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Neuromodulation of luteinizing hormone secretion in the mouse

Miller, Gregory Michael, Ph.D.

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U·M·I
300 N. Zeeb Rd.
Ann Arbor, MI 48106

NEUROMODULATION OF LUTEINIZING HORMONE SECRETION IN THE MOUSE

by

GREGORY M. MILLER

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Executive Officer

Ann-Judith Silverman

Joseph Goldfarb

Marilyn McGinnis

James Roberts

Supervisory Committee

THE CITY UNIVERSITY OF NEW YORK

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I dedicate this work to my wife Amy, who has always guided me and is the foundation of my life.

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CHAPTER 1: GENERAL INTRODUCTION

Gonadotropin-releasing hormone (GnRH) is the primary regulatory neuropeptide of reproductive physiology. The neurons which synthesize GnRH are born in the olfactory placode and migrate into the brain during embryonic development (1,2). In the adult rodent, a large percentage of GnRH neurons form a prominent network of cells, which are scattered in a loose continuum spanning the telencephalic diagonal band of Broca and more dorsal septal nuclei, the bed nucleus of the stria terminalis, the periventricular, medial and lateral preoptic areas, anterior hypothalamic areas, the retrochiasmatic zone medial to the optic tract, as well as the lateral hypothalamus and occasionally the supraoptic nucleus (3). In the mouse, the majority (but not all) of these neurons project to the median eminence (4).

GnRH is released into the hypophysial portal veins in pulses (5). These pulses stimulate coordinate pulses of the gonadotropins, Luteinizing Hormone (LH) and Follicle-stimulating Hormone (FSH), into the general circulation. Pulses of LH and FSH, in turn, stimulate gametogenesis and steroidogenesis in the gonads. Steroids and other substances secreted from the gonads feed back on the pituitary and brain to modulate further hormone secretion. The central feedback of gonadal steroids is at least partially via interneurons, since GnRH neurons themselves lack estrogen receptors (6). Likewise, other afferents communicate environmental, social and physiological information, necessary to coordinate GnRH neuronal activity to ensure maximal reproductive efficacy.

Recent findings suggest that pulsatility is intrinsic to GnRH neurons. Studies utilizing an immortalized GnRH-secreting cell line (GT1-7) derived from targeted tumorigenesis in transgenic mice (7) have demonstrated that these cells secrete distinct pulses of GnRH in perifusion chambers in vitro (8). This finding in transformed cells implies that GnRH neurons within the mouse brain may intrinsically be pulsatile, and that other neuronal systems which impinge on GnRH neurons may serve to modify the pattern or amplitude of these pulses. In this dissertation I present evidence that the actions of the excitatory amino acid analog N-methyl-D,L-aspartic acid (NMA) on LH secretion are mediated through afferents to GnRH neurons, and that opioid-containing afferents can modulate GnRH release in the mouse.

In my initial studies in normal (C3H/HeHx101H) mice, I found that the mouse differs from most other species in that the opiate antagonist Naloxone (NAL) fails to elicit LH release. I employed a paradigm of testing opiate antagonists in combination with NMA to reveal the role of endogenous opioids on LH secretion in the mouse. Chapter 2 describes experiments which demonstrate that endogenous opioid peptides modulate NMA-stimulated LH release in young adult but not older normal male mice by affecting GnRH release.

The remaining chapters investigate the neuromodulation of LH release in hypogonadal (HPG) mice with GnRH-secreting cell implants. Due to a truncated GnRH gene (9), HPG mice lack a functional network of GnRH-synthesizing neurons. The adult mouse

has infantile gonads and is infertile (10). Chapters 3 and 4 utilize HPG mice implanted with normal fetal preoptic area (POA) tissue into the third ventricle. These POA grafts provide a source of GnRH neurons, which can integrate with the host brain and restore many aspects of reproductive physiology (11). While the number of GnRH neurons in POA grafts varies considerably between individuals, immunocytochemistry for GnRH in the brains of HPG mice with POA brain grafts (HPG/POA) reveals that as few as one detectable GnRH neuron can support ovulation, pregnancy and maternal behavior in females (12). Chapter 5 utilizes female HPG mice implanted with immortalized GT1-7 cells to determine whether this tumorigenic cell line can support pulsatile LH release and/or NMA-stimulated LH secretion in situ.

My studies present the first evidence that the endogenous opioid system can modulate GnRH release in the mouse. The studies in HPG mice with GnRH-secreting cell grafts further establish that the effects of NMA on LH release are via afferents to GnRH neurons, which may also, in part, communicate information regarding steroid milieu.

CHAPTER 2: ENDOGENOUS OPIOID MODULATION OF N-METHYL-D,L-ASPARTIC ACID-STIMULATED LUTEINIZING HORMONE RELEASE IN YOUNG ADULT BUT NOT OLDER MALE MICE.

Studies in many species have demonstrated that the endogenous opioid system influences Luteinizing Hormone (LH) secretion. Immunocytochemical evidence in the rat indicates that endogenous opioid peptides derived from proenkephalin, prodynorphin and proopiomelanocortin are in close anatomical proximity to Gonadotropin-releasing Hormone (GnRH) neurons (1), and β-endorphin-containing synapses directly on GnRH neurons have been observed (2,3). Pharmacological studies in the rat suggest that opioid peptides act on other neurochemical systems, including catecholamines (4-9; however, see 10) and gamma-aminobutyric acid (GABA) (11-14), to modulate GnRH release. The opiate antagonist Naloxone (NAL) stimulates LH release in the gonadally intact adult male of many (15-23) but not all (24-26) species studied to date. However, the role of the endogenous opioid system in the regulation of LH release in the mouse has not been studied.

The excitatory amino acid analog, N-methyl-D,L-aspartic acid (NMA) induces LH secretion in a variety of species (27-38), including mice (39). NMA is believed to act via a hypothalamic mechanism which results in GnRH release, since in the rat, NMA does not elicit LH release from the pituitary <u>in vitro</u> (28,40) and a GnRH antagonist can block NMA-stimulated LH release (29,31). It is unknown whether GnRH neurons themselves possess functional receptors for this ligand, or whether the LH stimulatory response is due to the effects of NMA on afferents to GnRH neurons, or both.

Several studies suggest that the effects of NMA on GnRH release may be mediated by other neurotransmitter systems, rather than a direct effect on GnRH neurons themselves. Cicero, et al (29), demonstrated that morphine attenuates NMA-induced LH release in the male rat, and others have described a similar action of GABA (41) and baclofen (42). Saitoh, et al (43), showed that NMA induces cfos protein expression, a marker of neuronal activation, in the nuclei of several candidate neuromodulatory afferents to GnRH neurons, including catecholamine, corticotropin-releasing factor, and β-endorphin neurons, but not in GnRH neurons themselves, even though significant release of LH occurs. It is therefore likely that the action of NMA on LH release is the summation of both excitatory and inhibitory influences on GnRH neurons, mediated by afferents to GnRH neurons.

The following experiments examined the role of the endogenous opioid system in the regulation of LH release in adult male mice, and tested whether this system is a component of NMA-stimulated LH release. Since the LH response to NMA (29) and NAL (44) diminishes with age in the male rat, both young adult (10-to
14-week-old) and older (10- to 16-month-old) male mice were evaluated. I assessed whether opioid peptides were able to affect LH secretion at a location outside of the blood-brain barrier by using the quaternary derivative of NAL, naloxone methiodide (NALMI). Quaternary derivatives of NAL are

inefficient in crossing the blood-brain barrier (45), yet are just as effective as NAL at stimulating LH release (46) or antagonizing morphine inhibition of LH release (47) in the rat. Since I found an effect of opiate antagonists in young but not older male mice, I determined whether NAL could alter LH release at the level of the gonadotrope in the younger mice. I first established a dose-response of exogenous GnRH-induced LH release and then assessed the effects of NAL concurrent with low and high doses of GnRH on LH release. Finally, I administered GnRH challenges to older male mice to determine whether pituitary sensitivity to GnRH was different between the two age groups.

METHODS

Animals:

All mice (C3H/HeH x 101H) were housed in a 14:10 LD (lights out at 1500h) schedule with food and water available ad libitum. An initial group of intact male mice, ranging in age from 3 to 6 months, were used to assess the actions of NAL on LH secretion. For other experiments, young adult male mice were naive and were 10- to 14-weeks-old at the time of testing. Male siblings with similar bodyweights were divided among treatment groups. Older male mice were 10- to 16-months-old at the time of testing, and had a history of successful mating.

Intracardial cannulation:

Cannulae were prepared from 25cm lengths of Silastic tubing

(id, 0.030 cm; od, 0.0635 cm; Dow-Corning, Midland, MI) on which a ball of 100% medical grade silicone rubber was formed at 20mm and allowed to harden. At the time of surgery, this end of the cannula was shortened and beveled at 11-12mm, or up to 15mm for larger mice. The silicon rubber ball was then trimmed into a rectangular shape, such that the point of the bevel corresponded with a thickened section of the silicone rubber, and forceps could grip the ball during surgery. The cannula was then rinsed with sterile saline and zepharin chloride solution (1ml:750ml distilled H_2O). A 1-cc syringe with a blunted 26ga needle was filled with 3U heparin/ml bacteriostatic saline and attached to the long arm of the cannula. This assembly was then placed on a sterile surface.

All mice were fitted with an intracardial cannula under chloral hydrate anesthesia (360mg/kg, ip) during morning hours. Anesthetized mice were shaved and placed on an operating stage under a dissection microscope. Following isolation of the right jugular vein, the canula was slid into the vein through a small incision until the silicone rectangle reached the opening in the vein. The cannula was then adjusted and secured to the vein with sterile surgical silk tied in front of and behind the silicone rectangle. A stitch was ran through the silicone rectangle attaching it to adjacent skin and muscle tissue. The cannula was threaded subcutaneously behind the ear to exit the mouse dorsally through an incision at the base of the scalp. A modified 1-cc syringe top was secured to the base of the scalp

and served to house the cannula when it was not in use. The cannula was filled with 20U heparin/ml bacteriostatic saline, plugged and sealed with silicone rubber, wiped with an alcohol swab, and coiled into the syringe top housing. Parafilm was wrapped around the housing. Following surgery, each mouse was placed in a clean cage and returned to the colony room awake prior to lights out.

Blood sampling and drug administrations:

All blood sampling was done during morning hours. All 10- to 14-week-old males as well as 3- to 6-month-old males treated only with NAL were tested once on the day following surgery. Older males were tested repeatedly on consecutive days starting on the day following surgery. At the time of the experiment, a 1cc syringe filled with 3U heparin/ml bacteriostatic saline was attached to the cannula and then suspended above each cage, leaving the mouse completely unrestrained. Food and water were supplied.

Blood samples (100μl) were collected from awake and freely behaving mice in their home cages at 10 min intervals before and after drug challenges. After centrifugation, plasma fractions (60μl) were flash frozen in an ethanol and dry ice bath and stored at -20C until RIA. Throughout an experiment, blood cells from samples were resuspended in steroid free (50) human serum albumin (5%, 60 μl; Armour Pharmaceutical Co., Kankakee,IL) and introduced back into the host via the cannula immediately

following the next sample withdrawn or a drug administration.

All drugs were administered intravenously through the cannula following a blood sample. NMA (Sigma Chemical Co., St. Louis, MO) was administered at a dose of 20mg/4.0ml saline/kg body weight. NAL (Sigma Chemical Co., St. Louis, MO) and NALMI (Research Biochemicals Inc., Natick, MA) were administered at a dose of 3mg/2.0ml saline/kg bodyweight unless otherwise specified. Saline (SAL) was administered as 2.0ml/kg body weight. GnRH (Sigma Chemical Co., St. Louis, MO) doses were administered as ng GnRH/0.1ml saline.

Experimental procedures:

1. To assess whether NAL was capable of elevating LH levels in intact male mice, I administered NAL at a range of doses (0.1mg-20mg/2.0 ml saline/kg body weight), either once or repeatedly. Blood samples were collected at -20, -10, 0, +10, +20, +30, +45, +60, and +90 min relative to the NAL challenge.

2. To test whether opiate antagonists affected LH responses to NMA in 10- to 14-week-old male mice, separate groups (n=8, each group) were challenged with NAL, NALMI or SAL in combination with NMA. Each mouse received two NMA challenges 2h apart at times designated 0 and +120, and NAL, NALMI or SAL were administered following the +80 blood sample and again immediately prior to the second NMA challenge. Blood samples were collected at -10, 0, +10, +20, +80 +90, +100, +120, +130,

and +140 min.

3. To establish a dose response for exogenously administered GnRH, 10- to 14-week-old male mice were treated with a single GnRH challenge at one of the following doses; 0, 2.5, 25, 50, 100, and 200ng GnRH (n=5-13/dose). Blood samples were collected at -10, 0, +10, +20, and +30 min relative to the GnRH or vehicle challenge.

4. To evaluate whether opiate-antagonist-induced changes in LH release following NMA were due to an increase in GnRH secretion or to altered pituitary responsivity to GnRH, the effect of NAL combined with GnRH was evaluated. Separate groups of 10- to 14-week-old male mice (n=7-10/group) were challenged with NAL or SAL in combination with either 1.75ng GnRH or 50ng GnRH. Blood samples were collected at -40, -30, -20, 0, +10 and +20 min, relative to the GnRH challenge. NAL or SAL was administered immediately following the -40 blood sample and again immediately preceding the GnRH challenge.

5. To test whether opiate antagonists affected LH responses to NMA in 10- to 16-month-old male mice, each mouse (n=8) was tested on three consecutive days with NAL, NALMI or SAL in combination with NMA. Each mouse received all three treatments, and treatment order was randomized between mice. Each mouse received two NMA challenges 2h apart at times designated 0 and

+120, and NAL, NALMI or SAL were administered following the +80 blood sample and again immediately prior to the second NMA challenge. Blood samples were collected at -10, 0, +10, +20, +80 +90, +100, +120, +130, and +140 min.

6. To estimate whether enhanced LH secretion to NMA in 10- to 16-month-old male mice vs. 10- to 14-week-old male mice was due to increased GnRH secretion or to increased pituitary responsivity to GnRH, 1.75ng and 50ng GnRH challenges were given to 10- to 16-month-old male mice (n=9) on separate consecutive days, in a counterbalanced design. Blood samples were collected at -10, 0, +10, +20, and +30 min relative to the GnRH or vehicle challenge.

RIA for plasma LH

RIA of LH was performed with the kit for rat LH (provided by the NIDDK through the National Hormone and Pituitary Program), with rat (r) LH RP-2 as a reference standard. Iodinated rLH (Hazleton, Vienna, VA) was used as a tracer. Plasma samples (25μl) were assayed in duplicate. All samples from a given subject were in the same assay. Samples from mice in different treatment groups within an experiment were evenly represented across assays. Each assay was centrifuged in its entirety, and a standard curve and plasma pool was run in every assay. The mean intraassay coefficient of variation, calculated on a mouse plasma pool (n=7-10), was 9.8%, and the mean interassay

variation, calculated from values obtained by assaying this pool in each assay, was 9.3%.

Statistics

Significant differences between time points within a treatment group and between treatment groups were assessed by a one- or two-way ANOVA with repeated measures as appropriate, followed by Tukey's HSD post hoc comparisons when ANOVA was significant ($p<0.05$).

RESULTS

Effect of NAL on LH release:

NAL failed to elicit significant LH release in male mice. Representative data from male mice (n=8) treated with NAL (3mg/2.0ml saline/kg body weight) is shown in Fig. 2-1. Other male mice treated with other doses of NAL (0.1mg/kg to 20mg/kg, administered once or repeatedly) also failed to respond with elevations in plasma LH levels (data not shown).

Effect of opiate antagonists combined with NMA on LH release in 10- to 14-week-old male mice.

Significant increases in plasma LH levels occurred following each NMA challenge ($p<0.01$), but there were no changes in LH secretion after NAL, NALMI, or SAL administered alone (Fig. 2-2). When the second NMA challenge followed pretreatment with NAL or NALMI, LH release was significantly greater than that in

response to the first NMA challenge ($p<0.01$; Fig. 2-2a,2-2b). However, there was not a significant difference in LH responses to NMA with or without SAL pretreatment (Fig. 2-2c). Further, LH values in response to NMA in the presence of either opiate antagonist were significantly higher than the plasma LH level after NMA with SAL pretreatment ($p<0.01$).

Response to exogenously administered GnRH in 10- to 14-week-old male mice.

Significant elevations in plasma LH levels occurred at +10 min following all GnRH challenges in a dose dependent fashion ($p<0.01$; Fig. 2-3). Mice receiving a 25ng GnRH challenge had significantly greater plasma LH values measured at +10 ($p<0.05$) and +20 ($p<0.01$) min than mice which received 2.5ng GnRH, and significantly lower values than mice which received 50ng GnRH at +10 ($p<0.01$), +20 ($p<0.01$) and +30 ($p<0.05$) min. There were no significant differences in plasma LH values between mice treated with 50ng, 100ng or 200ng GnRH challenges at +10 or +20 min.

Effect of NAL combined with GnRH on LH release in 10- to 14-week-old male mice.

NAL or SAL administered alone at -40 min did not affect plasma LH levels, nor was there an effect of NAL pretreatment on GnRH-challenged LH release at either dose of GnRH tested (Fig. 2-4). Mice challenged with 50ng GnRH had significantly higher LH levels than mice treated with 1.75ng GnRH challenges at +10

and +20 min, regardless of NAL or SAL pretreatments ($p<0.01$).

