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Title of Thesis: Morphine Endogenous Opioid Peptides and Reproduction in the Male Rhesus Monkey.

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ABSTRACT

Title of Dissertation: Morphine, Endogenous Opioid Peptides, and Reproduction in the Male Rhesus Monkey

Pamela M. Gilbeau Scher, Doctor of Philosophy, 1983

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This study examines the effects of opioid agonists and antagonist on reproductive hormones in the male primate and explores the role of the endogenous opioid peptides (EOP) in the neuroendocrine control of primate reproductive function, specifically in the control of gonadotropin secretion. The prototype narcotic used was morphine sulfate, which acts via the mu opiate receptor type. The stable leucine-enkephalin analogue [D-Ala^2, D-Leu^5]-enkephalin (DADLE) and β-endorphin (β-end) were used as representatives of the EOP. DADLE acts via the delta type opioid receptor, whereas β-endorphin has mixed opiate receptor activities (both mu and delta). The opiate receptor blocker naloxone was used as a competitive antagonist to the pharmacologic effects of both the narcotic and the EOP.

These drugs or their vehicles were administered to adult male rhesus monkeys fitted with jugular catheters. The drugs were given intravenously and blood was drawn through the catheters to reduce the stress associated with these procedures. Blood was collected at 20 minute intervals for periods of four hours and the plasma retained for measurements of testosterone, luteinizing hormone (LH), and prolactin

(PRL) by radioimmunoassay.

Administration of morphine (1.0 mg/kg) and DADLE (10 μg/kg) produced marked decreases in LH levels within one hour which were followed by decreases in testosterone levels. Levels of both hormones remained depressed for approximately three hours. β-endorphin at 10 - 20 μg/kg had no effect on LH or testosterone levels. Naloxone (2.0 mg/kg) increased LH levels eight-fold and testosterone levels nine-fold; LH levels remained elevated for up to three hours and testosterone levels for up to two hours.

Both morphine and β-endorphin elicited immediate increases in prolactin levels, which remained elevated for up to three hours. In addition, naloxone was seen to decrease prolactin levels, but DADLE produced no significant prolactin changes.

Several of the drugs were shown to affect the amplitude or frequency of hormone level episodic fluctuations. Morphine lowered the amplitude of episodic LH fluctuation and the frequency of episodic testosterone fluctuations. DADLE lowered the episodic amplitudes of both LH and testosterone. β-end was seen to increase the episodic fluctuation amplitudes of both LH and testosterone without increasing their overall levels. Naloxone increased the episodic amplitudes of both LH and testosterone.

That the effect of these drugs on gonadotropin levels is mediated at the hypothalamus was demonstrated in reversal experiments in which the depressant effect of morphine or DADLE was completely reversed by administering gonadotropin releasing hormone (GnRH). In addition, the effects of these opioids on LH were shown to be reversible by naloxone, thus indicating an opiate receptor site of action.

These results show that the stimulation of the opiate receptors

by morphine or DADLE exerts a negative effect on LH and testosterone levels and a positive effect on PRL. Blocking the opiate receptors with naloxone produces the opposite of these effects. Since the changes in LH levels precede those of testosterone, and GnRH reverses the opioid effects on LH, it is likely that the opioids have their effect at the levels of the hypothalamus. In addition, the apparent selectivity of mu receptor agonists (morphine and β-end) for PRL indicate that PRL is modulated by mu opiate receptors, whereas both mu and delta receptors are involved in the regulation of LH secretion.

MORPHINE, ENDOGENOUS OPIOID PEPTIDES, AND REPRODUCTION IN THE MALE RHESUS MONKEY

by

Pamela M. Gilbeau Scher

Thesis submitted to the Faculty of the Department of Pharmacology
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Dedication

For all their help and support throughout my studies and during the preparation of this dissertation, I dedicate this dissertation to my husband, Robert B. Scher, Ph.D., my advisor, Carol Grace Smith, Ph.D., and my friend Deborah A. Mullen.

Pamela M. Gilbeau Scher

21 May 1983

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LIST OF ABBREVIATIONS

AA	acetaldehyde
ACTH	adrenocorticotropic hormone
AG	aminoglutethamide
AP	anterior pituitary
ARGG	anti-rabbit gamma globulin
B-BB	blood-brain barrier
β-end	beta-endorphin
β-LPH	beta-lipotropin
CNS	central nervous system
cyn	cynomolgus
DA	dopamine
DADLE	[D-Alanine^2,D-Leucine^5]-enkephalin
DAMME	D-Ala^2,MePhe^4,Met(o)-enkephalin-ol
Enk	enkephalin
EOP	endogenous opioid peptide
EP	epinephrine
FSH	follicle stimulating hormone
GnRH	gonadotropin releasing hormone
GTP	guanosine triphosphate
HCG	human chorionic gonadotropin
HCT	hematocrit
Leu-enk	leucine-enkephalin
LH	luteinizing hormone
Met-enk	methionine-enkephalin
MS	morphine sulfate
NAL	naloxone

NE	norepinephrine
NICHD	National Institute of Child Health and Development
PIF	prolactin inhibitory factor
PRF	prolactin releasing factor
PRL	prolactin
RIA	radioimmunoassay
rms	root mean squared
TRH	thyrotropin releasing hormone
5HT	serotonin

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Chapter 1

INTRODUCTION

Recent studies with endogenous opioid peptides (EOP) indicate that the EOP may be involved in the control of gonadotropin secretion. The current studies provide new information on the effects of the opiate drugs on reproductive endocrinology in the primate and on the mechanisms that produce these effects. The disruptive effects of narcotic abuse by men have been documented in the clinical literature. In male addicts, marked reductions in sexual activities, function, and fertility have been reported. Laboratory investigations in rodents, however, have provided the majority of the information on the mechanisms of these actions. The neuroendocrinology of reproduction differs in primate and non-primate animals. The vulnerability of the various species to drug actions also differs. The present studies were designed to distinguish between drug effects at the gonadal and pituitary-hypothalamic levels and to explore the role of EOP in neuroendocrine control of primate reproductive function. It is reasonable to assume that much additional information will be gained from studies of the effects and the mechanisms of these narcotic drugs' actions in the primate.

