in the MBH of gonadectomized monkeys (157).

The present findings show that a developmental increase in GnRH-II mRNA levels is prominent in the MBH even in gonad-intact males and females, however, previous data suggest GnRH-I is necessary for mammals to undergo puberty. This has been demonstrated both in the hypogonadal mouse (111) and humans with Kallmann's (180) syndrome; in each case, functional GnRH-I is not present in the

hypothalamus, and neither the mice nor the humans undergo puberty until they are treated with exogenous GnRH-I (180). Because GnRH-II immunoreactivity has been demonstrated in the hypogonadal mouse (111), this suggests that GnRH-II in the absence of GnRH-I is not sufficient to trigger puberty. However, existing data do not address whether GnRH-II is necessary for the onset of puberty. Furthermore, the distribution of the GnRH-II mRNA and peptide may allow GnRH-II to signal to GnRH-I cells. Therefore, this observed developmental increase of GnRH-II mRNA may play a permissive role in reproductive development or puberty.

In addition, the adult MBH expressing more GnRH-II than the juvenile MBH is interesting because it indicates that functional subpopulations of GnRH-II exist in the primate hypothalamus. GnRH-II expression in the SON and PVN was not significantly altered during development; these two nuclei vary widely from the remainder of the hypothalamus, because they both send major projections to the posterior pituitary where they release peptides into the peripheral circulation. Furthermore, gap junctions have been both observed in and demonstrated to increase following stimulation of either the SON or PVN (168), which would allow peptides to be coordinately released into the bloodstream, as has been observed for both VP (181) and OT (182). It is possible that circulating GnRH-II from the posterior pituitary could reach the pituitary gonadotropes; this is further supported by studies demonstrating that VP may impact LH release *in vivo* (179 - 181), although endogenous VP may reach the gonadotropes via a direct projection to the median eminence rather than through the peripheral bloodstream (168, 183). Another possibility, however, is that GnRH-II acts on peripheral tissues to have a nonreproductive function. Regardless, these data suggest that the SON and PVN populations utilize a different neuroendocrine pathway than the MBH population of GnRH-II.

Summary

Until recently, only a single molecular form of GnRH was thought to exist in the primate brain, so existing ideas about the neuroendocrine control of reproductive function in humans are still generally based on the involvement of only GnRH-I (183, 184). Therefore, the finding that in rhesus macaques GnRH-II mRNA is highly expressed in the hypothalamus suggests that both molecular forms of GnRH may be involved in the neuroendocrine control of the hpg axis. Furthermore, the marked difference in the distribution pattern of GnRH-I and GnRH-II expressing cells supports the view that these two neuropeptides are, to some extent, regulated differently and that they play different physiological roles. In addition, the GnRH-II distribution supports a histological capacity to modulate GnRH-I. Taken together, these findings raise the possibility that GnRH-II may contribute nontraditionally to modulate reproductive function and/or may play an important nonreproductive neuroendocrine role.

CHAPTER III

Relative Ability of GnRH-II to Modulate Gonadotropin Release

INTRODUCTION

Although 13 known forms of GnRH are believed to have reproductive functionality, it is largely unknown how these different forms interact to modulate the hpg axis in any vertebrate class, and even whether each "gonadotropin-releasing hormone" form functions physiologically to stimulate the release of LH and/or FSH. Although GnRH-II is both the most primitive and conserved form of GnRH phylogenetically, it is also unclear if GnRH-II acts as a gonadotropin-releasing factor and then whether it does so directly or indirectly.

When GnRH-I was first isolated and sequenced, controversy arose regarding the function, and hence the proper name, of the peptide. Bioassays indicated GnRH-I is able to stimulate both LH and FSH *in vitro* (3), and during Schally's isolation of GnRH-I using fractionation, a separate factor that selectively stimulates FSH was not found (5). Although many groups concluded at the time that only one gonadotropin-releasing hormone was sufficient, particularly in mammals, several discrete data indicate otherwise. Foremost among these is that the LH response to GnRH-I stimulation is much more robust than the FSH response. Furthermore, the FSH release pattern, extemporized from measurements of plasma FSH concentrations, appears to be uncoupled at least a third of the time from the measured GnRH-I release pattern (187). In addition, different hypothalamic extracts preferentially stimulate LH or FSH release, based on the region supplying the extract (31, 188, 189), suggesting that LH and FSH release may be separately regulated. Therefore, it is controversial whether GnRH-I is responsible for stimulating both LH and FSH secretion or whether a separate FSH-releasing hormone (FSHRH) exists (1, 2).

Apart from differential LH and FSH release, the pulsatile and surge release patterns of LH also appear to be regulated separately. Namely, estradiol has negative feedback on LH pulsatility but positive feedback on the LH surge (11, 190, 192), and

bolus progesterone attenuates the LH surge but does not affect LH pulsatile release in primates (41, 42), although continuous exposure to progesterone has been shown to decrease LH pulse frequency (43). In addition, the LH "pulse generator" and "surge generator" have distinct hypothalamic locations in the arcuate nucleus and around the suprachiasmatic nucleus, respectively (31, 68, 70).

Despite these dissenting data, it was long held that only one form of GnRH existed in the mammalian brain. As a result, the release patterns of LH and FSH were attributed nearly entirely to GnRH-I. However, based on recent evidence from HPLC (106), immunohistochemistry (80, 111, 112), Northern blots (110), and *in situ* hybridization (111), it is now clear that a second form of GnRH (GnRH-II) also exists in the brain of many mammals, including humans (80) and nonhuman primates (79, 110). Finding GnRH-II in the mammalian hypothalamus raises the question whether it may function as a gonadotropin-releasing factor, and if GnRH-II specifically accounts for any known discrepancies between LH and FSH and GnRH-I release patterns.

Research in representative non-mammalian vertebrates indicates that GnRH-II can be a potent gonadotropin-releasing factor, although it has yet to be determined whether the effect is physiologically relevant. In chickens, GnRH-II is six-fold more potent than chicken GnRH-I or mammalian GnRH-I *in vitro* (131) and approximately seven-fold more potent *in vivo.* (133). However, GnRH-II release is not detectable from the chicken median eminence, although chicken GnRH-I has a four-fold release from the median eminence following an identical stimulation (103). In seabream, GnRH-II is seven- to eight-fold more potent than seabream GnRH and two-fold more potent than salmon GnRH *in vivo*. Despite this, the seabream, which does not have a hypothalamic portal system but releases hypothalamic hormones directly into the pituitary, does not have GnRH-II in the pituitary (139). Worth noting, however, is that goldfish plasma contains a GnRH-binding protein

that is selective for GnRH-II and salmon GnRH, which suggests that GnRH-II may also be released into the peripheral circulation in fish (144). In tree-frogs, hypothalamic GnRH-II levels are lower than GnRH-I levels, but the GnRH-II concentration is higher in the plasma draining the hypothalamus; this may be due to extra-hypothalamic sources of GnRH-II, or GnRH-II may be more resistant to peptide degradation (97). Alternatively, GnRH-II also occurs in bullfrog dorsal root ganglia where it potentiates L-type calcium channels (99), and increases plasma catecholamine concentrations (114). In turtles, GnRH-II is approximately equi-potent to release LH *in vitro* (192), however the turtle median eminence expresses chicken GnRH-I at an eight-fold higher concentration than GnRH-II (100). Taken together, it appears that GnRH-II is a potent gonadotropin-releasing factor in non-mammalian vertebrates, but the GnRH-II distribution may not allow it to be the primary releasing factor. What is unknown are both whether the increased potency of GnRH-II compensates for its decreased presence at the pituitary gonadotropes and how these data relate to a GnRH-II function in mammals.

Work by Millar indicates that the mammalian GnRH-I receptor shows more selectivity for GnRH-I than the less discriminatory GnRH receptors in non-mammals (146); thus, it appeared that GnRH-II would not function directly to stimulate gonadotropin release in mammals. However, a preliminary study in rhesus macaques indicates exogenous GnRH-II does potently stimulate LH release (79). Furthermore, mapping the GnRH-II distribution also revealed that the rhesus hypothalamus expresses GnRH-II in the SON, PVN nuclei and the MBH, which may allow GnRH-II to reach the pituitary gonadotropes and stimulate LH and/or FSH release (110). This is underscored by the recent finding that specific GnRH-II receptors are expressed in the anterior pituitary of several mammalian species (147, 148). Taken together, these findings suggest that GnRH-II may contribute to regulating the primate reproductive axis and may be under separate neuroendocrine

control from GnRH-I. However, while it is known that GnRH-II can stimulate LH release *in vivo* (79), less is known about its potency compared to GnRH-I, or its ability to stimulate FSH release. Moreover, it is unclear whether the gonadotropin-releasing abilities of GnRH-II have physiological relevance.

This chapter, therefore, addresses if GnRH-II is sufficient to stimulate or modulate gonadotropin release *in vivo*, and whether this effect is physiologically relevant in the rhesus macaque.

MATERIALS & METHODS AND RESULTS

Relative ability to stimulate gonadotropin release in vivo

Animals

The Institutional Animal Care and Use Committee (IACUC) at the ONPRC approved this study, and animal care was provided by the ONPRC in accordance with the NIH *Guide for the Care and Use of Laboratory Animals*. For the physiological experiments, seven, regularly cycling female rhesus macaques (*Macaca mulatta*) were used to compare the gonadotropin-releasing ability of GnRH-I and GnRH-II; four of the animals were premenopausal (21-23 yr old) and three were young adults (8-12 yr old). Animals were surgically fitted with indwelling subclavian catheters as previously described (193) Silastic tubing to accommodate intravenous administration of GnRH and remote blood sampling. The catheter was extemporized around the mid-scapular region of the back where it was connected to PV-6 (Bolman Co; McKeesport, PA) tubing that ran to a blind sampling room next door. Animals were then fitted with a vest-and-tether system (Fig. 3-1), which protected the catheter while allowing each animal full range of movement within its cage. PVC tubing connected to a pump that infused heparinized saline (5 units/ml vol/vol) at a flow rate of 1 ml/hour. An attached three-way stopcock allowed infusions and blood sampling to occur, and this setup allowed the experiment to be performed without disturbing the animals.

Animals were allowed at least one month to recover from the surgery and become accustomed to the vest-and-tether before the experiments were performed.

Remote treatment and sampling

Luteal phase

Each of the seven animals was determined to be in their mid-luteal phase (days 18-24 of a ~28-day cycle) based on detailed menstruation records and by analyzing blood samples for estradiol and progesterone concentrations. They were given a bolus intravenous (iv) injection of saline, as negative control, or GnRH-I or GnRH-II at four doses, 0.01 µg, 0.1 µg, 1 µg, or 10 µg/kg body weight. Blood samples (0.5 ml) were collected in glass tubes coated with EDTA (50 µl of a 10% wt/vol; Ricca Chemical, Arlington, TX) at –10, 0, 10, 20, 30, 60, and 120 min following administration and centrifuged; the plasma supernatant was removed and stored at –20°C until assay for LH and FSH.

Follicular phase

Each of the seven animals was determined to be in their mid-follicular phase (days 4-10 of a ~28-day cycle) based on detailed menstruation records and by analyzing blood samples for estradiol and progesterone concentrations. They were given a bolus iv injection of saline, as negative control, or GnRH-I or GnRH-II at four doses, 0.01 µg, 0.1 µg, 1 µg, or 10 µg/kg body weight. Blood samples (0.5 ml) were collected, as before, at –10, 0, 10, 20, 30, 60, and 120 min following administration and centrifuged; the plasma supernatant was removed and stored at –20°C until assay, except samples were only assayed for FSH concentration to determine if the FSH response was more sensitive during the follicular, rather than luteal, phase.

Hormone assay

Samples were assayed for LH using a previously described mouse Leydig cell bioassay involving radioimmunoassay for testosterone (9); results are expressed in terms of a cynomolgus LH-RP1 standard (195) FSH was measured by radioimmunoassay using an anti-recombinant monkey FSH (NIDDK Lot # AFP782594) antibody; results are expressed in terms of the recombinant monkey FSH-RP1 (NIDDK Lot # AFP6940A) standard (19, 20).

Analytical methods

GnRH administrations were randomized and spaced 24-48 hours apart to prevent an interaction occurring between separate treatments. Basal LH and FSH concentrations were determined by averaging the values obtained at –10 and 0 minutes prior to GnRH treatment; these basal concentrations were then subtracted from experimental values to obtain the net LH and FSH concentrations at each time point. Net LH and FSH values over time for each GnRH dose were analyzed using Systat software (version 10) and two factor repeated measures analysis of variance (ANOVA), the two factors being time and treatment. The Huynh-Feldt (H-F) factor was used in lieu of the *P* value to minimize error due to non-uniform variance-covariance matrices (196). In addition, data were analyzed across the two age groups using two-factor ANOVA, and the *P* value was used to indicate any statistical significance.

Results

The responses of LH and FSH to a bolus dose of GnRH-II are shown in Figs. 3-2 and 3-3 The three highest doses of GnRH-II, 100-ng/kg, 1-μg/kg and 10-μg/kg, each produced a cumulative LH release that was significantly greater than both the lowest dose and vehicle treatment but not significantly different from one another. The time-course of release also appeared very similar between the three effective

doses; for the 1-μg/kg dose, the maximum response occurred approximately 20 minutes following treatment and then circulating LH levels fell gradually to baseline levels by 120 minutes. At 100 ng/kg and 10 μg/kg, which appeared less potent, LH levels rose to maximum levels by 10 minutes and remained high through 20 minutes before beginning to fall. The 10-ng/kg dose stimulated an LH release pattern similar to the higher GnRH-II doses, but the total LH release was not significantly different from vehicle alone. No treatment stimulated a significant amount of FSH release following a bolus injection during the follicular phase (Fig. 3-3) or the luteal phase (not shown); this was true for both GnRH-II and GnRH-I.

The LH response to GnRH-I treatment was very similar to that seen for GnRH-II (Fig. 3-2). Circulating LH levels peaked by approximately 10 minutes after each dose and then declined gradually, although the 1-μg dose of GnRH-I had not yet reached baseline concentrations by 120 minutes. In this aspect, the optimal GnRH-I dose, 1 μg/kg, displayed a faster time-course than that for GnRH-II to reach maximum stimulation, however the effect was not significant. However, all four doses of GnRH-I produced a significant increase in LH, although the lowest dose was significantly less effective than all other doses, which were not statistically different from one another. All in all, GnRH-I did not release significantly more LH than GnRH-II at any dose.

The ability of age to influence gonadotropin response was also analyzed for both GnRH-I and GnRH-II. Three of the seven rhesus monkeys used were young adults (8-12 yr old), and the remaining four were old but still premenopausal (21-23 yr old). Fig. 3-4 shows the responses of the two age groups to GnRH-I and GnRH-II treatment. Two-factor repeated measures ANOVA was performed using age as a grouping factor and suggests that the older animals were less sensitive to both

GnRH-I and GnRH-II (Fig. 3-4) treatment than the young animals, irrespective of dose ($P<0.05$).

Site of action

Animals

For the *in vitro* experiments, pituitaries were obtained from regularly cycling female rhesus monkeys that were euthanized as part of the ONPRC Tissue Distribution Program to provide tissue for this and other studies.

Pituitary cell dispersion

Four female rhesus were deeply anesthetized using ketamine/pentobarbital, according to procedures established by the Panel on Euthanasia of the American Veterinary Society, and fresh pituitaries were obtained. The anterior pituitary gland was separated from the posterior pituitary gland, and the cells were dispersed as previously described (15). Briefly, the tissue was incubated in 0.3% type IV collagenase (weight/vol, Sigma) and 0.1% DNase (weight/vol, Sigma) in calcium- and magnesium-free Hanks' balanced salt solution (HBSS; Gibco, Carlsbad, CA) at 37°C for one hour, centrifuged at 3000 rpm for five minutes, and then washed twice in Hanks BSS followed by centrifugation as before. Next, the tissue was incubated in 0.15% pancreatin (weight/vol, Sigma) and 0.1% DNase in HBSS at 37°C for 30 minutes, and centrifuged and washed as before. It was then triturated in 0.2% DNase in HBSS using a sterilized Pasteur pipette, centrifuged and washed, and resuspended in 1 ml DMEM/F12 (Sigma) containing 10% fetal calf serum (Sigma). The dispersed cells were counted using a histocytometer, transferred to coated 48-well tissue culture plates (Fisher Scientific) at 1.5×10^5 cells/well, and incubated at 37°Cwith 5% CO_2 for 48 hours to allow them to adhere to the plate.

Treatment *in vitro*

Plated cells were washed in serum-free DMEM/F12 media for first 60 min and then 5 min prior to treatment. GnRH-I and GnRH-II were separately diluted to doses ranging from 10^{-11} to 10^{-8} moles/well in serum-free media. Cells were then incubated with GnRH-I, GnRH-II, or serum-free media for two hours at 37°C. Following incubation, culture media was collected, centrifuged to remove any cells, and stored at -20°C until assay for LH and FSH concentration. Following media collection, cells were exposed to trypan blue and an average of 95% were able to exclude the dye, indicating their viability.

Analytical methods

Basal LH levels were obtained using media treatment alone and subtracted from experimental values to determine net LH release. In addition, experiments were performed in duplicate or triplicate for each pituitary, therefore the mean was calculated for each pituitary to determine experimental values. The net LH release for each pituitary was then analyzed using Systat software and two-way ANOVA, and the *P* value was used to indicate any statistical significance.

Results

It is possible that exogenous GnRH-II acts upstream of the pituitary and stimulates GnRH-I release rather than LH release directly. Therefore, the ability of GnRH-II to stimulate LH and FSH release from freshly dispersed pituitary cells *in vitro* was examined to determine whether the *in vivo* GnRH-II effect occurred upstream of the pituitary or could indeed be mediated directly at the gonadotrope level. To test this, GnRH-II was administered *in vitro* to primary cultures of dispersed anterior pituitary cells. Following a two-hour static incubation in media containing GnRH-I or GnRH-II at doses ranging between 10^{-11} and 10^{-8} M, the media was removed and assayed for LH and FSH concentrations. GnRH-I is known

to directly stimulate the pituitary gonadotropes to release LH, so it was used comparatively to assess the relative ability of GnRH-II to act at the pituitary level. As shown in Fig. 3-5, GnRH-II stimulated LH and FSH release *in vitro* with similar potency to GnRH-I.

Receptor signaling

To determine whether GnRH-II required the GnRH-I receptor to stimulate LH release, animals were treated with Antide® (Bachem; Torrance, CA), a potent GnRH-I receptor antagonist (196), either alone or in combination with GnRH-I and GnRH-II during the mid-luteal phase, determined as described in Experiment 1. GnRH forms were administered iv at a 1-μg/kg body weight dose and Antide was administered iv at 100 μg/kg body weight. Blood samples were collected, processed, stored, assayed for LH, and analyzed as described in Experiment 1.

Results

Recently, a second GnRH receptor was discovered in mammals with a high selectivity for GnRH-II over GnRH-I. This provoked the question whether the ability of GnRH-II to release LH is mediated by its own receptor or by cross-reactivity at the GnRH-I receptor. Therefore, a 100-μg/kg dose of Antide, a potent GnRH-I receptor inhibitor, was administered with GnRH-I or GnRH-II at 1 μg/kg or alone as a negative control. Administered with GnRH-I, Antide attenuated the cumulative LH release but did not affect the time-course of the response (Fig. 3-6). This treatment paradigm did not completely inhibit GnRH-I-stimulated LH release, likely due to the relative dissociation constants of Antide and GnRH-I. However, when administered with GnRH-II, Antide was able to completely block both the time-course and cumulative LH response (Fig. 3-6).

Modulatory effects

To determine the interaction of GnRH-I and GnRH-II, each animal received four different GnRH treatments: GnRH-I and GnRH-II both at 0.1 μg/kg body weight, GnRH-I at 0.1 μg and GnRH-II at 1 μg/kg body weight, GnRH-I at 1 μg and GnRH-II at 0.1 μg/kg body weight, and GnRH-I and GnRH-II both at 1 μg/kg body weight. All treatments were administered iv as a bolus injection during the mid-luteal phase, and blood samples were collected, processed, and stored as described in Experiment 1.

Results

Because exogenous GnRH-II was able to stimulate LH release through pituitary GnRH-I receptors and a population of GnRH-II containing cells occurs in the primate medial basal hypothalamus (MBH), it is possible that GnRH-II is released into the portal vasculature and may modulate the pituitary gonadotropes' response to GnRH-I stimulation. Therefore, GnRH-I and GnRH-II were administered concomitantly at two different doses to determine if GnRH-II exposure would potentiate or attenuate GnRH-I-stimulated LH release or if the time-course of LH secretion would be altered by exposure to GnRH-II. Because, the 1-μg dose appeared most effective for both forms, 1 μg was used as the optimal dose and 0.1 μg as the suboptimal dose for both forms.

As indicated in Fig. 3-7, the most effective treatments included GnRH-I at the optimal, 1-μg, dose. Therefore, GnRH-I at 1 μg and GnRH-II at 0.1 μg was more effective than GnRH-I at 0.1 μg and GnRH-II at 1 μg. However, combining both GnRH forms at optimal doses did not produce a statistically additive effect over either GnRH-I or GnRH-II alone at the 1-μg dose. Likewise, combining GnRH-II treatment with GnRH-I did not produce a subtractive effect from either form alone.

In addition, the time-course of LH secretion more closely resembled that seen with GnRH-I.

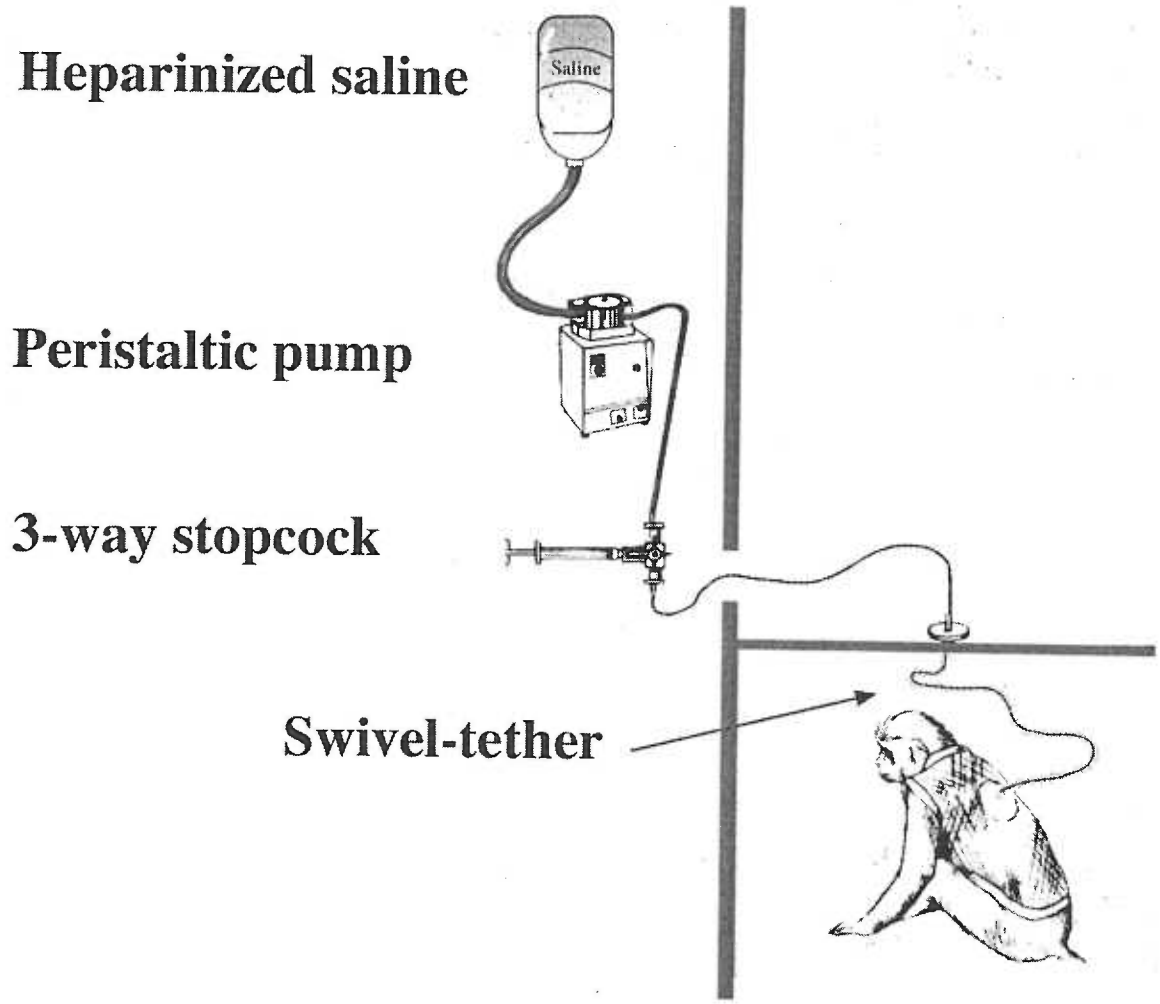

Figure 3-1. Diagram of a procedure that enables frequent blood samples to be remotely collected from conscious, undisturbed monkeys. Animals are surgically fitted with an indwelling vascular catheter that is exteriorized in the mid-scapula region of the back and the distal end connected via a swivel assembly to a sampling/ infusion port located in an adjacent room. An attached peristaltic pump is used to maintain catheter patency by continuously infusing a heparinized saline solution.