Effect of opiate antagonists on LH release and the LH response to NMA in 10- to 16-month-old male mice.

Significant elevations in plasma LH levels occurred following both NMA challenges on all three days ($p<0.01$). Neither opiate antagonist was effective at stimulating LH release or altering the LH secretory response to NMA (Fig. 2-5). There were no significant differences between the first and second NMA challenges for any pretreatment. NMA-induced increases in plasma LH were significantly larger in 10- to 16-month-old mice vs 10- to 14-week-old mice ($p<0.01$).

Effect of exogenous GnRH in old male mice.

A separate group of 10- to 16-month-old mice (n=9) were tested on two consecutive days with 1.75ng GnRH and 50ng GnRH in a counterbalanced design. Since treatment order was not significant at any time point measured, the data from day 1 and day 2 for the same treatments were combined. Plasma LH values at +10 min following either dose of GnRH were not significantly different. However, the 50ng GnRH challenge resulted in significantly higher plasma LH values measured at 20 and 30 min, compared with the 1.75ng challenge ($p<0.01$; Fig. 2-6). The LH response to 50ng GnRH was significantly lower in 10- to 16-month-old mice vs 10- to 14-week-old mice measured at +10 min ($p<0.01$), but was not significantly different when measured at

the +20 or +30 time points.

DISCUSSION

This is the first study to demonstrate an action of endogenous opioid peptides on the GnRH neuronal system in mice. My results of significant opiate-antagonist-induced elevations in NMA-stimulated LH release in 10- to 14-week-old male mice support the hypothesis that NMA stimulates opioidergic neurons, which release inhibitory opioid peptides capable of affecting GnRH release at a suprapituitary level outside of the blood-brain barrier. Further, a change in this neural system occurs with aging, such that the magnitude of GnRH release is increased and opioid inhibition is absent following an NMA challenge in older male mice. These findings are evidence that in the mouse, the actions of NMA on LH release are modulated via afferents to GnRH neurons.

In my initial studies, I tested the ability of a range of doses of NAL to stimulate LH release. Unlike many other species, male mice did not respond to opiate antagonists with elevations in plasma LH levels. This suggests that a tonic opioid inhibition on the GnRH neuronal system is absent in the male mouse. To further explore the endogenous opioid system in the mouse, I chose a single dose of NAL to use in combination with NMA and GnRH, based on studies in other species. Opiate antagonist pretreatment resulted in a significantly enhanced LH secretory response to NMA in 10- to 14-week-old male mice.

Since opiate antagonists were ineffective in stimulating LH release when administered alone, it is likely that one of the actions of NMA is the release of endogenous opioid peptides in an appropriate location to affect LH release.

Since in vitro studies have reported that NAL enhances (51-53) or inhibits (53) LH release from the pituitary, I determined whether the opiate-antagonist-induced potentiation of NMA-stimulated LH release in 10- to 14-week-old male mice was due to the actions of NAL at the pituitary. I first established a dose response for exogenously administered GnRH. This allowed me to define a dose of GnRH which would elicit LH release of the same magnitude as an NMA challenge in young male mice and one which elicited maximal LH secretion. NAL pretreatment had no effect on pituitary responses to systemic GnRH challenges at either dose of GnRH tested, supporting the hypothesis that NMA stimulated the release of endogenous opioids. These findings do not exclude the pituitary as a possible site of opioid action on LH release in the mouse, but rather, indicate that the observed action of NAL and NALMI enhancement of NMA-stimulated LH release is not at the level of the pituitary.

One explanation of these data is that opiate antagonists act to increase GnRH release, since larger doses of GnRH stimulated a greater magnitude of LH release. Since NALMI was equally as effective as NAL at potentiating NMA-stimulated LH release, a site of action of endogenous opioids may be outside of the blood-brain barrier at either the organum vasculosum of the

lamina terminalis, or at the GnRH terminals themselves in the median eminence. Opioid-containing fibers appear to terminate on or near GnRH neuronal elements in both of these locations (1), and there is evidence of opioid binding sites on GnRH terminals in the mediobasal hypothalamus of the male rat (54). However, the present studies utilized large doses (3mg/kg) of both opiate antagonists. It is possible that opiate-antagonist-induced potentiation of NMA-stimulated LH release would occur at much lower doses of NAL. In such a case, a sufficient amount of NALMI may cross into the brain to cause an effect at a location inside of the blood-brain barrier. While this issue remains unresolved, the current studies did find a similar statistically significant effect of both opiate antagonists in independent groups, attesting to the reliability of the significant finding of opiate-antagonist-induced potentiation of NMA-stimulated LH release.

The present studies did not address the issue of which opioid receptors are involved in the regulation of NMA-stimulated LH release. NAL, particularly at doses employed in the current studies, may antagonize a variety of opioid receptor subtypes. There are, however, studies which would suggest that the effects of NAL or NALMI are mediated through an action at mu-opioid receptors. Treatment of rat medial basal hypothalamic explants with NAL produced an increase in the release of GnRH which could be blocked by the mu-opioid specific agonist sufentanil, but not by the delta-opioid receptor agonist D-

Alanine,D-Leucine-enkephalin (DADL) or the kappa-opioid receptor agonist Ethylketocyclazocine (EKC) (55). Likewise, NAL stimulated LH release in young adult male rats, whereas ICI 174864 or MR2266, which block delta-opioid and kappa-opioid receptors, respectively, were unable to stimulate LH release (56).

Opiate-antagonist-induced elevations in NMA-stimulated LH release were absent in 10- to 16-month-old male mice. Further, these older mice had significantly greater LH responses to NMA as compared with 10- to 14-week-old male mice, and changes in pituitary responsivity to GnRH did not account for these higher LH responses. One explanation is that in older mice, the endogenous opioid component of NMA-stimulated LH release is lost. This hypothesis is congruent with reports of age-related decreases in proopiomelanocortin gene expression in the arcuate nucleus (57) and in the number of mu-opioid receptors in the hypothalamus (58) in the male rat brain. The loss of inhibitory regulation of the GnRH system could result in greater GnRH release in response to NMA in older mice.

Aside from endogenous opioid involvement in NMA-stimulated LH release, it is likely that other neurochemical systems stimulated by NMA mediate the excitatory action of NMA on GnRH release. Saitoh, et al (43) suggest that an excitatory component of NMA-stimulated LH release may be due to norepinephrine. Although there are no pharmacological reports of catecholamine involvement in LH release in the mouse, Saitoh, et

al (43) have reported that NMA induces cfos in a large percentage of neurons containing norepinephrine and dopamine in the mouse brain, in regions likely to project to areas containing GnRH neurons. A much smaller percentage of β-endorphin-immunoreactive neurons were also cfos positive, and these neurons are a likely source of opioid peptide during NMA stimulation.

The apparent inability of opiate antagonists to effect LH release when administered alone to male mice may be due to a species difference, or may be due to my experimental conditions. Although mice recovered from surgery quickly and remained behaviorally active during testing, I cannot exclude that the stress of blood sampling may be greater in mice than in other larger species, and may affect pharmacological responses. On the contrary, NAL administered subcutaneously to mice without any previous surgery fails to elicit LH secretion (as measured from trunk blood following decapitation (unpublished observations). Further, endogenous LH pulsatility has been demonstrated in cannulated male (59) and female (48) mice undergoing a more prolonged period of blood sampling, and these studies utilized heparinized saline rather than human serum albumin to resuspend red blood cells for return to the mouse during sampling.

In summary, this is the first study which illustrates a role for the endogenous opioid system in the modulation of the hypothalamic-pituitary-gonadal axis in the mouse. The opiate

antagonists tested in these studies did not stimulate LH release when administered alone, yet potentiated the LH secretory response to NMA in young adult male mice. It is not yet known whether endogenous opioid activation is involved in the physiological modulation of GnRH release in the male mouse. I hypothesize that NMA stimulates opioidergic neurons, and that opioid release decreases the amount of GnRH secreted at the median eminence in response to NMA. With age, either the sensitivity of this population of opioidergic neurons to NMA is diminished, or the afferent influence of these neurons on the GnRH neuronal system is no longer viable. These studies add further evidence that NMA-stimulated LH release is modulated via afferents to GnRH neurons.

Figure Legends

Fig. 2-1. Effect of NAL (3mg/2.0 ml saline/kg body weight) on plasma LH levels in 3- to 6-month old male mice (n=8). There was no significant effect of NAL on plasma LH levels. This data is representative of other male mice challenged with other doses of NAL (0.1mg/kg to 20mg/kg).

Fig. 2-2. Effect of NAL (3mg/2.0ml saline/kg body weight), NALMI (3mg/2.0ml saline/kg body weight) or SAL (2.0ml/kg body weight) administered alone and combined with NMA (20mg/4.0ml saline/kg body weight) on plasma LH levels in 10- to 14-week-old male mice. Separate groups (n=8/group) received two NMA challenges, following the 0 and +120 blood samples. NAL (a), NALMI (b), or SAL (c) was administered following the +80 blood sample and again immediately prior to the second NMA challenge. There were no significant effects of NAL or NALMI alone on plasma LH levels. **: $p<0.01$ vs. baseline values. ϕ: $p<0.01$ vs. all other time points.

Fig. 2-3. Effect of different doses of GnRH on plasma LH levels in 10- to 14-week-old male mice. Separate groups of mice were challenged with 0ng (n=13), 2.5ng (n=8), 25ng (n=13), 50ng (n=5), 100ng (n=5) and 200ng (n=5) GnRH/0.1ml saline following the 0 blood sample. See text for significance.

Fig. 2-4. Effect of NAL or SAL combined with a GnRH challenge on plasma LH levels in 10- to 14-week-old male mice. Separate groups were pretreated with NAL (n=10) or SAL (n=10) and were challenged with 1.75ng GnRH. Two other groups were pretreated with NAL (n=7) or SAL (n=7) and challenged with 50ng GnRH. There were no significant effects of NAL alone or in combination with either dose of GnRH on LH secretion.

Fig. 2-5. Effect of NAL, NALMI or SAL administered alone and combined with NMA on plasma LH levels in 10- to 16-month-old male mice. Each mouse (n=8) received two NMA challenges, following the 0 and +120 blood samples on three consecutive days. On each day, mice were pretreated with NAL (a), NALMI (b), or SAL (c), administered following the +80 blood sample and again immediately prior to the second NMA challenge. Each mouse received all treatments, and treatment order was randomized among mice. There were no significant effects of NAL or NALMI alone on plasma LH levels. There were no significant differences between LH responses to the first vs. second NMA challenge for any of the three treatments. **: $p<0.01$ vs. baseline values.

Fig. 2-6. Effects of a 1.75ng or 50ng GnRH challenge on plasma LH levels in 10- to 16-month-old mice. Each mouse (n=9) was tested with both doses on separate consecutive days in a counterbalanced design, and data were combined. **: $p<0.01$ vs.

same time point after lower dose.

Fig. 2-1

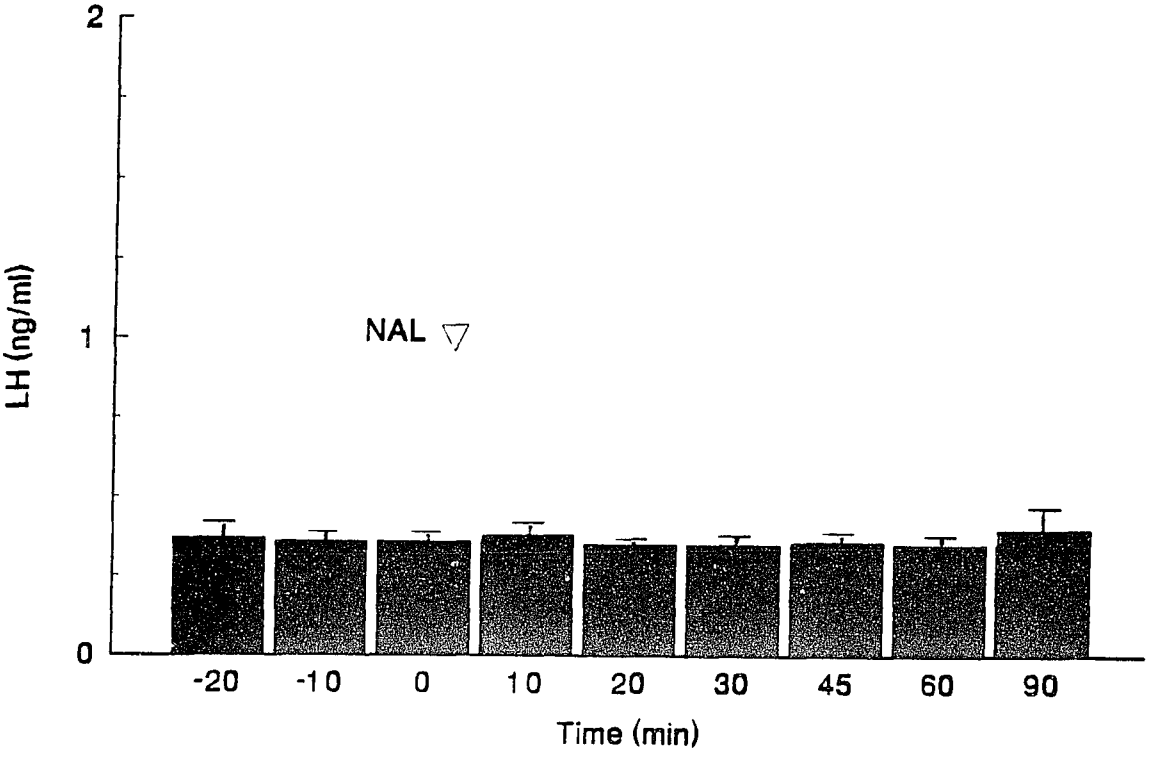
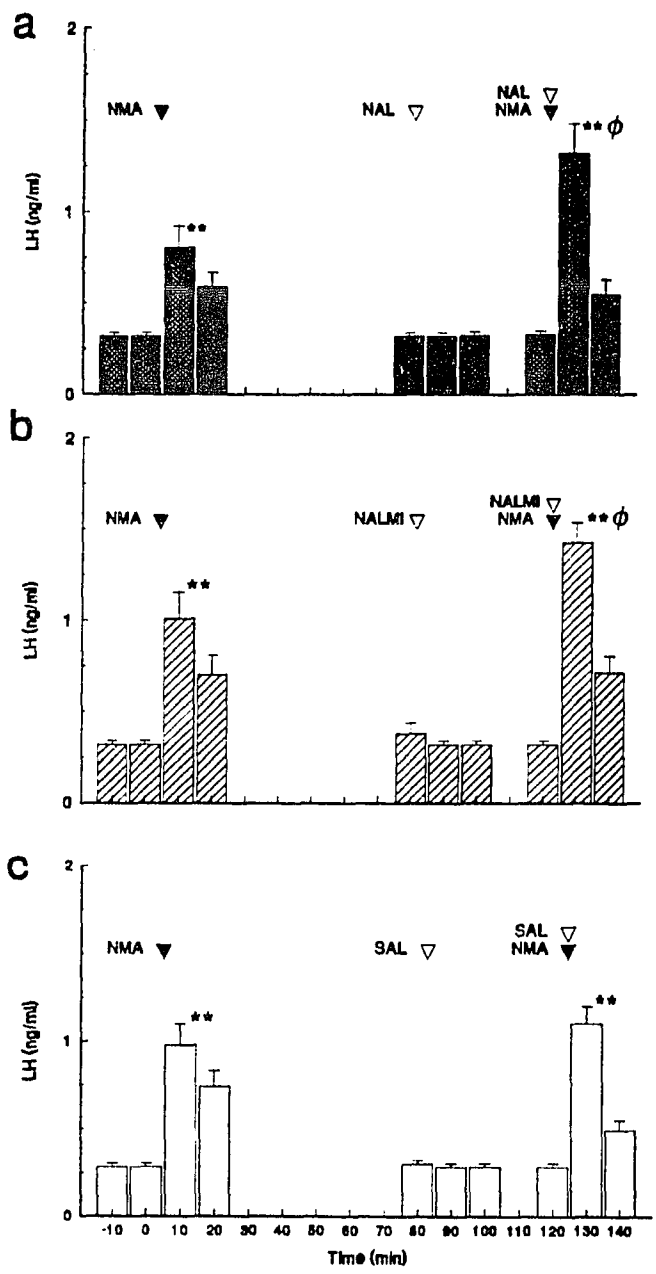


Fig. 2-2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SAL
LH (ng/ml)
Time (min)
-10 0 10 20 30 40 50 60 70 80 90 100 110 120 130 140