Under normal conditions, the hypothalamus secretes gonadotropin releasing hormone (GnRH) and prolactin inhibitory factor (PIF). These hormones cause the release of the gonadotropic hormone luteinizing hormone (LH) and prolactin (PRL) by the pituitary gland (Figure 1). This is complex unit is termed the hypothalamic - pituitary axis. LH and PRL are carried by the bloodstream to the testes where they stimulate secretion of the sex steroid testosterone. Testosterone is carried in

Chapter 2

BACKGROUND

Hypothalamic-Pituitary-Gonadal Function

Under normal conditions, the hypothalamus secretes gonadotropin-releasing hormone (GnRH) and prolactin-inhibitory factor (PIF). These hormones regulate the release of the gonadotropin hormones luteinizing hormone (LH) and prolactin (PRL) by the pituitary gland (Figure 1). This complex unit is termed the hypothalamic-pituitary axis. LH is carried by the bloodstream to the testes where it stimulates secretion of testosterone. PRL is released from the pituitary axis into the bloodstream and carried to the gonads where it potentiates the action of LH on testosterone release. This effect of PRL has been demonstrated in rodents, but has not been studied in primates. Testosterone is carried in the blood to the sex accessory organs (e.g. seminal vesicles and prostate) where it stimulates growth and development, and maintains function of these organs. PRL synergizes with testosterone to stimulate these agents (Bartke, 1980). Testosterone also exerts feedback control on hypothalamic-pituitary function. Prolactin secretion appears to be influenced by thyrotropin-releasing hormone (TRH), as well as PIF. PRL secretion by normal pituitaries is increased by TRH through a direct stimulation of the lactotropes' adenylate cyclase system (Jackson, 1982). Although this increase in secretion occurs in man as well as in laboratory animals, TRH does not appear to play an important physiological role in PRL regulation (Besser, et al., 1979).

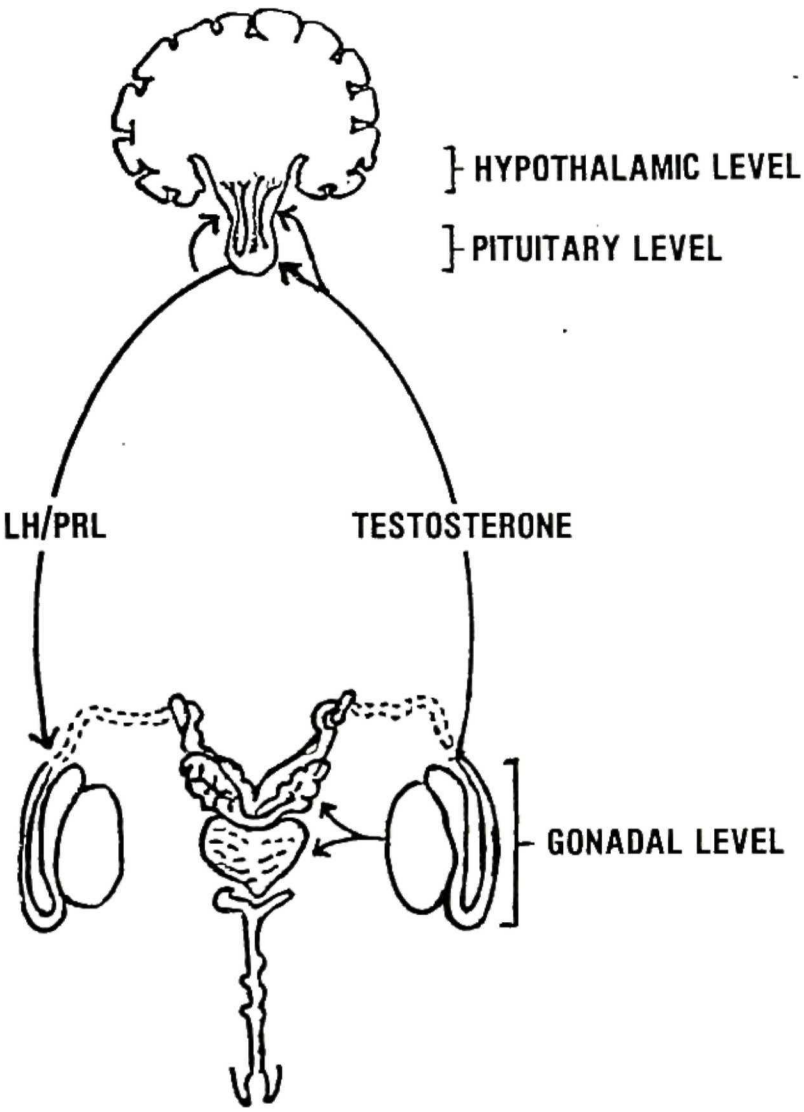

Figure 1. The normal hypothalamic-pituitary-gonadal relationships that control male reproductive function.

The Endogenous Opioid System

In 1964, C. H. Li published information describing a new biologically active peptide isolated from pituitary glands. This peptide, discovered in the process of the isolation of adrenocorticotropin (ACTH), possessed very low adrenocorticotropic activity, and had melanocyte-stimulating activity similar to ACTH (Li, 1964). The peptide, containing 91 amino acids, was named β-lipotropin (β-LPH). Later, two pentapeptides with potent opiate-agonist properties were isolated from brain and named methionine-enkephalin (Met-enk) and leucine-enkephalin (Leu-enk) (Hughes, 1975; Hughes et al., 1975). At this time it was noted that the amino acid sequence of Met-enk was identical to that of amino acids 61 to 65 of β-LPH. Further investigation of fragments of β-LPH led to the isolation of β-LPH 61-91 or β-endorphin (β-end). This fragment displayed opiate-like activity pharmacologically similar to but far more potent than, the classical opiate-agonist, morphine (Loh et al., 1976).