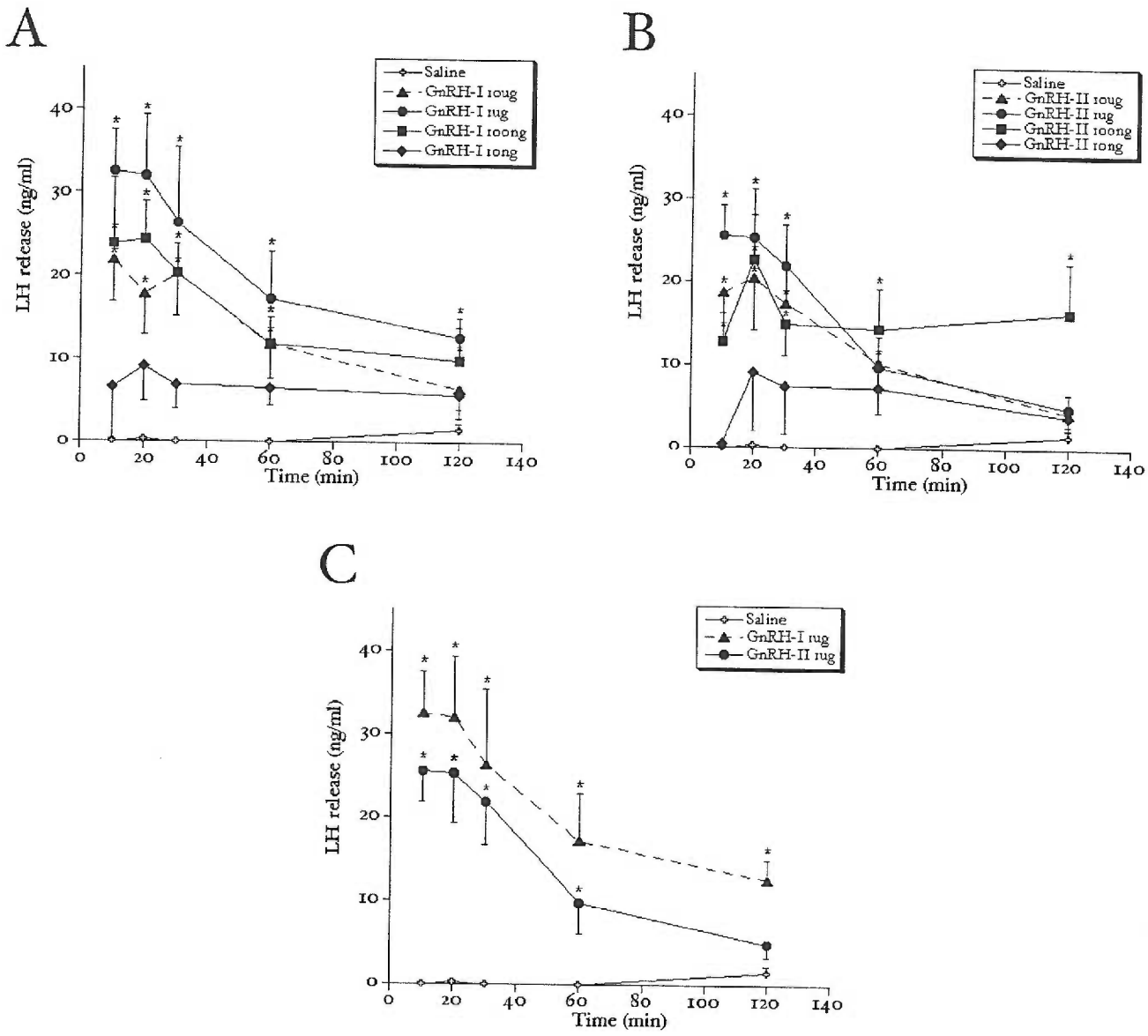

Figure 3-2. Comparison of the LH-releasing abilities of various doses of GnRH-I and GnRH-II in the rhesus monkey *in vivo*. Mean increase of plasma LH concentrations above baseline levels following iv administration at time 0 of saline or GnRH-I (A) or GnRH-II (B) at four doses. C, Comparison of the optimal dose, 1 μg/kg body weight, of GnRH-I and GnRH-II to stimulate LH release *in vivo*. Each *data point* represent the mean and SEM of seven animals. *, H-F<0.05 compared to saline treatment.

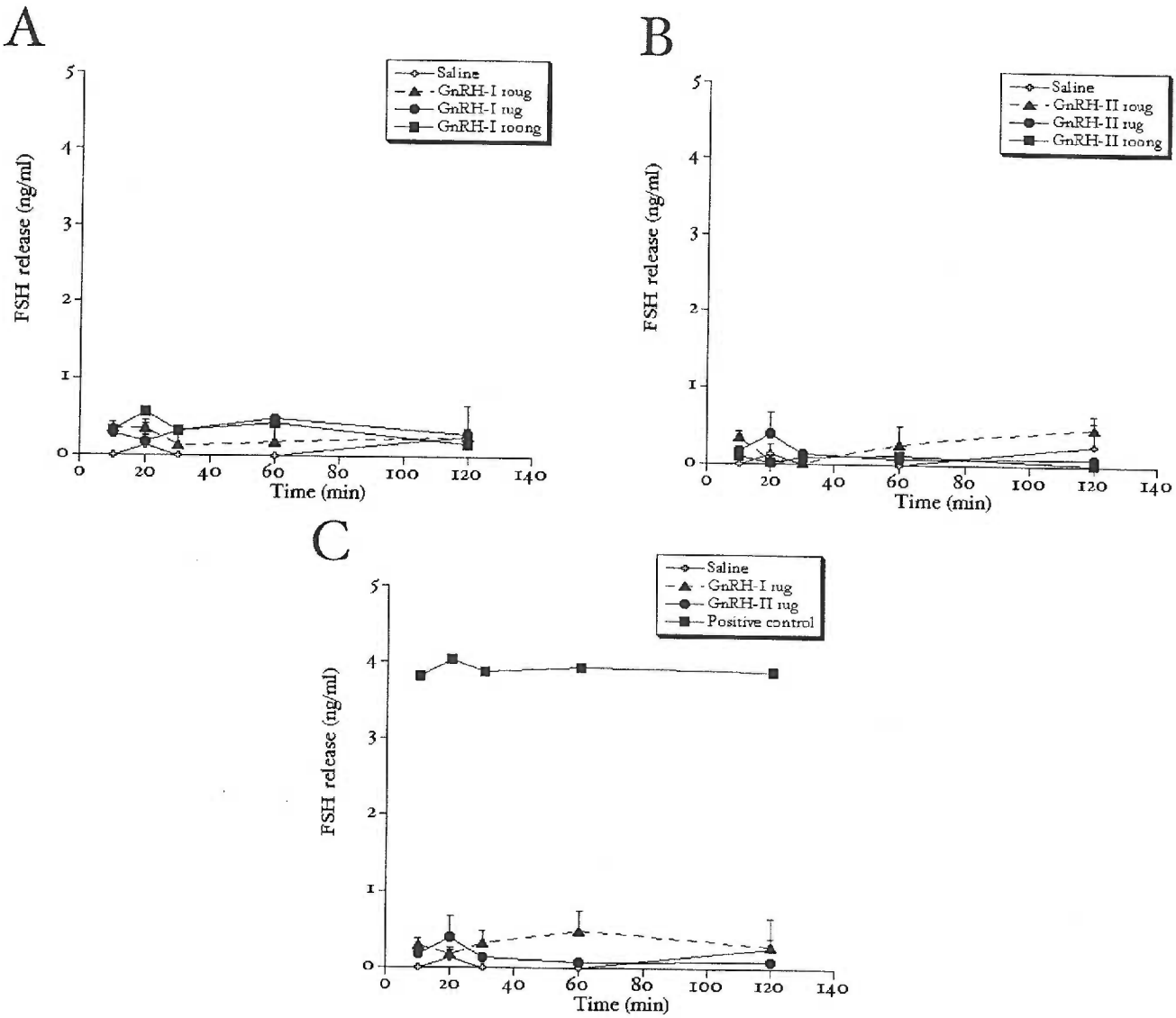

Figure 3-3. Comparison of the FSH-releasing abilities of various doses of GnRH-I and GnRH-II in the rhesus monkey *in vivo*. Mean increase of plasma FSH concentrations above baseline levels following administration at time 0 of saline or GnRH-I (A) or GnRH-II (B) at four doses. C, Comparison of the 1 μg/kg body weight dose of GnRH-I and GnRH-II to stimulate FSH release *in vivo*. Absolute values of circulating FSH from a post-menopausal monkey are also shown as a marker of expected FSH concentrations. Data points represent the mean and SEM of seven animals. No significant increase in plasma FSH observed after treatment with either GnRH-I or GnRH-II, regardless of dose.

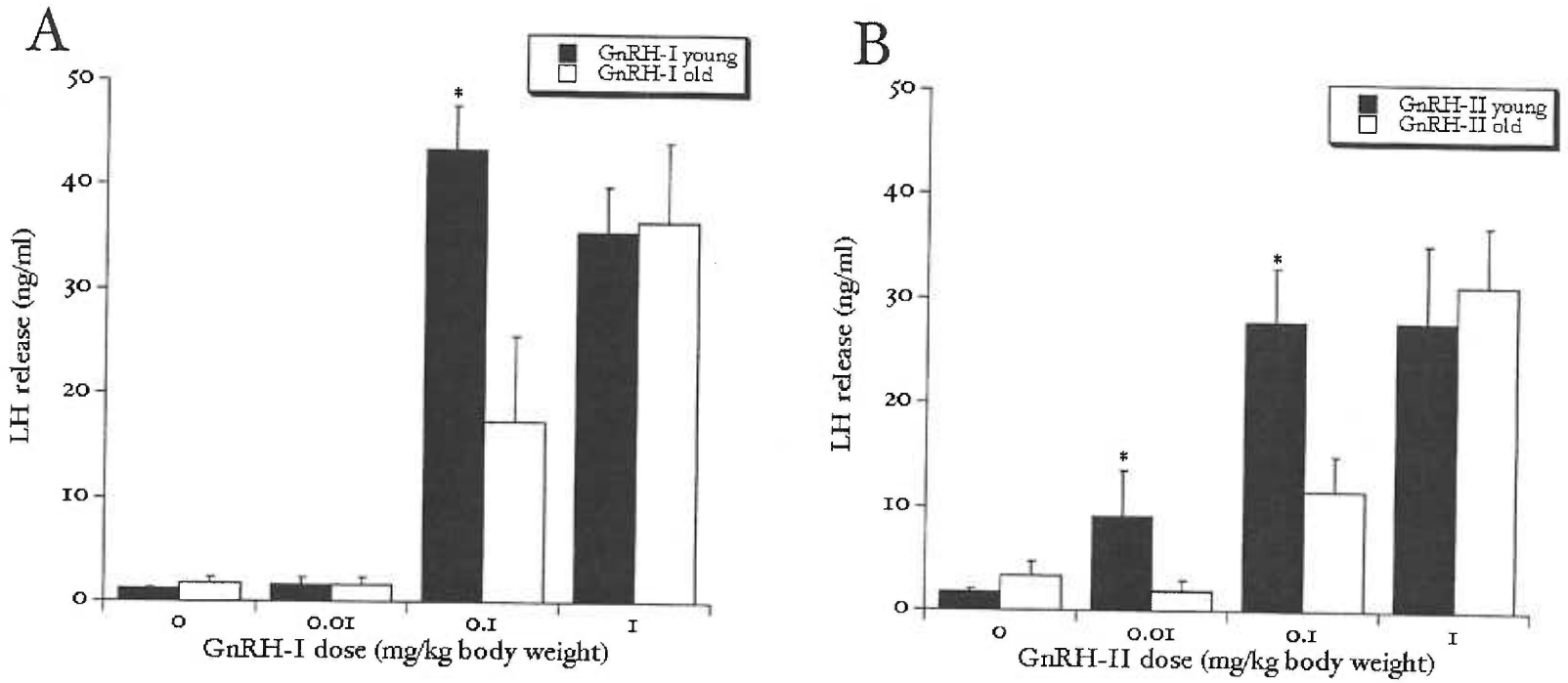

Figure 3-4. Comparison of the net LH release (ng/ml) between young (8-12 yrs) and old (21-23 yrs) rhesus monkeys at ten minutes following iv administration of three doses of GnRH-I (A) or GnRH-II (B). Each *bar* represents the mean value from three (young) or four (old) animals and the SEM are represented by the vertical lines. *, $P<0.05$ compared to old animals.

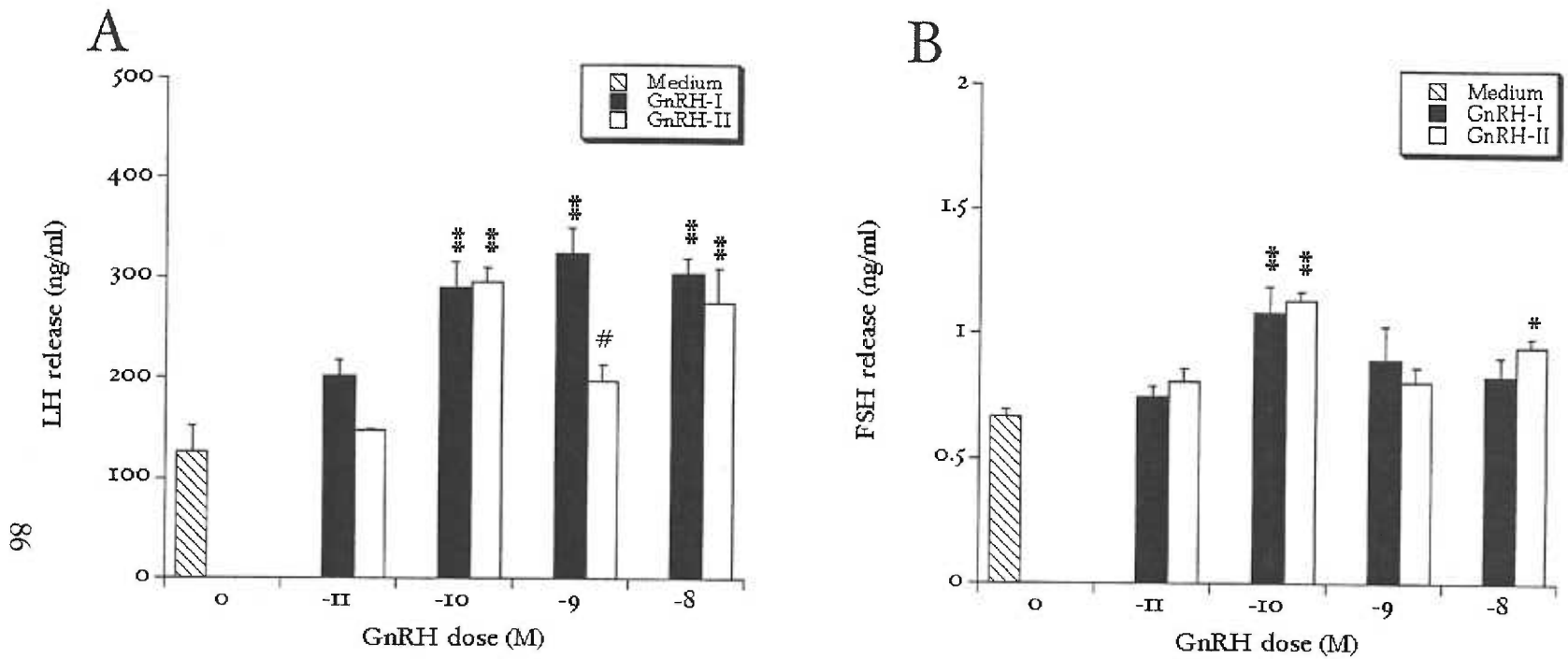

Figure 3-5. Comparison of the *in vitro* gonadotropin-releasing abilities of various doses of GnRH-I and GnRH-II on primary cultures of rhesus monkey anterior pituitary cells. A, Representative histogram of total LH output following two-hour static incubations of media and GnRH-I or GnRH-II at four doses. B, Representative histogram of total FSH output following two-hour static incubations of media alone and GnRH-I or GnRH-II at four doses. Each dose was tested in duplicate or triplicate per pituitary culture, and each experiment was repeated in four pituitaries. Each *bar* represents the mean and SEM from one representative pituitary. *, $P<0.05$ compared to media; **, $P<0.01$ compared to media; #, $P<0.05$ compared to GnRH-I

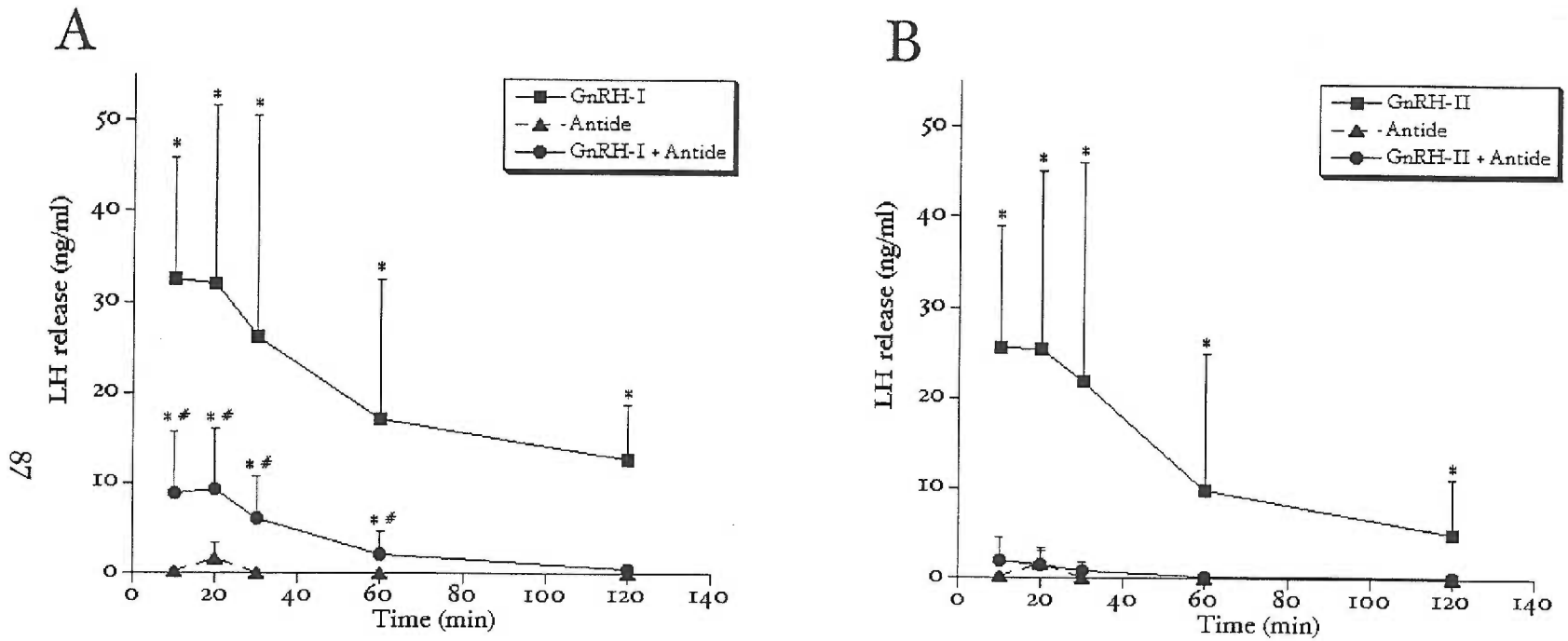

Figure 3-6. Net increase of plasma LH concentrations following treatment with GnRH-I (A) or GnRH-II (B) combined with the potent GnRH-I receptor antagonist, Antide. Values represent the mean and SEM of seven animals. *, P<0.05 compared to Antide alone. #, P<0.05 compared to GnRH alone.

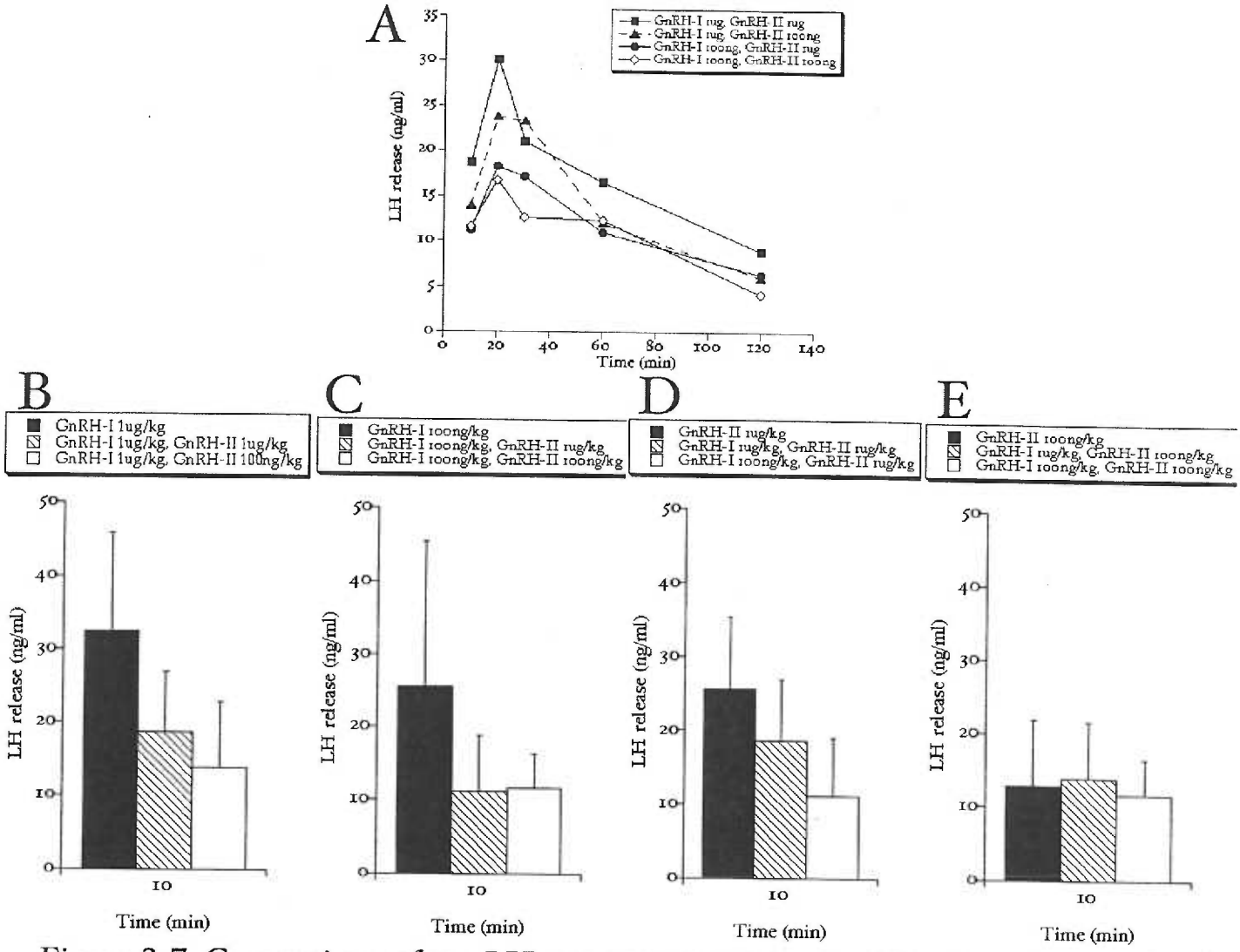

Figure 3-7. Comparison of net LH concentrations *in vivo* following the combined treatment of GnRH-I and GnRH-II at two different doses, 100 ng/kg and 1 μg/kg body weight. A, Time-course of net LH response to all four possible combinations of GnRH-I and GnRH-II. B, Comparison at ten minutes following treatment with GnRH-I at 1 μg/kg either alone or combined with either dose of GnRH-II. C, Comparison at ten minutes following treatment with GnRH-I at 100 ng/kg either alone or combined with either dose of GnRH-II. D, Comparison at ten minutes following treatment with GnRH-II at 1 μg/kg either alone or combined with either dose of GnRH-I. E, Comparison at ten minutes following treatment with GnRH-II at 100 ng/kg either alone or combined with either dose of GnRH-I. Each *bar* represents the mean and SEM of seven animals. No statistically significant differences in plasma LH concentrations were observed after treatment with the various combinations of GnRH.