Fig. 2-3

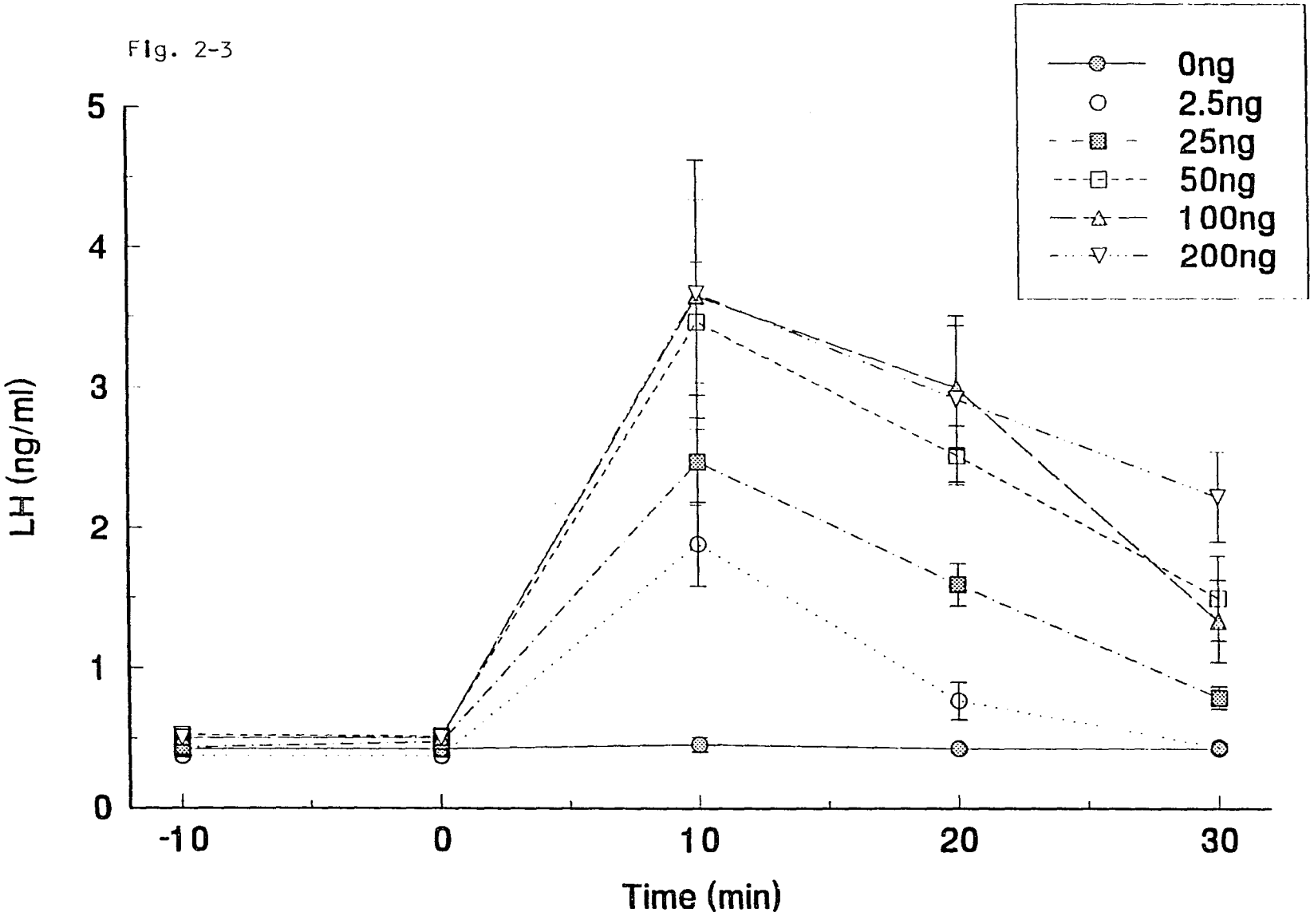

0ng
2.5ng
25ng
50ng
100ng
200ng
LH (ng/ml)
5
4
3
2
1
0
-10
0
10
20
30
Time (min)

Fig. 2-4

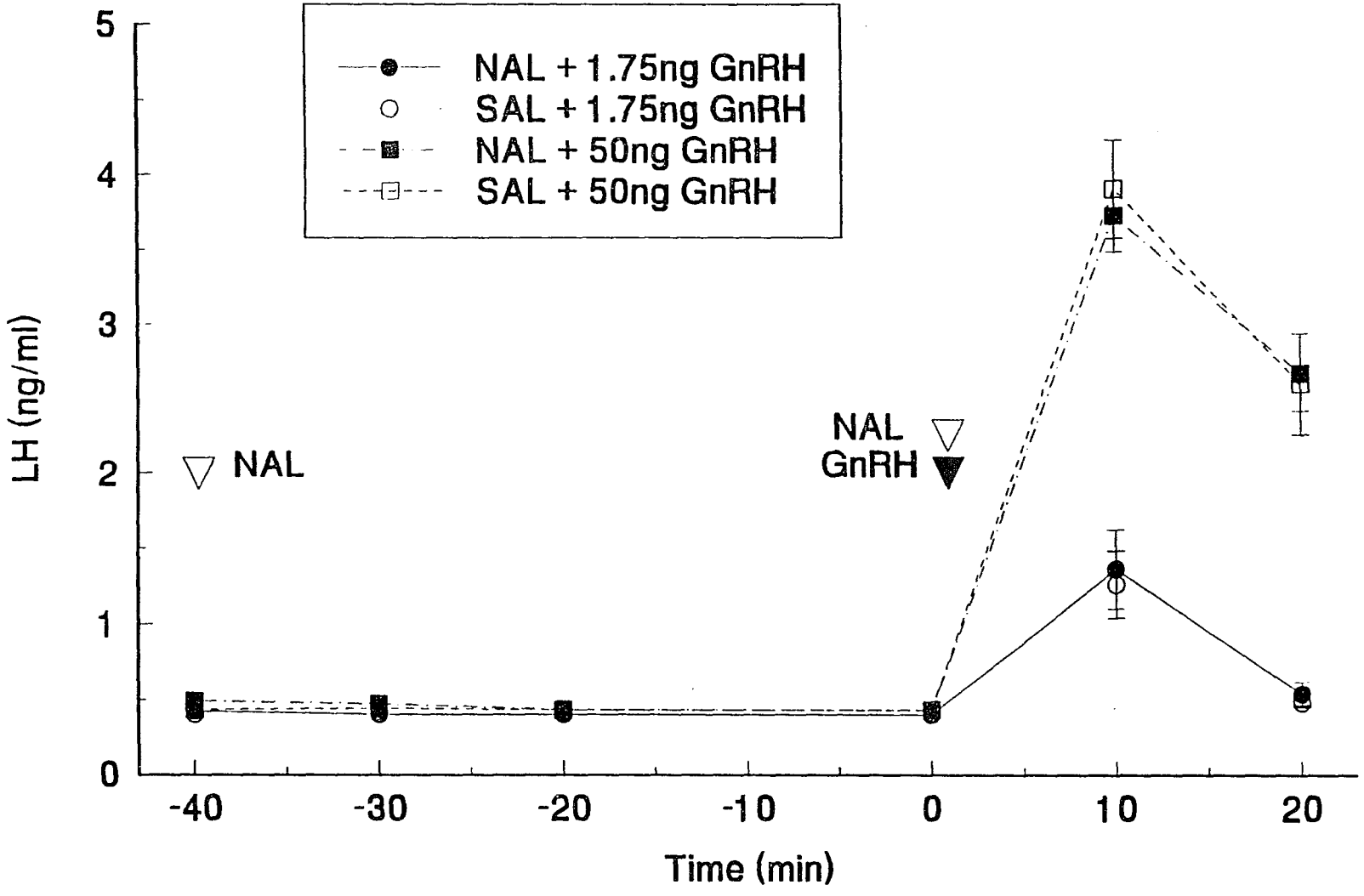
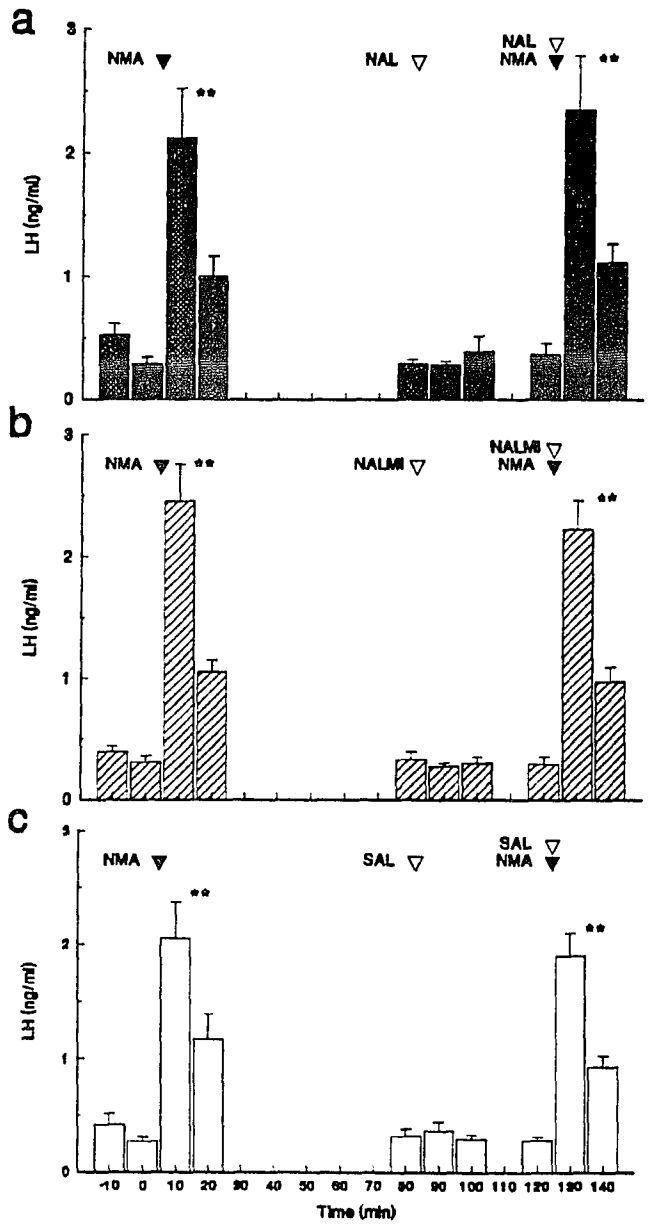

NAL + 1.75ng GnRH
SAL + 1.75ng GnRH
NAL + 50ng GnRH
SAL + 50ng GnRH
LH (ng/ml)
5
4
3
2
1
0
NAL
NAL
GnRH
-40
-30
-20
-10
0
10
20
Time (min)

Fig. 2-5


a
NMA
NAL
NAL
NMA
**
LH (ng/ml)
b
NMA
NALMI
NALMI
NMA
**
LH (ng/ml)
c
NMA
SAL
SAL
NMA
**
LH (ng/ml)
-10 0 10 20 30 40 50 60 70 80 90 100 110 120 130 140
Time (min)

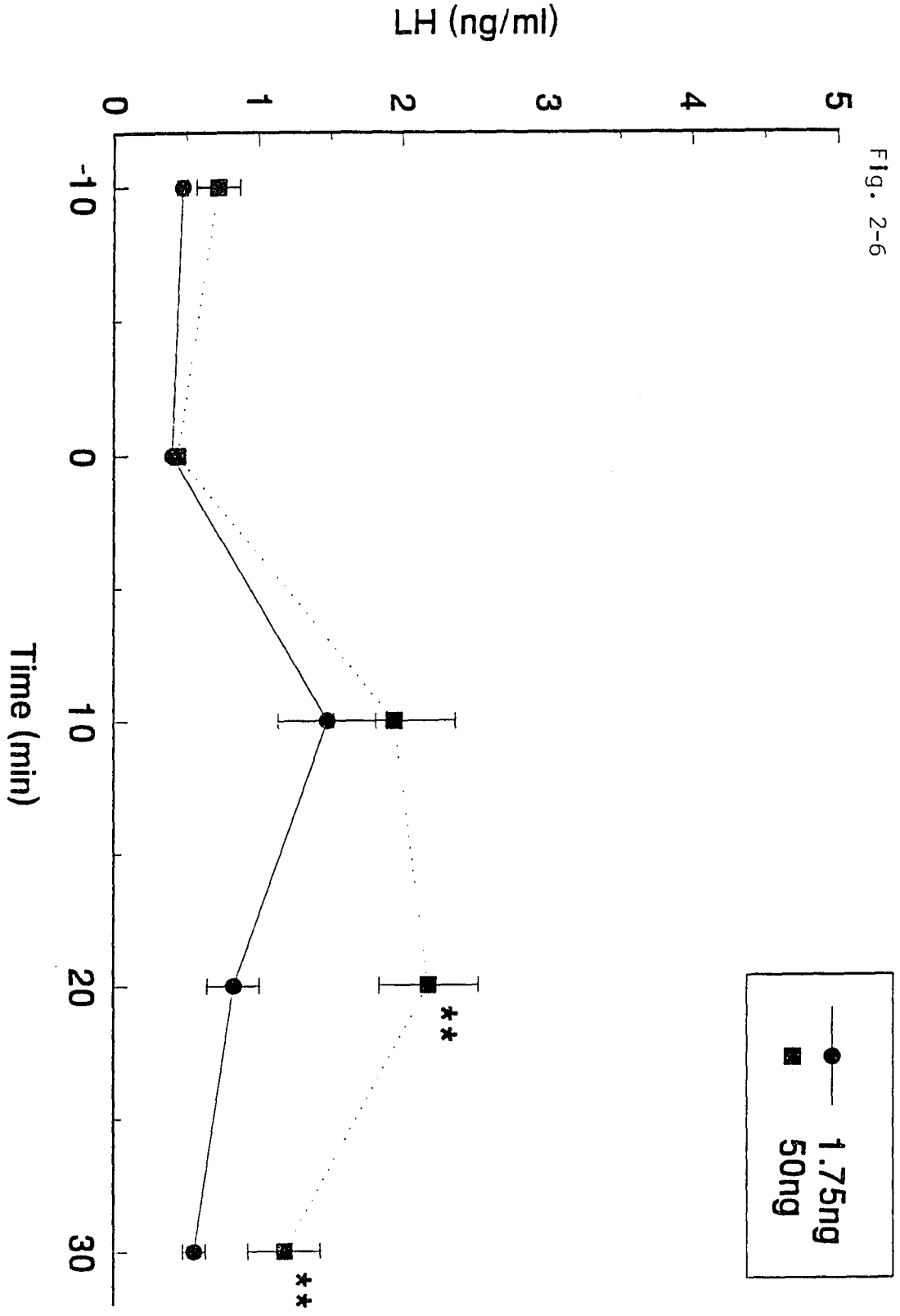


Fig. 2-6

CHAPTER 3: NEUROMODULATION OF TRANSPLANTED GONADOTROPIN-RELEASING HORMONE NEURONS IN MALE AND FEMALE HYPOGONADAL MICE WITH PREOPTIC AREA BRAIN GRAFTS.

Due to an autosomal recessive mutation in the gonadotropin-releasing hormone (GnRH) gene (1), hypogonadal (HPG) mice are unable to synthesize GnRH and are infertile (2). Hypogonadism in the adult HPG mouse can be reversed by implantation of normal fetal preoptic area (POA) tissue into the third ventricle (3,4). Transplanted GnRH neurons within POA brain grafts have axons which selectively innervate the host median eminence (5,6), where GnRH is released in a manner sufficient to stimulate pulsatile Luteinizing Hormone (LH) secretion and reproductive development in both male (7) and female (8) HPG mice with POA brain grafts (HPG/POA). Female HPG/POA enter a state of continuous vaginal estrus, can ovulate reflexively after mating, and can have successful pregnancies (4,9). Some females may then begin to ovulate spontaneously, implying a plasticity of the hypothalamic-pituitary-ovarian axis (10).

Pharmacological and anatomical studies have revealed that GnRH neurons are influenced by numerous neurotransmitter and peptidergic signals (11,12). Saitoh, et al (13), reported that the excitatory amino acid analog, N-methyl-D,L-aspartic acid (NMA), rapidly stimulated LH release in normal male and female mice, as has been described in other species (14-22). NMA is believed to act via a hypothalamic mechanism to stimulate GnRH release, since NMA does not elicit LH release from the pituitary in vitro (15,23), and a GnRH antagonist can block NMA-stimulated LH release (16,18). It is still unclear whether NMA acts directly on GnRH neurons or on afferents to GnRH neurons, or both.

Along with excitatory amino acids, endogenous opioid peptides are also involved in the regulation of LH release. Although the opiate antagonist naloxone (NAL) increases LH secretion in many species, including gonadally intact male (24) and female (25) rats, it failed to do so in male mice (26; described in Chapter 2). However, opiate antagonists significantly potentiated NMA-induced LH release in young adult male mice, suggesting that NMA triggers the release of inhibitory endogenous opioid peptides.

This chapter evaluates the physiological and anatomical integration of the POA grafts with the HPG host brain. I tested whether endogenous opioidergic inhibition modulates NMA-stimulated LH release in HPG/POA. Both male and female HPG/POA were challenged with NMA alone and in conjunction with NAL, NAL methiodide (NALMI), a quaternary derivative of NAL which is inefficient at crossing the blood-brain barrier (27), or saline (SAL). Other studies employing quaternary derivatives of NAL have demonstrated their effectiveness at eliciting LH release (28) or antagonizing morphine inhibition of LH release (29) in the rat, suggesting that opioid modulation of GnRH release occurs at a circumventricular location, such as the organum vasculosum of the lamina terminalis or the median eminence. I also tested older male HPG/POA with grafts of longer duration, since older normal male mice had significantly larger LH responses to NMA than young adult normal male mice (Chapter 2). Immunocytochemistry for β-endorphin was performed on the brains

of seven representative female HPG/POA to provide preliminary information on a possible anatomical substrate for the endogenous opioid component of NMA-induced LH release. To assess the longevity of GnRH neurons within POA grafts, immunocytochemistry for GnRH was performed on the brains of the two oldest male HPG/POA.