A large amount of attention has been focused on the opioid receptor itself. Opioids interact with stereospecific, saturable receptors. These receptors are present in high concentrations in the hypothalamus, thalamus, midbrain, and limbic system (Snyder, 1979). Investigations using CNS slice preparations and cell cultures have shown that opioid agonists act by decreasing adenylate cyclase activity. This effect is reversible by administration of naloxone and is thought to be mediated by stimulation of GTP hydrolysis through enhanced GTPase activity (Sharma et al., 1975; Koski and Klee, 1981).

Two important questions concern the number of types or classes of of opioid receptors and their distribution within the CNS. In 1976,

Martin et al., postulated existence of three types of opioid receptors in the CNS. Their theory was based on in vivo observations that various natural and synthetic opioids elicited different pharmacological effects and that certain analogues could not substitute for each other to supress withdrawal in addicted chronic spinal dogs. These three receptor types were distinguished by and named for their prototypical agonist: μ for morphine, σ for N-allylnormetazocine (SKF 10047), and κ for ketocyclazocine.

In vitro studies using preparations of guinea pig ileum and mouse vas deferens again revealed different opioid receptors, based on agonist activities (Lord et al., 1977). Receptors in the guinea pig ileum were more sensitive to morphine (μ-receptors), while the mouse vas deferens contained receptors with greater sensitivity to enkephalins (δ-receptors). These findings are supported by cross-competition binding studies between opiate alkaloids and enkephalins in guinea pig brain membrane preparations (Simon et al., 1980).

Morphine is thought to exert its pharmacologic activity primarily by interaction with the μ opiate receptor type. Beta-endorphin has mixed narcotic activities (μ and δ), whereas Leu-enk exerts its pharmacologic activities primarily by interaction with δ receptors (Kosterlitz and Hughes, 1978). Due to rapid proteolysis of Leu-enk in the blood, a stable analogue [D-Ala^2, D-Leu^5]-enkephalin (DADLE) was used in these studies. Naloxone is a competitive antagonist to the pharmacological effects of both narcotic drugs and the EOP. This receptor blocker has been used to gain information on whether the EOP act endogenously as neuroendocrine modulators, and it has also been used as a pharmacological tool. Specificity of receptor antagonism by naloxone is is thought to be dependent upon the dose of the naloxone used. μ and δ opiate receptors

are both antagonized at high dose levels, while at low doses, naloxone antagonizes only μ-receptors (Kosterlitz, 1980).

Studies of a possible regional distribution of opioid receptor types in the CNS have utilized cross-competition studies. Bonnet et al., (1981) suggest that in human brain the thalamus contains a preponderance of μ-receptors, while the frontal cortex is enriched with δ-receptors. A similar distribution is observed in the rhesus monkey brain (Lewis et al., 1981).

The Postulated Role of the EOP in Anterior Pituitary Function

Since discovery of these endogenous opiate peptides and receptors (see above), they have been the center of much scientific investigation. A possible physiological role for the EOP involves regulation of pituitary hormone secretion. Most studies of effects of morphine and EOP on various aspects of the male hypothalamic-pituitary-gonadal axis have focused primarily on rodents. Very little is known about the effects of opiates on primate reproductive function.

The anterior pituitary is regulated by hypothalamic releasing and inhibiting hormones (Figure 2). These hormones are secreted into the hypophyseal portal system at the level of the median eminence. They are transported to the adenohypophysis where they act on specific cells to either stimulate or inhibit release of specific pituitary hormones.

Release of these hormones is not maintained as a steady release, but appears as episodic pituitary discharges. Control mechanisms ultimately responsible for these episodic discharges are as of yet unknown. However, evidence does exist to suggest that pulsatile release of LH is mediated by episodic release of GnRH from the hypothalamus (Carmel et al., 1976). This intermittent supply of GnRH is necessary to maintain