DISCUSSION

The gonadotropin-releasing hormone family is literally defined by its members' abilities to stimulate gonadotropin release, however whether each individual form has this physiological function has not been determined. The recent discovery of a second form of GnRH (GnRH-II) in the human and non-human primate, however, raises the possibility that control of gonadotropin secretion may be coordinated between GnRH-I and GnRH-II. This idea is certainly plausible given that GnRH-II is the most primitive form of GnRH, and phylogenetic studies have demonstrated it is a potent gonadotropin-releasing factor in nonmammalian vertebrates (133, 139, 192). In many vertebrates, however, the distribution pattern of GnRH-II counters this ability to stimulate LH release. Furthermore, whether GnRH-II might physiologically function to stimulate gonadotropin release hasn't been examined in much detail for any species. In rhesus macaques, the GnRH-II distribution may allow the peptide to reach the gonadotropes, either by diffusion from the posterior pituitary, or more directly if the MBH cell population releases GnRH-II into the portal vasculature. In addition, preliminary work suggests GnRH-II can potently stimulate LH release *in vivo* (79), but the question remains, "Does GnRH-II function as a gonadotropin-releasing hormone?"

Relative ability to stimulate gonadotropin release in vivo

GnRH-II and GnRH-I stimulated an equivalent LH response when they were administered intravenously at three different bolus doses to adult, luteal-phase rhesus monkeys. Because a previous study reported that the mammalian GnRH-I receptor had a ten-fold higher affinity for GnRH-I compared to GnRH-II (146), these results suggested that GnRH-II did not act directly at the GnRH-I receptor to stimulate LH secretion. However, two alternate explanations were that GnRH-II might stimulate nerve terminals in the median eminence to release GnRH-I into the

portal vasculature or that GnRH-II might be acting through the recently discovered GnRH-II receptor (147, 148), which was shown to be colocalized with LH in pituitary gonadotropes (148).

In addition, the time-course of response to GnRH-II appeared slower than that for GnRH-I. Namely, LH release peaked at 10 minutes following GnRH-I treatment but did not peak until 20 minutes following lower doses of GnRH-II. Although this difference was not statistically significant, this apparent delay may imply at the mechanisms by which the GnRH forms signal. For example, the GnRH-II receptor has a cytoplasmic tail (147), unlike the GnRH-I receptor, so perhaps this slows signal transduction. Or perhaps the substitution of histidine for glycine at position 5 in GnRH-II hinders proteolytic degradation by alpha-chymotrypsin (145), giving GnRH-II a longer circulating half-life when it could transduce its effects. Alternatively, GnRH-II could be stimulating GnRH-I release upstream of LH, which might postpone the LH response. Or perhaps GnRH-II binds the GnRH-I receptor, and its lower affinity causes an apparent delay in stimulating LH release. To sort through these possibilities, the obvious questions became whether GnRH-II acts directly on the pituitary gonadotropes and which receptor subtype mediates the GnRH-II effect.

In contrast to the LH response, neither GnRH-I nor GnRH-II stimulated FSH secretion *in vivo*. However, a few factors complicate the interpretation of these results. Among them, it is questionable whether the existing assay is entirely reliable to accurately detect FSH (149). Although the assay is reproduceable with a standard curve, multiple isoforms of FSH are known to exist (150). Furthermore, the secretion pattern of FSH is somewhat unclear because baseline concentrations of FSH are markedly elevated compared to LH, but it is unclear if this is due to a tonic release pattern, a longer circulating half-life,or another unknown factor. As a result, determining the FSH response is, at best, a relative measurement. However, a recent

study in sheep demonstrated that administering GnRH-I or GnRH-II every two hours for 24 hours stimulated FSH release *in vivo* (151). In addition, the present study showed GnRH-I and GnRH-II were equally effective to stimulate FSH secretion *in vitro*. Taken together, this suggests that the treatment paradigm sufficient to stimulate LH release was not optimal to produce an FSH response, but either or both forms may still be responsible to stimulate FSH release. Alternatively, FSH may be regulated by a different neuroendocrine factor, or the pattern of FSH release may largely be determined by the pituitary gland itself (197).Although the present data fail to distinguish either form as a definitive FSHRH, they do support the theory that LH and FSH are not controlled by identical mechanisms and/or factors.

Site of action

The distribution of GnRH-II cells in the hypothalamus might allow the peptide to modulate LH release by acting at the level of GnRH-I cell bodies or nerve terminals, or the pituitary gonadotropes. To ascertain GnRH-II's site-of-action, it was prudent to initially determine if GnRH-II could stimulate LH release directly from the pituitary. Following static incubation of freshly-dispersed anterior pituitary cells with GnRH-II, LH concentrations were significantly elevated compared to treatment with medium alone. Furthermore, the LH response to different doses of either GnRH-I or GnRH-II was similar, indicating that exogenous GnRH-II acted directly at the pituitary to stimulate LH secretion. In combination with Millar's data that a population of LH gonadotropes expressed the GnRH-II receptor (148), these findings suggested GnRH-II might be acting through its own receptor to stimulate LH release. However, it is also possible that the delayed peak of LH release following GnRH-II treatment was due to interactions between GnRH-II and the GnRH receptor(s) to which the peptide binds. Because this interaction might be complicated by the receptor's cytoplasmic tail or the circulating half-life of GnRH-II, the preliminary question to be addressed was whether GnRH-II binds to the

GnRH-I and/or GnRH-II receptor to stimulate LH release. This is made further relevant because the anterior pituitary expresses both GnRH-I and GnRH-II receptors (198).

Receptor signaling

The GnRH-II receptor recently discovered in mammals has approximately a 400-fold selectivity for GnRH-II over GnRH-I (147, 148, Neill review). This is in contrast to the ten-fold selectivity of the mammalian GnRH-I receptor for GnRH-I over GnRH-II (146). Taken together, the evidence suggests GnRH-II would stimulate LH release through the GnRH-II receptor, however, a potent GnRH-I receptor antagonist, Antide, prevented LH secretion in response to GnRH-II treatment *in vivo*. A previous study has demonstrated that the GnRH-II receptor has little to no affinity (IC_{50} > 10,000 nM) for Antide (194), so it is unlikely that this effect was mediated at the GnRH-II receptor, despite the pharmacological dose of Antide (100 μg/kg body weight) used. Therefore, this finding indicates that a bolus dose of GnRH-II requires the GnRH-I receptor to stimulate LH release directly from the pituitary gland *in vivo*.

Modulatory effects

However, the LH and FSH data call into question the physiological relevance of GnRH-II as a gonadotropin-releasing hormone. Does endogenous GnRH-II duplicate the action of GnRH-I or was the treatment paradigm masking the true function of GnRH-II? It is possible that GnRH-II modulates pituitary responsivenss to GnRH-I Previous studies have shown the GnRH-I receptor became desensitized when exposed to continuous, rather than episodic, GnRH-I (17, 27, 200). In addition, GnRH-II has a substantially longer circulating half-life than GnRH-I (97, 140). Therefore, if GnRH-II were able to bind the GnRH-I receptor over a longer period of time, it is possible that GnRH-II treatment could lead to the

eventual densitization of the GnRH-I receptor. Alternatively, episodic GnRH-I exposure is known to prime the GnRH-I receptor, and potentiate the LH response to a bolus GnRH-I treatment (203). However, it is unclear whether GnRH-II might also act to prime the GnRH-I receptor. Because a population of GnRH-II containing cells occurs in the primate MBH, it is possible that GnRH-II is released into the portal vasculature and may modulate the pituitary gonadotropes' response to GnRH-I stimulation.

To address this, GnRH-I and GnRH-II were administered together at two different doses to determine if GnRH-II exposure has an inhibitory or stimulatory effect on GnRH-I-stimulated LH release. GnRH-II did not potentiate nor attenuate GnRH-I-stimulated LH secretion, which suggested that the only interaction between the GnRH-I and GnRH-II treatments occurred due to competition at the GnRH-I receptor. These results corresponded to the earlier findings that GnRH-II required the GnRH-I receptor to stimulate LH secretion. However, because previous investigations found the GnRH-I receptor has a higher affinity for GnRH-I than GnRH-II (146, 148, Neill review), GnRH-II may not affect GnRH-I signaling unless the pituitary is exposed to the two forms sequentially, rather than simultaneously. Furthermore, because GnRH-I and GnRH-II demonstrated equal potency, it is likely that an endogenous, bolus secretion of GnRH-II does not significantly affect GnRH-I release, rather the equal potencies of GnRH-I and GnRH-II nullify any competition that might occur at the GnRH-I receptor. Alternatively, endogenous GnRH-II might signal to GnRH-I cell bodies to alter GnRH-I secretion, but the present experimental design could not address this possibility. Taken together, these data indicate that exogenous GnRH-II signaled using the established GnRH-I pathway rather than a unique neuroendocrine pathway.

However, although GnRH-II administration was able to stimulate LH release, anterior pituitary cells did not show immunoreactivity to GnRH-II (Fig. 2-7). In contrast to this, GnRH-I did show immunoreactivity in the anterior pituitary, where it may have been internalized after binding to GnRH receptors (26). This ability to detect GnRH-I but not GnRH-II in the anterior pituitary suggests that an appreciable amount of endogenous GnRH-II does not reach the anterior pituitary, further suggesting that GnRH-II may not regularly signal to the gonadotropes.

In addition to the relative potency of GnRH-I and GnRH-II, the effect of age on the LH response to GnRH treatment was also examined. Unexpectedly, the results demonstrated that aged but regularly-cycling rhesus monkeys secreted less LH following GnRH treatment compared to young adult rhesus monkeys. This was observed for both GnRH-I and GnRH-II at the 10-ng/kg and 100-ng/kg doses, but was not noticeable at 1 μg/kg, which appeared to be the most effective dose for both GnRH forms. It is possible that these results stem from a change in GnRH receptor numbers or a smaller releasable pool of LH stored in the gonadotropes. However, I am hesitant to make more than a preliminary conclusion due to the small number of animal subjects per group.

Summary & Physiological Relevance

In summary, the present study finds that exogenous GnRH-II is able to stimulate LH release from the anterior pituitary using the GnRH-I receptor. Furthermore, GnRH-II demonstrates equal potency to GnRH-I at stimulating LH release, although a single dose of either form failed to stimulate FSH release *in vivo*. Therefore, it is possible that neither GnRH-II nor GnRH-I plays a significant physiological role as an FSHRH in primates. Moreover, because the hpg axis of rhesus macaques closely resembles that of humans, these results may provide further understanding of the neuroendocrine mechanisms that regulate human reproduction.

CHAPTER IV

Interaction of GnRH-II and Estradiol: Influence of Estradiol on GnRH-II Expression, Release, and Signaling

INTRODUCTION

Traditionally, the hpg axis is thought to be composed of a triad of hormones that feed-back on one another to coordinate a functional reproductive system; these three groups of hormones are the gonadotropin-releasing hormones, GnRH-I and possibly GnRH-II; the gonadotropins, LH and FSH; and the gonadal sex steroids, estrogens, progestins, and androgens. Although several estrogens and progestins exist in the female, the majority of studies have been performed using 17 β-estradiol or estradiol benzoate (estradiol) and progesterone. Estradiol exhibits both positive and negative (11, 12) feedback on the reproductive axis, and to achieve the end result of either potentiated or attenuated gonadotropin release, this feedback occurs at multiple levels. However, the signaling mechanisms used by estrogens are differentially understood, primarily due to the existence of multiple estrogen receptors (ER), ERα and ERβ, whose distributions and specific functions require further definition. In addition, a general consensus has not been achieved to explain the mechanisms underlying the switch from negative to positive feedback. However, it is also possible that another impediment to explaining how estradiol modulates gonadotropin release has been the assumption that only one form of GnRH existed in the mammalian hypothalamus.

Estradiol may influence GnRH at the level of its production, secretion, and/or its signaling to the gonadotropes; any or a combination of these can eventually alter LH and FSH release. Comparing the volume of literature devoted to each of these feedback mechanisms, the effect of estradiol on GnRH-I expression has been the least studied. One possible explanation is that until the discovery of a second estrogen receptor, ERβ, (59) it was widely theorized that GnRH-I cells did not contain estrogen receptors in any appreciable amounts (51 - 56), therefore estradiol would not be expected to directly affect GnRH-I expression. Despite the limited

number of studies, ovariectomy did result in increased GnRH-I expression in rats (46, 199), and this was reversed by estradiol replacement (46). Contrary to this, a similar study carried out in ferrets did not demonstrate statistically different GnRH-I expression following ovariectomy and estradiol replacement (47)., and ovariectomy in sheep did not induce Fos expression in GnRH-I cells (203). In addition to the effects on expression, other investigations have shown a direct effect of estradiol on GnRH-I neurons. Ovariectomy attenuated GnRH neurons responsiveness to prostaglandins in rats (48) and increased glial ensheathment of GnRH neurons in primates (48). In addition, a subpopulation of arcuate nucleus neurons were shown to both contain GnRH immunoreactivity and to rapidly hyperpolarize after treatment with estradiol (50). Taken together, these data suggest that estradiol can have direct negative feedback on GnRH-I expression, as well as GnRH-I neuron morphology, and GnRH-I cell tone.

The evidence is stronger, however, that estradiol might affect GnRH-I release, rather than its expression. In terms of positive feedback, a GnRH pre-ovulatory surge has been detected in the peripheral plasma of women (201), and in the portal plasma of sheep (14, 16). Furthermore, an estradiol-induced surge in ovariectomized sheep induced Fos expression in 41% of GnRH-I cells (203). The majority of studies, however, used radiofrequency or knife lesions to demonstrate that estradiol could act on the hypothalamus to generate the gonadotropin surge (45, 66, 68, 70). Similarly, when an impermeable Teflon barrier was inserted into the pituitary stalk, estradiol treatment failed to initiate a gonadotropin surge, suggesting that estradiol was acting on the hypothalamus (37). In addition, evoked LH release was potentiated when estradiol replacement was combined with electrical stimulation of the rostral hypothalamus, but LH release was attenuated following the same paradigm when the MBH was stimulated (69). Although most investigations examine the positive feedback effects of estradiol, this study also shows negative

feedback effects of estradiol on GnRH release from the MBH. In addition, direct portal sampling demonstrated a decreased amplitude of GnRH release following estradiol treatment in ewes (202), however push-pull perfusion in rhesus macaques did not show altered GnRH-I release following estradiol treatment (206). Taken together, the data are more consistent that estradiol primarily has positive feedback on GnRH release.

Similar to studies investigating whether estradiol could affect GnRH release, most examinations of estradiol's affects on pituitary responsiveness to GnRH used hypothalamic lesions. However, because how the pituitary responded to GnRH was being studied, exogenous GnRH-I treatments were used, and this allowed the investigators to observe both negative and positive feedback effects of estradiol. In these cases, treatment with estradiol at concentrations above 200 pg/ml for 36 hours or more resulted in an initial decline of LH secretion followed by an LH surge, demonstrating estradiol also acted at the level of the pituitary to modulate GnRH signaling (31, 34 - 36). In addition, pituitary sensitivity to intravenous GnRH treatment was decreased following estradiol treatment in women (44), and LH sensitivity was decreased to intrapituitary GnRH infusion following estradiol treatment in rhesus macaques (45).

In examining whether estradiol altered GnRH release or pituitary responsiveness, however, the majority of studies used LH as a marker and thus did not establish that estradiol affected GnRH-I signaling in lieu of another GnRH form. In particular, the lack of ERα in GnRH-I neurons complicated attempts to map what mechanisms mediated estradiol positive and negative feedback. Although recently, ERβ was found to occur in GnRH-I neurons of several species (60 - 62), it remains unclear how estradiol feedback switches from negative to positive prior to the pre-ovulatory surge. Preliminary data suggests ERβ may mediate positive

feedback because its mRNA expression increases just prior to ovulation (63). Furthermore, an estrogen receptor antagonist blocked the LH surge without affecting tonic LH release (65), and ERα has been shown to be necessary for estradiol negative feedback (64). Taken together, this suggests that the positive and negative feedback of estradiol may be controlled by ERβ and ERα, respectively.

Considering that both GnRH-I and GnRH-II occur in the primate hypothalamus, it is plausible that two forms of GnRH may also contribute to estradiol signaling to the reproductive axis. Correspondingly, recent studies have begun to examine whether GnRH-II concentrations change after removal of the sex steroids, however, the data is inconsistent measuring GnRH-II mRNA following gonadectomy. Castration and subsequent steroid replacement has no effect on GnRH-II expression in the male (108, 123). In the female, ovariectomy both stimulates (108) and inhibits (104) GnRH-II expression, however, progesterone treatment decreases GnRH-II mRNA levels (129) while estradiol has no effect (124), suggesting that the variable results following ovariectomy may reflect progesterone, rather than estradiol, effects. However, many of these studies examined GnRH-II expression in the midbrain, rather than the hypothalamus. In addition, the paucity of direct evidence that GnRH-I mediates estradiol feedback warrants investigation of how estradiol treatment affects GnRH-II expression, release, and signaling.

To help resolve the issues regarding the roles each GnRH form plays to help regulate gonadotropin secretion in response to changing estrogen milieu, this chapter examines how ovariectomy and steroid replacement in the primate alter the expression of GnRH-II mRNA, the amount of GnRH-II secreted, and pituitary responsiveness *in vitro* to GnRH-II administration. Comparing these results to existing data on GnRH-I during the same events may help clarify the respective

influences of GnRH-II and GnRH-I. In addition, the ability of estradiol to signal directly to GnRH-II cells via either ERα or ERβ is examined.

MATERIALS & METHODS AND RESULTS

Regional overlap of estrogen receptors and GnRH-II

Animals

Tissues from three intact female rhesus macaques (*Macaca mulatta*) were obtained from the ONPRC Tissue Distribution Program to provide tissue for this and other studies. Based on detailed menstruation records, one of the three adult females was in the early follicular phase of the menstrual cycle and the other two were in the luteal phase. Animal care was provided by the ONPRC in accordance with the NIH *Guide for the Care and Use of Laboratory Animals*. They had been housed under controlled lighting (12 hours of light and 12 hours of darkness per day) and temperature (23 ± 2°C), and had been provided a diet consisting of Purina monkey chow and fresh fruit, with unlimited access to drinking water.

Tissue preparation

The animals were deeply anesthetized using ketamine/pentobarbital according to procedures established by the Panel on Euthanasia of the American Veterinary Society. Their brains were fixed and the hypothalami were blocked, cryoprotected and sectioned as described in Chapter II. Sections were stored free-floating in cryoprotectant at –20°C until use, which was a maximum of 6 months after sectioning to prevent undue degradation of the mRNA.

Immunohistochemistry

IHC was performed as described above using RNase-free reagents and solutions. Furthermore, sections were incubated overnight at 4°C with a previously characterized (207) polyclonal ERβ or monoclonal ERα (Santa Cruz Biotech; Santa

Cruz, CA) at a 1:800 or 1:100 dilution, respectively, in Tris buffer In addition, 1 μg of RNase inhibitor (vol/vol; Promega Corp; Madison, WI) was added per ml Tris buffer. The secondary antibodies used were biotinylated goat anti-sheep or horse anti-mouse IgG (Vector Laboratories), respectively. Following IHC for ERα or ERβ, sections were mounted on glass microscope slides (Fisherbrand SuperFrost/Plus; Fisher Scientific). After being air-dried for 30 min and vacuum-dried overnight, the mounted sections were stored at -85°C for ISH.

In situ hybridization

ISH was performed as described above using a ^{35}S-labeled antisense riboprobe against GnRH-II precursor cDNA.

To further quantitate the expression of GnRH mRNA in the different groups, following ISH, the hypothalamic sections were subsequently dehydrated, defatted, and dipped in photographic emulsion as described above. Silver grain density was examined under a microscope using a X40 objective lens. Only cells that had an obvious round or fusiform silver grain deposition pattern around a thionin counter-stained nucleus were counted and analyzed. The images were digitized, as described above, and the NIH Image program (version 1.59) was then used to determine the total number of positive cells per section. Two-way ANOVA was used to assess statistical differences in regional GnRH-I and GnRH-II mRNA expression between the treatment groups.

Results

Representative autoradiographs (Fig. 4-1) suggest that the estrogen receptor β (ERβ) distribution may overlap that for GnRH-II in the SON and PVN. Therefore, IHC for ERβ, or ERα as a positive control, was combined with ISH for GnRH-II, and photographic emulsion to induce silver grain deposition was used to allow microscopic examination for double labeling. Using NIH Image and a digital frame

grabber, cells were individually analyzed to determine if silver grain deposition exceeded 5 times above background, the parameters used to define a positive cell. As shown in Figs. 4-2, GnRH-II mRNA did occur together with ERβ immunoreactivity in subpopulations in the PVN and MBH but not in the SON. The incidence of colocalization was approximately 50% for the PVN and 40% for the MBH (Table 4-1). However, no coexpression was observed for GnRH-II and ERα.

The monoclonal ERα is commercially available and has been previously characterized, however characterization for the polyclonal ERβ had not been performed in the rhesus macaque brain. Therefore, IHC for ERβ was combined with ISH for ERβ; as shown in Fig. 4-3, ERβ immunoreactivity occurred in cells expressing ERβ mRNA, demonstrating the antibody was specific for ERβ in the hypothalamus.

Estradiol feedback on GnRH-II expression

Animals

Tissues from nine adult (6-12 yr old) female rhesus macaques (*Macaca mulatta*) were obtained from the ONPRC Tissue Distribution Program to provide tissue for this and other studies. Three of the animals were gonad-intact, three were ovariectomized (ovx), and three were ovariectomized but treated with estradiol replacement (ovx+E). Animals were bilaterally ovariectomized at least 1 month prior to euthanasia. To restore circulating estradiol back to physiological levels, ovx animals were implanted with a 3-cm Silastic capsule filled with crystallized 17 β-estradiol (Sigma). Using radioimmunoassay, estradiol levels were determined to be 60 pg/ml, which is within physiological parameters for this species (189). Animal care was provided as described above by the ONPRC in accordance with the NIH *Guide for the Care and Use of Laboratory Animals.*

Tissue preparation

The animals were deeply anesthetized using ketamine/pentobarbital according to procedures established by the Panel on Euthanasia of the American Veterinary Society. Their brains were fixed by perfusion as described above. Hypothalami were blocked just rostral to the optic chiasm and rostral to the mammillary bodies. The tissue blocks were frozen, sectioned, and stored as described in Chapter II.

In situ hybridization (ISH)

ISH involved the use of ^{35}S-labeled antisense riboprobes to macaque GnRH-I mRNA or macaque GnRH-II mRNA. The riboprobes were 224- or 430-base-pairs long, respectively, and spanned the complete decapeptide coding region as well as most of the GnRH-associated peptide (GAP) coding region. Because the GAP region is unique for each GnRH precursor form, the probes specifically identified only those cells that express GnRH-I or GnRH-II mRNA, respectively.