METHODS

Animals:

HPG mice used in this experiment were F1 hybrids derived from C3H/HeH X 101/H. Mice were housed in a 14:10 LD (lights out at 1500h) schedule with food and water available ad libitum. Female HPG mice (n=11) were tested 3 to 7 months following graft surgery and were 7 to 11 months of age at the time of the experiment (Table 1). Daily vaginal lavages confirmed that females entered persistent estrus following POA implantation. All but one female HPG/POA (U23) was paired with a fertile normal male on at least one occasion, and U45 and Y10 conceived and delivered live young 4 and 2 months prior to testing, respectively. Male HPG mice (n=12) were tested 3 to 12 months following graft surgery and were 7 to 16 months of age at the time of the experiment (Table 2). Only HPG/POA with significant gonadal development following graft surgery, as determined at the time of sacrifice, were included in this study.

Surgery:

Fetal POA brain grafts:

Graft tissue was derived from normal mouse fetuses (E15 or later), or day 1 neonates from the same colony as HPG mice. Previous studies have found that grafts derived from day 1 neonates, whether male or female, are just as effective as those of fetal origin in supporting reproductive development in HPG/POA (30,31). The POA was dissected from the ventral forebrain with a coronal incision at the bifurcation of the anterior cerebral artery, a parallel cut 0.7mm caudal to this, sagittal cuts 0.5mm lateral to the midline, and an undercut at 0.5mm. Each POA segment was then placed in a drop of sterile saline prior to implantation.

HPG hosts were anesthetized with chloral hydrate (360mg/kg, ip), and placed in a Kopf stereotaxic apparatus fitted with a mouse adaptor. Tissue from one or two fetuses was implanted with a 20ga needle into the anterior third ventricle of HPG hosts. With the skull level between lambda and bregma, stereotaxic coordinates were -1.0mm posterior from bregma at the midline, and -5.2mm from the surface of the brain (32).

Intracardial Cannulation:

Each mouse was fitted with an intracardial cannula under chloral hydrate anesthesia (360mg/kg, ip) during morning hours. The intracardial cannulation procedure has been described in Chapter 2, and was adapted from Weeks (33). Following surgery,

each mouse was placed in a clean cage and returned to the colony room awake prior to lights out at 1500h.

Blood Sampling:

All blood sampling was done during morning hours, beginning on the day following cannulation. Methods for blood sampling and plasma collection were identical to that described in Chapter 2. Throughout an experiment, blood cells from samples were resuspended in steroid free (13) human serum albumin (5%, 60μl; Armour Pharmaceutical Co., Kankakee, IL) and introduced back into the host via the cannula immediately following the next sample withdrawn or a drug administration.

Experimental design:

I assessed the action of NMA alone, opiate antagonists alone, and opiate antagonists combined with NMA on LH release in male and female HPG/POA. Blood samples were collected at -10, 0, +10, +20, +80 +90, +100, +120, +130, and +140 min. Each mouse received two NMA challenges 2h apart immediately following the 0 and +120 blood samples, and NAL, NALMI or SAL were administered following the +80 blood sample and again immediately prior to the second NMA challenge. Each mouse received NAL, NALMI and SAL treatments on separate consecutive days, and treatment order was randomized between mice of the same sex. All drugs were administered intravenously through the cannula and always followed a blood sample. NMA (Sigma Chemical Co.,

St. Louis, MO) was administered at a dose of 20mg/4.0ml saline/kg body weight. NAL (Sigma Chemical Co., St. Louis, MO) and NALMI (Research Biochemicals Inc., Natick, MA) were administered at a dose of 3mg/2.0ml saline/kg body weight. Saline (SAL) was administered as 2.0ml/kg body weight.

Perfusion and gonadal collection:

Animals were sacrificed following all testing with an overdose of chloral hydrate. Selected HPG/POA were deeply anesthetized with chloral hydrate and perfused through the heart with saline and Zamboni's fixative. The brain was removed, placed in Zamboni's for one to four days, and stored in cryoprotectant (35) at -20C until immunocytochemistry was performed. At the time of perfusion, the descending aorta was clamped to prevent fixation of gonadal tissues. Gonadal tissue was removed and weighed following sacrifice (Tables 3-1 and 3-2).

Immunocytochemical Methods:

All immunocytochemistry was performed in the laboratory of Dr. A-J. Silverman at Columbia University, College of Physicians and Surgeons in New York. Brains were sectioned on a vibratome at 40μm. Immunocytochemical detection of GnRH was carried out with the LR1 antiserum (courtesy of R. Benoit) at a 1:20,000 dilution and of β-endorphin with antibody (36,37) in a 1:4000 dilution. A commercial biotinylated secondary antibody (Vector Lab., Burlingame, CA), and an avidin-biotin-HRP complex (Vector

Lab.) using a nickel intensification of 3,3' diaminobenzidine (20mg DAB, 80mg $NiSO_4(NH_4)_2SO_4$, 100ml 0.05M TRIS pH 7.6) was used as the chromogen. Sections were dehydrated, cleared, coverslipped and photographed.

Radioimmunoassay for LH:

Methods for radioimmunoassay are identical to those described in Chapter 2. The mean intraassay coefficient of variation (CV) was 9.2%, and the interassay CV was 9.9%.

Statistics:

A "detectable LH value" is defined as any measurable increase in plasma LH above baseline and is not significant. Determination of a significant endogenous LH pulse or LH increase following a drug challenge in an individual mouse was based on the following criteria: 1) an initial elevation of plasma LH greater than 20% of the previous LH value, and 2) coefficient of variation of the peak value greater than twice the intraassay coefficient of variation (8). For each sex, significant differences in time point means between treatment conditions and within treatments were assessed by a two-way randomized blocks ANOVA or a one-way repeated measures ANOVA, followed by Tukey's HSD post hoc comparisons when $p<0.05$. Gonadal development was considered significant if testes or ovarian weights were at least two standard deviations heavier than untreated HPG mice.

RESULTS

All HPG/POA mice included in this study had significant gonadal development (Tables 3-1 and 3-2). All mice remained healthy and behaviorally active throughout the experiment. Unlike previous reports utilizing HPG/POA (4,5,7-10,13,31,38,-40), most individuals in this study received a POA segment from a single donor, rather than POA segments from two donors. Many of these mice had robust gonadal responses, and two females had healthy litters, demonstrating that a single POA segment can support these reproductive functions.

Effect of NMA and opiate antagonists on LH release in female HPG/POA:

Ten of 11 female HPG/POA had detectable variations in plasma LH levels, and responded to at least one of the three NMA challenges with significant increases in plasma LH (Table 3-1). In several other cases elevations in LH followed NMA treatment but were not significant with our criteria. As a group, female HPG/POA always responded to the first NMA challenge with significant elevations in plasma LH levels at the +10 time point ($p<0.01$ vs. the 0 time point, Fig. 3-1). No female HPG/POA responded to either of the opiate antagonists alone with significant LH release. There was an overall significant difference between the three treatment groups when all time points were compared (ANOVA, $p<0.05$;Fig. 3-1).

Pretreatment with either NAL or NALMI resulted in: 1) a

significantly enhanced LH secretory response to NMA at +130 (NAL,$p<0.05$; NALMI,$P<0.01$) and +140 (NAL,$p<0.01$;NALMI,$p<0.01$) as compared to the +10 and +20 time points, respectively, for each data set; and 2) a significantly enhanced LH secretory response to NMA at +130 (NAL,$p<0.05$; NALMI,$P<0.01$) and +140 (NAL,$p<0.01$; NALMI,$p<0.01$) as compared to the same time points following SAL pretreatment, respectively. There was no significant difference in LH values in response to the first vs second NMA challenge with SAL pretreatment.

The two HPG/POA (U45, Y10) which had litters prior to testing failed to respond to NMA with elevated LH levels on the first day of testing. Both of these females however had significant LH responses to NMA following opiate antagonist pretreatment but not saline pretreatment (Table 3-1).

Immunocytochemistry for β-endorphin:

Immunocytochemistry for β-endorphin was performed on the brains of seven of the 11 female HPG/POA to provide preliminary information on a possible anatomical substrate for the opioid component of NMA-induced LH release. All seven female HPG/POA brains studied had at least modest β-endorphin innervation of the graft. Some had robust innervation by β-endorphin-immunoreactive fibers throughout the dorsal-ventral extent of the graft. Fibers entered the graft in areas where it appeared to merge with the host brain (Fig. 3-3). Fibers were also seen entering the graft via small bridges of tissue between graft and host

(Fig. 3-4). A few grafts had large areas completely devoid of immunoreactive fibers (Fig. 3-5). Occasionally fibers were seen at the lateral boundary of the graft along the third ventricle. β-endorphin cell bodies were contained entirely within the host brain and concentrated in the medial basal hypothalamus (Fig. 3-5).

Effect of NMA and opiate antagonists on LH release in male HPG/POA:

Seven of 12 male HPG/POA had detectable variations in plasma LH levels. Five males responded to NMA alone with significant increases in plasma LH levels following at least one of three NMA challenges, and three of these mice responded to the first NMA challenge on the first day of testing (Table 3-2). As a group, male HPG/POA never responded to the first NMA challenge with significant elevations in plasma LH levels at the +10 time point ($p>0.05$ vs. the 0 time point, Fig. 3-2). T42 had significant endogenous LH pulses just prior to the first NMA challenge on all three days of testing. T04 failed to respond to NMA, either alone or following any pretreatment, but had detectable LH values at other time points during the three days of testing. LH values remained below the limit of detection in the remaining five males on all three days of testing, despite robust gonadal development (Table 3-2).

T04, T13 and W21 had significant elevations in plasma LH following NAL alone, and T04 and W21 also had significant in-

creases in LH secretion following NALMI alone. However, since all three mice had similar changes in plasma LH following SAL alone, it is unlikely that these increases in LH secretion represented true responses to the drugs. Rather, these increases may represent ongoing endogenous pulsatility.

There was no overall significant difference between the three treatment groups when all time points were compared (ANOVA, $p=.74$). LH responses to the first vs. second NMA challenge were not significantly different from each other for any pretreatment (Fig. 3-2).

Immunocytochemistry for GnRH

To assess the long term viability of GnRH neurons within the grafts of aging mice, the brains of the two oldest male HPG/POA were processed for immunocytochemistry for GnRH. These two mice (W22 and W21) were 16 months old at the time of the experiment, and had POA grafts for a period of one year. Both of these mice responded repeatedly to NMA (Table 3-2), implying that GnRH neurons within their POA grafts were still viable. GnRH immunoreactive neurons and fiber innervation of the median eminence were detected in the grafts of both of these male HPG/POA (Fig. 3-6).

DISCUSSION

This study demonstrates that endogenous opioid peptides can modulate the activity of transplanted GnRH neurons within a POA

graft. Female HPG/POA had significantly greater LH secretory responses to NMA when an opiate antagonist was present. The finding that β-endorphin-immunoreactive fibers originating in the host brain innervated all seven POA grafts examined suggests a possible anatomical substrate for opiate-antagonist-induced elevations in the LH response to an NMA challenge. In contrast, male HPG/POA responded to NMA only occasionally with significant elevations in LH and were unaffected by opiate antagonist pre-treatment (Table 3-2). These results imply that NMA-induced LH release may be dependent upon critical afferent connectivity to GnRH neurons, which may be sexually dimorphic in HPG/POA, or effective only within the context of an appropriate steroid milieu.

In addition to their action in female HPG/POA in the current study, opiate antagonists also potentiated NMA-stimulated LH release in normal young adult male mice, as described in Chapter 2. The ability of NALMI to modulate NMA-stimulated LH secretion in both normal mice and female HPG/POA may indicate that opioidergic modulation of LH release can occur at or in close proximity to a circumventricular location, most likely the median eminence, as this is the only circumventricular location innervated by GnRH fibers in HPG/POA (unpublished observation). The consistent finding of GnRH fiber innervation of the median eminence in HPG/POA (e.g. 3,5,6,9,13,38,this study) supports the hypothesis that the median eminence is a principal site of host regulation of GnRH neurons within a POA graft.

I propose that NMA stimulates both excitatory and inhibitory afferents to the GnRH neuronal system, and that one of the effects of NMA is the activation of inhibitory opioidergic afferents to the GnRH neuronal system in female HPG/POA mice. β-endorphin-containing afferents to GnRH neurons or via an interneuron may underlie this action of NMA. NMA induces cfos protein expression, a marker of neuronal activation, in many neurons including β-endorphin-containing neurons in the medial basal hypothalamus of the normal mouse (38). β-endorphin-immunoreactive terminals are known to synapse directly on GnRH cell bodies in male (36) and female (37) rats, and to be sexually dimorphic in that species (37). In the present study, β-endorphin-immunoreactive fibers were seen entering the graft, particularly in areas of graft-host fusion (Fig. 3-3,3-4), while β-endorphin-containing cell bodies were entirely within host hypothalamic tissue adjacent to the graft (Fig. 3-5). The absence of β-endorphin-immunoreactive cell bodies in the grafts was not surprising, as the POA tissue is excised rostral to the medial basal hypothalamus, the hypothalamic site of β-endorphin neurons in the rodent (39). The present findings are in agreement with our previous observations of β-endorphin and arcuate nucleus-derived innervation of the graft identified with immunocytochemistry and DiI (1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate) tracing (40). Further analysis awaits double label ultrastructural studies for GnRH and β-endorphin in the HPG/POA mouse.

Integration of a POA implant with the host brain has also been demonstrated with electrophysiology. Some neurons within POA grafts which can be fired with antidromic stimulation from the median eminence can also be excited or inhibited when host hypothalamus lateral to the graft is stimulated (41). However, these neurons have not been identified as GnRH neurons.

As previously reported (13), there was a general deficit in the ability of many male HPG/POA with robust gonadal development to respond to NMA (e.g. T11, W16, W33, Table 2). This was despite the selection of older HPG/POA males, which concurrent observations in older normal male mice (described in Chapter 2) suggested should be more responsive to NMA. Of interest in this regard, the two oldest male HPG/POA both repeatedly responded to NMA. Similar to my findings in older normal males, HPG/POA males here also lacked opiate-antagonist-induced potentiation of NMA-stimulated LH release.

Immunocytochemistry for GnRH was performed on the brains of the two oldest male HPG/POA responders (W21 and W22), and both had GnRH neurons within their POA grafts, which robustly innervated the median eminence. Both cell survival and pharmacological responses, along with significant gonadal development in these mice demonstrate that GnRH neurons within a POA graft can survive and function for at least one year following implantation, and may be subject to aging processes of the host.

Although there are no apparent sex differences in GnRH fiber innervation of the median eminence or GnRH cell survival

within POA grafts, it is conceivable that afferent connectivity to grafted GnRH neurons is sexually dimorphic. It is clear that the inability of many male HPG/POA to respond to NMA with elevated LH secretion is not due to excessive opioidergic inhibition of the GnRH system, although other inhibitory afferent systems sensitive to NMA may be responsible. Conversely, NMA-induced LH release may be dependent upon critical excitatory afferents which are absent or inactive in many male HPG/POA.

From another perspective, the ability to respond to NMA may be dependent upon an appropriate steroid milieu at the time of challenge. In the present study all mice tested were gonadally intact. Heterogeneity among individual HPG/POA in gonadal development, LH pulsatility (7,8), positive feedback and reproductive ability (4,10) suggests that endogenous steroid levels in intact HPG/POA are also variable among individuals. It is possible that gonadal steroids affect the proportion of inhibitory and excitatory influences on GnRH release in HPG/POA. In Chapter 4, findings are presented which demonstrate that estrogen treatment of castrated male HPG/POA facilitates NMA-stimulated LH release.

In conclusion, most female HPG/POA but few male HPG/POA responded to NMA challenges with elevations in plasma LH. Female HPG/POA had significantly greater responses to NMA when an opiate antagonist was present, demonstrating that endogenous opioids can modulate GnRH neurons within POA brain grafts. β-endorphin innervation of POA grafts is a likely candidate as a

regulatory element of the GnRH neuronal system in HPG/POA. The wide variety of physiological responses to POA grafts in HPG/POA may be due to differences in the pattern of afferent innervation to the GnRH neuronal system or the activation of these afferents.

Figure Legends

Fig. 3-1. Effect of NAL (3mg/2.0ml saline/kg body weight), NALMI (3mg/2.0ml saline/kg body weight) or SAL (2.0ml/kg body weight) administered alone and combined with NMA (20mg/4.0ml saline/kg body weight) on plasma LH levels in female HPG/POA. Each mouse (n=11) received two NMA challenges, immediately following the 0 and +120 blood samples on three consecutive days. On each day, mice were pretreated with NAL (top), NALMI (middle), or SAL (bottom), administered following the +80 blood sample and again immediately prior to the second NMA challenge. There were no significant effects of NAL, NALMI or SAL alone on plasma LH levels. Pretreatment with either NAL or NALMI resulted in: 1) a significantly enhanced LH secretory response to NMA at the +130 and +140 time points as compared to the +10 and +20 time points within the treatment, respectively; and 2) a significantly enhanced LH secretory response to NMA at the +130 and +140 time points as compared to the same time points with SAL pretreatment, respectively. There was no significant difference in LH values in response to the first vs second NMA challenge with SAL pretreatment. *: $p<0.05$, **: $p<0.01$; applicable to both comparisons.

Fig. 3-2. Effect of NAL, NALMI or SAL administered alone and combined with NMA on plasma LH levels in male HPG/POA (n=12). See legend for Fig. 1 for treatments and doses. There were no significant effects of NAL or NALMI alone on plasma LH levels. There were no significant differences between LH responses to the first vs. second NMA challenge for any of the three treatments.