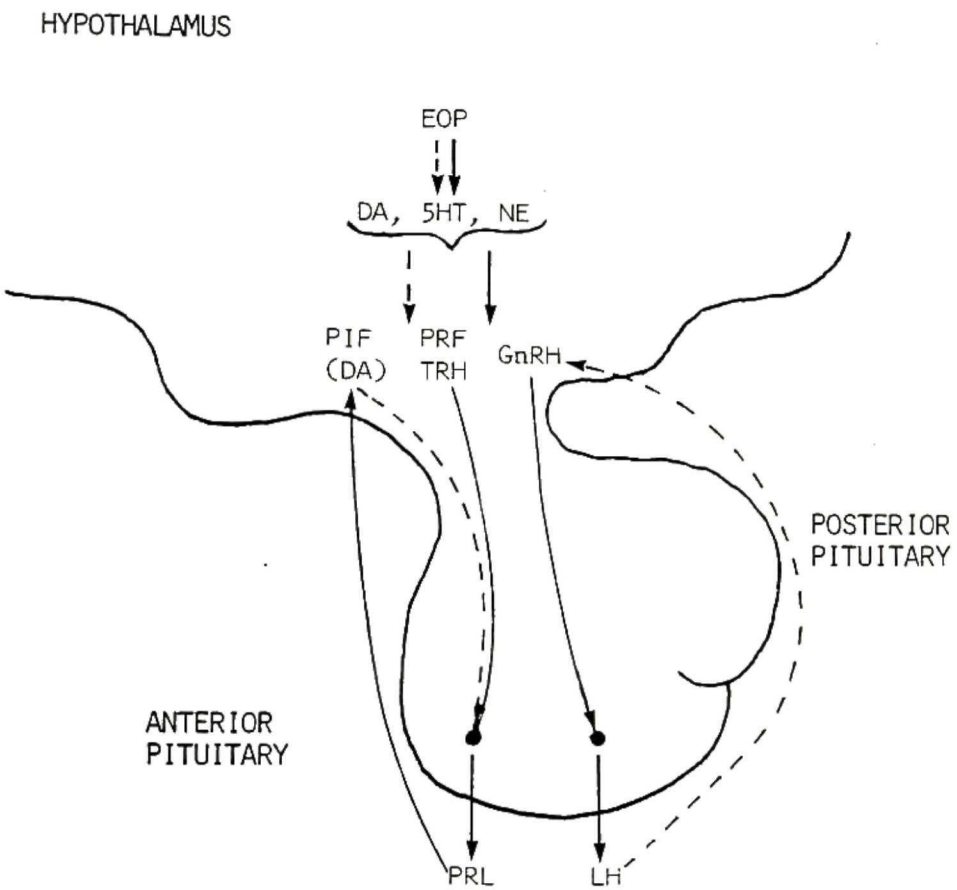

Figure 2. The relationships between hypothalamic hormones, neurotransmitters, endogenous opioid peptides, and anterior pituitary hormones. Hormone secretion is modulated by stimulatory (——) and inhibitory (---) effects. DA, dopamine; EOP, endogenous opioid peptides; GnRH, gonadotropin releasing hormone; LH, luteinizing hormone; PRL, prolactin; PRF, prolactin releasing factor; PIF, prolactin inhibitory factor; 5HT, serotonin; NE, norepinephrine; TRH, thyrotropin releasing hormone.

functional integrity of the adenohyphyoid gonadotropes (Belchetz et al., 1978). Ropert et al., (1981) investigated the effects of naloxone infusion on the frequency and amplitude of LH secretion in women during the luteal phase of the menstrual cycle. These studies suggested that the EOP may modulate GnRH pulsatile release.

The hypothalamic neurons which synthesize and secrete releasing/inhibiting hormones are controlled by various putative synaptic transmitters (neurotransmitters). Neurotransmitters for which significant evidence exists regarding their role in the complex neural circuitry of hypothalamic releasing/inhibiting hormone regulation include dopamine (DA), epinephrine (EP), norepinephrine (NE), and serotonin (5HT). It is possible that narcotics and EOP elicit their actions through modulation of these neurotransmitters.

1. Effects of Opioids on Luteinizing Hormone, Prolactin, and Testosterone Secretion

Narcotics. Effects of narcotics, especially morphine, on male rodent gonadal functions and sex hormone levels have been well documented. Chronic narcotic users have reported decreased fertility resulting in atrophy of male accessory sex organs. Marked decreases in seminal vesicle and prostate weights (Tokunaga et al., 1977; Cicero et al., 1977; Meites et al., 1979) have been observed in narcotic treated rats. The onset of effects produced by morphine (100 mg/kg) is very rapid. Significant reductions of serum testosterone levels within two hours of drug administration were reported (Muraki et al., 1978). Normal levels of testosterone returned within 48 hours of narcotic withdrawal. This effect on serum testosterone levels has been shown to be inhibited by administration of the opiate-antagonist naloxone (Meites

et al., 1979).

Some information concerning effects of narcotics in human subjects is available; however, since most subjects were chronic users of narcotics, the acute or short-term narcotic effects have not been appropriately evaluated. In studies involving male addicts, marked reductions in sexual activities, function, and fertility have been reported (Cicero et al., 1976). Significant reductions in the serum testosterone levels of male addicts has consistently been reported (Wang et al., 1978). Administration of narcotics increases prolactin levels in rodents (Meites et al., 1979) and humans (Kley et al., 1977). In rodents, this increase in PRL is reversed by administration of naloxone (Shaar and Clemens, 1980). Data on other sex hormones is conflicting. Serum levels of follicle stimulating hormone and luteinizing hormone in male addicts are reported as normal (Wang et al., 1978), significantly decreased (Brambilla et al., 1979) or slightly increased (Kley et al., 1977).

Several mechanisms for the effects of narcotics on plasma LH, FSH, and PRL levels have been proposed. Recent investigations in rodents have indicated that narcotics act via the hypothalamus and not through a direct pituitary effect, since addition of morphine to an incubation system containing rat anterior pituitary tissue had no effect on gonadotropin release (Meites et al., 1979). The majority of current evidence concerning the narcotic-induced decrease in plasma testosterone levels provides for a CNS-mediated action, although a concomitant direct peripheral action cannot be excluded. Evidence for a direct testicular action has not been found for morphine, but does exist for other narcotics such as methadone (Jakubovic et al., 1979; Purohit et al., 1978). These inhibitory effects on testosterone production were not antagonized by naloxone, thus indicating actions not involving specific opiate recep-

tors.

Endogenous Opioid Peptides. Recent studies with endogenous opioid peptides (EOP), both enkephalins and endorphins, indicate that they are intricately involved in the regulation of sex hormone secretion. Administration of EOP to male rodents results in significant depression of serum LH levels with no effect of FSH (Meites et al., 1979; Cicero et al., 1979). Serum PRL levels increase significantly following EOP administration to rodents (Lien et al., 1976; Shaar and Clemens, 1980).

In normal human male volunteers, a consistant decrease in basal serum levels of LH has been shown with administration of ß endorphin, Leu-enkephalin, Met-enkephalin, or [D-Ala^2, MePhe^4, Met(o)-ol] enkephalin (DAMME, a long-acting analog of Met-enk) (Stubbs et al., 1978; Grossman et al., 1981; Reid et al., 1981). Serum levels of FSH are decreased (Stubbs et al., 1978) or not affected (Grossman et al., 1981) by DAMME. Neither Met- nor Leu-enkephalin were observed to alter basal FSH levels (Golstein et al., 1981). Administration of DAMME to normal male volunteers produces a marked increase in circulating PRL (Stubbs et al., 1978) while neither Met- nor Leu-enkephalin affected serum PRL levels (Golstein et al., 1981). In studies with non-human primates ß-endorphin was shown to significantly increase PRL blood levels (Wardlaw et al., 1981).