ISH was performed as described above on a series of six coronal hypothalamic sections from each animal, collected at approximately 200-µm intervals. Sections were then washed for 5 min in distilled water and dipped in thionin (Sigma) for 10 min followed by two 5-min washes in water. Sections were then dehydrated with ethanol, cleared with xylenes, and finally coverslipped using DPX mounting medium. For quantitation, the autoradiographs were uniformly transilluminated, and the images captured, digitized, and analyzed as described above. Two-way ANOVA was used to assess statistical differences in regional GnRH-I and GnRH-II mRNA expression between the treatment groups.

Results

Because the silver grain analysis for the developmental series closely corroborated what was observed using the autoradiographs (Chapter II), I chose to

not use autoradiographic analysis to examine GnRH-II expression during changing sex steroid milieu. GnRH-I ISH was performed on sections adjacent to those used for GnRH-II ISH in order to determine that the estradiol concentration used as replacement was physiological to achieve negative feedback on the hypothalamus. In addition, because GnRH-I cells are scattered throughout the hypothalamus rather than clustered, the hypothalamus was compared as a unit between treatment groups instead of comparing individual hypothalamic nuclei or regions for GnRH-I analysis. The ovx hypothalamus expressed significantly more GnRH-I mRNA than either the intact or ovx+E hypothalamus (Fig. 4-4). However, treating ovariectomized animals with estradiol reduced GnRH-I mRNA expression to the level found in gonad-intact animals. In both cases, these changes in expression were reflected in the total number of cells expressing GnRH-I, rather than silver grains per cell (Fig. 4-4, 4-5).

In contrast to GnRH-I expression, GnRH-II mRNA levels appeared greatest in the ovx+E animals (Fig. 4-2). Upon analysis, silver grain deposition revealed that significantly more MBH cells expressed GnRH-II in the ovx+E animals compared to either the intact or the ovx animals (Fig. 4-5). Furthermore, the ovx animals expressed similar levels of GnRH-II mRNA in the MBH compared to the intact animals MBH (Fig. 4-5). However, the SON and PVN GnRH-II populations were not significantly different between any treatment groups (Fig. 4-5).

Estradiol feedback on GnRH-II signaling

Animals

Pituitaries were obtained from 19 adult (6-12 yr old) female rhesus macaques (*Macaca mulatta*) that were euthanized as part of the ONPRC Tissue Distribution Program to provide tissue for this and other studies. Four of the animals were gonad-intact, six were ovx, and six were ovx+E. Animals were bilaterally ovariectomized at least 5 months prior to euthanasia. To restore circulating estradiol back to

physiological levels, ovx animals were implanted with a 3-cm Silastic capsule filled with crystallized 17 β-estradiol (Sigma). Using radioimmunoassay, estradiol levels were determined to be 60 pg/ml, which is within physiological parameters for this species (191). Animal care was provided as described above by the ONPRC in accordance with the NIH *Guide for the Care and Use of Laboratory Animals.*

Pituitary cell dispersion

Animals were deeply anesthetized using ketamine/pentobarbital, according to procedures established by the Panel on Euthanasia of the American Veterinary Society, and fresh pituitaries were obtained. The anterior pituitary was isolated, and the cells were dispersed and cultured as described in Chapter III.

Treatment *in vitro*

Plated cells were washed twice in serum-free DMEM/F12 media for first 60 min and then 5 min prior to treatment. GnRH-I and GnRH-II were separately diluted to doses ranging from 10^{-12} to 10^{-5} moles/well in serum-free media. Cells were then incubated with GnRH-I, GnRH-II, or serum-free media for two hours at 37°C. Following incubation, media was collected, centrifuged to remove any cells, and stored at -20°C until assay. To measure their viability, cells were exposed to 0.4% trypan blue (Sigma) and then washed in media; the number of live cells able to exclude were approximately 95%.

Results

To examine whether estradiol alters pituitary responsiveness to GnRH-II, freshly dispersed cells were cultured from intact, ovx, or ovx+E rhesus macaque anterior pituitaries. These primary cell cultures were then statically incubated with GnRH-II at doses ranging from 10^{-12} to 10^{-5} molar for two hours, and the media assayed for LH and FSH concentrations. As demonstrated in Fig. 4-8, the net amount of LH released was consistently lower in the ovx and ovx+E pituitaries

compared to the intact control group. This pattern of responsiveness was also characteristic of LH secretion following an identical paradigm of treatment with GnRH-I.

Dissimilar to that seen for LH, pituitaries from ovx+E animals secreted less FSH than pituitaries from intact or ovx animals (Fig. 4-9). This was evident at every dose of GnRH-II except 10^{-9} molar, when the response was equivalent rather than attenuated for the ovx+E. Furthermore, examining the FSH response to GnRH-I revealed a similar pattern (Fig. 4-9).

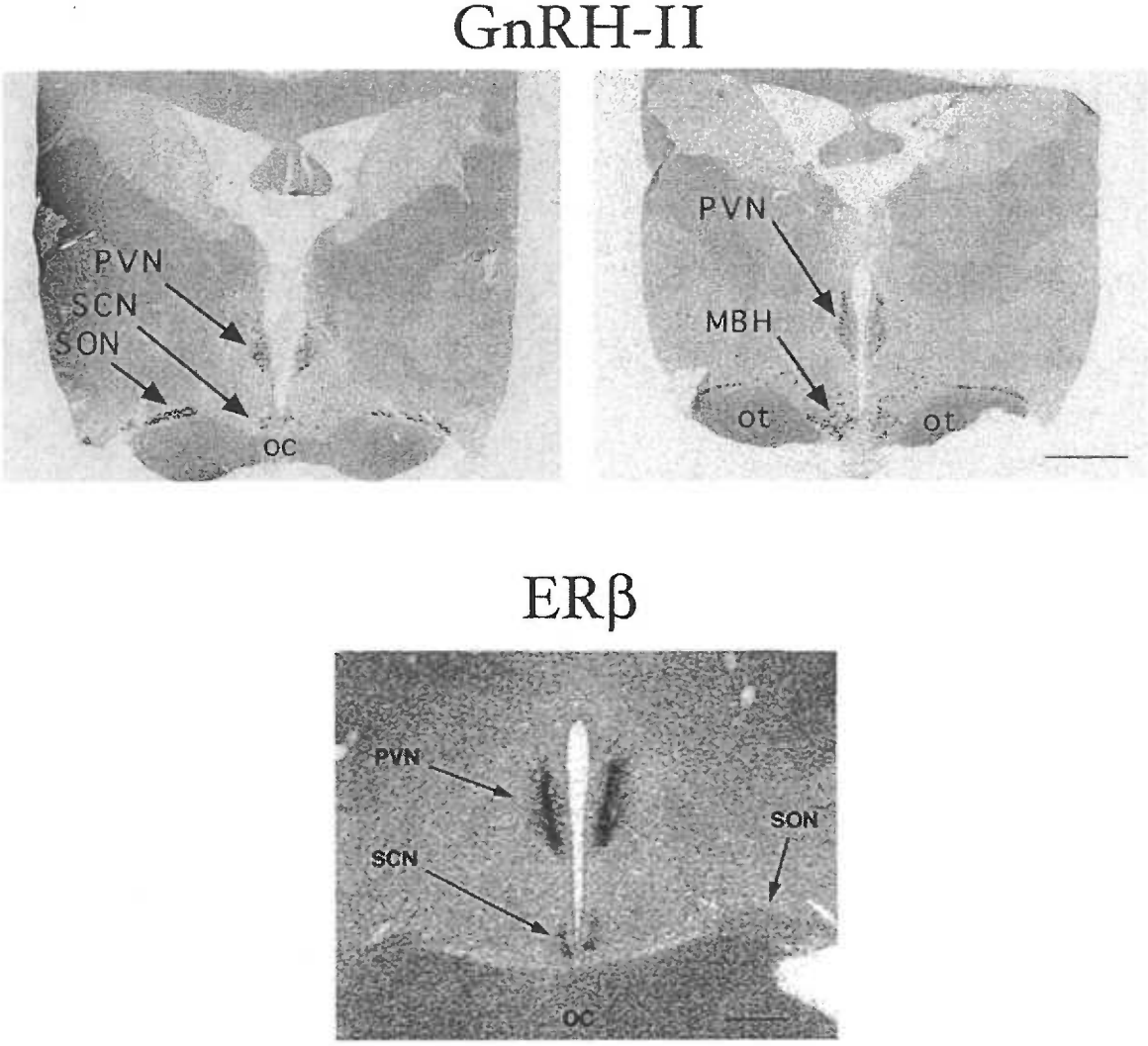

Figure 4-1. Regional expression of GnRH-II and ERβ mRNA in the rhesus macaque brain as revealed by radioisotopic *in situ* hybridization. Autoradiographs of coronal brain sections represent hybridization of an antisense GnRH-II or ERβ riboprobe in the rostral (*left panel* and ERβ)and caudal hypothalamus. Notice that the ERβ signal, while overlapping with GnRH-II, shows the strongest hybridization in the PVN. *Scale bars*, 5 mm (*upper panels*), 10 mm (*lower panel*). oc=optic chiasm, ot = optic tract.

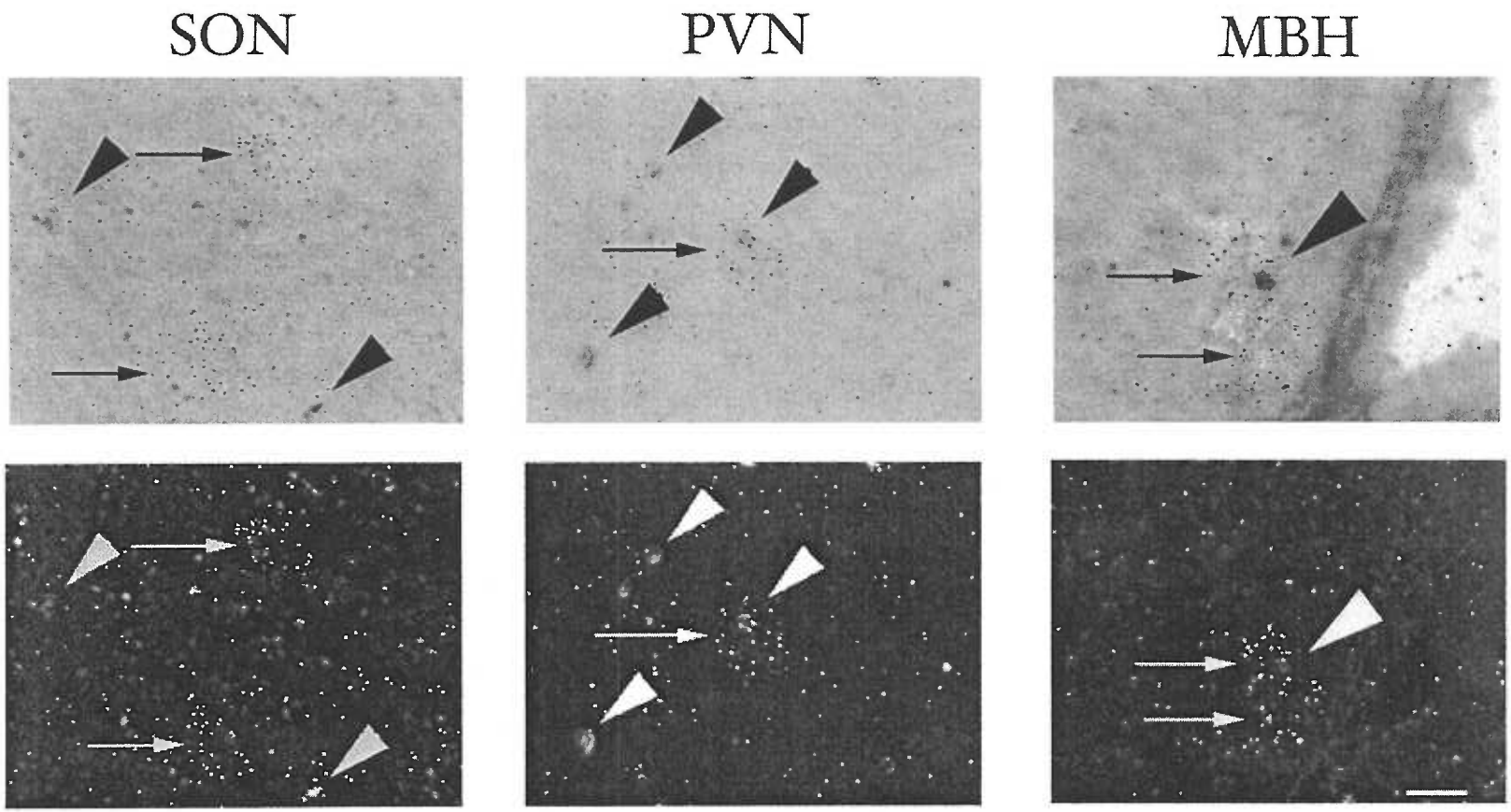

Figure 4-2. Representative darkfield photomicrographs of hypothalamic sections, double-labeled using a procedure that combined immunohistochemistry (IHC) with *in situ* hybridization (ISH). The sections were initially processed for ERβ IHC and then for ISH using a riboprobe to GnRH-II mRNA. ERβ-immunopositive cells are indicated by *arrowheads*, and cells expressing GnRH-II mRNA are identified by regions of clustered silver grains, indicated by *arrows*. Note the colocalization of ERβ and GnRH-II in the MBH and PVN but not SON. *Scale bar* = 10 μm.

Nucleus	Number of cells		Percentage of cells	
	GnRH-II	Double-label	GnRH-II	Double-label
SON	200	19	93	7
PVN	51	28	60	40
MBH	40	41	50	50

Table 4-1. Number and percentage of GnRH-II cells that expressed ERβ in the SON, PVN, and MBH. ERβ and GnRH-II cells were identified in the SON, PVN, and MBH by immunohistochemistry and *in situ* hybridization, respectively. Numbers represent the total number of positive cells detected in the sections that were examined. 6 sections per animal, n =3 .

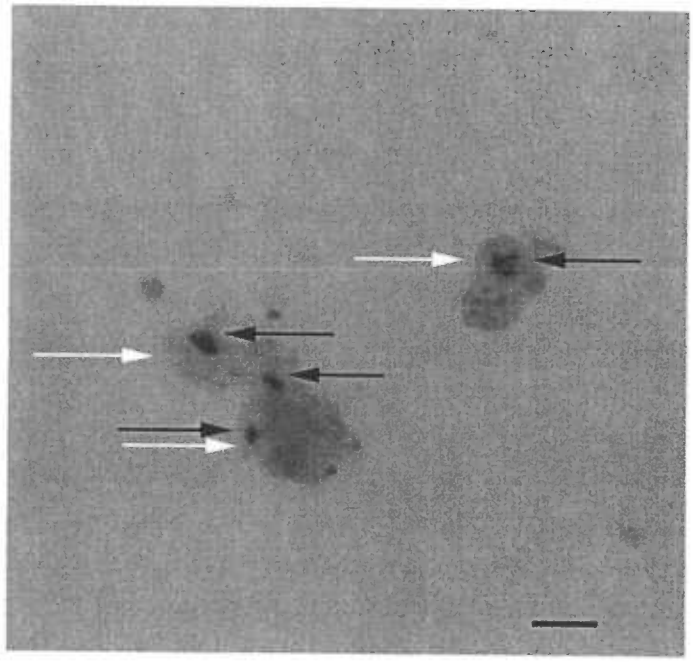

Figure 4-3. Representative brightfield photomicrograph demonstrating that the ERβ antibody used was specific for cells expressing ERβ mRNA. Immunohistochemistry for ERβ was combined with non-isotopic *in situ* hybridization using an antisense riboprobe specific for ERβ. ERβ immunopositive cells are labeled with *black arrows*. Cells expressing ERβ mRNA are labeled with *white arrows*. *Scale bar*=10 μm.

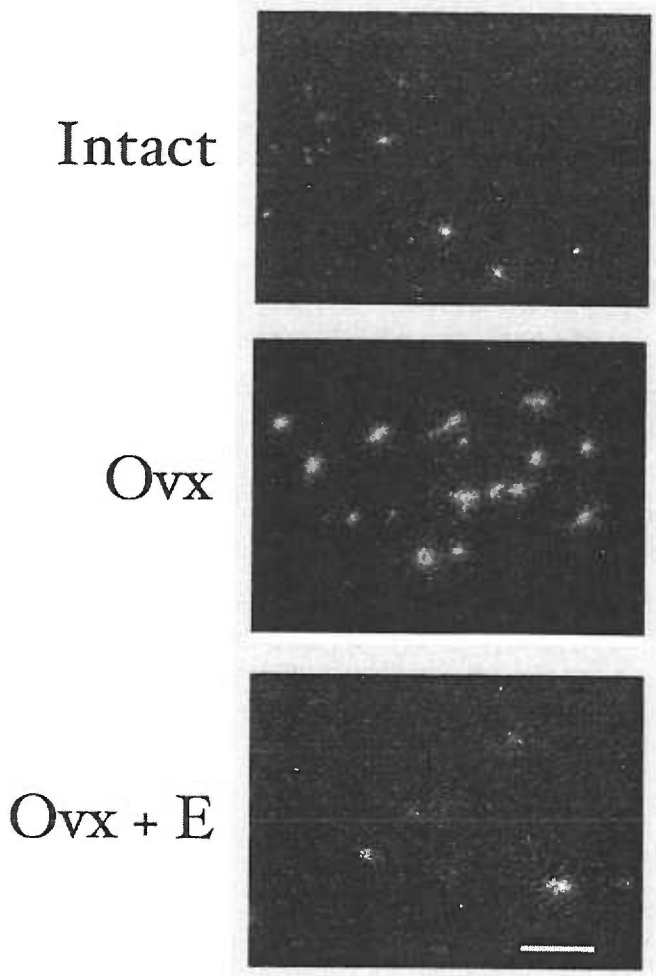

Figure 4-4. Hypothalamic GnRH-I expression levels following ovariectomy and estrogen replacement. Representative darkfield photomicrographs demonstrating GnRH-I expression in the hypothalamus of intact, ovariectomized, and estrogen-treated ovariectomized animals as revealed by *in situ* hybridization. Hypothalamic GnRH-I expression increased following ovariectomy, estrogen treatment attenuated GnRH-I expression back to intact levels. *Scale bar*=15 μm.

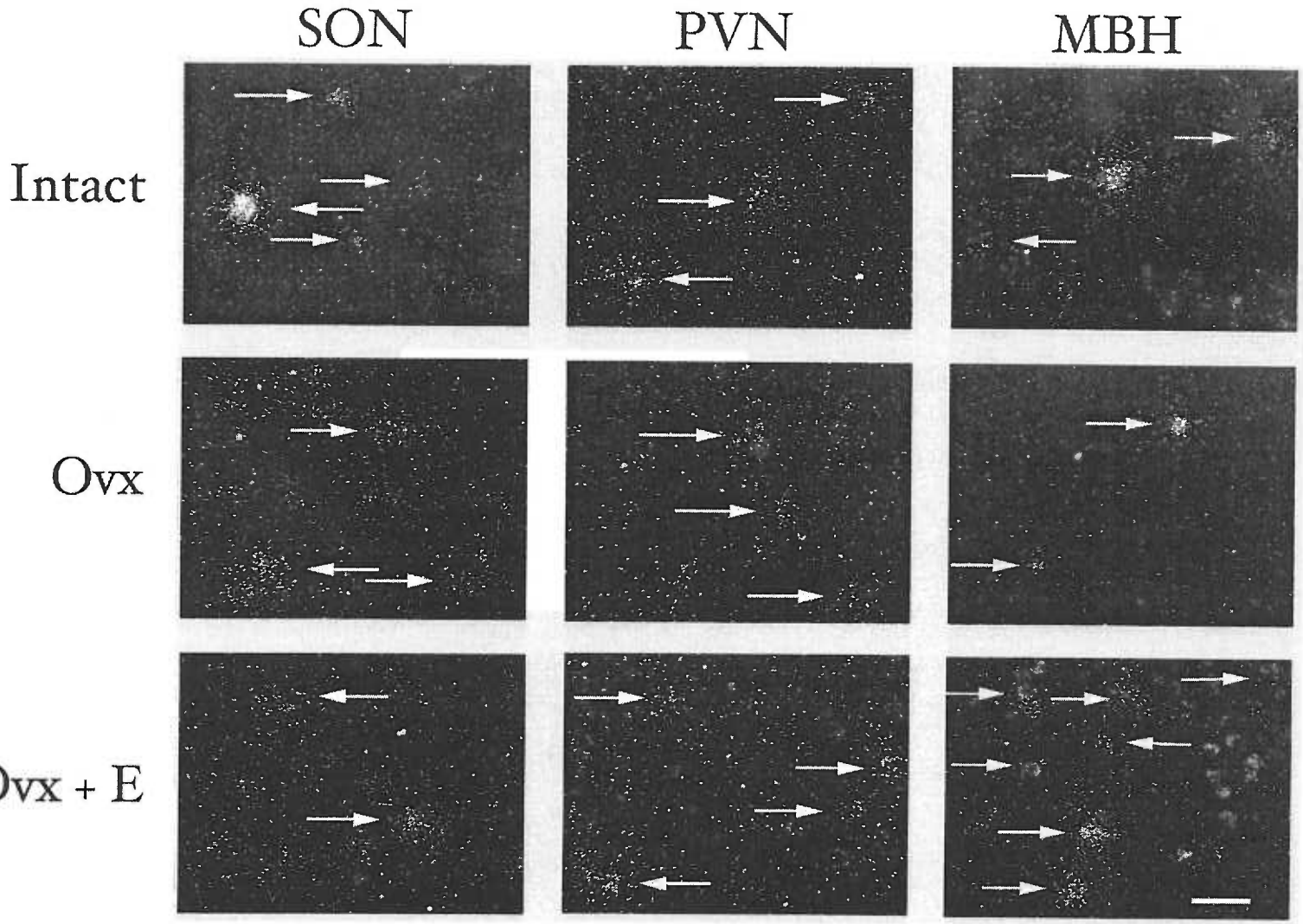

Figure 4-5. Hypothalamic GnRH-II expression levels following ovariectomy and estrogen replacement. Representative darkfield photomicrographs demonstrating GnRH-II expression (*white arrows*) in the SON, PVN, and MBH in the intact, spayed, and estrogen-treated spayed animals as revealed by *in situ* hybridization. MBH GnRH-II expression increased following estrogen treatment in the spayed animal, but no expression changes were seen in the SON or PVN. * $P<0.05$. *Scale bar*=15 μm.

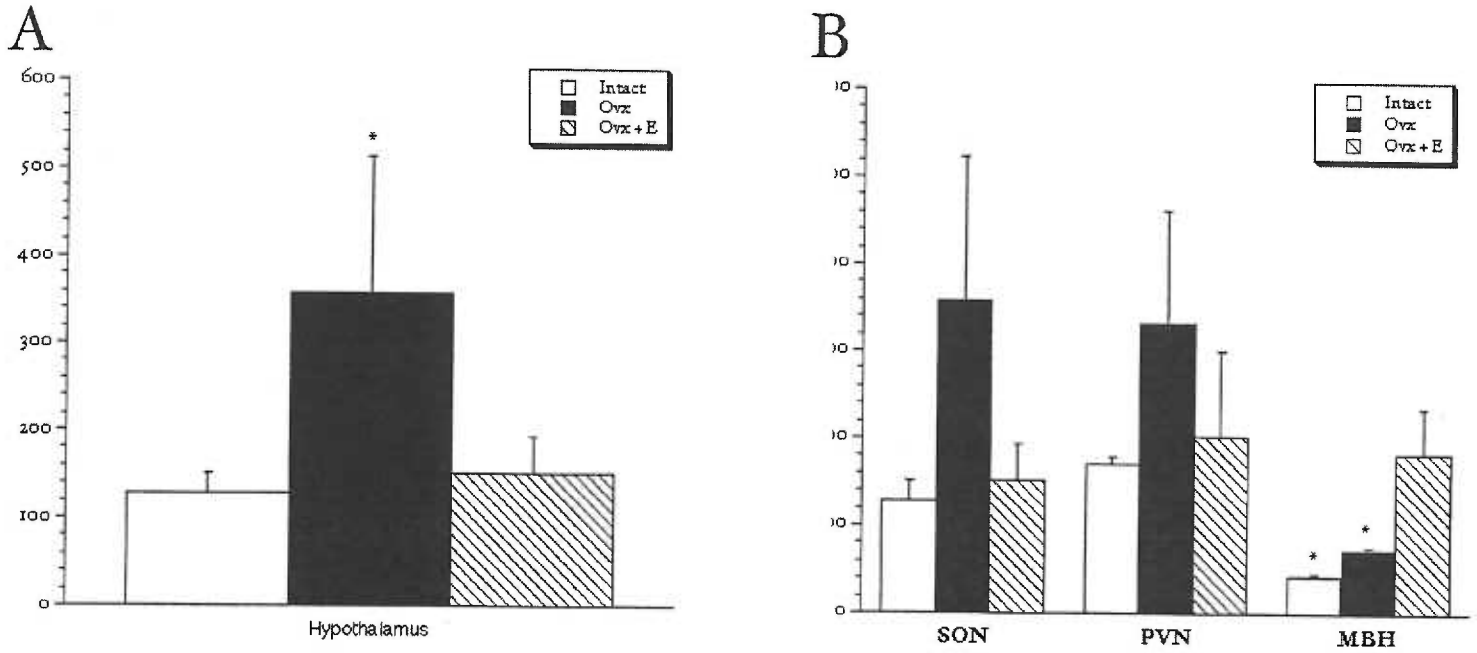

Figure 4-6. Hypothalamic GnRH expression levels following ovariectomy and estrogen replacement. A, *Histogram* quantitating number of cells expressing GnRH-I in the hypothalamus of intact, ovariectomized, and estrogen-treated ovariectomized animals as revealed by *in situ* hybridization. Hypothalamic GnRH-I expression increased following ovariectomy, estrogen treatment attenuated GnRH-I expression back to intact levels. B, *Histogram* quantitating number of cells expressing GnRH-II in the SON, PVN, and MBH of intact, ovariectomized, and estrogen-treated ovariectomized animals as revealed by *in situ* hybridization. MBH GnRH-II expression increased following estrogen treatment in the ovariectomized animal, but no expression changes were seen in the SON or PVN. n=3/group * $P<0.05$.