Fig. 3-3. Illustration of the β-endorphin fiber innervation of both host (H) and graft (G) at the level of the hypothalamic paraventricular nucleus. Fibers enter the graft from the dorsal aspect (arrowheads) where donor and host merge. Animal Y01.

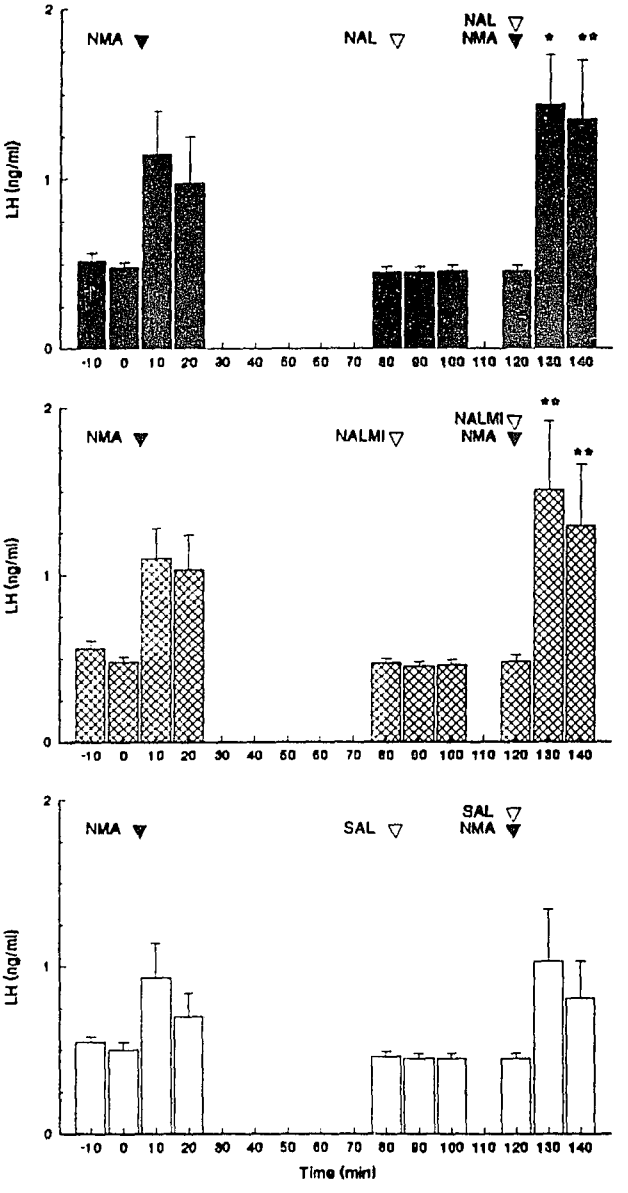
Fig. 3-4. β-endorphin fiber (arrowhead) is seen crossing between host (H) and graft (G) over a bridge of tissue. v = third ventricular space. Animal U45.

Fig. 3-5. β-endorphin cell bodies are numerous in the host medial basal hypothalamus (arrowheads) and are absent from the graft (G) at this and at all levels. Although the graft in this particular section is devoid of β-endorphin-positive axons, this animal (Y24) did receive some β-endorphin innervation in other regions of the donor tissue.

Fig. 3-6. GnRH innervation of the median eminence of W22. Fibers are concentrated above the host (H) tuberoinfundibular sulci (arrows) and continue down into the pituitary stalk (S). This animal was 16 months old at the time of sacrifice and the graft (G) had been in place for one year prior to pharmacologi-

cal testing.

Fig. 3-1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NALMI
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SAL
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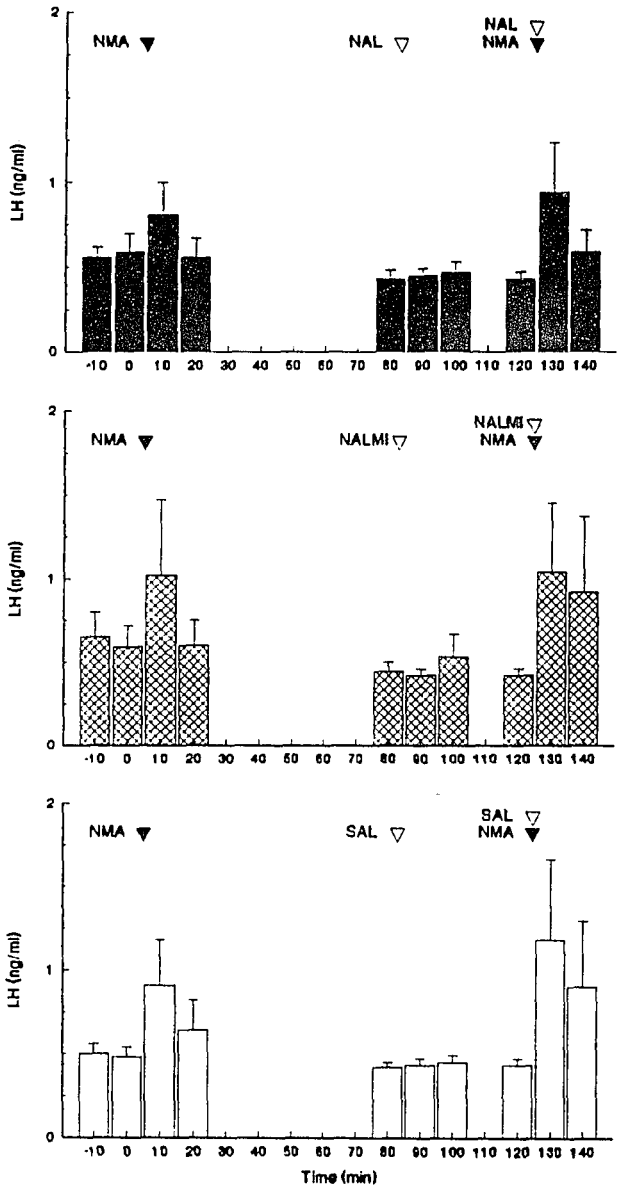
Fig. 3-2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Time (min)

Fig. 3-3

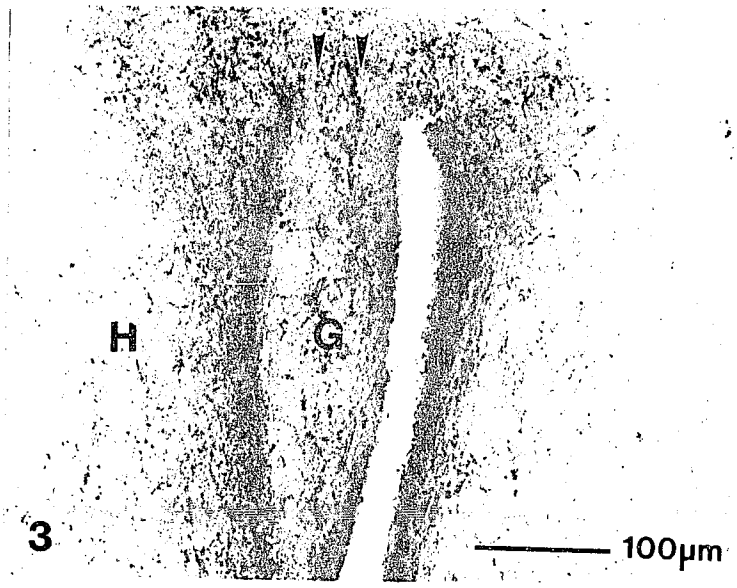

Fig. 3-4

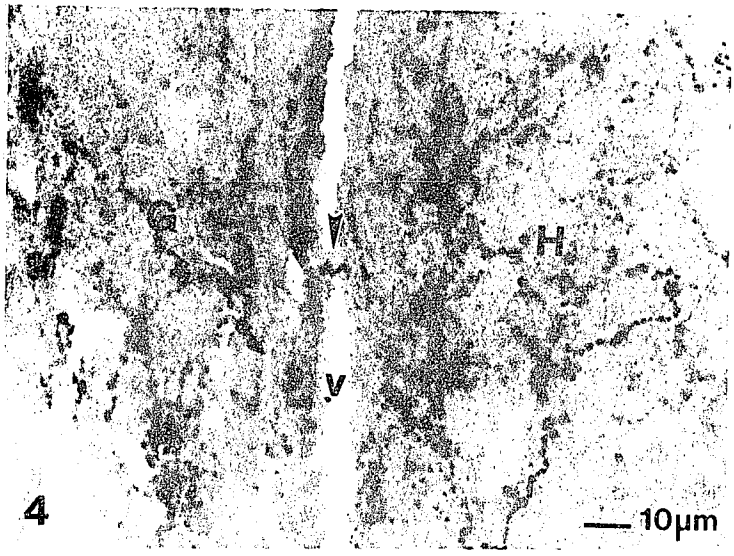

Fig. 3-5

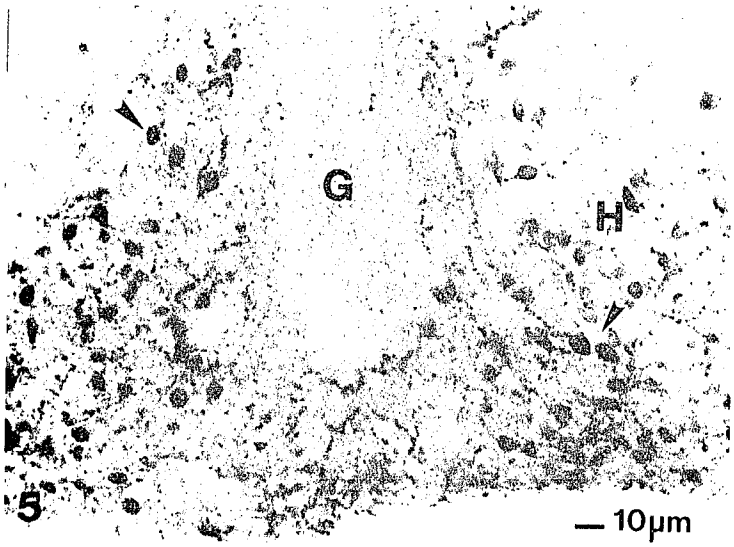

Fig. 3-6

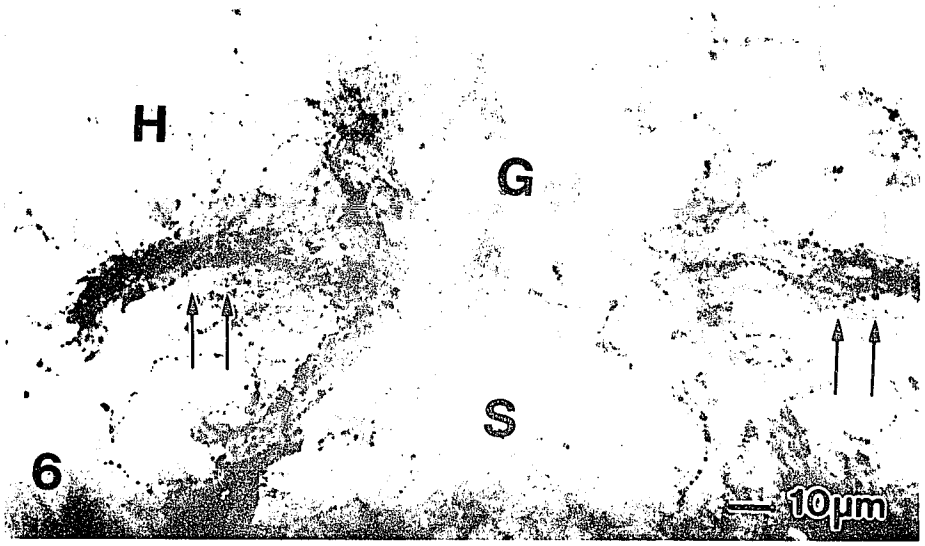

Table 3-1. Female HPG/POA mice challenged with NMA alone or following pretreatment with NAL (N), NALMI (M) or SAL (S).

HPG/POA	AGE OF HOST (mos)	AGE OF GRAFT (mos)	OVARIAN WEIGHT (mg)	UTERINE WEIGHT (mg)	NMA[a] ALONE	NMA WITH[b] PRETREATMENT (N,M,S)
U7	8.5	5	10.4	160.4	2	NMS
U8	9.5	5	16.7	135.7	3[c]	MNS
U23	7.5	3	4.1	98.9	1	N
U36	8	3.5	4.1	24.8	0	
U40	8.5	3.5	8.4	50.3	3[c]	NMS
U45	11	6.5	11.2	288.2	1	NM
Y01	8	5	7.0	50.2	3[c]	NS
Y10	7	5	8.5	244.4	2	MN
Y24	8.5	5.5	9.0	115.7	3[c]	S
Y31	8	4	8.9	66.3	1	MS
Y50	8	5.5	5.4	81.3	3[c]	MNS
Normal female[d]			15.3+/-0.9	195.5+/-27.1		
Untreated female HPG[d]			0.7+/-0.2	10.7+/-1.5		

[a] Number of significant LH responses to three NMA challenges

[b] Significant responses to NMA following pretreatment with NAL, NALMI or SAL are denoted by N, M or S in order of decreasing magnitude of response. The absence of a letter denotes the lack of a significant LH response to NMA with that pretreatment.

[c] responded to the first NMA challenge on the first day.

[d] reference (43)

Table 3-2. Male HPG/POA mice challenged with NMA alone or following pretreatment with NAL (N), NALMI (M) or SAL (S).

HPG/POA	AGE OF HOST (mos)	AGE OF GRAFT (mos)	TESTES WEIGHT (mg)	SEMINAL VESICLE WEIGHT (mg)	NMA[a] ALONE	NMA WITH[b] PRETREATMENT (N,M,S)
T04	10.5	8	83.2	24.8	0	
T11	11	8	89.4	265.0	0	
T13	10	6.5	50.0(1)	89.7	2	NSM
T14	11	7.5	143.7	184.0	3[c]	SMN
T19	10	6.5	33.9	26.8	1[c]	
T42	10	6.5	120.8	107.6	0	M
W16	7	3	68.2	106.6	0	
W21	16	12	43.7	56.3	3[c]	NMS
W22	16	12	61.6	37.2	2	MS
W30	11	5	21.2	105.2	0	
W32	7	3	51.0	45.6	0	
W33	7	3	53.8(1)	101.2	0	
Normal male[d]			213.2+/-6.4	235.1+/-13.0		
Untreated male HPG[d]			6.4+/-1.2	2.1+/-0.5		

[a] Number of significant LH responses to three NMA challenges

[b] Significant responses to NMA following pretreatment with NAL, NALMI or SAL are denoted by N, M or S in order of decreasing magnitude of response. The absence of a letter denotes the lack of a significant LH response to NMA with that pretreatment.

[c] responded to the first NMA challenge on the first day.

[d] reference (43)

CHAPTER 4: ESTROGEN FACILITATES N-METHYL-D,L-ASPARTIC ACID STIMULATION OF LUTEINIZING HORMONE SECRETION IN MALE HYPOGONADAL MICE WITH PREOPTIC AREA BRAIN GRAFTS.

Mutant hypogonadal mice lack a functional gonadotropin-releasing hormone (GnRH) gene (1) and are infertile (2). Implantation of normal fetal preoptic area (POA) tissue into the third ventricle of the adult HPG mouse stimulates reproductive development (3) via a population of transplanted GnRH neurons, which innervate the host median eminence (4,5) and release GnRH in a sufficient manner to stimulate pulsatile Luteinizing Hormone (LH) secretion (6,7).

Previous studies have shown that male HPG mice with POA brain grafts (HPG/POA) seldom respond to intravenously administered N-methyl-D,L-aspartic acid (NMA) with increased LH secretion, whereas female HPG/POA do so with much greater frequency (8,9). This sex difference in the NMA-stimulated LH response was not accounted for by differences in GnRH neuronal survival within POA transplants or GnRH fiber innervation of the median eminence, which was present in individuals of both sexes (8). The lack of significant LH secretion following NMA challenge in male HPG/POA, as well as other recent findings in the mouse (9,10) and rat (11), suggest that NMA elicits LH release indirectly via afferent input to GnRH neurons. The organization or activation of afferents to grafted GnRH neurons may therefore determine whether or not an individual HPG/POA can respond to NMA. Similarly, the central actions of estrogen on the GnRH neuronal system are also thought to be indirect, since GnRH neurons themselves do not generally contain intracellular estrogen receptors (12). Gonadal steroids, then, may enhance or

inhibit NMA-stimulated LH release by modulating critical afferents to GnRH neurons.

In this chapter I describe experiments which tested whether steroid milieu affects the ability of male HPG/POA to secrete LH in response to NMA challenges. I also examined whether various steroid treatments affected GnRH fiber outgrowth to the median eminence.

METHODS

Animals:

Male HPG mice were obtained from Dr. Harry Charlton, Oxford, England. These mice are F1 hybrids of C3H/HeH and 101H. All mice were housed in a 14:10 LD (lights out at 1500h) schedule with food and water available ad libitum. Mice were 3-5 months of age at the time of graft implantation and were tested with NMA challenges two months later.

POA implantation:

Male HPG mice were implanted with a POA segment, derived from one normal mouse fetus of the same stock. Methods for POA implantation were described in chapter 3.