Regulation of sex hormone secretion by EOP appears to be at the level of the hypothalamus rather than at the pituitary. Decreases in serum LH and FSH brought on by DAMME can be reversed and even increased over basal levels by gonadotropin releasing hormone (GnRH) administration in humans (Grossman et al., 1981). ß-endorphin stimulation of PRL release in the intact non-human primate is not observed in

the pituitary stalk-sectioned monkey (Wardlaw et al., 1981). In addition, in vitro incubation of rodent pituitaries with EOP produced no effect (Shaar et al., 1977; Rivier et al., 1977). Recent investigations indicate that the EOP may act via hypothalamic neurotransmitters.

Opioid Antagonists. To further assess effects of EOP on the male reproductive system, a number of studies have been undertaken using the opioid antagonists naloxone and naltrexone. Intravenous injections of naloxone into normal human male volunteers resulted in a significant rise in the plasma level of LH followed by an increase in plasma FSH (Lightman et al., 1981; Morley et al., 1980). This increase in plasma LH is supported by studies in women (Quigley and Yen, 1980; Ropert et al., 1981) and male rats (Cicero et al., 1980). These results suggest a tonic inhibitory effect of EOP on LH and FSH.

The effect of opioid antagonists on PRL levels is controversial. In man, PRL levels following naloxone infusion are unchanged (Blankstein et al., 1979; Lightman et al., 1981; Morley et al., 1980) or decreased (Rubin et al., 1979). A consistent decrease in plasma PRL has been seen in rodents treated with naloxone (Bruni et al., 1977; Meites et al., 1979). This effect on PRL concentration may prove to be species specific.

Chapter 3

METHODS

This section is divided into two major areas: Description of Techniques and General Research Protocol.

1. Description of Techniques

Animal Care and Housing. Adult male rhesus monkeys (Macaca mulata) were caged individually and housed in the Department of Laboratory Animal Medicine primate facilities. These animals were exposed to environmental conditions of constant temperature 75±2°F with 50% relative humidity and a 14-hour light, 10-hour dark cycle. Bi-daily rations of Charles River primate formula (Agway) were provided ad libitum together with fresh fruit or peanuts once daily. Water was available ad libitum.

Drugs and Hormones. Morphine sulfate (MS), [D-Ala^2,Leu^5]-enkephalin (DADLE), β-endorphin (β-end), naloxone-HCl (NAL), and GnRH were solubilized in sterile normal saline and sterilized through a Nalgene 20 micron filter unit. These drugs or their respective vehicles were administered through an indwelling intravenous catheter. Human chorionic gonadotropin (HCG) was solubilized according to the manufacturer's instructions and given intramuscularly. Aminoglutethamide tablets were administered orally to monkeys under light ketamine-HCl anesthesia. Previous studies in our laboratory showed that light ketamine anesthesia had no effect in the hormones studied.

Blood Drawing. An indwelling intravenous catheter/conduit system was developed in order to facilitate blood drawing. This system consists of a silastic catheter surgically inserted into the monkey's internal jugular vein and passed through the vein into the pre-cava.

The catheter was fastened in place by dacron glitches and runs subcutaneously over the animal's shoulder to mid-back, where it exits through the skin into a stainless steel conduit connected to a close fitting jacket. This catheter-conduit combination then continues through the back of the cage and into the adjoining room, where it is connected to a swivel/pump set-up (Figure 3). A constant infusion of heparinized saline (2.5 IU/ml) maintains the catheter's patency. The catheter was left in place for as long as patency could be maintained (currently for up to 15 months). This system eliminates the stress associated with the handling of monkeys during blood drawing and drug administration. It also makes possible blood drawing from sleeping animals. The elimination of the stress variable is important in endocrine studies because sex hormone levels can be altered by stress. Blood samples (3.0 ml) were drawn into sterile syringes through the indwelling catheter and placed in heparinized blood collection tubes. Blood samples were centrifuged and the plasma stored at -20°C until assayed.

Testosterone Assay. Testosterone plasma levels were measured by radioimmunoassay (RIA) using a tritium labeled antigen and dextran-coated charcoal absorption for separation of bound and free hormone. Testosterone antisera, produced in rabbits in response to testosterone-3-oximealbumin, testosterone standards, and ^{3}H-testosterone were obtained from Wien Laboratories, Inc. Assay components were incubated for one hour at 4°C before the addition of charcoal. Prior to the actual RIA, plasma samples were extracted in methylene chloride and distilled water. Steroid recovery averaged 96%. The intraassay coefficient of variation averages 6.3% and the average interassay variation was 10.1%. The lower level of sensitivity of this assay was 5.0 ng/dl. Testosterone values for non-treated male rhesus ranged from 200-1500 ng/dl.