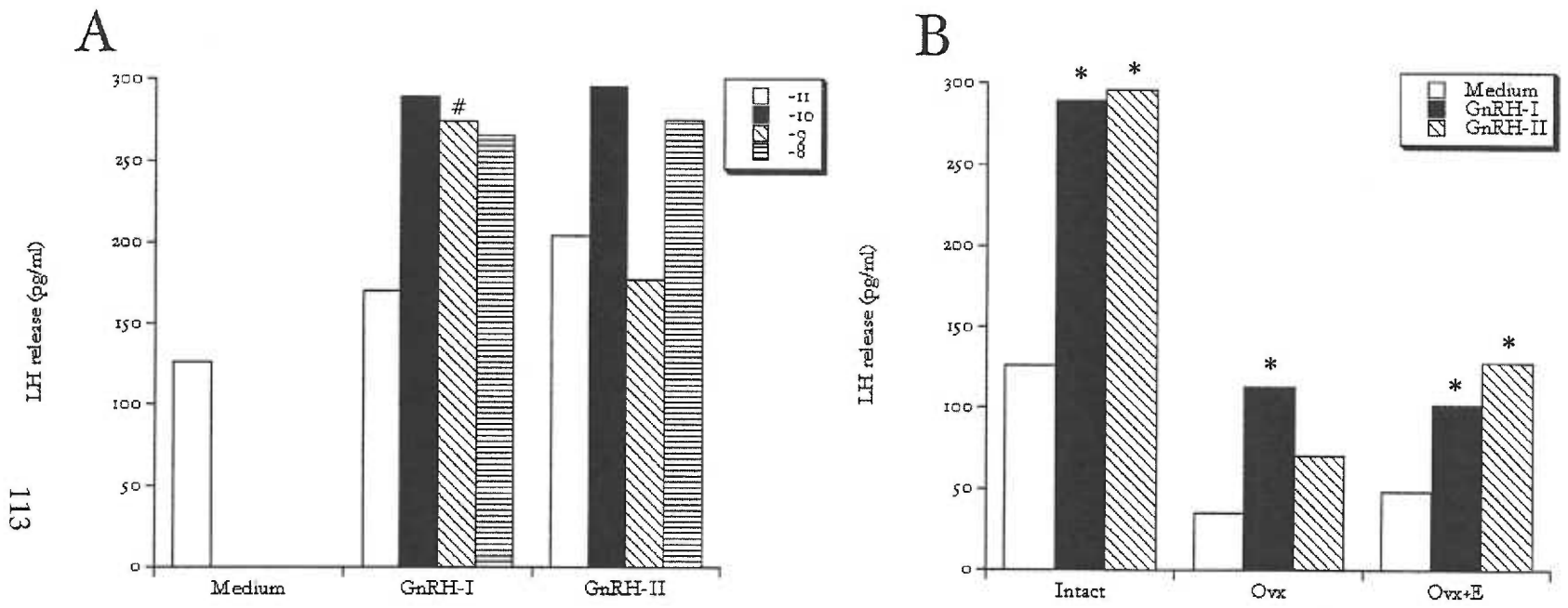

Figure 4-7. Comparison of the *in vitro* gonadotropin-releasing abilities of various doses of GnRH-I and GnRH-II on primary cultures from intact, ovariectomized, or estrogen-treated ovariectomized rhesus macaque anterior pituitary cells. A, Representative histogram from an intact animal of total LH output following two-hour static incubations of medium and GnRH-I or GnRH-II at four doses. B, Representative histogram of total LH output from pituitaries from intact, ovx, and ovx+E animals following two-hour static incubations of medium alone and GnRH-I or GnRH-II at -10 M dose. Each dose was tested in duplicate or triplicate per pituitary culture, and each experiment was repeated in three pituitaries. Each *bar* represents the mean from one representative pituitary. *, $P<0.05$ compared to media; #, $P<0.05$ compared to GnRH-II.

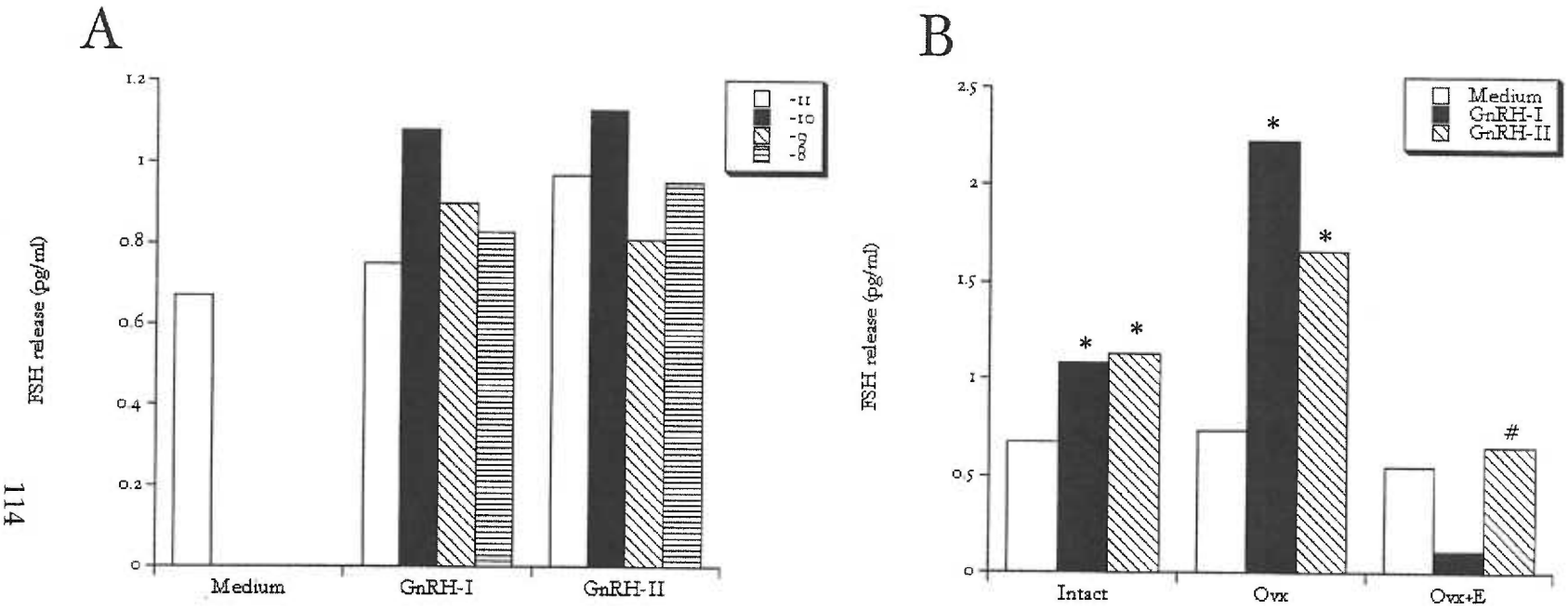

Figure 4-8. Comparison of the *in vitro* gonadotropin-releasing abilities of various doses of GnRH-I and GnRH-II on primary cultures from intact, ovariectomized, or estrogen-treated ovariectomized rhesus macaque anterior pituitary cells. A, Representative histogram from an intact animal of total FSH output following two-hour static incubations of medium and GnRH-I or GnRH-II at four doses. B, Representative histogram of total FSH output from pituitaries from intact, ovx, and ovx+E animals following two-hour static incubations of medium alone and GnRH-I or GnRH-II at -10 M dose. Each dose was tested in duplicate or triplicate per pituitary culture, and each experiment was repeated in three pituitaries. Each *bar* represents the mean from one representative pituitary. *, $P<0.05$ compared to media; #, $P<0.05$ compared to GnRH-I.

DISCUSSION

Estradiol feedback on the hpg axis is key to modulate tonic GnRH and LH secretion and to help entrain the menstrual cycle to a regular rhythm. During most of the cycle, estradiol exerts negative feedback to attenuate the amplitude of GnRH (207) and gonadotropin (31) secretion. The elevated estradiol levels at midcycle, however, result in a surge of both GnRH (14) and gonadotropin (35) release; this ability to significantly potentiate GnRH and LH release has led some investigators to view estrogen as a gonadotropin-releasing hormone in its own right (38). These data showing estradiol affecting both LH and GnRH release confirm that estradiol signals both at the hypothalamic and pituitary level to regulate gonadotropin release, but the mechanisms underlying estradiol action and the estradiol site-of-action for positive and negative feedback require further investigation.

Regional overlap of estrogen receptors and GnRH-II

GnRH-II provides a new potential target to mediate estradiol feedback effects to the reproductive axis. GnRH-II mRNA has a clustered distribution in SON and PVN magnocellular cells, which secrete peptides into the peripheral circulation, and a GnRH-II cell population occurs in the MBH, which may send projections to the POA (73). GnRH-II axons may also terminate on the hypothalamo-pituitary portal vessels, which vascularize the anterior pituitary. Moreover, exogenous GnRH-II is a potent LH-releasing hormone, so taken together, GnRH-II may reach the pituitary gonadotropes and/or GnRH-I cells where it can modulate gonadotropin signaling. Furthermore, because there is an apparent overlap of expression between GnRH-II and estrogen receptor β (ERβ) in the rhesus macaque hypothalamus (110, 208), GnRH-II is in a position to act downstream of estradiol to influence either or both GnRH-I and gonadotropin secretion.

GnRH-II and estradiol in the rostral hypothalamus

These data demonstrate that GnRH-II expression is influenced by estrogen milieu and that estradiol may directly exert feedback on the decapeptide. A GnRH-II subpopulation of cells were found to express ERβ, however, although 40% of GnRH-II cells in the PVN expressed ERβ, the degree of overlap was minimal to non-existent in the SON. This variability suggests that GnRH-II is involved in multiple neuroendocrine pathways. However, gap junctions, which allow electrical activity to be transmitted directly between cells, are known to exist in the SON and PVN and to increase in numbers when these nuclei are electrically stimulated (170). Therefore the lack of ERβ in GnRH-II cells in the SON may represent an economy of expression rather than the existence of functional subpopulations.

Despite PVN cells coexpressing GnRH-II and ERβ, ovariectomy with or without estradiol replacement did not affect GnRH-II expression in the SON or PVN. Therefore it appears that chronic changes in estrogen milieu do not feedback on GnRH-II expression.

GnRH-II and estradiol in the caudal hypothalamus

In addition to estradiol effects on GnRH-II in the rostral hypothalamus, approximately 50% of GnRH-II cells in the MBH expressed ERβ, the receptor subtype that may mediate estradiol's positive feedback (63). In keeping with this, treating ovariectomized animals with estradiol resulted in greater GnRH-II expression specifically in the MBH. This indicates that estradiol is able to directly exert positive feedback on GnRH-II, and provides some impetus to examine whether GnRH-II is involved in the preovulatory surge.

It is questionable whether GnRH-I provides the entire hypothalamic stimulus for a gonadotropin surge. Although recent data demonstrates that GnRH-I

cells express ERβ, estradiol exerted negative, rather than positive, feedback on GnRH-I expression. Furthermore, a GnRH-I antisera administered to rhesus macaques decreased pulsatile LH but was unable to block the estradiol-induced LH surge (18). It should be noted that controversy exists whether a GnRH pulse is even necessary to generate a surge, however a study blocking neuroendocrine inputs to the pituitary with a Teflon shield was able to obliterate the surge (37), and lesions that destroyed the arcuate nucleus and MBH blocked the estradiol-induced surge in rhesus macaques (70). Moreover, elevated GnRH-I has been detected in portal plasma around the time of the surge (14, 16, 39, 201). These findings strongly suggest a neuroendocrine input is necessary for the pre-ovulatory surge and that GnRH-I contributes to this input, however it is unclear how a surge of GnRH-I is coordinated. The present findings provide circumstantial evidence that suggest MBH GnRH-II cells may play an important role in mediating feedback from estradiol to the hypothalamus and in augmenting the central stimulus for the preovulatory gonadotropin surge.

However, the question remains why MBH GnRH-II expression was not attenuated in ovx animals, which lack the estradiol stimulus. One possibility is that a gonadal signal separate from estradiol exerts negative feedback on GnRH-II expression; therefore GnRH-II levels in the intact animal are achieved by coordinating estrogenic positive feedback with a gonadal negative-feedback signal. Based on this hypothesis, it would be expected that ovariectomy removes both the positive and negative signals, leaving basal GnRH-II expression to be similar to intact levels. Such a system would allow for GnRH-II expression to be modulated by the relative concentrations of feedback signals. A potential candidate to have negative feedback is progesterone, because previous work has demonstrated that progesterone treatment will decrease hypothalamic GnRH-II levels (129). Moreover, early studies showed that progesterone was able to selectively block the preovulatory

and estradiol-stimulated LH surge without affecting pulsatile LH release (42, 43). Therefore, if progesterone does apply negative feedback, this provides further support to hypothesize that GnRH-II from the MBH may help mediate the gonadotropin surge.

Estradiol feedback on GnRH-II signaling

In examining estradiol feedback on GnRH-II, it is also important to determine whether estrogen milieu affects the gonadotropes' sensitivity to GnRH-II. Several studies have used hypothalamic lesions to demonstrate that acute exposure to estradiol concentrations above 200 pg/ml increases pituitary sensitivity to GnRH-I in rhesus macaques (31, 35, 36). However, the estradiol levels typical of the luteal or early follicular phases attenuate the pituitary response to GnRH-I in primates (44, 45). In contrast to these studies, which examined pituitary sensitivity following short-term exposure to estradiol, LH release following GnRH-I treatment was less in pituitaries from chronically ovx compared to ovx+E rats (210). The present data, however, suggest that estradiol does not exert feedback on the *in vitro* LH response to GnRH-II, but estradiol does attenuate the FSH response to GnRH-II. This was identical to the results obtained following GnRH-I treatment, which supports the earlier data in Chapter III that GnRH-II stimulated gonadotropin release using the GnRH-I receptor. Taken together, long-term estradiol exposure exerts either no or negative feedback on both GnRH-I and GnRH-II signaling to LH and FSH, respectively.

Summary

In summary, the results from this study show that GnRH-II may mediate the positive estradiol signal to the reproductive axis. The MBH cell population expresses more GnRH-II in response to estradiol alone, and this modulation may occur directly as a cell population in the MBH express both GnRH-II and ERβ.

Furthermore, these cells may project to regions containing GnRH-I neurons, potentially allowing them to communicate the estradiol signal to GnRH-I. Therefore, these findings give credence to the view that reproductive function in higher primates may be regulated by more than one GnRH neuronal system.

In addition, estradiol exerted negative feedback on GnRH-II release into the peripheral circulation. It is possible that cells in the PVN that express both GnRH-II and ERβ are responsible for this observed negative feedback. What is unlikely is that plasma GnRH-II signals to the pituitary gonadotropes, because the highest concentrations observed did not reach the necessary levels to stimulate LH release (Fig. 3-2 and Chapter III), however, the peripheral target for GnRH-II remains unknown. Taken together, then, this latter data opens new avenues to understand how estradiol signals to the neuroendocrine system, although not necessarily the reproductive axis.

CHAPTER V

Summary and Conclusions: how GnRH-II Modulates the HPG Axis

HPG axis

Reproduction is regulated by a variety of events and signals that ultimately exert their influence via one or more members of the hypothalamo-pituitary-gonadal (hpg) axis, which governs the reproductive system. The hpg axis maintains homeostasis through a triad of hormones: gonadotropin-releasing hormone (GnRH); the gonadotropins, LH and FSH; and the gonadal sex steroids, estrogens, progestins, and androgens. These hormones modulate each other's release to form an interconnected regulatory system. Furthermore, because there are several external stimuli that influence reproduction compared to the key players in the hpg axis, the frequency and amplitude of secretion provide additional signaling mechanisms. However, it is controversial whether this carefully orchestrated interconnectivity can fully account for the release patterns observed during the primate menstrual cycle.

Gonadotropin-releasing hormone

GnRH-I is distributed in isolated cells dispersed throughout the hypothalamus from the POA to the median eminence (165), and it is secreted into the hypothalamic portal blood vessels, which vascularize the anterior pituitary. GnRH-I has a high efficacy to stimulate LH release, which earned it the name luteinizing hormone-releasing hormone (LHRH), although bioassays demonstrated it is also capable of stimulating the release of another gonadotropin, FSH (3 - 6). Further complicating the interpretation of these data, measuring GnRH-I release, either by sampling portal blood (14, 16) or by push-pull perfusion (39), has proved to be a difficult and delicate task, therefore there is limited data demonstrating GnRH-I release patterns. Results to date, however, show GnRH-I release follows a pulsatile pattern that corresponds to LH, but not always to FSH, release (24). These difficulties have also plagued determining whether GnRH-I undergoes a surge prior

to ovulation, although data from the sheep (14, 16) and rhesus macaque (39) indicate this is the case.

In addition to regulating gonadotropin release during the ovulatory or menstrual cycle, the pattern of GnRH-I release during puberty is also unclear. Although nocturnal LH pulses are an early indicator of the onset of puberty, the trigger for nocturnal GnRH-I release is unknown. GnRH-I expression has not been observered to change developmentally in the intact primate (156), therefore the stimulus for the onset of puberty must instead regulate GnRH-I release, and has therefore been theorized to be either an excitatory or inhibitory neurotransmitter, such as GABA (211) or glutamate (171, 173), respectively. In addition, the absence of NPY, an orexigenic signal that inhibits GnRH-I release, has also been postulated to trigger puberty by releasing GnRH-I from inhibitory feedback (157). Regardless, several details of the mechanisms underlying GnRH-I's control of the reproductive system remain unclear under the paradigm of one gonadotropin-releasing hormone in the hpg axis.

Gonadotropins

For the gonadotropins, the second tier of the hpg axis, LH is secreted in pulses occurring once per hour, or in a circhoral rhythm, during the follicular phase and once every 2-3 hours during the luteal phase (10 - 12, 20). These observed LH pulses correspond to pulsatile secretion of GnRH-I in hypothalamic portal blood (14 - 16); this intermittent, rather than continuous, exposure to GnRH is obligatory to maintain LH release (17). Moreover, the gonadotropes require GnRH pulses within a narrow frequency range to maintain the observed physiological amplitude of LH and FSH release (27, 28), as Knobil's data demonstrated that a slower pulse frequency will more selectively stimulate FSH release (28).

Establishing a clear FSH secretory pattern has been difficult. Although FSH secretion appears similar to LH, the FSH pulses are less distinct (20 - 22) and appear to overlay a tonic FSH release (23). Also less clear is what regulates FSH release. A prevailing theory is that the GnRH pulse frequency provides the signal for selective LH or FSH secretion in lieu of separate LH- and FSH-releasing hormones. However, although a slower pulse frequency has been demonstrated to increase FSH release compared to LH release (28), the decreased endogenous frequency of GnRH-I during the primate luteal phase results in an increased amplitude for both FSH and LH release (190). Alternatively, this may result from increased releasable stores of the gonadotropins (29).

Additional data also contradict the conclusion that only one gonadotropin-releasing hormone is sufficient to regulate both LH and FSH secretion. LH release following GnRH-I stimulation is much more robust than the FSH response, which appears to be uncoupled at least a third of the time from the measured GnRH-I release pattern (189). Furthermore, LH or FSH release are preferentially stimulated by extracts from different hypothalamic regions (30, 189, 190), suggesting that LH and FSH release may also be regulated by different hormones. As a result, a unique FSH-releasing hormone (FSHRH) has long been postulated (1, 2).

Separate from the tonic release patterns, plasma gonadotropin concentrations also surge, and this occurs approximately 37 hours prior to ovulation in the rhesus macaque (32). Circulating estradiol levels above 200 pg/ml for over 36 hours are necessary for a gonadotropin surge to occur in primates (34), however, a hypothalamic contribution to the surge is both controversial and less clear. The controversy springs from attempts to prevent endogenous GnRH-I from reaching pituitary gonadotropes followed by treatment with pre-surge concentrations of estradiol and exogenous pulses of GnRH-I (29, 35, 36, 38). Although pre-ovulatory surges have been observed under these conditions, it is often difficult to conclude

that all hypothalamic input to the pituitary is blocked. This is particularly the case because the experimental design is considered successful when tonic gonadotropin release no longer occurs, however, other studies have demonstrated that separate hypothalamic areas are responsible for tonic and surge gonadotropin release (31, 68 - 70), thus bringing into question the methodology used. In dissention with these conclusions, blocking the pituitary stalk and portal blood vessels with an impermeable barrier prevented the generation of a gonadotropin surge (37), providing strong evidence that hypothalamic input is necessary for the preovulatory surge. Furthermore, elevated GnRH concentrations have been observed in portal blood temporally corresponding to the surge (14, 16, 39). However, a GnRH-I antiserum failed to prevent the surge, although it abolished pulsatile gonadotropin release (18). Taken together, it appears that both elevated estradiol and a hypothalamic signal are obligatory for the surge. It is possible, however, that the hypothalamic signal is an alternate form of GnRH than the one required to generate tonic gonadotropin secretion.

Estradiol

Estradiol influences GnRH production and secretion, and also affects LH and FSH release by altering the gonaodtrope's sensitivity to GnRH-I. In the rhesus macaque, plasma estradiol concentrations are low during the early follicular phase, which attenuates GnRH-I and LH release. However, with ongoing LH stimulus, circulating estradiol levels rise until they reach 200 pg/ml or higher for at least 36 hours, and this stimulates the surge of GnRH and the gonadotropins, which trigger ovulation. Therefore, estradiol has negative feedback on tonic LH and FSH secretion but positive feedback on the gonadotropin surge (11, 190, 192).

Estradiol exerts negative feedback on gonadotropin release using multiple mechanisms (41). Removing endogenous estrogens by ovariectomy will increase GnRH-I gene expression in rats (46, 202), although ovariectomy in sheep did not

induce Fos expression in GnRH-I cells (203). A similar variation occurs for estradiol negative feedback on GnRH-I secretion. Direct portal sampling demonstrated a decreased amplitude of GnRH release following estradiol treatment in rhesus macaques (205), however push-pull perfusion did not show altered GnRH-I release following estradiol treatment (206). In addition, ovariectomy and estradiol treatment have been shown to alter GnRH-I neurons activity by attenuating their responsiveness to prostaglandins (48) for the former and for estradiol treatment to cause rapid hyperpolarization (50).