Experimental protocol:

Four groups of HPG/POA received NMA challenges. Five HPG/POA remained gonadally intact throughout the experiment. Two other groups (n=5/group) were castrated prior to graft implanta-

tion, and received a subcutaneous capsule containing either 100μg 17-β-estradiol (E2)/ml sesame oil (SO) or SO. As previously described (8), capsules were made from 10mm of Silastic tubing (id, 0.0102cm; od, 0.0216cm; Dow-Corning, Midland, MI), with the ends sealed with Silastic adhesive. These capsules were replaced every 2-3 weeks under Isoflurane (Anaquest, Madison, WI) anesthesia, such that at the time of testing capsules had been replaced 2-3 weeks earlier. The fourth group (n=8) was comprised of male HPG/POA which were castrated and given an E2 capsule at 1 week prior to NMA testing.

Catheterization and blood sampling:

One day prior to testing, each mouse was fitted with an intracardial cannula under chloral hydrate anesthesia (360mg/kg, ip) during morning hours. The methods for intracardial cannulation were described in Chapter 2. Following surgery, each mouse was placed in a clean cage and returned to the colony room awake prior to lights out.

At the time of the experiment, a 1cc syringe filled with 3U heparin/ml bacteriostatic saline was attached to the cannula and then suspended above each cage, leaving each mouse completely unrestrained. Food and water were supplied. Blood samples (100μl) were collected as described in Chapter 2. Throughout an experiment, blood cells from samples were resuspended in steroid free (13) human serum albumin (5%, 60μl; Armour Pharmaceutical Co., Kankakee, IL) and introduced back into the host via the

cannula immediately following the next sample withdrawn or a drug administration.

To assess the action of NMA (Sigma Chemical Co., St. Louis, MO) on LH release, blood samples were collected at -10, 0, +10, and +20 min relative to each of six NMA challenges, administered intravenously through the cannula at a dose of 20mg/4.0ml saline/kg body weight. NMA challenges were administered immediately following the 0 blood samples, at 1000h, 1200h, and 1400h on two consecutive days.

Immunocytochemistry for GnRH

All immunocytochemistry was performed in the laboratory of Dr. A-J. Silverman at Columbia University, College of Physicians and Surgeons in New York. Animals were sacrificed following all testing with an overdose of chloral hydrate. All HPG/POA which were castrated at the time of POA implantation along with selected HPG/POA from the other groups were deeply anesthetized with chloral hydrate and perfused through the heart with saline and Zamboni's fixative. Each brain was removed, placed in Zamboni's for one to 4 days, sunk in 20% sucrose, and then sunk in cryoprotectant (14) and frozen at -20C until immunocytochemistry for GnRH was performed. Gonadal tissue obtained from intact mice and those castrated one week prior to testing was removed and weighed. Brains were sectioned on a vibratome at 40μm. Immunocytochemical detection of GnRH was carried out with LR1 antiserum at a 1:20,000 dilution. A commercial biotinylated

secondary antibody (Vector), and an avidin-biotin-HRP complex (Vector) using a nickel intensification of 3,3' diaminobenzidine (20mg DAB, 80mg $NiSO_4(NH_4)_2SO_4$, 100ml 0.05M TRIS pH 7.6) was used as the chromogen. Sections were dehydrated, cleared, coverslipped and photographed.

Radioimmunoassay for LH:

Methods for RIA were described in chapter 2. The mean intraassay coefficient of variation (CV) was 10.5%, and the interassay CV was 11.0%.

Statistics:

Determination of a significant LH increase following an NMA challenge in an individual mouse was based on the following criteria: 1) an initial elevation of plasma LH greater than 20% of the previous LH value, and 2) coefficient of variation of the peak value greater than twice the intraassay coefficient of variation. To determine whether plasma LH values measured before and after NMA challenges were significantly affected by the time of day that animals were tested or the day on which animals were tested, a randomized blocks ANOVA was performed on all the data for each group. To determine whether the different treatments affected the propensity of subjects having significant LH secretion following NMA challenges, the percent of subjects in each group with significant increases in LH was determined for each NMA challenge. Significant differences

between groups were assessed with a Kruskal-Wallis non-parametric ANOVA. Comparisons between individual groups were then assessed with multiple Mann-Whitney U-tests. Gonadal development was considered significant if testes weights were at least two standard deviations heavier than untreated HPG mice.

RESULTS

In this study, mice were challenged with NMA at different times of day on two consecutive days. Plasma LH values measured prior to and following NMA challenges were not significantly affected by the time of day or the day on which animals were tested for any group of HPG/POA.

Both the intact HPG/POA and those which were castrated and treated with E2 at 1 wk prior to testing had testicular development which could be assessed as a measure of graft efficacy. All five intact HPG/POA had extremely robust testicular and seminal vesicle development (Table 4-1). Those HPG/POA which were castrated and treated with E2 at 1 week prior to testing were selected at the time of castration on the basis of significant testes weights (Table 4-1). Therefore, all of these mice had significant gonadal development and were considered "responders". The two groups of HPG/POA which were castrated prior to POA implantation could not be classified as "responders" using the criteria of gonadal development. Therefore, immunocytochemistry for GnRH was necessary to confirm graft survival and GnRH fiber innervation of the host median eminence.

Of these ten HPG/POA, nine had immunoreactive GnRH soma within the graft and GnRH fiber innervation of the median eminence.

The finding that all five intact male HPG/POA had robust gonadal development implied that a functional GnRH neuronal system was present in these mice. Further, GnRH immunocytochemistry revealed GnRH neurons within the POA graft and fiber innervation of the host median eminence in all of these brains. An example of GnRH immunocytochemistry on a section from an intact male HPG/POA (Z24) brain is shown in Fig. 4-1A, and corresponding plasma LH data for this animal is shown in Fig. 4-2A. Despite these functional POA implants, there were just two intact HPG/POA which responded to one of six NMA challenges with significantly elevated plasma LH levels (Table 4-1). Similarly, only one of five HPG/POA castrated prior to graft implantation and treated with SO responded to one of six NMA challenges with significantly elevated plasma LH levels. GnRH immunocytochemistry on a brain section from a castrated HPG/POA treated with SO (Z33) is shown in Fig. 4-1B, and corresponding plasma LH data for this animal is shown in Fig. 4-2B. One animal in this group (Z04) had no detectable GnRH immunoreactivity.

A significantly greater proportion of individuals in the E2-treated groups responded to NMA challenges ($p \leq 0.025$ vs. intact or SO; Table 4-2). Four of five HPG/POA castrated and treated with E2 prior to graft implantation responded to at least two of six NMA challenges with significant elevations in plasma LH (Table 4-1). The brains of these four NMA responders

had GnRH neurons within the POA transplant and fiber innervation of the host median eminence. An example of GnRH immunocytochemistry on a brain section from a male HPG/POA treated with E2 prior to graft implantation (Z21) is shown in Fig. 4-1C, and corresponding plasma LH data for this animal is shown in Fig. 4-2C. The brain of the non-NMA-responder had only a couple of detectable fibers in the host median eminence.

Six of eight HPG/POA castrated and treated with E2 at one week prior to testing responded to at least one of the six NMA challenges, and three of these mice responded to all six NMA challenges (Table 4-1). One animal in this group (BB12) had LH values following NMA challenges which were extremely large (Fig. 4-2D). The brain of this HPG/POA had GnRH immunoreactive cell bodies just dorsal to the host median eminence (Fig. 4-1D).

DISCUSSION

The present results suggest that E2 facilitates NMA-stimulated LH release in male HPG/POA mice. The effect of E2 treatment was similar in HPG/POA male mice treated prior to surgery or just one week prior to NMA challenge testing. I suggest that the action of E2 is via estrogen receptor-mediated effects on afferent neurons to the GnRH neuronal system, which might be similar in both male and female HPG/POA. One mechanism for this action may be diminution by E2 of the influence of dominant inhibitory afferents on the GnRH neuronal system which are activated by NMA. Conversely, enhancement of excitatory affer-

ent influences by E2 may permit NMA to stimulate GnRH release in HPG/POA.

Estrogen is known to selectively accelerate and enhance neuritic proliferation and branching in organotypic cultures of newborn mouse hypothalamus and preoptic area (15). However, these effects may be limited to those neurons containing estrogen receptors, which GnRH neurons lack (12). In the present study, altered steroid milieu at the time of graft implantation did not obviously alter GnRH fiber outgrowth to the median eminence or GnRH cell survival within the graft. HPG/POA castrated at the time of graft implantation and treated with sesame oil had innervation of the median eminence and failed to respond to NMA, similar to their intact controls. Likewise, male HPG/POA treated with E2 prior to graft implantation had GnRH immunoreactivity comparable to males with other treatments. Therefore, the present findings suggest that GnRH neuronal survival and branching may not be affected by gonadal steroids in HPG/POA.

One HPG/POA (BB12) which was castrated and treated with E2 at 1 week prior to NMA challenge testing had extremely large LH responses to NMA. Examination of this brain revealed the presence of GnRH cell soma within the median eminence, illustrating a possible anatomical correlation for this aberration. In the case of this one animal, it is possible that NMA was able to trigger a much greater release of GnRH. A GnRH neuron within the median eminence may be accessible to afferent inputs which

GnRH neurons at more superior location within the graft in the third ventricle lack. However, a direct effect of NMA at the level of the GnRH cell body cannot be excluded.

In a previous study by Saitoh, et al (8), female HPG/POA "responders", which were ovariectomized prior to testing, were able to respond to a single NMA challenge with significant elevations in plasma LH levels. Therefore, estrogen is not essential at the time of NMA challenge for female HPG/POA to have significant LH responses. This finding suggests that estrogen might have an effect on the adult brain which persists following its removal. Future studies will examine whether NMA-stimulated LH release occurring in male HPG/POA treated with E2 can continue to occur in male HPG/POA following the removal of E2 treatment.

These findings provide further evidence that afferent modulation of grafted GnRH neurons modulates NMA-stimulated LH release in HPG/POA, and that at least some of these same afferents can convey information to grafted GnRH neurons regarding alterations in steroid milieu. Further, gonadectomy or estrogen treatment did not obviously alter the pattern of GnRH fiber innervation of the median eminence. Future studies will examine the role of steroids in the regulation of the GnRH release in HPG/POA mice.

Figure Legends

Fig. 4-1A. Photomicrograph of GnRH fiber innervation of the host median eminence (me) in an intact male HPG/POA (Z24). In this section, the POA graft with GnRH immunoreactive cell soma is located in more dorsal regions of the third ventricle (V) and is not shown. arrows: GnRH fiber innervation of the host median eminence.

Fig.4-2A. Graph of plasma LH values before and after six separate NMA challenges (20mg/4.0ml saline/kg body weight) in the same intact male HPG/POA (Z24). Four blood samples were collected at 10 min intervals starting at the times shown, on two consecutive days. NMA was administered following the second blood sample. There were no significant plasma LH increases following any of the six NMA challenges.

Fig. 4-1B. Photomicrograph of GnRH fiber innervation of the host median eminence (me) in a castrated male HPG/POA treated with SO at the time of POA implantation (Z33). In this section, the POA graft (G) fills most of the third ventricle (V). arrows: GnRH fiber innervation of the host median eminence. H: host hypothalamus.

Fig. 4-2B. Graph of plasma LH values before and after six separate NMA (20mg/4.0ml saline/kg body weight) challenges in the same in the same castrated male HPG/POA treated with SO at the time of graft implantation (Z33). Four blood samples were collected at 10 min intervals starting at the times shown, on two consecutive days. NMA was administered following the second blood sample. Note a spontaneous LH pulse detected at 1210 h on day 1, just prior to an NMA challenge. *: This mouse responded to the last NMA challenge with a modest but significant increase in plasma LH levels at 20 min following the challenge.

Fig. 4-1C. Photomicrograph of GnRH fiber innervation of the host median eminence (me) in a castrated male HPG/POA treated with E2 at the time of POA implantation (Z21). In this section, the POA graft (G) fills the entire the third ventricle. arrows: GnRH fiber innervation of the host median eminence. H: host hypothalamus.

Fig. 4-2C. Graph of plasma LH values before and after six separate NMA challenges (20mg/4.0ml saline/kg body weight) in the same castrated male HPG/POA treated with E2 at the time of graft implantation (Z21). Four blood samples were collected at 10 min intervals starting at the times shown, on two consecutive days. NMA was administered following the second blood sample. *: This mouse responded to all three NMA challenges on the second day of testing with significant elevations in plasma LH levels.

Fig. 4-1D. Photomicrograph of GnRH fiber innervation of the host median eminence (me) and GnRH cell soma (arrowheads) in a castrated male HPG/POA treated with E2 at one week prior to testing (BB12). In this section, GnRH cell bodies with the graft (G) are directly dorsal to the host median eminence. The graft is in a ventral portion of the third ventricle (V). arrows: GnRH fiber innervation of the host median eminence. H: host hypothalamus.

Fig. 4-2D. Graph of plasma LH values before and after six separate NMA (20mg/4.0ml saline/kg body weight) challenges in the same castrated male HPG/POA treated with E2 at one week prior to testing (BB12). Four blood samples were collected at 10 min intervals starting at the times shown, on two consecutive days. NMA was administered following the second blood sample. *: This mouse responded to all six NMA challenges with extreme elevations in plasma LH levels. Note the scaling change for the Y axis.