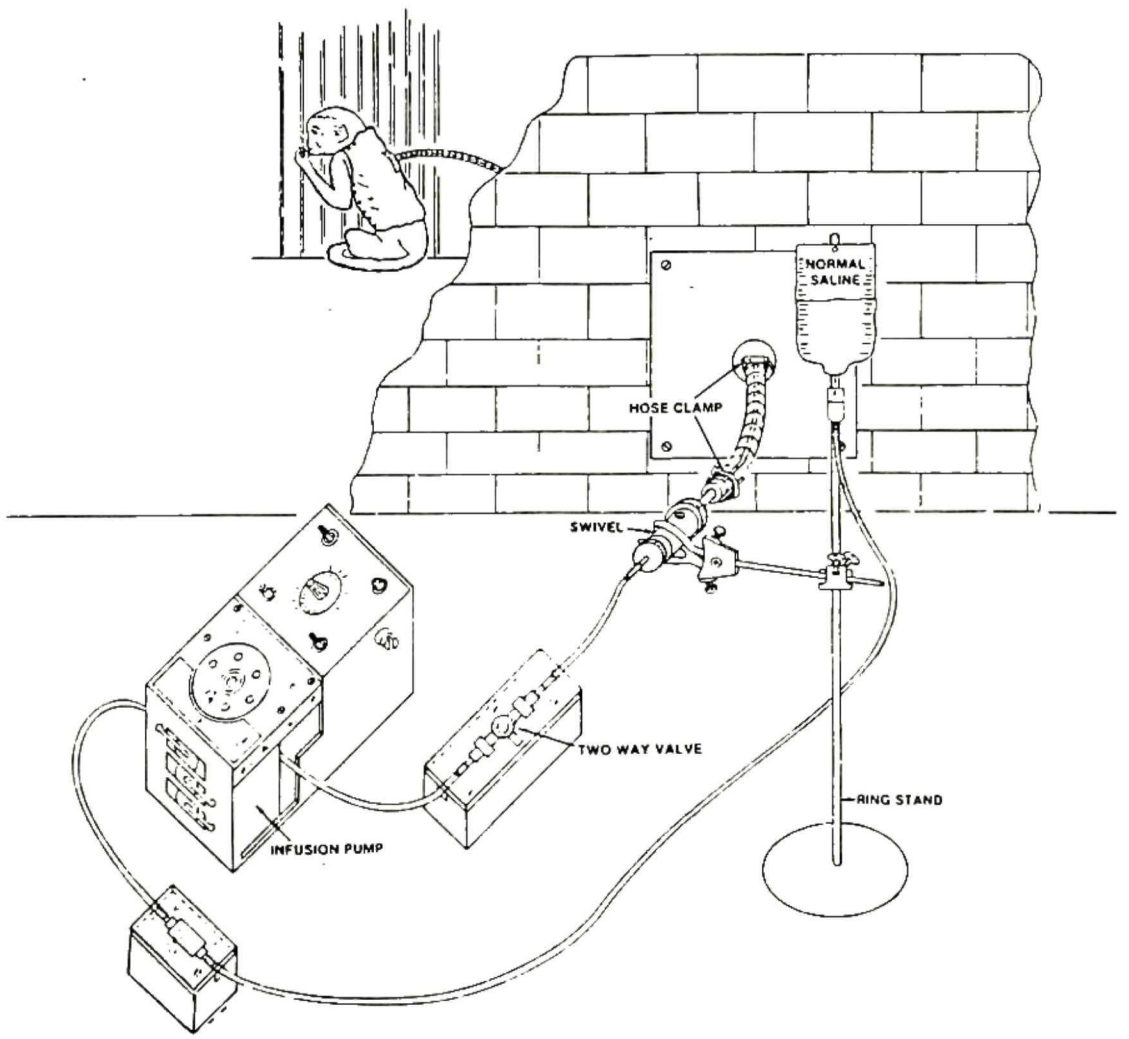

Figure 3. Indwelling catheter system. The rhesus monkeys are jacketed, tethered, and caged in an adjacent room. The apparatus for for maintaining a patent catheter and obtaining blood samples is shown.

Gonadotropin Assays.

(a) Luteinizing Hormone. Monkey LH was measured by a double antibody RIA using cynomolgus (cyn) monkey antigen. The components of the assay were prepared by Dr. Gary Hodgen and provided by NICHD. A highly purified cynLH (W2-XV-63) was used for radioiodination. The lactoperoxidase method of iodination was employed as it yields relatively undamaged antigen of high specific activity, ranging from 65-112 μCi/μg. This iodination was done by Hazelton Laboratories,Inc., Vienna, VA. Rabbit antiserum to HCG (R13, pool D) was used at a final dilution of 1:20,000 at which 30-40% of the tracer was bound. Rhesus LH reference preparation (NICHD-rhLH; also known as WP-XV-20) was used to provide a standard curve with concentrations ranging from 1-80 ng/tube. Separation of the bound and unbound hormone was through the addition of a second antibody, sheep anti-rabbit gamma globulin (ARGG). The sensitivity of the assay was increased at least two-fold by increasing the time of incubation prior to the addition of the labeled antigen. All reactions were carried-out at 4°C. The intraassay coefficient of variance averaged 2.7% and the average interassay coefficient of variance was 6.1%. The lower level of sensitivity of this assay was 2.5 ng/ml. Average LH values for male rhesus ranged from non-detectable to 30.0 ng/ml.

(b) Prolactin. The PRL assay was a double antibody RIA. Assay components were provided to our laboratory by the Pituitary Hormones and Antisera Center of the National Institute of Health. A highly purified human PRL antigen (AFP-2284C2) was iodinated through a chloramine-T process in which the amounts of radioactivity added to this antigen was monitored throughout the process and thereby controlled so as to yield an undamaged antigen with specific activity. This process yielded an antigen with specific activity from 30 - 40 μCi/μg. The iodination was

was done as needed by Hazelton Laboaratories, Inc., Vienna, VA. Rabbit anti-hPRL (AFPC11580) was used at a final dilution of 1:400,000 which yielded total binding of 45-55%. A hPRL reference preparation (AFP--2312C) was used as to construct standard curves with concentrations ranging from 0.39 - 100.0 ng/ml. Normal horse serum was added to each tube of the standard curve for stabilization. The reagents were incubated for 24 hours prior to the addition of the labeled antigen. Following a second 24 hour incubation, titered sheep-ARGG was added to precipitate bound from free hormone. All incubations were carried out at 4°C. The interassay coefficient of variance averaged 10.0% and the average intra-assay coefficient of variance was 12.5%.

Morphine Assay. Plasma levels of morphine were measured by RIA. Morphine antiserum (goat), morphine standards, and ^{125}I-morphine derivative were obtained from Roche Diagnostics. Assay components were incubated at ambient temperature for 10 minutes. The free and bound antigens were separated by precipitation with ammonium sulfate. The lower level of sensitivity of this assay was 10 ng/ml. The intraassay coefficient of variance averaged 3.3% and the average intraassay coefficient of variance was 8.7%. Acute i.v. administration of 1.0 mg/kg morphine sulfate elicited morphine plasma levels ranging from 600 to 900 ng/ml at 0.5 to 3.0 hours.