In addition to studies investigating whether estradiol affects GnRH expression or release, there have been several examinations of estradiol's affects on pituitary responsiveness to GnRH, most using hypothalamic lesions. Because how the pituitary responded to GnRH was being studied, exogenous GnRH-I treatments were used, and this allowed the investigators to observe both negative and positive feedback effects of estradiol. In these cases, treatment with estradiol at concentrations above 200 pg/ml for 36 hours or more resulted in an initial decline of LH secretion followed by an LH surge, demonstrating estradiol also acted at the level of the pituitary to modulate GnRH signaling (31, 34 - 36). Pituitary sensitivity to GnRH-I has also been shown to decrease following estradiol treatment in mammals (31, 44, 45).

Estradiol also exerts positive feedback using multiple mechanisms of action. An estradiol-induced surge in ovariectomized sheep induced Fos expression in 41% of GnRH-I cells (203). Furthermore, several studies have used radiofrequency or knife lesions to demonstrate that estradiol can act on the hypothalamus to generate the gonadotropin surge (45, 66, 68, 70). In addition, a GnRH pre-ovulatory surge has been detected in the peripheral plasma of women (204), and the portal plasma of sheep (14, 16).

Among other sites-of-action, these data suggest estradiol can act directly on GnRH-I neurons, but until recently, estrogen receptors were either not found on GnRH neurons (51 - 56) or the techniques used to find receptors were of questionable stringency (57, 58). However, a second estrogen receptor, ERβ, was recently discovered (60) and found to colocalize with GnRH-I neurons in several species (60 - 62). Moreover, preliminary data has since suggested that ERβ may help control the pre-ovulatory surge due to its distribution and upregulated mRNA expression prior to ovulation (63), suggesting estradiol might directly signal positive feedback. In contrast, ERα is necessary for estradiol negative feedback (64), and the majority of evidence suggests ERα and GnRH-I have separate distributions.

Therefore, the members of hpg axis form a system of checks and balances, which largely orchestrates reproduction; however growing evidence suggests an additional element or elements may exist within the hpg axis to help mediate individual members' signals. With this in mind, a second form of GnRH, GnRH-II, was recently demonstrated to occur in a majority of vertebrates, including human and non-human primates. This conservation of expression suggests that GnRH-II may play an important role within the hpg axis, although its physiological interaction with each member of the axis was unclear. The current results address how GnRH-II interacts in the female rhesus macaque with the hpg triad: GnRH and the hypothalamus, the gonadotropins in the pituitary, and the sex steroids estradiol and to a certain extent, progesterone.

How GnRH-II modulates the hpg axis

In the mammalian hpg axis, GnRH-I is regarded as the primary neuroendocrine link between the brain and the reproductive system. In essence, then, GnRH-I transmits signals that have been relayed to the hypothalamus to the

remainder of the hpg axis. However, the mechanisms underlying how these extrahypothalamic signals modulate GnRH-I in addition to how specific gonadotropin release patterns are regulated remain less clear. The discovery that GnRH-II is expressed by almost every vertebrate examined for it thus raises the possibility that at least two forms of GnRH coordinately regulate reproduction. Furthermore, because GnRH-II is so conserved phylogenetically, it is worth considering that GnRH-II may have an essential or conserved function. Therefore, it is tantalizing to hypothesize that GnRH-II modulates the hpg axis, as this may resolve some unanswered questions regarding how physiological events impact reproductive function. A summary diagram is provided (Fig. 5-1) to aid the discussion of GnRH-II's possible involvement with the hpg axis.

A primary question to be addressed is whether the distribution pattern of GnRH-II within each vertebrate class would allow it to influence the hpg axis, either at the level of GnRH-I or the gonadotropes. In fish, GnRH-II occurs predominately in the midbrain tegmentum (83, 89 - 92), although a few species have sparse GnRH-II immunoreactivity in the pituitary (91). In amphibians and reptiles, GnRH-II occurred more in the forebrain-spinal cord system than the hypothalamic-pituitary system (96, 100). In addition, it was discovered that a population of GnRH-II fibers terminate in the spinal cord near motorneurons, suggesting GnRH-II might function as a neurotransmitter/neuromodulator (99, 115). In birds, GnRH-II also occurs predominately in the midbrain compared to the hypothalamus (102, 103). Prior to the current studies, GnRH-II was also commonly found in the midbrain in mammals with a major terminal field in the medial habenula (107, 111, 112). Taken together, although GnRH-II has a wide distribution in the brain (91, 96 - 98, 100, 103, 108, 111), the majority of its cell bodies usually occur in the midbrain and spinal cord.

GnRH-II and GnRH-I

Using *in situ* hybridization, the current study found that GnRH-II mRNA is also expressed in the midbrain of rhesus macaques. However, clustered expression was also found in the rostral and caudal hypothalamus, specifically in the SON, SCN, PVN, and MBH. While this pattern of expression varied greatly from the scattered distribution of GnRH-I cells (165), some general overlap did occur in the MBH. Upon further examination, however, the cells that expressed GnRH-II were completely separate from the cells that were immunoreactive for GnRH-I. In addition, when the distribution study was extended using immunohistochemistry, GnRH-II immunoreactive fibers were found in the median eminence and the ventral hypothalamic tract as well as in the posterior pituitary, which is the presumed terminal field for the magnocellular GnRH-II cells in the SON and PVN. However, GnRH-II was not detectable in the plasma of luteal- or follicular-phase rhesus macaques, suggesting that the SON and PVN cell populations of GnRH-II did not have a tonic function during the follicular or luteal phase.

In addition to mapping where the GnRH-II mRNA and peptide were expressed, the pattern of expression was compared between adult and juvenile rhesus macaques to determine whether GnRH-II might play a role during the onset of puberty. In nonmammalian vertebrates, GnRH-II expression during development has been observed to increase (128), decrease (127), and not change (125, 126), depending on the species. However, GnRH-I expression does not change during development in intact primates (156 - 159). The current investigation demonstrated that considerably more cells expressed GnRH-II mRNA in the adult MBH than in the juvenile MBH. Although this finding suggests that a subpopulation of GnRH-II may help modulate reproductive development, previous studies indicate GnRH-II would not be sufficient as a trigger for puberty. Specifically, disorders occur in both humans and mice in which GnRH-I is non-functional, and these subjects fail to

undergo puberty, although there are no indications that GnRH-II is not functional in these cases (111, 180). However, the MBH population of GnRH-II may project to GnRH-I cells in the POA (73).

Multiple approaches are available to examine whether the GnRH-II increase predicts or follows the induction of puberty. However, determining if changing sex steroid milieu impacts GnRH-II expression appeared to be an economical choice, because it informed both whether estradiol exerts positive or negative feedback on GnRH-II expression and whether the developmental increase might be due to rising estradiol concentrations. Taken together, the current results suggest that the observed increase of GnRH-II expression developmentally occurred due to rising estradiol levels during puberty.

Therefore, when these data are considered together, they suggest that GnRH-II neurons may have functional subpopulations: while the SON and PVN populations likely signal to extra-neuronal tissues, the MBH cell population may play a neuroendocrine role. In addition, the SON and PVN populations may not function to regulate reproduction during the follicular or luteal phase. Furthermore, because previous studies have found the MBH neurons send projections to the POA (73), GnRH-II from the MBH may modulate GnRH-I expression and/or secretion.

GnRH-II and the gonadotropins, LH & FSH

The recent discovery of GnRH-II in the human and non-human primate raises the possibility that neuroendocrine control of the reproductive axis may be coordinated by GnRH-I and GnRH-II. This possibility gains further credence because the existence of a specific FSH-releasing hormone has long been hypothesized (6, 8), but no potential candidates are unequivocally supported (23). As a result, the presence of a highly conserved form of GnRH in the hypothalamus raises questions regarding whether it functions to stimulate gonadotropin secretion.

Furthermore, it is possible that the MBH population secretes GnRH-II into the hypothalamic portal vessels, thus allowing it to affect gonadotropin release from the pituitary gland.

The relative ability of GnRH-II to stimulate gonadotropin secretion both *in vitro* and *in vivo* has been examined in almost every vertebrate class. An *in vitro* investigation in birds demonstrated that GnRH-II has a much greater potency than mammalian GnRH-I or chicken GnRH-I, the native form, to stimulate both LH and FSH release (130 - 133). This ability was further confirmed in birds *in vivo* (134). Similar studies in fish *in vivo* corroborated the evidence that GnRH-II was the most potent gonadotropin-releasing hormone examined (135, 136, 139). Moreover, several separate examinations suggested that GnRH-II might be responsible for ovulation in fish (88, 137, 138). Studies in reptiles (140) and amphibians (97, 141) also demonstrated GnRH-II could stimulate LH release more potently *in vivo* than other GnRH forms present in each respective species. Taken together, it appears that GnRH-II can potently stimulate gonadotropin release in non-mammalian vertebrates, but it remains possible that GnRH-II does not function *physiologically* to stimulate LH and/or FSH release.

In contrast to the relative potency of GnRH-II in other vertebrates, GnRH-II initially appeared less effective to stimulate LH release in mammals. An early study that examined the ability of GnRH-II to release LH and bind pituitary gonadotropes *in vitro* found GnRH-II to be approximately ten-fold less effective than GnRH-I in sheep and rats, respectively (130). Furthermore, GnRH-I stimulated significantly more musk shrews to ovulate than GnRH-II (107). However, the ability of GnRH-II to stimulate LH release in the primate was reported to be similar to that for GnRH-I, although the LH-releasing abilities of the two GnRH forms were not directly compared (79). Moreover, the gonadotropin-releasing properties of GnRH-II were based on its ability to bind the mammalian GnRH-I receptor.

Work by Millar indicates that the mammalian GnRH-I receptor shows more selectivity for GnRH-I than the less discriminatory GnRH receptors in non-mammals (146). However a specific GnRH-II receptor was recently cloned from primates and found in the anterior pituitary of several mammalian species (147, 148). Therefore, it is possible that GnRH-II might act as a gonadotropin-releasing hormone.

The present investigation found that GnRH-II was able to potently stimulate LH release *in vivo* similar to that seen following GnRH-I treatment. Moreover, the relative LH-releasing ability of GnRH-II was also comparable to that for GnRH-I *in vitro*. However, neither GnRH-I nor GnRH-II treatment resulted in significant FSH secretion *in vivo*, and the two forms showed equal potency to stimulate FSH release *in vitro*. Therefore, the current data support the theory that FSH secretion may be regulated by a neuroendocrine factor other than GnRH-I or GnRH-II, or FSH release may be coordinated between GnRH and an inhibitory factor derived from the brain or gonads. This latter possibility exists because both GnRH-I and GnRH-II were able to release FSH in culture, so it is possible that using a different treatment paradigm, GnRH-I and/or GnRH-II would stimulate FSH release *in vivo*. In dissention with this hypothesis, existing data have demonstrated that plasma FSH concentrations are not fully synchronized with observed GnRH-I release patterns (23), nor has the receptor selective for GnRH-II been demonstrated on FSH-containing gonadotropes (148). Taken together, another hormone or factor may be primarily responsible to regulate FSH release, the pattern of FSH release may largely be determined by the pituitary gland itself (197), or relatively low concentrations of GnRH may be sufficient to stimulate tonic FSH release. Therefore, it is probable that the gonadotropins are not controlled by identical mechanisms and/or factors.

Although GnRH-I and GnRH-II were equally able to stimulate LH release, the time-course of LH secretion appeared delayed following lower doses of GnRH-

II compared to GnRH-I. Although this difference was not statistically significant, the observation raised questions about the mechanisms underlying GnRH-II-induced LH secretion. GnRH-II demonstrated equal potency to stimulate LH release from cultured anterior pituitary cells, suggesting it could act directly at the pituitary, however, examining the distribution of GnRH-II cells in Chapter II suggested GnRH-II could also modulate LH release by acting at the level of GnRH-I cell bodies or nerve terminals.

To ascertain the site-of-action for exogenous GnRH-II, a two-pronged approach was taken. An earlier study in birds demonstrated that suboptimal doses of GnRH-II attenuated LH release stimulated by chicken GnRH-I (212), therefore it was possible that GnRH-II might modulate GnRH-I secretion. Because of the difficulty in directly measuring GnRH-I release, GnRH-I and GnRH-II were administered concomitantly at two different doses to determine if GnRH-II exposure had an effect on GnRH-I-stimulated LH release. However, this treatment paradigm did not potentiate nor attenuate GnRH-I-stimulated LH secretion, nor did it alter the LH release pattern, suggesting that any interaction between GnRH-I and GnRH-II administration occurred at the level of the pituitary.

Existing studies had mapped the GnRH-II receptor to pituitary gonadotropes immunoreactive for LH (17). Therefore, the second step was to determine whether exogenous GnRH-II required the GnRH-I receptor to stimulate LH release. Administering a GnRH-I receptor antagonist, Antide, with GnRH-I and GnRH-II blocked the ability of GnRH-II to stimulate LH release *in vivo*. Because the GnRH-II receptor has little to no affinity (IC_{50} > 10,000) for Antide (28), the data indicate this effect could only have been mediated at the level of the GnRH-I receptor. Taken together, under the treatment paradigm of a bolus exposure, exogenous GnRH-II requires the GnRH-I receptor to stimulate LH release *in vivo*. However, endogenous GnRH-II may act at GnRH-I cell bodies in the rostral

hypothalamus, ultimately resulting in the modulation of GnRH-I release. The current data do not rule out this possibility because the blood-brain barrier likely prevented exogenous GnRH-II from reaching GnRH-I cell bodies in the rostral hypothalamus.

Considering whether GnRH-II functions to stimulate gonadotropin release, the current results suggest that GnRH-II signaled using the established GnRH-I pathway rather than a unique neuroendocrine pathway. In addition, an investigation in Chapter II failed to find GnRH-II in the anterior pituitary, although GnRH-I was readily apparent. Furthermore, studies in the hypogonadal mouse and in patients with Kallmann's syndrome indicate that GnRH-I is necessary for both the onset of puberty and tonic gonadotropin release (111, 178). Taken together, the evidence supports the hypothesis that GnRH-II may act upstream of GnRH-I to modulate gonadotropin secretion.

GnRH-II and Estradiol

Completing the hpg axis in the female are the gonadal sex steroids, estrogens and progestins. Of these, estradiol has been more heavily investigated, because it is known to exhibit both positive and negative feedback on the reproductive axis (11, 12). This feedback occurs at multiple levels to result in either potentiated or attenuated gonadotropin release. However, the mechanisms underlying estradiol feedback remain unclear, partially because until recently, estrogen receptors were not found on GnRH-I neurons (50 - 56). Following the discovery of a second estrogen receptor, ERβ (59), it was discovered that GnRH-I cells in the rostral hypothalamus do express an estrogen receptor (60 - 62), but it still remains unclear how estradiol feedback changes from negative to positive. However, a possible impediment to understanding estradiol feedback might be the assumption that only GnRH-I exists in the mammalian hypothalamus.

In addition to potentially modulating GnRH-I and/or gonadotropin secretion, the GnRH-II distribution pattern also suggests that GnRH-II may help mediate sex-steroid feedback onto the reproductive axis. In particular, a GnRH-II cell population occurs in the MBH; this hypothalamic region has been shown to send projections to the POA (73) in the vicinity of GnRH-I neurons. Furthermore, because there is an apparent overlap of expression between GnRH-II and ERβ in some regions of the rhesus macaque hypothalamus (110, 208), GnRH-II may be histologically capable to mediate estradiol signals to GnRH-I and/or the gonadotropins.

Several investigators have investigated whether sex-steroid milieu affects GnRH-II content or expression in different vertebrate classes. In fish, GnRH-II content was observed to remain constant during conditions when the sex-steroid milieu changes naturally (127, 126). In contrast, ovariectomy in birds attenuated GnRH-II content (104), and this effect was increased following im treatment with progesterone (129), suggesting that progesterone has negative feedback, and estradiol may have positive feedback. Furthermore, GnRH-II expression increased in reptiles during development (100). The existing data from mammals also correspond to the reptile data, as ovariectomy decreases GnRH-II cell number in musk shrews (108).

The current investigation corroborates the findings in birds, reptiles, and mammals. Specifically, GnRH-II expression increased in concert with rising estradiol concentrations, both following estradiol replacement in ovx animals and during development. Furthermore, this change in mRNA concentration was restricted to the MBH population, where approximately 40% of GnRH-II cells expressed ERβ, the receptor subtype implicated in estradiol's positive feedback (64). However, MBH GnRH-II expression was not attenuated in ovx animals, which lack the estradiol stimulus. In the case of this latter finding, a gonadal signal separate

from estradiol may exert negative feedback on GnRH-II expression; therefore GnRH-II levels in the intact animal would be achieved by coordinating estrogenic positive feedback with a gonadal negative feedback signal. This hypothesis corresponds to the earlier finding in birds, which show decreased GnRH-II content after treatment with progesterone (129). Early studies have also demonstrated that progesterone could selectively block the spontaneous and estradiol-stimulated surge without attenuating LH pulses (42, 43). Taken together, these observations and considerations suggest that a functional subpopulation of GnRH-II cells in the MBH may play an important role in mediating positive feedback from estradiol to the hypothalamus, and this provides some impetus to examine whether GnRH-II is involved in the preovulatory gonadotropin surge.

It is questionable whether GnRH-I provides the entire hypothalamic stimulus for a gonadotropin surge because estradiol exerted negative, rather than positive, feedback on GnRH-I mRNA expression. Although the positive feedback required for a gonadotropin surge may only affect GnRH-I secretion, an additional study demonstrated that GnRH-I antisera capable of decreasing pulsatile LH did not block the estradiol-induced LH surge (18). Existing data do strongly suggest that GnRH-I contributes to the necessary neuroendocrine input for the pre-ovulatory surge (14, 16, 37, 70, 204), but it is unclear how a surge of GnRH-I is coordinated. However, GnRH-II appears to contribute to ovulation in several nonmammalian vertebrates (88, 137, 138). Because GnRH-II required the GnRH-I receptor to stimulate LH release and elevated GnRH-I has been observed during the rhesus macaque LH surge (39), this suggests GnRH-I signals the pre-ovulatory surge directly to the gonadotropes. However, GnRH-II may mediate estradiol's positive feedback to GnRH-I cells in the POA.

Summary

In summary, the results from this study show that GnRH-II may mediate the positive estradiol signal to the reproductive axis. The MBH cell population expresses more GnRH-II in response to estradiol alone, and this modulation may occur directly as a cell population in the MBH express both GnRH-II and ERβ. Furthermore, these cells may project to regions containing GnRH-I neurons, potentially allowing them to communicate the estradiol signal to the GnRH-I release mechanism. Alternatively, GnRH-II was able to stimulate LH release equally well as GnRH-I, which might allow GnRH-II to exert positive feedback directly on the gonadotropes. Therefore, these findings give credence to the view that reproductive function in higher primates may be regulated by more than one GnRH neuronal system.

Future studies

Several future studies would further elucidate whether GnRH-II might mediate estradiol positive feedback to GnRH-I neurons. Recently, a specific GnRH-II receptor was discovered and antibodies have since been generated to detect it using IHC (147, 148). This antibody would allow immunohistochemical analysis to examine whether GnRH-I cells express the GnRH-II receptor and therefore whether GnRH-II can directly signal to GnRH-I neurons. In addition, GnRH-II cells could be examined for increased Fos expression both during the spontaneous pre-ovulatory surge and following administration of surge-like concentrations of estradiol. This could be further compared to Fos expression in animals that were also treated with progesterone to block the spontaneous or estradiol-stimulated LH surge. Taken together, the resulting data would suggest whether GnRH-II cells are stimulated by the spontaneous or estradiol-stimulated surge and if progesterone inhibits this activation. In addition, the receptor study would indicate whether

GnRH-II could directly modulate GnRH-I release following estradiol positive feedback. The generation of GnRH-II receptor-specific agonists and antagonists would also help to address the specific role of GnRH-II in modulating the hpg axis. However, a previous study has demonstated the feasibility to actively immunize rats against GnRH-II (161), but it is debatable whether mice or rats express GnRH-II. Therefore, another future study would involve active immunization in rhesus monkeys to elucidate any reproductive functions for GnRH-II and potentially nonreproductive functions for the SON and PVN populations.

The role of the SON and PVN populations of GnRH-II are less clear, however, it would be useful to determine whether GnRH-II occurred together with either OT or VP in these hypothalamic nuclei. Determining this would then suggest further studies to examine whether GnRH-II secretion patterns were altered by stimuli that affect OT and VP release, including lactation or a hyperosmotic challenge, respectively. This would begin to test the hypothesis that GnRH-II in the SON and PVN populations may not act primarily to modulate reproduction. Data demonstrating that immunizing rats against GnRH-II resulted in mild hypertrophy of the kidney (161) supports this view that the peptide may also act to control water balance or have a similar neuroendocrine function.

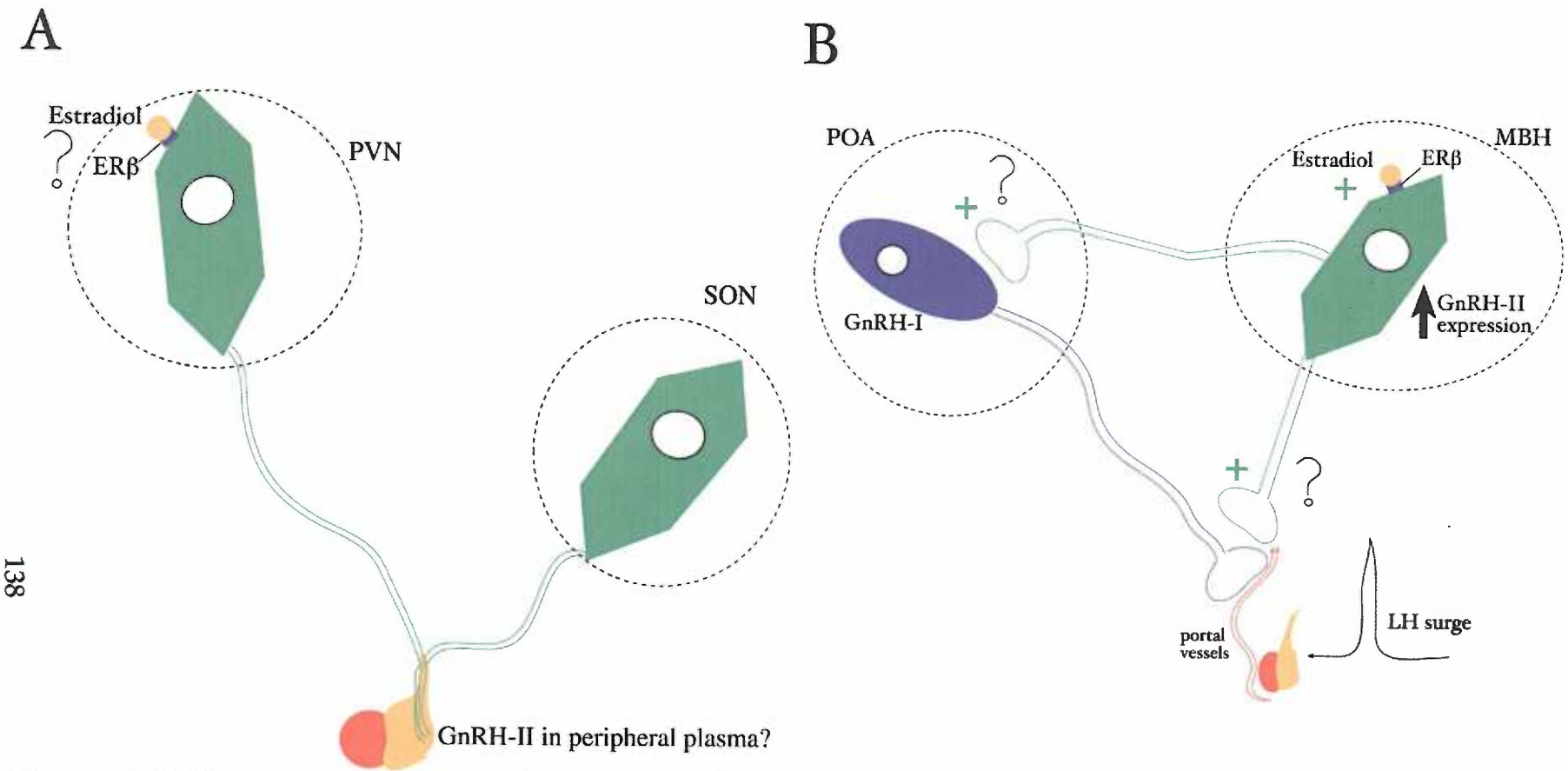

Figure 5-1. Summary diagram of potential functions for GnRH-II from the rostral (A) and caudal (B) hypothalamus. (A) GnRH-II occurred in magnocellular cells in the SON and PVN, and GnRH-II fibers were observed in the posterior pituitary. However, GnRH-II was not detected in peripheral plasma from follicular- or luteal-phase rhesus macaques. (B) GnRH-II cells in the MBH expressed ERβ, and estradiol exerted positive feedback on GnRH-II mRNA expression. These cells in the MBH may contact GnRH-I cell bodies or nerve terminals to communicate estradiol's positive feedback and help mediate the preovulatory surge.