Fig. 4-1

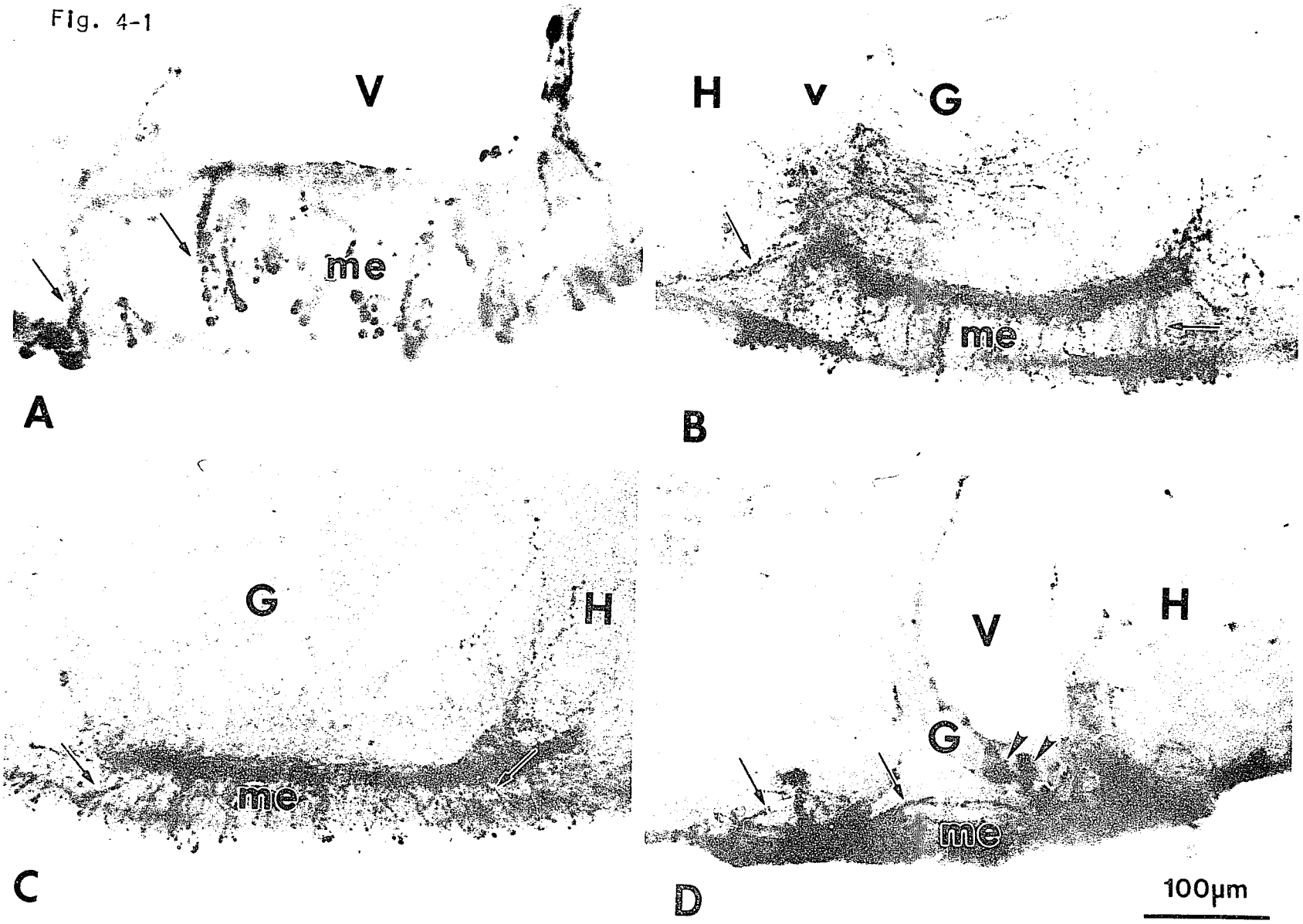

Fig. 4-2

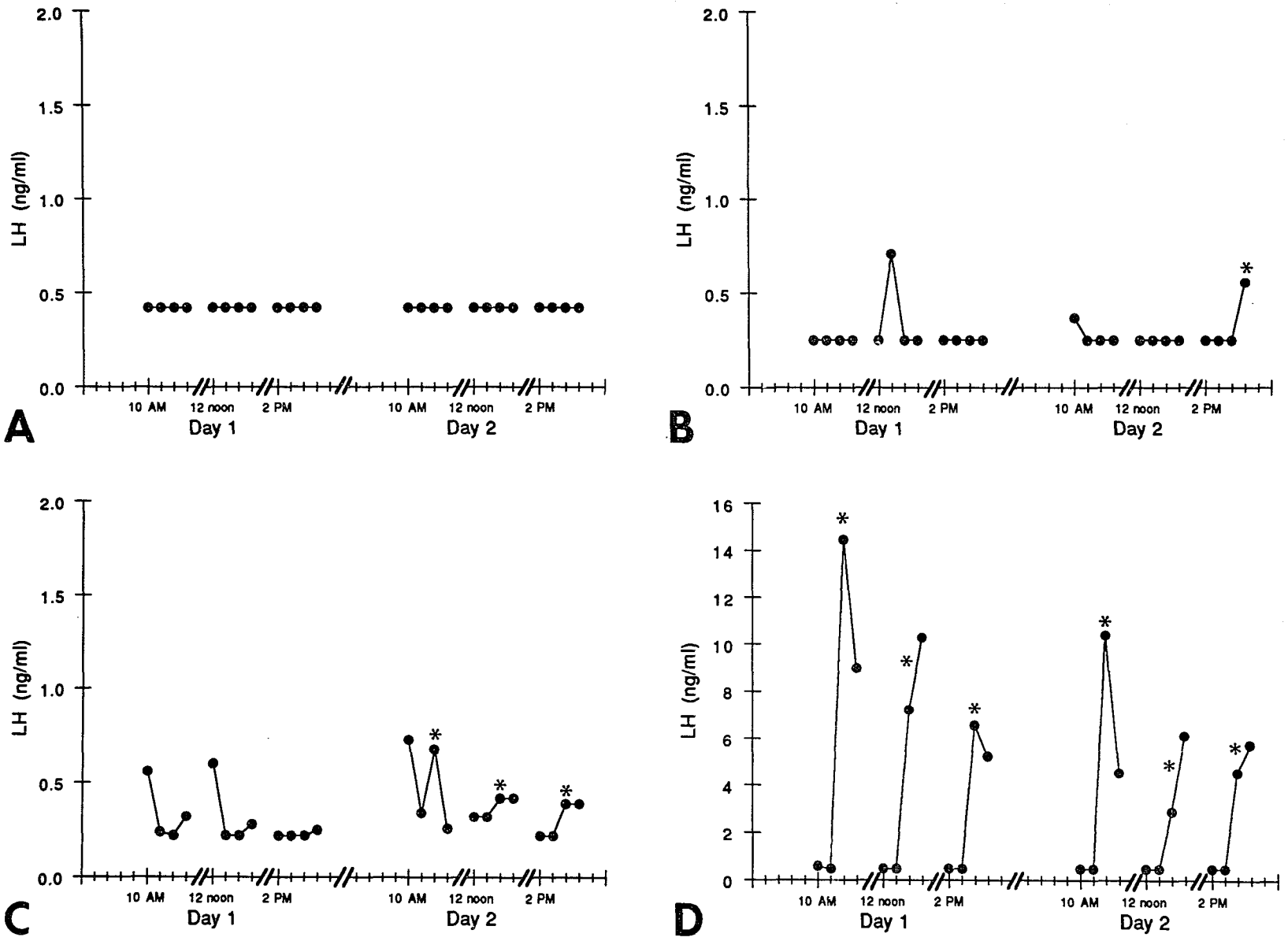
A
B
C
D
LH (ng/ml)
2.0
1.5
1.0
0.5
0.0
16
14
12
10
8
6
4
2
0
10 AM
12 noon
2 PM
Day 1
Day 2
*

Table 4-1: Summary of gonadal development and the number of significant plasma LH increases following six NMA challenges in groups of individual HPG/POA with different treatments.

HPG/POA	Testes wt. (mg)	Seminal Vesicles wt. (mg)	X = Significant response to NMA challenge challenge #: 1	2	3	4	5	6
Intact:								
Z06	128.8	164.8						
Z07	84.7	49.7		X				
Z12	92.1	62.2	X					
Z23	106.1	286.6						
Z24	90.0	47.0						
Castrated and SO treated prior to graft implantation:								
Z00								
Z01								
Z04								
Z33								
Z40				X				
Castrated + E2 treated prior to graft implantation:								
Z02			X	X		X	X	X
Z11								
Z15						X	X	
Z21						X	X	X
Z26				X	X			
Castrated + E2 treated at 1 week prior to testing:								
BB11	51.7							
BB12	107.6		X	X	X	X	X	X
BB13	49.2							X
BB14	85.2		X	X	X	X	X	X
BB16	55.6		X	X				
BB23	64.3					X		
B25	61.0							
BB33	57.2		X	X	X	X	X	X

	Testes wt. (mg)	Seminal vesicle wt. (mg)
*Normal male	213.2+/-6.4	235.1+/-13.0
*Untreated male HPG	6.4+/-1.2	2.1+/-0.5

*reference 16

Table 4-2. Mean percent of male HPG/POA having significant plasma LH increases following NMA challenges.

Group	n	mean %
Intact	5	6.7+/-4.2
Castrate + SO	5	3.3+/-3.3
Castrate + chronic E2	5	36.7+/-9.5**
Castrate + E2; 1 week	8	45.8+/-2.6**

**: $p \leq 0.025$ vs. Intact or SO.

CHAPTER 5: FUNCTIONAL ASSESSMENT OF INTRAHYPOTHALAMIC IMPLANTS OF IMMORTALIZED GONADOTROPIN-RELEASING HORMONE-SECRETING CELLS IN FEMALE HYPOGONADAL MICE.

Miller GM, Silverman A-J, Roberts JL, Dong KW, Gibson MJ 1993 Functional assessment of intrahypothalamic implants of immortalized gonadotropin-releasing hormone-secreting cells in female hypogonadal mice. Cell Transplantation 2:251-257.
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The immortalized cell line, designated GT1, was developed through genetically targeted tumorigenesis directed at Gonadotropin-releasing Hormone (GnRH) neurons in transgenic mice (1). Clones of this cell line have a functional GnRH gene, process GnRH prohormone, and release GnRH decapeptide. They possess a neuronal phenotype, as markers of neuronal, neurosecretory and synaptic specializations are present and glial-specific markers are absent (2). They can exhibit a neuronal morphology, both in culture in the absence of serum (1), and within the brain of an hypogonadal (HPG) host (3).

The HPG mouse is a mutant which lacks a functional GnRH gene (4). When normal fetal preoptic area (POA) tissue is transplanted into the third ventricle of adult HPG hosts, reproductive development is supported by GnRH neurons within the transplant. The capacity to maintain the pituitary-gonadal axis is dependent on graft-derived GnRH axons exiting the graft and innervating the host median eminence (ME) (5,6).

In a recent study (3), either GT1-3 or GT1-7 cells were injected bilaterally into the hypothalami of HPG hosts at various cell densities and for variable durations. It was observed that these cells migrate into the host brain, differentiate morphologically, and process proGnRH <u>in situ</u>. In addition, several HPG mice with GT1-7 cell grafts had modest but significant gonadal development, suggesting that GnRH was released in an appropriate amount and pattern to stimulate gonadotropin secretion.

The current experiments were to determine whether intrahypothalamic GT1-7 cells in adult female HPG mice can support spontaneous (i.e., pulsatile) and/or pharmacologically stimulated elevations in plasma Luteinizing Hormone (LH) concentrations. As GnRH release from perifused GT1-7 cells is reported to be pulsatile in vitro (7), plasma samples were collected at 10 min intervals to assess the temporal pattern of LH secretion. In addition, all mice were challenged with the excitatory amino acid analog, N-methyl-D,L-aspartic acid (NMA), which stimulates LH release via a hypothalamic mechanism in both normal mice and female HPG mice with POA grafts (HPG/POA) (10).

METHODS

Cell culture and collection:

GT1-7 cells (1) were cultured on 100mm plates in Dulbecco's Modified Eagle's Medium (DME) with 10% fetal calf serum. Cells were harvested by trypsinization of a monolayer, washed with phosphate buffered saline (PBS, pH 7.0), collected by centrifugation at 4C, and resuspended in 1ml cold PBS. Cells were counted using a hemocytometer, and appropriate dilutions were made with PBS to yield 1000 cells/μl PBS. Cells were then stored on ice until the time of intracranial injection.

Animals:

Female HPG mice were obtained from Dr. Harry Charlton, Oxford University, England. Upon arrival, mice were maintained

in our colony in a 14:10 LD schedule (lights off at 15:00h) with food and water available ad libitum.

GT1-7 cell injections:

Eight HPG mice were anesthetized with chloral hydrate (360mg/kg, ip). After the scalp was shaved and sterilized with alcohol, mice were placed in a Kopf small animal stereotaxic apparatus fitted with a mouse adaptor. A Kopf needle driver holding a 10μl Hamilton Syringe was used to make bilateral, 1μl injections of 1000 GT1-7 cells/μl PBS into the hypothalamus. Each injection was made over a 1 min period with the needle left in place for an additional 1-2 min per side. With the skull horizontal between lambda and bregma, stereotaxic coordinates were: Anterior/Posterior = -1.0; Lateral = +/-0.6; Depth = -5.2 from the top of the brain (8).

An additional mouse, S75, also received bilateral 1μl injections of 1000 GT1-7 cells/μl PBS into the hypothalamus. Results of GnRH immunocytochemistry on this brain were previously reported (3). This mouse received an NMA challenge and was treated in a similar manner to all other subjects in this study. After cell implantation, vaginal cytology was assessed daily in all mice.

Jugular cannulation, blood sampling, and NMA challenges:

The methods for jugular cannulation were described in Chapter 2. For all experiments, cannulations were conducted in

the morning, designated day 0, under chloral hydrate anesthesia. All mice were then housed individually, and returned to the colony awake prior to lights out. On the morning of day 1, the cannula was removed from its housing and attached to a 1cc syringe filled with 3U heparin/ ml saline. Syringes were suspended above each home cage.

Blood samples were collected and plasma samples were isolated and stored as described in Chapter 2. Throughout an experiment, blood cells from samples were resuspended in 3U heparin/ ml human serum albumin (5%, 60-80μl; Armour Pharmaceutical Co., Kankakee, IL) and introduced back into the host via the cannula immediately following the next sample's withdrawal or an NMA challenge. NMA (20mg/4.0ml saline/kg body weight) was administered through the same cannula immediately following a blood sample.

RIA for LH:

The methods for RIA were identical to that described in Chapter 2. The intraassay coefficient of variation (CV) ranged from 10.5-14.8%, and the interassay CV was 7.2%.

Immunocytochemical Methods:

All immunocytochemistry was performed in the laboratory of Dr. A-J. Silverman at Columbia University, College of Physicians and Surgeons in New York. Animals were anesthetized with an overdose of chloral hydrate and perfused transcardially with

saline followed by Zamboni's fixative. Ovaries and uteri were removed at the time of perfusion and weighed. Brains were removed from the cranium and post-fixed for 4-24 hrs prior to sectioning on a vibratome (Oxford Instruments) at 40μm. Immunocytochemical detection of GnRH was carried out as described in Chapter 4. Sections were dehydrated, cleared, coverslipped and photographed.

Statistics:

Elevations in plasma LH values were measured using identical criteria as that used to assess LH pulsatility (9) and NMA-stimulated LH responses (10) in both normal female mice and HPG/POA female mice. First, LH values were evaluated by the Pulsar program (11) adapted for the PC computer (12). The cut-off criteria for pulse determination, G1, G2, G3, G4 and G5, were 3.1, 3.0, 2.6, 1.6, and 1.6, respectively. Second, LH values were evaluated by the following criteria: 1) initial elevation in plasma LH greater than 20% of the previous LH value, and 2) CV of the ascending and descending limbs of a putative pulse greater than two times the assay CV. Results using either criteria were identical.

RESULTS

As in a previous report (3), all mice remained healthy and showed no behavioral evidence of neurological damage. There were occasional cornified and nucleated cells in the vaginal

lavages, but none of the females entered persistent vaginal estrus. Only modest gonadal and/or uterine development occurred in the HPG hosts (Table 5-1).

Plasma samples for LH determinations were obtained from nine (including S75) female HPG mice with GT1-7 cell grafts. There were no significant spontaneous LH pulses in any of the nine mice, according to the criteria defined above. Three of these nine mice, however, showed significant increases in plasma LH secretion at 10 min following an NMA challenge (Y15, Y09 and S75, see Fig. 5-1). Y15 responded significantly to each of two NMA challenges administered 30 min apart, Y09 responded significantly to one of four NMA challenges, and S75 responded significantly to a single NMA challenge. An additional animal, Y32, exhibited episodes of spontaneous LH secretion that began prior to each of two NMA challenges (Fig. 5-1). All LH values from the other five female HPG mice remained below the assay limit of detection.

GnRH immunoreactive cells were apparent in 6/8 HPG mice with GT1-7 cell grafts studied 42-48 days following cell implantation. Four of these mice had large bilateral hypothalamic tumors (Y08, Y09, Y14 and Y32), one developed a midline thalamic tumor (Y27), and one had about 1200 well differentiated GnRH positive neurons bilaterally in the piriform cortex and olfactory tubercle at the level of the diagonal band of Broca, but no evidence of tumor (Y15, Fig. 5-2). When tumors were observed, they consisted of a mass of undifferentiated cells from which

additional cells migrated into the host tissue (Fig. 5-3). The GnRH immunoreactive cells which were not part of the tumor tended to assume a more neuronal phenotype (Fig. 5-3) and elaborate axonal processes (data not shown; see Fig. 1b in ref. 3). GnRH axons did not grow towards or into the ME in any of these eight animals. S75, which was studied 68 days after cell implantation, had several hypothalamic tumors and axons from one did reach the ME (Fig. 4 in ref. 3).

DISCUSSION

This study provides the first evidence that intrahypothalamic GT1-7 cells can, in some cases, support spontaneous LH secretion and NMA-stimulated elevations in plasma LH concentrations. As in our previous report (3), GT1-7 cells are capable of surviving and differentiating within the brains of HPG mice. In many cases, cells proliferate into large tumors. Cells within tumor masses are spherical or flattened, whereas differentiated neurons are seen at the periphery of tumor masses or as isolated cells. Occasionally, GnRH positive cells with complex dendritic arbors are present in the absence of tumors. Although GT1-7 cells elaborate an axon, there is a general failure, unlike normal GnRH neurons, of the axons from transformed cells to target the ME. A similar finding was reported in the transgenic progenitors (13).

GT1-7 cells <u>in vitro</u> secrete GnRH in a pulsatile fashion (7). Significant spontaneous LH pulses were not observed in the

present study, although detectable increases in LH were apparent in some subjects. As in our previous study (3), modest gonadal development in the HPG hosts infers that GT1-7 cells are capable of stimulating spontaneous LH secretion (Table 5-1).

Tumors of GT1-7 cells were not necessary for the response of increased LH release to an NMA challenge. Although Y09 (Fig. 5-3) and S75 (see Figs. 3 and 4 in ref. 3) both had large tumors as well as cells with a neuronal morphology, i.e. dendrites and axons, Y15 had differentiated cells with variable neuronal morphology but no tumor.

The mechanism by which NMA stimulates GnRH neurons is currently unknown. Either GnRH neurons possess functional receptors for this ligand, or afferents to GnRH neurons mediate the response to NMA, or both. Although there is now a growing list of factors which directly stimulate or inhibit GnRH release from GT1 cells in vitro (14-20), there are currently no reports of NMA-stimulated GnRH release, nor is there published evidence of NMDA-type glutamate receptor mRNA (NMDAR1) in any GnRH-secreting cells.

Demonstration of NMA-stimulated GnRH release from GT1-7 cells in vitro would suggest that NMA responsiveness is an inherent property of these cells, and that integration of implanted cells with the host brain is not necessary for NMA-stimulated LH release in HPG mice with GT1-7 cell grafts. However, several lines of evidence suggest that GnRH release in response to an NMA challenge is via afferents to GnRH neurons.

Saitoh, et al (10), have shown that both normal mice and female HPG/POA respond to NMA with significant elevations in plasma LH, with the same timing and magnitude of response reported in this study. However, in male HPG/POA, NMA administration did not result in LH release even though robust GnRH fiber innervation of the ME was apparent. These observations lead me to hypothesize that the action of NMA on GnRH release is not directly on GnRH neurons, but is dependent upon appropriate afferent connectivity. In support of this hypothesis, NMA administration results in cfos protein expression, a marker of neuronal activation, in the nuclei of several candidate neuromodulatory afferents to GnRH neurons, but not in the GnRH neurons themselves (21).

Clearly, chemical stimulation of GT1-7 cells <u>in situ</u> can drive LH secretion. Since innervation of the ME was rarely apparent, it is unclear how secreted GnRH was delivered to the anterior pituitary gonadotrophs. One mechanism may be diffusion of GnRH through the brain parenchyma to the portal circulation in the ME. The large numbers of GnRH immunoreactive cells present following tumor formation might be capable of producing and releasing sufficient peptide to diffuse to the portal capillaries; however this mechanism does not account for the finding obtained in the case of Y15, where no tumor was present in the brain. Although only a few GnRH neurons in POA grafts are necessary to support reproductive development in HPG mice, in these cases GnRH axons always innervate the host ME.