Analysis of Data - Drug Effects on Hormone Levels. The conventional method of statistical analysis of time course effects relies on the drug effect being comparable in magnitude to, or larger than, the amount of variability among individual subjects at any sample time in either a drug or control series. The conventional method was not appropriate for the present work, however, as numerous experiments on rhesus

monkeys using the indwelling catheter system have indicated: 1. extreme short-term variability of hormone levels; 2. major variations at exact 24 hour intervals in a single animal; and, consequently, 3. significant variability among subjects at any individual sampling point.

The following method, which can be instituted in either a "paired" or "unpaired" scheme, was used to determine if statistically significant differences existed between "control" and "drug" series. This method employs two basic concepts: smoothing (averaging over multiple samples, and comparison of changes occurring over time in the control and drug sets rather than comparison of absolute levels.

The purpose of smoothing is to remove, at least partially, the obscuring effects of extreme short-term variability, e.g., pulsatile behavior in LH and its effects. Thus the data analysis method allows each original time series of samples to be replaced by a moving average series, the averaging window size being a selectable parameter. (A window size of one sample simply reproduces the original time series.)

The process of change comparison is to first compute from each smoothed series its changes relative to its initial value. This amounts to subtracting the first value from all the others. The initial value represents pretreatment hormone levels. Following this procedure, point to point comparisons can be made between vehicle and drug series. Such comparisons enable us to detect the statistical significance level of the differences between drug-treated and vehicle series with respect to their changes from pretreatment levels.

As already mentioned, the method described lends itself to either a "paired" or "unpaired" format. In some of the experiments performed with the indwelling catheter system, the groups of animals available for for the control and drug experiments were not identical. Thus, it is

necessary in these cases to make unpaired comparisons. As is done in the conventional analysis method, the statistical test applied to the "smoothed change" time series point-by-point is Student's t-test in its paired or unpaired form as appropriate. Null hypotheses were rejected at the (two-tailed) 95% significance level ($p < 0.05$).

Analysis of Data - Effects on Episodic Release Patterns. Episodic hormone release and level fluctuation patterns were studied by essentially classical techniques for the spectral analysis of time series (Grenander and Rosenblatt, 1957). To characterize episodic release patterns for a group of animals subject either to drug or vehicle, each animal's hormone levels were de-trended by computing and removing the best (least-squares) second-order polynomial fit to the raw sample values. The periodogram (squared magnitude of the discrete Fourier transform) of the residuals was computed as a spectrum estimate. (This estimate was selected in lieu of the cosine-transform of the Hammingwindowed sample autocovariance (Blackman and Tukey, 1959) because: 1. the time series were relatively short (10 samples); and 2. tests on pure sinusoidal time series of up to 50 samples indicated that errors of the sample autocovariance from the true function could result in some negative spectral values and exaggeration of the significance of the peak.) The spectrum estimate was scanned for peak value and, in addition, a "weighted average frequency" (discrete spectral estimate frequencies weighted by their respective spectral values) was computed. This weighted average frequency was found to closely approximate the peak frequency when the latter is very dominant, but unlike the peak frequency, always changes smoothly for small changes in the time series (e.g., for small changes in the frequency of a pure sinusoid). The weighted average frequency was therefore adopted as a representative measure of the episodic (residual) time

series' dominant frequency.

Averages and standard errors of both dominant frequency and associated amplitude were computed for all experimental groups of vehicle-treated and drug-treated animals. Grand averages of dominant frequency and amplitude were computed over all groups of vehicle-treated animals. In addition, t-test comparisons of dominant frequencies and amplitudes were made between drug-treated groups and their associated second-order polynomial fit to the raw sample values. The sample autocovariance of the residuals was computed and multiplied by a so-called Hamming window function. The resulting function was then cosine-transformed to yield the spectrum estimate.

For time series consisting of typically 10 samples, spectrum estimates at four frequencies ("spectral frequencies") could be estimated. (The zero-th spectral frequency always showed zero amplitude since trend removal left residuals with zero average value.) The spectral frequency having the highest amplitude was found, and of the two neighboring frequencies, that having the higher amplitude was also identified. An attempt was made to refine the estimate of the periodic component in the neighborhood of the spectral peak by finding that intermediate frequency between the spectral peak and its higher amplitude neighbor which could account for the observed ratio of amplitudes at the two spectral frequencies. The dominant frequency and associated amplitude were thus estimated for each time series. Averages and standard errors of both dominant frequency and associated amplitude were computed for all experimental groups of vehicle-treated and drug-treated animals. Grand averages of dominant frequency and amplitude were computed over all groups of vehicle-treated animals. In addition, t-test comparisons of dominant frequencies and amplitudes were made between drug-treated groups and

their associated vehicle (control) groups. In this way it was sometimes possible to discover apparent drug effects on the amplitude or dominant frequency of quasi-periodic hormone level fluctuations or "episodes."

2. General Research Protocol

The research project was designed to study effects of opioids and their antagonist on the adult male rhesus monkey reproductive system. In these studies morphine sulfate was used as a prototypical opiate, and leu-enkephalin and ß-endorphin as representatives of the endogenous opioid peptides. Naloxone was the opioid antagonist used as it exerts no agonistic effect of its own.

Studies of Acute Drug Effects on Gonadal Steroid Levels. In order to evaluate the acute effects of opioid agonists and naloxone on gonadal steroid levels, studies were designed to define the dose and time course relationships between the drugs administered and blood testosterone levels *in vivo*. Drug or vehicle was administered in an i.v. bolus through an indwelling jugular catheter and blood samples drawn back through the catheter. Due to constant vacillation of plasma sex hormone levels, frequent blood samplings were necessary. Blood was drawn at 20 minute intervals for a period of four hours. With frequent sampling the volume of blood drawn was limited to 3.0 ml.

The drawing interval of 20 minutes and post-treatment period of three hours were determined from our previous studies that showed a short blood sampling interval was necessary due to the normal episodic fluctuations in plasma testosterone levels. An interval of 20 minutes was decided upon because it allowed for at least three samples during a testosterone episode and one sample during a testosterone half-life ($t_{1/2}$

is 20 - 30 minutes). The three hour period allowed sufficient time to observe the drug effects while also allowing for 20 minute blood drawings without compromising the monkeys' blood volume. The four samples drawn during the hour before drug treatment were useful in establishing each monkey's pre-treatment levels, which could vary considerably from one day to the next. Drug or vehicle was administered after the fourth blood drawing (time zero minutes). All studies began at 0700 with drug or vehicle administration at 0800 EST.