References

1. Matsuo H, Baba Y, Nair RM, Arimura A, Schally AV 1971 Structure of the porcine LH- and FSH-releasing hormone. I. The proposed amino acid sequence Biochem Biophys Res Comm 43:1334-1339

2. Baba Y, Matsuo H, Schally AV 1971 Structure of the porcine LH- and FSH-releasing hormone. II. Confirmation of the proposed structure by conventional sequential analyses. BiochemBiophys Res Comm 44:459-463

3. Burgus R, Butcher M, Amoss M, Ling N, Monahan M, Rivier J, Fellows R, Blackwell R, Vale W, Guillemin R 1972 Primary structure of the ovine hypothalamic luteinizing hormone-releasing factor (LRF) (LH-hypothalamus-LRF-gas chromatography-mass spectrometry-decapeptide-Edman degradation). Proc Natl Acad Sci USA 69:278-282

4. Harris GW 1955 Neural control of the pituitary gland. Monographs of the Physioogical Society3 London 298

5. Schally AV, Arimura A, Bowers CY, Kastin AJ, Sawano S, Reeding TW 1968 Hypothalamic neurohormones regulating anterior pituitary function. Rec Prog Horm Res 24:497-588

6. Schally AV, Arimura A, Kastin AJ, Matsuo H, Baba Y, Redding TW, Nair RM, Debeljuk L, White WF 1971 Gonadotropin-releasing hormone: one polypeptide regulates secretion of luteinizing and follicle-stimulating hormones. Science 173:1036-1038

7. Ratner A, Dhariwal AP, McCann SM 1967 Hypothalamic factors in gonadotropic hormone regulation. Clin Obstr Gyn 10:106-118

8. Watanabe S, McCann SM 1968 Localization of FSH-releasing factor in the hypothalamus and neurohypophysis as determined by in vitro assay. Endocrinology 82:664-673

9. Monroe SE, Peckham WD, Neill JD, Knobil E 1970 A radioimmunoassay for rhesus monkey luteinizing hormone (RhLH). Endocrinology 86:1012-1018

10. Monroe SE, Atkinson LE, Knobil E 1970 Patterns of circulating luteinizing hormone and their relation to plasma progesterone levels during the menstrual cycle of the Rhesus monkey. Endocrinology 87:453-455

11. Atkinson LE, Bhattacharya AN, Monroe SE, Dierschke DJ, Knobil E 1970 Effects of gonadectomy on plasma LH concentration in the rhesus monkey. Endocrinology 87:847-849

12. Dierschke DJ, Bhattacharya AN, Atkinson LE, Knobil E 1970 Circhoral oscillations of plasma LH levels in the ovariectomized rhesus monkey. Endocrinology 87:850-853

13. Santen RJ, Bardin CW 1973 Episodic luteinizing hormone secretion in man. Pulse analysis, clinical interpretation, physiologic mechanisms. J Clin Invest 52:2617-2628

14. Carmel PW, Araki S, Ferin M 1976 Pituitary stalk portal blood collection in rhesus monkeys: evidence for pulsatile release of gonadotropin-releasing hormone (GnRH). Endocrinology 99:243-248

15. Eskay RL, Mical RS, Porter JC 1977 Relationship between luteinizing hormone releasing hormone concentration in hypophysial portal blood and luteinizing hormone release in intact, castrated, and electrochemically-stimulated rats. Endocrinology 100:263-270

16. Neill JS, Patton JM, Dailey RA, Tsou RC, Tindall GT 1977 Luteinizing hormone releasing hormone (LHRH) in pituitary stalk blood of rhesus monkeys: relationship to level of LH release. Endocrinology 101:430-434

17. Belchetz PE, Plant TM, Nakai Y, Keogh EJ, Knobil E 1978 Hypophysial responses to continuous and intermittent delivery of hypothalamic gonadotropin-releasing hormone. Science 202:631-633

18. McCormack JT, Plant TM, Hess DL, Knobil E 1977 The effect of luteinizing hormone releasing hormone (LHRH) antiserum administration on gonadotropin secretion in the rhesus monkey. Endocrinology 100:663-667

19. Midgley AR 1967 Radioimmunoassay for human follicle-stimulating hormone. J Clin Endocrinol Metab 27:295-299

20. Faiman C, Ryan RJ 1967 Radioimmunoassay for human follicle stimulating hormone. J Clin Endocrinol Metab 27:444-447

21. Wallace JM, McNeilly AS 1986 Changes in FSH and pulsatile secretion of LH during treatment of ewes with bovine follicular fluid throughout the luteal phase of the oestrous cycle. J Endocrinol 111:317-327

22. Gross KM, Matsumoto AM, Bremner WJ 1987 Differential control of luteinizing hormone and follicle-stimulating hormone secretion by luteinizing hormone-releasing hormone pulse frequency in man. J Clin Endocrinol Metab 64:675-680

23. Padmanabhan V, McNeilly AS 2001 Is there an FSH-releasing factor? Reproduction 121:21-30

24. Labhsetwar AP 1969 Pituitary levels of FSH and LH at various intervals after ovariectomy in the rat. J Reprod Fert 18:531-533

25. Yen SS, Tsai CC 1971 The effect of ovariectomy on gonadotropin release. J Clin Invest 50:1149-1153

26. Peckham WE, Yamaji T, Dierschke DJ, Knobil E 1973 Gonadal function and the biological and physicochemical properties of follicle stimulating hormone. Endocrinology 92:1660-1666

27. Wildt L, Hausler A, Marshall G, Hutchison JS, Plant TM, Belchetz PE, Knobil E 1981 Frequency and amplitude of gonadotropin-releasing hormone stimulation and gonadotropin secretion in the rhesus monkey. Endocrinology 109:376-385

28. Pohl CR, Richardson DW, Hutchison JS, Germak JA, Knobil E 1983 Hypophysiotropic signal frequency and the functioning of the pituitary-ovarian system in the rhesus monkey. Endocrinology 112:2076-2080

29. Frawley LS, Dailey RA, Tindall GT, Neill JD 1981 Increased LH secretory response to LHRH after hypophyseal stalk-transection of monkeys. Neuroendocrinol 32:14-18

30. Lumpkin MD, McDonald JK, Samson WK, McCann SM 1989 Destruction of the dorsal anterior hypothalamic region suppresses pulsatile release of follicle stimulating hormone but not luteinizing hormone. Neuroendocrinol 50:229-235

31. Nakai Y, Plant TM, Hess DL, Keogh EJ, Knobil E 1978 On the sites of negative and positive feedback actions of estradiol in the control of gonadotropin secretion in the rhesus monkey. Endocrinology 102:1008-1014

32. Weick RF, Dierschke DJ, Karsch FJ, Butler WR, Hotchkiss J, Knobil E 1973 Periovulatory time courses of circulating gonadotropic and ovarian hormones in the rhesus monkey. Endocrinology 93:1140-1147

33. Ferin M, Zimmering PE, Vande Wiele RL 1969 Effects of antibodies to estradiol-17-beta on PMS-induced ovulation in immature rats. Endocrinology. 84:893-900

34. Karsch FJ, Weick RF, Butler WR, Dierschke DJ, Krey LC, Weiss G, Hotchkiss J, Yamaji T, Knobil E 1973 Induced LH surges in the rhesus monkey: strength-duration characteristics of the estrogen stimulus. Endocrinology 92:1740-1747

35. Ferin M, Rosenblatt H, Carmel PW, Antunes JL, Vande Wiele RL 1970 Estrogen-induced gonadotropin surges in female rhesus monkeys after pituitary stalk section. Endocrinology 104:50-52

36. Knobil E, Plant TM, Wildt L, Belchetz PE, Marshall G 1980 Control of the rhesus monkey menstrual cycle: permissive role of hypothalamic gonadotropin-releasing hormone. Science 207:1371-1373

37. Norman RL, Gliessman P, Lindstrom SA, Hill J, Spies HG 1982 Reinitiation of ovulatory cycles in pituitary stalk-sectioned rhesus monkeys: evidence for a specific hypothalamic message for the preovulatory release of luteiniaing hormone. Endocrinology 111:1874-1882

38. Wildt L, Hausler A, Hutchison JS, Marshall G, Knobil E 1981 Estradiol as a gonadotropin releasing hormone in the rhesus monkey. Endocrinology 108:2011-2013

39. Pau KY, Berria M, Hess DL, Spies HG 1993 Preovulatory gonadotropin-releasing hormone surge in ovarian-intact rhesus macaques. Endocrinology 133:1650-1656

40. Gore AC, Roberts JL 1995 Regulation of gonadotropin-releasing hormone gene expression in the rat during the luteinizing hormone surge. Endocrinology 136:889-896

41. Yamaji T, Dierschke DJ, Bhattacharya AN, Knobil E 1972 The negative feedback control by estradiol and progesterone of LH secretion in the ovariectomized rhesus monkey. Endocrinology 90:771-777

42. Spies HG, Niswender GD 1971 Blockade of the surge of preovulatory serum luteinizing hormone and ovulation with exogenous progesterone in cycling rhesus (*Macaca mulatta*) monkeys. J Clin Endocrinol 32:309-316

43. Dierschke DJ, Yamaji T, Karsch FJ, Weick RF, Weiss G, Knobil E 1973 Blockade by progesterone of estrogen-induced LH and FSH release in the rhesus monkey. Endocrinology 92:1496-1501

44. Keye WR Jr, Jaffe RB 1974 Modulation of pituitary gonadotropin response to gonadotropin-releasing hormone by estradiol. J Clin Endocrinol Metab 38:805-810

45. Spies HG, Norman RL 1975 Interaction of estradiol and LHRH on LH release in rhesus females: evidence for a neural site of action. Endocrinology 97:685-692

46. Zoeller RT, Seeburg PH, Young 3rd WS 1988 In situ hybridization histochemistry for messenger ribonucleic acid (mRNA) encoding gonadotropin-releasing hormone (GnRH): effect of estrogen on cellular levels of GnRH mRNA in female rat brain. Endocrinology 122:2570-2577

47. Bakker J, Baum MJ 2000 Effect of gonadal steroids on pituitary LH secretion and mediobasal hypothalamic GnRH mRNA in ferrets. J Reprod Fert 119:315-321

48. Ojeda SR, Urbanski HF, Katz KH, Costa ME 1986 Activation of estradiol-positive feedback at puberty: estradiol sensitizes the LHRH-releasing system at two different biochemical steps. Neuroendocrinol 43:259-265

49. Witkin JW, Ferin M, Popilskis SJ, Silverman A-J 1991 Effects of gonadal steroids on the ultrastructure of GnRH neurons in the rhesus monkey: synaptic input and glial apposition. Endocrinology 129:1083-1092

50. Lagrange AH, Ronnekleiv OK, Kelly MJ 1995 Estradiol-17 beta and mu-opioid peptides rapidly hyperpolarize GnRH neurons: a cellular mechanism of negative feedback? Endocrinology 136:2341-2344

51. Shivers BD, Harlan RE, Morrel JI, Pfaff DW 1983 Absence of oestradiol concentration in cell nuclei of LHRH-immunoreactive neurones. Nature 304:345–347

52. Lehman ML, Karsch FJ 1993 Do GnRH, tyrosine hydroxylase and b-endorphin-immunoreactive neurons contain estrogen receptors? A double-label immunocytochemical study in the Suffolk ewe. Endocrinology 133:887–895

53. Herbison AE, Robinson JE, Skinner DC 1993 Distribution of estrogen receptors-immunoreactive cells in the preoptic area of the ewe: co-localization with glutamic acid decarboxylase but not LHRH. Neuroendocrinol 57:751–759

54. Herbison AE, Horvath TL, Naftolin F, Leranth C 1995 Distribution of estrogen receptor-immunoreactive cells in monkey hypothalamus: relationship to neurons containing LHRH and tyrosine hydroxylase. Neuroendocrinol 61:1–10

55. Sullivan KA, Witkin JW, Ferin M, Silverman A-J 1995 Gonadotropin-releasing hormone neurons in the rhesus macaque are not immunoreactive for the estrogen receptor. Brain Research 685:198-200

56. Warembourg M, Leroy D, Peytevin J, Martinet L 1998 Estrogen receptor and progesterone receptor-immunoreactive cells are not co-localized with gonadotropin-releasing hormone in the brain of the female mink (*Mustela vison*). Cell Tiss Res 291:33-41

57. Butler JA, Sjoberg M, Coen CW 1999 Evidence for oestrogen receptor alpha-immunoreactivity in gonadotrophin-releasing hormone-expressing neurones. J Neuroendocrinol 11:331-335

58. Skynner MJ, Sim JA, Herbison AE 1999 Detection of estrogen receptor alpha and beta messenger ribonucleic acids in adult gonadotropin-releasing hormone neurons. Endocrinology 140:5195-5201

59. Mosselman S, Polman J, Dijkema R 1996 ER beta: identification and characterization of a novel human estrogen receptor. FEBS Lett 392:49-53

60. Hrabovszky E, Shughrue PJ, Merchenthaler I, Hajszan T, Carpenter CD, Liposits Z, Petersen SL 2000 Detection of estrogen receptor-β messenger ribonucleic acid and ^{125}I-estrogen binding sites in luteinizing hormone-releasing hormone neurons of the rat brain. Endocrinology 141:3506-3509

61. Kallo I, Butler JA, Barkovics-Kallo M, Goubillon ML, Coen CW 2001 Oestrogen receptor beta-immunoreactivity in gonadotropin releasing hormone-expressing neurones: regulation by oestrogen. J Neuroendocrinol 13:741-748

62. Herbison AE, Pape JR 2001 New evidence for estrogen receptors in gonadotropin-releasing hormone neurons. Frontiers Neuroendocrinol 22:292-308

63. Orikasa C, Kondo Y, Hayashi S, McEwen BS, Sakuma Y 2002 Sexually dimorphic expression of estrogen receptor beta in the anteroventral periventricular nucleus of the rat preoptic area: implication in luteinizing hormone surge. Proc Natl Acad Sci USA 99:3306-3311

64. Lindzey J, Wetsel WC, Couse JF, Stoker T, Cooper R, Korach KS 1998 Effects of castration and chronic steroid treatments on hypothalamic gonadotropin-releasing hormone content and pituitary gonadotropins in male wild-type and estrogen receptor-alpha knockout mice. Endocrinology 139:4092-4101

65. Ordog T, Goldsmith JR, Chen MD, Connaughton MA, Hotchkiss J, Knobil E 1998 On the mechanism of the positive feedback action of estradiol on luteinizing hormone secretion in the rhesus monkey. J Clin Endocrinol Metab 83:4047-4053

66. Krey LC, Butler WR, Knobil E 1975 Surgical disconnection of the medial basal hypothalamus and pituitary fnction in the rhesus monkey. I. Gonadotropin secretion. Endocrinology 96:1073-1087

67. Antunes JL, Carmel PW, Zimmerman EA, Ferin M 1979 Regeneration of the magnocellular system of the rhesus monkey following hypothalamic lesions. Annals of Neurology 5:462-469

68. Norman RL, Resko JA, Spies HG 1976 The anterior hypothalamus: how it affects gonadotropin secretion in the rhesus monkey. Endocrinology 99:59-71

69. Spies HG, Norman RL, Quadri SK, Clifton DK Effects of estradiol-17β on the induction of gonadotropin release by electrical stimulation of the hypothalamus in rhesus monkeys. Endocrinology 100:314-324

70. Plant TM, Krey LC, Moosy J, McCormack JT, Hess DL, Knobil E 1978 The arcuate nucleus and the control of gonadotropin and prolactin secretion in the female rhesus monkey (*Macaca mulatta*). Endocrinology 102:52-62

71. Caraty A, Fabre-Nys C, Delaleu B, Locatelli A, Bruneau G, Karsch FJ, Herbison A 1998 Evidence that the mediobasal hypothalamus is the primary site of action of estradiol in inducing the preovulatory gonadotropin releasing hormone surge in the ewe. Endocrinology 139:1752–1760

72. Clarke IJ, Pompolo S, Scott CJ, Rawson JA, Caddy D, Jakubowska AE, Pereira AM 2001 Cells of the arcuate nucleus and ventromedial nucleus of the ovariectomized ewe that respond to oestrogen: a study using fos immunohistochemistry J Neuroendocrinol 13:934-941

73. Walsh JP, Clarke IJ 1998 Blockade of the oestrogen-induced luteinizing hormone surge in ovariectomized ewes by a highly selective opioid μ-receptor agonist: evidence for site of action. Neuroendocrinol 67:164-170

74. Miyamoto K, Hasegawa Y, Noura M, Igarashi M, Kangawa K, Matsuo H 1984 Identification of the second gonadotropin-releasing hormone in chicken hypothalamus: evidence that gonadotropin secretion is probably controlled by two distinct gonadotropin-releasing hormones in avian species. Proc Natl Acad Sci USA 81:3874-3878

75. Yu KL, Sherwood NM, Peter RE 1988 Differential distribution of two molecular forms of gonadotropin-releasing hormone in discrete brain areas of goldfish (*Carassius auratus*). Peptides. 9:625-630

76. Powell RC, Millar RP, King JA 1986 Identification of diverse molecular forms of GnRH in reptile brain. Gen Comp Endocrinol. 63:77-85

77. King JA, Mehl AE, Tyndale-Biscoe CH, Hinds L, Millar RP 1989 A second form of gonadotropin-releasing hormone (GnRH), with chicken GnRH II-like properties, occurs together with mammalian GnRH in marsupial brains. Endocrinology 125:2244-2252

78. Dellovade TL, King JA, Millar RP, Rissman EF 1993 Presence and differential distribution of distinct forms of immunoreactive gonadotropin-releasing hormone in the musk shrew brain. Neuroendocrinol 58:166-177

79. Lescheid DW, Terasawa E, Abler LA, Urbanski HF, Warby CM, Millar RP, Sherwood NM 1997 A second form of gonadotropin-releasing hormone (GnRH) with characteristics of chicken GnRH-II is present in the primate brain. Endocrinology 138:5618-5629

80. White RB, Eisen JA, Kasten TL, Fernald RD 1998 Second gene for gonadotropin-releasing hormone in humans. Proc Natl Acad Sci USA. 95:305-309

81. Lovejoy DA, Stell WK, Sherwood NM 1992 Partial characterization of four forms of immunoreactive gonadotropin-releasing hormone in the brain and terminal nerve of the spiny dogfish (Elasmobranchii; *Squalus acanthias*). Reg Peptides 37:39-48

82. King JA, Steneveld AA, Millar RP, Fasano S, Roman G, Spagnuolo A, Zanetti L, Pierantoni R 1992 Gonadotropin-releasing hormone in elasmobranch (electric ray, *Torpedo marmorata*) brain and plasma: chromatographic and immunological evidence for chicken GnRH II and novel molecular forms. Peptides 13:27-35

83. Montero M, Vidal B, King JA, Tramu G, Vandesande F, Dufour S, Kah O 1994 Immunocytochemical localization of mammalian GnRH (gonadotropin-releasing

hormone) and chicken GnRH-II in the brain of the European silver eel (*Anguilla anguilla L.*). J Chem Neuroanat 7:227-241

84. Sherwood NM, Grier HJ, Warby C, Peute J, Taylor RG 1993 Gonadotropin-releasing hormones, including a novel form, in snook *Centropomus undecimalis*, in comparison with forms in black sea bass *Centropristis striata*. Reg Peptides 46:523-534

85. Powell J.FF, Zohar Y, Elizur A, Park M, Fischer WH, Craig AG, Rivier JE, Lovejoy DA, Sherwood NM 1994 Three forms of gonadotropin-releasing hormone characterized from brains of one species. Proc Natl Acad Sci USA 91:12081-12083

86. Zandbergen MA, Kah O, Bogerd J, Peute J, Goos HJTh 1995 Expression and distribution of two gonadotropin-releasing hormones in the catfish brain. Neuroendocrinol 62:571-578

87. King JA, Millar RP, Vallarino M, Pierantoni R 1995 Localization and characterization of gonadotropin-releasing hormones in the brain, gonads, and plasma of a dipnoi (lungfish, *Protopterus annectens*). Reg Peptides 57:163-174

88. Carolsfeld J, Powell JFF, Park M, Fischer WH, Craig AG, Chang JP, Rivier JE, Sherwood NM 2000 Primary structure and function of three gonadotropin-releasing hormones, including a novel form, from an ancient teleost, herring. Endocrinology 141:505-512

89. Amano M, Oka Y, Aida K, Okumoto N, Kawashima S, Hasegawa Y 1991 Immunocytochmical demonstration of salmon GnRH and chicken GnRH-II in the brain of masu salmon, *Oncorhynchus masou*. J Comp Neurol 314:587-597

90. Yamamoto N, Oka Y, Amano M, Aida K, Hasegawa Y, Kawashima S 1995 Multiple gonadotropin-releasing hormone (GnRH)-immunoreactive systems in

the brain of the dwarg gouramis, *Colisa lalaia*: immunohistochemistry and radioimmunoassay. J Comp Neurol 355:354-368

91. Kima M-H, Oka Y, Amano M, Kobayashi M, Okuzawa K, Hasegawa Y, Kawashima S, Suzuki Y, Aida K 1995 Immunocytochemical localization of sGnRH and cGnRH-II in the brain of goldfish, *Carassius auaratus*. J Comp Neurol 356:72-82

92. Parhar IS, Soga T, Ishikawa Y, Nagahama Y, Sakuma Y 1998 Neurons synthesizing gonadotropin-releasing hormone mRNA subtypes have multiple developmental origins in the medaka. J Comp Neurol 401:217-226

93. Bogerd J, Zandbergen T, Andersson E, Goos H 1994 Isolation, characterization and expression of cDNAs encoding the catfish-type and chicken-II-type gonadotropin-releasing-hormone precursors in the African catfish. European J Biochem 222:541-549

94. Lin X-W, Peter RE 1997 Cloning and expression pattern of a second [$His^5Trp^7Tyr^8$] gonadotropin-releasing hormone (chicken GnRH-II) mRNA in goldfish:evidence for two distinct genes. Gen Comp Endocrinol 170:262-272

95. Yu K-L, He M-L, Chik C-C, Lin X-W, Chang JP, Peter RE 1998 mRNA expression of gonadotropin-releasing hormones (GnRHs) and GnRH receptor in goldfish. Gen Comp Endocrinol 112:303-311

96. Muske LE, Moore FL 1990 Ontogeny of immunoreactive gonadotropin-releasing hormone neuronal systems in amphibians. Brain Res 534:177-187

97. Licht P, Tsai P, Sotowska-Brochocka J 1994 The nature and distribution of gonadotropin-releasing hormones in brains and plasma of ranid frogs. Gen Comp Endocrinol 94:186-198

98. Di Matteo L, Vallarino M, Pierantoni R 1996 Localizaiton of GnRH molecular forms in the brain, pituitary, and testis of the frog, *Rana esculenta*. J Exp Zoo 274:33-40

99. Chartrel N, Collin F, Montero M, Dufour S, Vandesande F, Vaudry H 1998 biochemical characterization and regional distribution of mGnRH and cGnRH-II in the spinal cord of the frog *Rana ridibunda*. Ann NY Acad Sci 839:361-362

100. Tsai P-S, Licht P 1993 Differential distribution of chicken-I and chicken-II GnRH in the turtle brain. Peptides. 14:221-226

101. Lescheid DW, Rosen GJ, Bridge AE, Jones RE, Warby CM, Sherwood NM 1997 Immunoreactive gonadotropin-releasing hormone (GnRH) is detected only in the form of chicken GnRH-II within the brain of the green anole, *Anolis carolinensis*. Gen Comp Endocrinol 108:247-257