I cannot exclude the possibility that some GT1-7 cells escape the brain and take up residence in the vicinity of the anterior pituitary. This scenario would suggest that GT1-7 cells are inherently responsive to NMA. This issue was examined in animals where the injection cannula reached the ventral pial surface but we found no evidence of GT1-7 cells in the anterior pituitary (Silverman and Gibson, unpublished). Clarification of the effects of NMA on GnRH secretion from GT1-7 cells *in vitro* will be useful in assessing whether integration with the host brain is essential for the NMA responses witnessed in this study.

Figure Legends

Fig. 5-1. Plasma LH values determined from sequential ten minute blood samples in four female HPG mice bearing GT1-7 cell grafts. ↓: NMA (20mg/4.0ml saline/kg body weight was administered following a blood sample as shown. *: significant elevation in plasma LH. ●: LH values. o: limit of detection of LH assay.

Fig. 5-2. GnRH positive neurons in the olfactory tubercle of Y15. In the low magnification (A) one can appreciate that these cells are dispersed through the host parenchyma and are not tumorous. At higher magnification (B) it is clear that the GnRH containing cells have a diverse morphology from multipolar (arrow) to a simple ovoid shape (arrowhead). All staining in this figure is due to the Ni-DAB reaction product; the sections were not counterstained. A = 416x B = 832x

Fig. 5-3. An example of a GT1-7 cell injection that resulted in the formation of a large tumor (T) from which GnRH positive cells have migrated (M) into the host. This photomicrograph of Y09 represents a very small portion of this tumor which extended throughout midline thalamic and hypothalamic regions. All staining in this figure is due to the Ni-DAB reaction product; the sections were not counterstained. 208x

Fig. 5-1

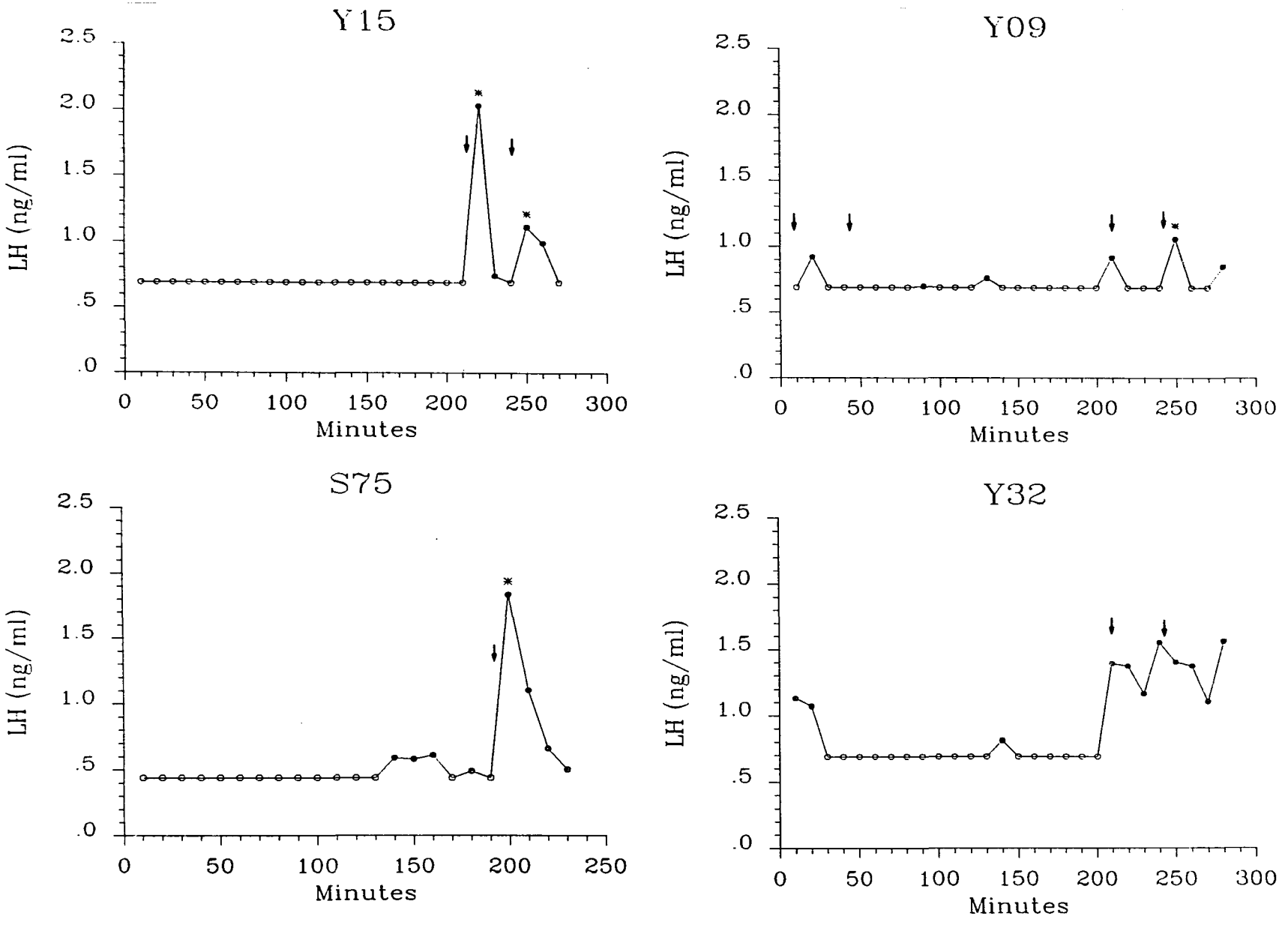
Y15
Y09
S75
Y32
LH (ng/ml)
Minutes
2.5
2.0
1.5
1.0
.5
.0
0
50
100
150
200
250
300

Fig. 5-2

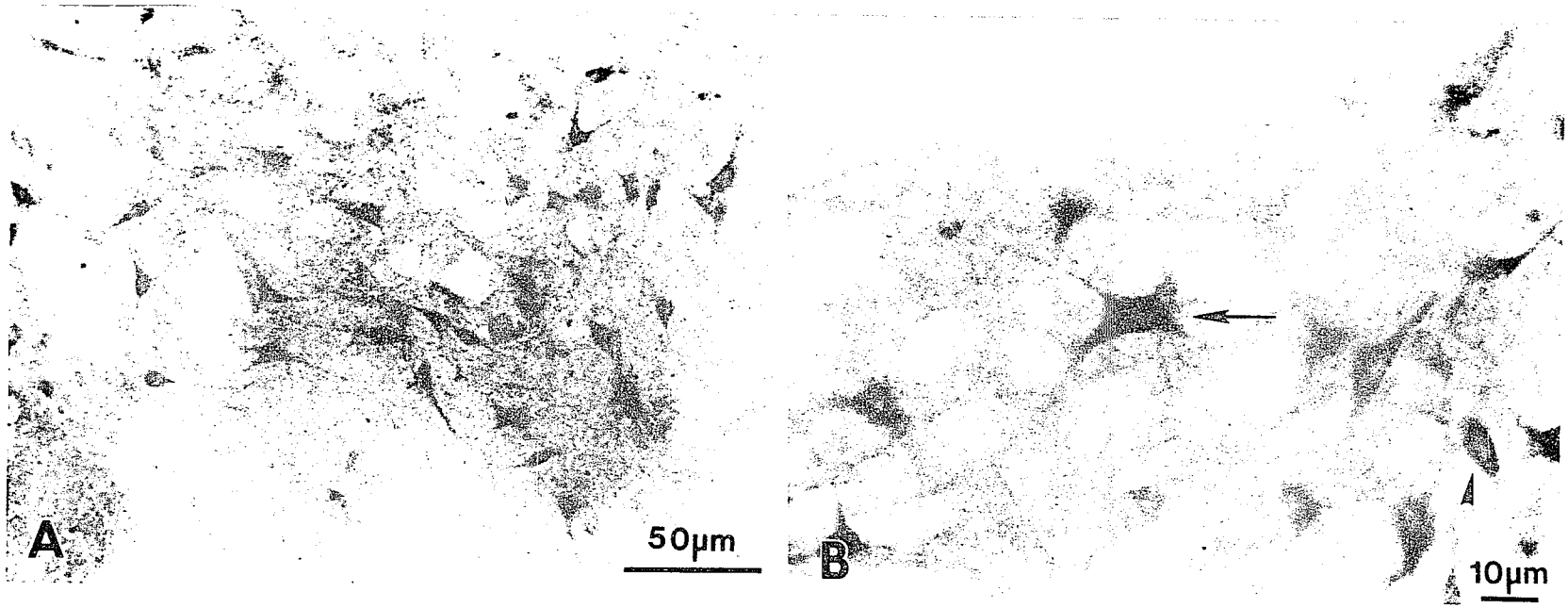

Fig. 5-3

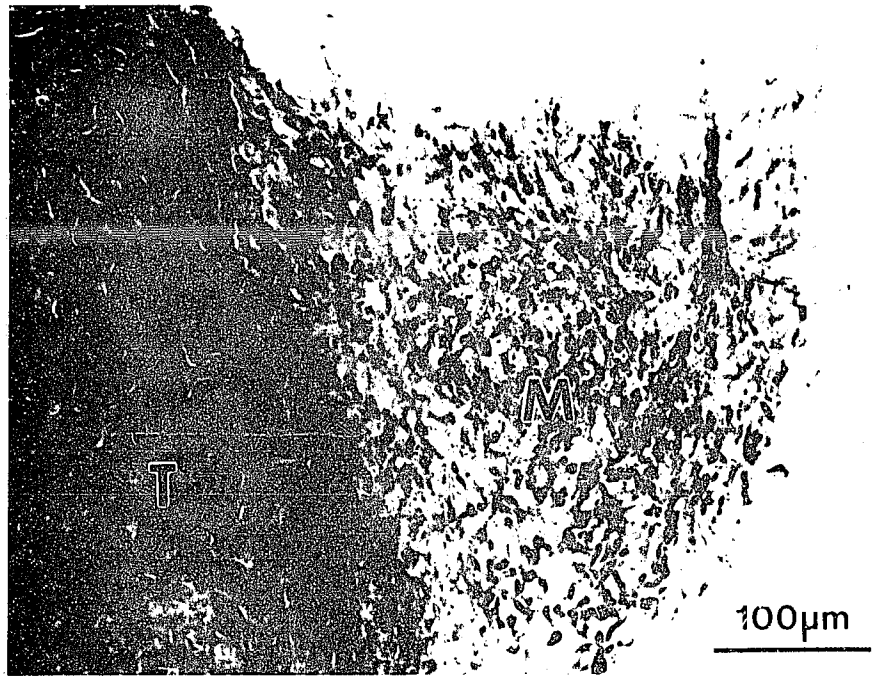

Table 5-1. Ovarian and uterine weights of female HPG mice bearing GT1-7 cell grafts.

	ovarian weight (mg)	uterine weight (mg)
Y08	1.6 (1)	23.7
Y09	1.6	18.1
Y14	<1.0	20.9
Y15	1.1	16.7
Y27	0.8	11.4
Y32	1.2	12.8
Y34	<1.0	16.8
Y35	1.0	8.6
S75	1.8	36.0
Untreated HPG	<1.0	5-12

Data for S75 was previously reported (3).

SUMMARY

In chapter 2, endogenous opioid interactions with N-methyl-D,L-aspartic acid (NMA) were studied in normal male mice. Although the opiate antagonist Naloxone (NAL) induces Luteinizing Hormone (LH) secretion in many species, there are no reports of an effect of NAL on LH release in mice. In my studies, the role of the endogenous opioid system in the regulation of LH release in adult male mice was assessed by testing whether this system was a component of the NMA-stimulated LH response. NAL (3mg/2.0ml saline/kg body weight), its quaternary derivative NAL methiodide (NALMI; 3mg/2.0ml saline/kg body weight), or saline (2.0ml/kg body weight) were administered alone and in combination with NMA (20mg/4.0ml saline/kg body weight) via intravenous catheters. Although neither opiate antagonist stimulated LH release when administered alone, each significantly potentiated the LH response to NMA in young adult (10- to 14-week-old) male mice ($p<0.01$) but not in older (10- to 16-month-old) male mice. The equivalent action of the two opioid blockers suggested an action outside of the blood-brain barrier.

I then ascertained whether opioid blockade altered pituitary sensitivity to Gonadotropin-releasing Hormone (GnRH). A dose response for exogenously administered GnRH was first determined, and low and high doses of GnRH were tested in combination with NAL or saline. Neither treatment was effective in altering the LH response to GnRH, indicating that the action

of the opiate antagonists was at a suprapituitary location. Ten- to 16-month-old male mice had significantly greater LH responses to NMA than 10- to 14-week-old male mice ($p<0.01$). GnRH challenges indicated that the increased LH secretion to NMA seen in the older males was not due to an increased pituitary sensitivity to GnRH. These findings support the hypothesis that NMA stimulates opioidergic neurons which contribute an inhibitory influence on GnRH secretion at a central location outside of the blood-brain barrier, such as the median eminence, and that inhibitory influences on the GnRH neuronal system diminish with aging. These are the first studies to demonstrate a role of endogenous opioid peptides on the hypothalamic-pituitary-gonadal axis in the mouse.

In chapter 3, the endogenous opioid system and its interaction with NMA was assessed in hypogonadal (HPG) mice with preoptic area (POA) brain grafts (HPG/POA). The HPG mutant mouse lacks GnRH-containing neurons and is infertile. Transplantation of normal fetal POA tissue into the third ventricle of the adult HPG mouse provides a source of GnRH neurons which innervate the host median eminence and stimulate reproductive development. To further explore how POA grafts integrate with the HPG host brain, I assessed the actions of NMA and opiate antagonists on LH secretion in male and female HPG/POA. Ten of 11 female and five of 12 male HPG/POA responded to at least one of three intravenous NMA challenges with significantly increased LH secretion. Opiate antagonists administered alone were

ineffective in stimulating LH release. However, opiate antagonist pretreatment significantly potentiated and prolonged the LH response to NMA in female HPG/POA but not in male HPG/POA. β-endorphin immunocytochemistry performed on seven of the female HPG/POA brains revealed fibers of host origin which innervated the graft. These data suggest that NMA activates host-derived opioidergic afferents which may impinge directly or indirectly on the GnRH neuronal system within the graft.

Among the individual HPG/POA males that responded to NMA challenge with significantly increased LH secretion were two animals that had received their grafts one year prior to testing. Immunocytochemistry for GnRH confirmed the continued presence of GnRH neurons and GnRH fiber innervation of the host median eminence in both of these long-term functional grafts. The findings in chapter 3 further establish that the effects of NMA on LH release are modulated by afferents to GnRH neurons, and illustrate a potential pathway for host-mediated modulation of grafted GnRH neurons in HPG mice.

Chapter 4 assessed whether steroid milieu modulates NMA-stimulated LH release in male HPG/POA. Previous studies had revealed a sex difference in the ability of HPG/POA mice to respond to an NMA challenge with elevations in plasma LH. Despite gonadal development and GnRH fiber innervation of the host median eminence in both male and female HPG/POA, fewer males than females had significant LH responses to NMA. My studies tested whether male HPG/POA could respond to NMA if

repeatedly challenged, and whether steroid milieu could affect the ability of male HPG/POA to secrete LH in response to NMA challenges. I found that a greater proportion of male HPG/POA responded to NMA challenges with increased LH secretion when castrated and treated with 17-β-estradiol (E2) either prior to graft implantation or at 1 wk prior to NMA challenge testing, in contrast to intact male HPG/POA or castrated male HPG/POA treated with vehicle. Further, there were no obvious differences in graft derived GnRH innervation of the host median eminence among the treatment groups. These data support the hypotheses that the action of NMA on LH release is mediated by estrogen-sensitive afferents to transplanted GnRH neurons, and that the sex difference in the ability of HPG/POA to respond to NMA with elevated plasma LH levels is due to a sex difference in steroid milieu.

In chapter 5, female HPG mice received bilateral intra-hypothalamic implants of an immortalized GnRH-secreting cell line (GT1-7). Nine mice were tested 42-65 days after implantation to determine whether these cells could support spontaneous and/or NMA-stimulated LH secretion. When sampled via intravenous catheters, four mice had measurable LH secretion. Three of these mice responded to NMA challenges with significant increases in circulating LH. GnRH immunocytochemistry revealed that GT1-7 cells were present in these four mice and three others in which LH values were not detectable. There were about 1200 GnRH-immunoreactive cells dispersed within the piroform cortex

and olfactory tubercle and no tumor found in one of the HPG mice that responded to NMA, whereas the other NMA responders had large bilateral hypothalamic tumors. The presence or absence of such tumors did not predict the capacity to respond to the NMA challenge with alterations in LH secretion. This study provides the first evidence that intrahypothalamic GT1-7 cells can support LH release in the HPG mouse, and that this secretion can be modified by pharmacological agents.

In this dissertation I have shown that the endogenous opioid system can modulate GnRH release in the mouse. I have presented evidence in the normal mouse that the action of NMA on LH secretion is modulated through afferents to GnRH neurons, and that opioid-containing afferents stimulated by NMA modulate GnRH release. The studies in HPG mice with GnRH-secreting cell grafts further establish that the effects of NMA on LH release are modified via afferents to GnRH neurons which may also, in part, communicate information regarding steroid milieu. In this manner, the excitatory amino acids and the endogenous opioid peptides serve as important neuromodulators of the GnRH neuronal system in the mouse.

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