102. Mikami S, Yamada S, Hasegawa Y, Miyamoto K 1988 Localization of avian LHRH-immunoreactive neurons in the hypothalamus of the domestic fowl, *Gallus domesticus*, and the Japanese quail, *Coturnix coturnix*. Cell Tiss Res 251:51-58

103. Katz IA, Millar RP, King JA 1990 Differential regional disribution and release of two forms of gonadotropin-releasing hormone in the chicken brain. Peptides. 11:443-450

104. Rozenboim I, Silsby JL, Tabibzadeh C, Pitts GR, Youngren OM, El Halawani ME 1993 Hypothalamic and posterior pituitary content of vasoactive intestinal peptide and gonadotropin-releasing hormones I and II in the turkey hen. Biol Reprod 49:622-626

105. King JA, Hinds LA, Mehl AEI, Saunders NR, Millar RP 1990 Chicken GnRH II occurs together with mammalian GnRH in a south American species of marsupial (*Monodelphis domestica*). Peptides 11:521-525

106. King JA, Stenevedl AA, Curlewis JD, Rissman EF, Millar RP 1994 Identification of chicken GnRH II in brains of metatherian and early-evolved eutherian species of mammals. Reg Peptides 54:467-477

107. Rissman EF, Alones VE, Craig-Veit CB, Millam JR 1995 Distribution of chicken-II gonadotropin-releasing hormone in mammalian brain. J Comp Neurol 357:524-531

108. Rissman EF, Li X 1998 Sex differences in mammalian and chicken-II gonadotropin-releasing hormone immunoreactivity in musk shrew brain. Gen Comp Endo 112:346-355

109. Kasten TL, White SA, Norton TT, Bond CT, Adelman JP, Fernald RD 1996 Characterization of two new preproGnRH mRNAs in the tree shrew: first direct evidence for mesencephalic GnRH gene expression in a placental mammal. Gen Comp Endocrinol 104:7-19

110. Urbanski HF, White RB, Fernald RD, Kohama SG, Garyfallou VT, Densmore VS 1999 Regional expression of mRNA encoding a second form of gonadotropin-releasing hormone in the macaque brain. Endocrinology. 140:1945-1948

111. Chen A, Yahalom D, Ben-Aroya N, Kaganovsky E, Okon E, Koch Y 1998 A second isoform of gonadotropin-releasing hormone is present in the brain of human and rodents. FEBS Lett 435:199-203

112. Gestrin ED, White RB, Fernald RD 1999 Second form of gonadotropin-releasing hormone in mouse: immunocytochemistry reveals hippocampal and perventricular distribution. FEBS Lett 448:289-291

113. Jones SW 1987 Chicken II luteinizing hormone-releasing hormone inhibits the M-current of bullfrog sympathetic neurons Neuroscience Letters 80:180-184

114. Wilson JX, Butchey JK, Deshpande AA 1988 Chicken gonadotropin-releasing hormone II increases plasma catecholamines in the bullfrog Neurosci Lett 95:354-358

115. Collin F, Chartrel N, Fasolo A, Conton JM, Vandesande F, Vaudry H 1995 Distribution of two molecular forms of gonadotropin-releasing hormone (GnRH) in the central nervous system of the frog *Rana ridibunda*. Brain Res 703:111-128

116. Oka Y, Satou M, Ueda KJ 1986 Descending pathways to the spinal cord in hime salmon (landlocked red salmon, *Oncorhynchus nerka*) Comp Neurol 254:91-103

117. Riskand P, Moss RL 1983 Midbrain LHRH infusions enhance lordotic behavior in ovariectomized estrogen-primed rats independently of a hypothalamic response to LHRH. Brain Res Bull 11:481-485

118. Modianos DT, Hitt JC, Flexman J 1974 Habenular lesions produce decrements in feminine, but not masculine, sexual behavior in rats Behav Biol 10:75-87

119. Mora S, Diaz-Veliz G 1986 Pharmacological evidence of catecholaminergic involvement in the behavioral effects of luteinizing hormone releasing hormone in rats. Pharm, Biochem & Beh 24:433-438

120. Smith MT, Mason RT 1997 Gonadotorpin antagonist modulates courtship behavior in male red-sided garter snakes, *Thamnophis sirtalis parietalis*. Physiol Beh 61:137-143

121. Maney DL, Richardson RD, Wingfield JC 1997 Central administration of chicken gonadotropin-releasing hormone-II enhances courtship behavior in a female sparrow. Horm Behav 32:11-18

122. Volkoff H, Peter RE 1999 Actions of two forms of gonadotropin releasing hormone and a GnRH antagonist on spawning behavior of the goldfish *Carassius auratus*. Gen Comp Endocrinol 116:347-355

123. Parhar IS, Soga T, Sakuma Y 1998 Quantitative *in situ* hybridization of three gonadotropin-releasing hormone-encoding mRNAs in castrated and progesterone-treated male tilapia. Gen Comp Endo 112:406-414

124. Montero M, Le Belle N, King JA, Millar RP, Dufour S 1995 Differential regulation of the two forms of gonadotropin-releasing hormone (mGnRH and cGnRH-II) by sex steroids in the European female silver eel (*Anguilla anguilla*). Neuroendo 61:525-535

125. Parhar IS, Soga T, Sakuma Y 1996 In situ hybridization for two differentially expressed GnRH genes following estrogen and triiodothyronine treatment in the brains of juvenile tilapia (cichlid). Neurosci Lett 218:135-138

126. Holland MCH, Gothilf Y, Meiri I, King JA, Okuzawa K, Elizur A, Zohar Y 1998 Levels of the native forms of GnRH in the pituitary of the gilthead seabream, *Sparus aurata*, at several characteristic stages of the gonadal cycle. Gen Comp Endo 112:394-405

127. Okuzawa K, Amano M, Kobayashi M, Aida K, Hanyu I, Hasegawa Y, Miyamoto K 1990 Differences in salmon GnRH and chicken GnRH-II contents in discrete brain areas of male and female rainbow trout according to age and stage of maturity. Gen Comp Endocrinol 80:116-126

128. Wilson SC, Gladwell RT, Cunningham FJ 1990 Differential responses of hypothalamic LHRH-I and –II to castration and gonadal steroid or tamoxifen treatment in cockerels. J Endocrinol 125:139-146

129. Wilson SC, Chairil A, Cunningham FJ, Gladwell RT 1990 Changes in the hypothalamic contents of LHRH-I and –II and in pituitary responsiveness to synthetic chicken LHRH-I and –II during the progesterone-induced surge of LH in the laying hen. J Endocrinol 127:487-496

130. Millar RP, DEL Milton RC, Follett BK, King JA 1986 Receptor binding and gonadotropin-releasing activity of a novel chicken gonadotropin-releasing hormone ([His^5, Trp^7, Tyr^8] GnRH) and a D-Arg^6 analog. Endocrinology 119:224-231

131. King JA, Davidson JS, Millar RP 1988 Interaction of endogenous chicken gonadotrophin-releasing hormone-I and -II on chicken pituitary cells. J Endocrinol 117:43-49

132. Connolly PB, Callard IP 1987 Luteinizing hormone secretion from the quail pituitary in vitro. Bio Reprod 36:1238-1246

133. Nakamura T, Nagata T, Tanabe Y, Yanaihara N, Hasegawa Y 1991 Comparison of *in vivo* biological activities of luteinizing hormone releasing hormone (LHRH) analogues in 60-day-old cockerels. Gen Comp Endocrinol 83:290-296

134. Guemene D, Williams JB 1992 In-vitro and in-vivo responses to chicken LHRH-I and chicken LHRH-II in male turkeys (*Meleagris gallopavo*). J Endocrinol 132:387-393

135. Schulz RW, Bosma PT, Zandbergen MT, Van Der Sanden MCA, Van Dijk W, Peute J, Bogerd J, Goos HJTh 1993 Two gonadotropin-releasing hormones in the African catfish, *Clarias gariepinus*: localization, pituitary receptor binding, and gonadotropin release activity. Endocrinology 133:1569-1577

136. Schulz RW, van der Sanden MCA, Bosma PT, Goos HJTh 1994 Effects of gonadotrophin-releasing hormone during the pubertal development of the male African catfish (*Clarias gariepinus*): gonadotrophin and androgen levels in plasma. J Endocrinol 140:265-273

137. Ngamvongchon S, Sherwood NM, Warby CM, Rivier JE 1992 Gonadotropin-releasing hormone from Thai catfish: chromatographic and physiological studies. Gen Comp Endocrinol 87:266-274

138. Kohayashi M, Amano M, Kim M, Furukawa K, Hasegawa Y, Aida K 1994 Gonadotropin-releasing hormones of terminal nerve origin are not essential to ovarian development and ovulation in goldfish. Gen Comp Endocrinol 95:192-200

139. Zohar Y, Elizur A, Sherwood NM, Powell JFF, Rivier JE, Amora N 1995 Gonadotropin-releasing activities of the three native forms of gonadotropin-releasing hormone present in the brain of gilthead seabream, *Sparus aurata*. Gen Comp Endocrinol 97:289-299

140. Tsai P, Licht P 1993 *In vivo* GnRH responsiveness of LH secretion in the female turtle, *Trachemys scrpta*, in relation to the reproductive stage. Gen Comp Endocrinol 90:328-337

141. King JA, Millar RP 1986 Identification of His^5, Trp^7, Tyr^8-GnRH (chicken GnRH II) in amphibian brain. Peptides 7:827-834

142. Siler-Khodr TM, Grayson M 2001 Action of chicken II GnRH on the human placenta. J Clin Endo Metab 86:804-810

143. Advis JP, Contijoch AM, Urbanski HF, Ojeda SR 1988 N-Tosyl-L-Phenylalanine Chloromethyl Ketone Inhibits LHRH-degrading activity and increases *in vitro* LHRH release from the immature rat median eminence. Neuroendocrinol 47:102-108

144. Huang YP, Guo DC, Peter RE 1991 Isolation and characterization of a gonadotropin-releasing hormone binding protein in goldfish serum. Gen Comp Endocrinol 84:58-66

145. Lovejoy DA, King JA, Sherwood NM Peter RE 1993 Identification of gonadotropin-releasing hormone and associated binding substances in the blood serum of holocephalan (*Hydrolagus colliei*). Peptides 14:1237-1243

146. Millar RP, Flanagan CA, Milton RCdeL, King JA 1989 Chimeric analogues of vertebrate gonadotropin-releasing hormones comprising substitutions of the varian amino acids in positions 5, 7, and 8. J Biol Chem 264:21007-21013

147. Neill JD, Duck LW, Sellers JC, Musgrove LC 2001 A gonadotropin-releasing hormone (GnRH) receptor specific for GnRH II in primates. Biochem Biophys Res Comm 282:1012-1018

148. Millar R, Lowe S, Conklin D, Pawson A, Maudsley S, Troskie B, Ott T, Millar M, Lincoln G, Sellar R, Faurholm B, Scobie G, Kuestner R, Terasawa E, Katz A. 2001 A novel mammalian receptor for the evolutionarily conserved type II GnRH. Proc Natl Acad Sci USA. 98:9636-9641

149. Shiraishi M 1970 Further investigations on specificity and precision in the Igarashi-McCann FSH assay method. Endocrinologia Japonica 17:585-598

150. Phillips DJ, Moore LG, Hudson NL, Lun S, McNatty KP 1995 Effects of ovariectomy and FecBB genotype on the median charge and circulating half-life of pituitary FSH isoforms of ewes. J Reprod Fert 104:27-31

151. Gault PM, Lincoln GA Gonadotrophin releasing effects of exogenous GnRH II are mediated by GnRH I receptor. Proc 5th International Congress of Neuroendocrinology, Bristol, UK, 2002, P291

152. Fernald RD, White RB 1999 Gonadotropin-releasing hormone genes: phylogeny, structure, and functions. Frontiers Neuroendo 20, 224–240

153. Steiner RA, Clifton DK, Spies HG, Resko JA 1976 Sexual differentiation and the feedback control of luteinizing hormone secrtetion in rhesus monkeys. Biol Reprod 15:206-212

154. Wildt L, Marshall G, Knobil E. 1980 Experimental induction of puberty in the infantile female rhesus monkey. Science 207:1373-1375

155. El Majdoubi M, Sahu A, Plant TM 2000 Changes in hypothalamic gene expression associated with the arrest of pulsatile gonadotropin-releasing hormone release during infancy in the agonadal male rhesus monkey (*Macaca mulatta*). Endocrinology. 141:3273-3277

156. Ma YJ, Costa ME, Ojeda SR 1994 Developmental expression of the genes encoding transforming growth factor alpha and its receptor in the hypothalamus of female rhesus macaques. Neuroendocrinol 60:346-359

157. El Majdoubi M, Sahu A, Ramaswamy S, Plant TM 2000 Neuropeptide Y: A hypothalamic brake restraining the onset of puberty in primates. Proc Natl Acad Sci USA 97:6179-6184

158. Plant TM 2001 Neurobiological bases underlying the control of the onset of puberty in the rhesus monkey: a representative higher primate. Front Neuroendo 22:107-139

159. Urbanski HF 1991 Monoclonal antibodies to luteinizing hormone-releasing hormone: production, characterization, and immunocytochemical application. Bio Reprod 44:681-686

160. Franzoni MF, Viglietti-Panzica C, Ramieri G, Panzica GC 1984 A Golgi study on the neuronal morphology in the hypothalamus of the Japanese quail (*Coturnix coturnix japonica*). I. Tuberal and mammillary regions. Cell Tiss Res 236:357-364

161. Ferro VA, Khan AH, Latimer VS, Brown D, Urbanski HF, Stimson WH 2001 Immunoneutralisation of GnRH-I, without cross-reactivity to GnRH-II, in the development of a highly specific anti-fertility vaccine for clinical and veterinary use. J Repro Immuno 51:109-129

162.Benkirane M, Delori P, Lebec S, Cordeil M, Delaage M 1988 Production of monoclonal antibodies complementary to an antibody-antigen complex use in an immunoradiometric assay for follitropin. J Immunol Methods 111:189-194

163. Tougard C, Picart R, Tixier-Vidal A 1980 Immunocytochemical localization of glycoprotein hormones in the rat anterior pituitary. A light and electron microscope study using antisera against rat beta subunits: a comparison between preembedding and postembeeding methods. J Histochem Cytochem 28:101-114

164. Latimer VS, Rodrigues SM, Garyfallou VT, Kohama SG, White RB, Fernald RD, Urbanski HF 2000 Two molecular forms of gonadotropin-releasing hormone (GnRH-I and GnRH-II) are expressed by two separate populations of cells in the rhesus macaque hypothalamus. Mol Brain Res 75:287-292

165. Standish LJ, Adams LA, Vician L, Clifton DK, Steiner RA 1987 Neuroanatomical localization of cells containing gonadotropin-releasing hormone messenger ribonucleic acid in the primate brain by *in situ* hybridization histochemistry. Molec Endo 1:371-376

166. Conover CD, Kuljis RO, Rabii J, Advis J-P 1993 Beta-endocrphin regulation of luteinizing hormone-releasing hormone at the median eminence in ewes: immunocytochemical and physiological evidence. Neuroendocrinol 57:1182-1195

167. Russell JT, Brownstein MJ, Gainer H 1980 Biosynthesis of vasopressin, oxytocin, and neurophysins: isolation and characterization of two common precursors (propressophysin and prooxyphysin). Endocrinology. 107:1880-1891

168. Swanson LW, Sawchenko PE 1983 Hypothalamic integration: organization of the paraventricular and supraoptic nuclei. Ann Rev Neurosc 6:269-324

169. Amico JA, Hempel J 1990 An oxytocin precursor intermediate circulates in the plasma of humans and rhesus monkeys administered estrogen. Neuroendocrinol 51:437-43

170. Brownstein MJ, Russell JT, Gainer H 1980 Synthesis, transport, and release of posterior pituitary hormones. Science. 207:373-378

171. The Physiology of Reproduction, 2nd Ed. New York: Raven Press; 453-485 1994

172. Terasawa E 1995 Control of luteinizing hormone-releasing hormone pulse generation in nonhuman primates. Cell Molec Neurobio 15:141-164

173. Goldsmith PC, Thind KK, Perera AD, Plant TM 1994 Glutamate-immunoreactive neurons and their gonadotropin-releasing hormone-neuronal interactions in the monkey hypothalamus. Endocrinology 134:858-868

174. The Neurobiology of Puberty; Bristol: Journal of Endocrinology; 139-151 1995

175. Kasuya E, Nyberg CL, Mogi K, Terasawa E 1999 A role of gamma-amino butyric acid (GABA) and glutamate in control of puberty in female rhesus monkeys: effect of an antisense oligodeoxynucleotide for GAD67 messenger ribonucleic acid and MK801 on luteinizing hormone-releasing hormone release. Endocrinology. 140:705-712

176. Mitsushima D, Hei DL, Terasawa E 1994 Gamma-Aminobutyric acid is an inhibitory neurotransmitter restricting the release of luteinizing hormone-releasing hormone before the onset of puberty. Proc Natl Acad Sci USA 91:395-399

177. Khorram O, Pau KY, Spies HG 1988 Release of hypothalamic neuropeptide Y and effects of exogenous NPY on the release of hypothalamic GnRH and pituitary gonadotropins in intact and ovariectomized does in vitro. Peptides 9:411-417

178. Beck B, Jhanwar-Uniyal M, Burlet A, Chapleur-Chateau M, Leibowitz SF, Burlet C 1990 Rapid and localized alterations of neuropeptide Y in discrete hypothalamic nuclei with feeding status. Brain Res 528:245-249

179. Gore AC, Mitsushima D, Terasawa E 1993 A possible role of neuropeptide Y in the control of the onset of puberty in female rhesus monkeys. Neuroendocrinol 58:23-34

180. Seminara SB, Oliveira LM, Beranova M, Hayes FJ, Crowley WF Jr 2000 Genetics of hypogonadotropic hypogonadism. J Endocrinol Invest 23:560-565

181. Forsling ML, Akerlund M, Stromberg P 1981 Variations in plasma concentrations of vasopressin during the menstrual cycle. J Endo 89:263-266

182. Bealer SL, Crowley WR 1999 Stimulation of central and systemic oxytocin release by histamine in the paraventricular hypothalamic nucleus: evidence for an interaction with norepinephrine. Endocrinology. 140:1158-1164

183. Lado-Abeal J, Norman RL 1998 Absence of an inhibitory vasopressinergic tone on LH release in pubertal male rhesus macaques. Neuroendocrinol 67:236-243

184. Palm IF, van der Beek EM, Wiegant VM, Buijs RM, Kalsbeek A 2001 The stimulatory effect of vasopressin on the luteinizing hormone surge in ovariectomized, estradiol-treated rats is time-dependent. Brain Res 901:109-116

185. Recht LD, Hoffman DL, Haldar J, Silverman AJ, Zimmerman EA 1981 Vasopressin concentrations in hypophysial portal plasma: insignificant reduction following removal of the posterior pituitary gland. Neuroendocrinol 33:88-90

186. Lopez FJ, Merchenthaler IJ, Moretto M, Negro-Vilar A 1998 Modulating mechanisms of neuroendocrine cell activity: the LHRH pulse generator. Cell Molec Neurobio 18:125-146

187. Padmanabhan V, McFadden K, Mauger DT, Karsch FJ, Midgley AR Jr 1997 Neuroendocrine control of follicle-stimulating hormone (FSH) secretion. I. Direct evidence for separate episodic and basal components of FSH secretion. Endocrinology. 138:424-432

188. Samson WK, Snyder G, Fawcett CP, McCann SM. 1980 Chromatographic and biologic analysis of ME and OVLT LHRH. Peptides 1:97-102

189. Mizunuma H, Samson WK, Lumpkin MD, McCann SM 1983 Evidence for an FSH-releasing factor in the posterior portion of the rat median eminence. Life Sci 33:2003-2009

190. Knobil E 1974 On the control of gonadotropin secretion in the rhesus monkey. Rec Progr. Horm. Res. 30:1-46, 1974.

191. Karsch FJ 1987 Central actions of ovarian steroids in the feedback regulation of pulsatile secretion of luteinizing hormone. Ann Rev Physiol 49:365-382

192. Tsai PS, Licht P 1993 GnRH-induced desensitization of *in vitro* luteinizing hormone secretion in the turtle, *Trachemys scripta*. Gen Comp Endocrinol. 89:238-247

193. Urbanski HF, Garyfallou VT, Kohama SG, Hess, DL 1997 Alpha-adrenergic receptor antagonism and N-methyl-D-aspartate (NMDA) induced luteinizing hormone release in female rhesus macaques. Brain Res 744:96-104

194. Ljungqvist A, Feng DM, Hook W, Shen ZX, Bowers C, Folkers K 1988 Antide and related antagonists of luteinizing hormone release with long action and oral activity. Proc Natl Acad Sci USA. 85:8236-40

195. Ellinwood WE, Resko JA 1980 Sex differences in biologically active and immunoreactive gonadotropins in the fetal circulation of rhesus monkeys. Endocrinology 107:902-907

196. Huynh H & Feldt LS 1970 Conditions under which mean square ratios in repeated measurements designs have exact *F*-distributions. J Am Stats Assoc. 65:1582–1589

197. Findlay JK, Clarke IJ 1987 Regulation of the secretion of FSH in domestic ruminants. J Reprod Fert 34:27-37

198. Jennes L, Eyigor O, Janovick JA, Conn PM 1997 Brain gonadotropin releasing hormone receptors: localization and regulation Rec Prog in Horm Res. 52:475-491

199. Neill JD 2002 GnRH and GnRH receptor genes in the human genome. Endocrinology 143:737-43

200. Smith MA, Perrin MH, Vale WW 1983 Desensitization of cultured pituitary cells to gonadotropin-releasing hormone: evidence for a post-receptor mechanism Mol and Cell Endocrinol 30:85-96

201. Pieper DR, Gala SR, Regiana SR, Marshall JC 1982 Dependence of pituitary gonadotropin-releasing hormone (GnRH) receptors on GnRH secretion from the hypothalamus. Endocrinology 110:749-753

202. Gore AC, Roberts JL 1995 Regulation of gonadotropin-releasing hormone gene expression in the rat during the luteinizing hormone surge. Endocrinology. 136:889-896

203. Moenter SM, Karsch FJ, Lehman MN 1993 Fos expression during the estradiol-induced gonadotropin-releasing hormone (GnRH) surge of te ewe: induction in GnRH and other neurons. Endocrinology 133:896-903

204. Arimura A, Kastin AJ, Schally AV 1974 Immunoreactive LH-releasing hormone in plasma: midcycle elevation in women. J Clin Endocrinol Metab 38:510-513

205. Ferin M, Van Vugt D, Wardlaw S 1984 The hypothalamic control of the menstrual cycle and the role of endogenous opiod peptides. Rec Progr Horm Res 40:441-485

206. Pau K-Y, Gliessman PM, Hess DL, Ronnekleiv OK, Levine JE, Spies HG 1990 Acute administration of estrogen suppresses LH secretion without altering GnRH release in ovariectomized rhesus macaques. Brain Res 517:229-235

207. Gundlah C, Lu NZ, Mirkes SJ, Bethea CL 2001 Estrogen receptor beta (ERbeta) mRNA and protein in serotonin neurons of macaques. Brain Res Mol Brain Res 91:14-22

208. Gundlah C, Kohama SG, Garyfallou VT, Urbanski HF, Bethea CL 2000 Distribution of estrogen receptor β (ER β) mRNA in hypothalamus, midbrain and temporal lobe of spayed macaques: continued expression with hormone replacement. Mol Brain Res 76:191-204

209. Goodman RL, Karsch FJ 1980 Pulsatile secretion of luteinizing hormone: differential suppression by ovarian steroids. Endocrinology 107:1286-1290

210. Liu T-C, Pu H-F, Wang PS 1993 Unimpaired postreceptor regulation of luteinizing hormone secretion by gonadotropin-releasing hormone and estrogen in aged rat anterior pituitary cells. Endocrinology 132:1189-1194.

211. Richter TA, Terasawa E 2001 Neural mechanisms underlying the pubertal increase in LHRH release in the rhesus monkey. Trends Endocrinol Metab 12:353-9

212. Bosma PT, Rebers FEM, vanDijk W, Willems PHGM, Goos HJTh, Schulz RW 2000 Inhibitory and stimulatory interactions between endogenous gonadotropin-releasing hormones in the African catfish (*Clarias gariepinus*). Bio Reprod 62:731-738