The following drugs and dosages were administered:

Morphine Sulfate	0.25 mg/kg body weight
	0.50 mg/kg body weight
	1.00 mg/kg body weight

These doses gave a range of morphine blood levels equivalent to those observed in human patients administered morphine in analgesic doses.

Leucine-Enkephalin	5.0 µg/kg body weight
[D-Ala^2,D-Leu^5]-Enk	10.0 µg/kg body weight
	20.0 µg/kg body weight

Due to the extensive proteolysis of Leu-enk in the blood, the stable analogue [D-Ala^2,D-Leu^5]-enkephalin (DADLE) was administered. Substitution of D-alanine for glycine at the 2-position and D-leucine for leucine at the 5-position protects against degradation by an aminopeptidase and a carboxypeptidase, respectively (Pert et al., 1976; Bajusz et al., 1976). The dose levels were adapted from studies in humans by Golstein et al., (1981) and Stubbs et al., (1978).

ß-Endorphin	10.0 µg/kg body weight
	20.0 µg/kg body weight

The above doses were based on investigations by Spies et al., (1980) in female rhesus monkeys and Reid et al., (1981) in humans.

Naloxone	0.5 mg/kg body weight
	1.0 mg/kg body weight
	2.0 mg/kg body weight

These bolus dosages were adapted from naloxone infusion studies in female rhesus monkeys by Spies et al., (1980).

Vehicle series was run on the day prior to each drug series. Each monkey served as his own control. In order to rule out any error caused by dilution of blood, hematocrits (HCT) were obtained at 0, 20, 80, 140, 200, and 240 minutes. If a depression in HCT of greater than 10% occurred, that animal was disqualified from the study.

In order to assess if changes in blood testosterone levels are due to a direct gonadal drug effect, testosterone production was stimulated by administration of HCG (500 IU) to intact male monkeys. HCG has a half-life of 23 hours and testosterone stimulation can be observed for up to 48 hours post-administration. Opioids were given four hours later in an attempt of counteract the increased testosterone production. Aminogluthethamide (α-ethyl-p-aminophenyl-glutarimide), a compound which inhibits the first step of steroidogenesis from cholesterol, was administered as a positive control.

Morphine Sulfate	(1.0 mg/kg)
DADLE	(20.0 µg/kg)
Aminogluthethamide	(75 mg/monkey)

Studies of Acute Drug Effects on Pituitary Sex Hormones. In these studies the effects of MS, DADLE, β-end, naloxone and their vehicles on blood LH and PRL in vivo were defined. The blood sampling techniques and drug dose levels described previously in this section were used. Dose and time course relationships between the drugs administered and blood LH and PRL levels were established.

Studies of the Mechanisms of Drug Effects on Reproductive Hormones. To further characterize the location of opioid effects, exogenous GnRH was administered 60 minutes after opioid administration which was at the time of maximal opioid LH depression. Pituitary response was evaluated by the monitoring of LH blood levels. For this study the following i.v. drug doses were used:

Morphine Sulfate	(1.0 mg/kg)	+	GnRH (100.0 µg)
DADLE	(20.0 mg/kg)	+	GnRH (100.0 µg)

The amount of GnRH administered was based on work by Krey, et al., (1973).

To establish whether the opioids under consideration are acting through opiate receptors, naloxone was administered at two dose levels 100 minutes after opioid injection. The following drug combinations were given intravenously:

Morphine Sulfate	(1.0 mg/kg)	+	Naloxone (0.03, 1.0 mg/kg)
DADLE	(20.0 µg/kg)	+	Naloxone (0.03, 1.0 mg/kg)

As previously described, blood was drawn through indwelling catheters at at intervals of 20 minutes for four hours with intravenous drug administration at one hour. Testosterone, LH, and PRL blood levels were determined as appropriate.

Chapter 4

RESULTS

The experimental results are organized and presented in three major sections. First, results of acute administration of opioids and naloxone on testosterone are described. These results are presented in two subsections: studies of opioid effects on stimulated Leydig cells, and studies of acute drug effects on normal plasma testosterone levels. The former studies investigate the possibility that opioid and naloxone effects on testosterone plasma levels are due to direct drug action on the testes. The second major section describes acute drug effects on plasma testosterone levels of LH and PRL, which provides information on pituitary effects. The third major section presents results from experiments designed to study the mechanisms and site of acute opioid and naloxone effects on reproductive hormones. This section describes effects of opioid pretreatment on LH and testosterone responses to exogenous administration of GnRH, as well as the effects of opioid pretreatment on naloxone's ability to alter plasma LH, testosterone, and PRL levels. These experiments, besides illustrating the basic drug effects presented in the first two major sections, create conditions under which possible opioid competition with substances (naloxone, GnRH) of known sites of action could be observed and thereby give further indications of primary sites of opioid action on sex hormones. In addition, results of analyzing frequencies and amplitudes of hormone episodic release patterns in both vehicle-treated and opioid- or naloxone-treated animals are presented. This part of the study involved a more detailed characterization of hormone level time courses than simply the determination of overall

upward or downward trends. Frequencies and amplitudes of fluctuations in hormone levels about their trend lines have been tabulated. Drug effects on episodic release characteristics, when considered together with overall drug effects on hormone levels (i.e., effects on trends), may have implications for the way in which the EOP enter into the regulatory mechanisms of LH, testosterone, and PRL plasma levels, as is discussed later.

As described in Chapter 3, Methods, statistical analysis of overall drug effects on hormone levels was accomplished by computing hormone level change relative to pre-administration for each animal in both drug-treated and control groups and then comparing the sets of changes relative to pre-administration by a paired or unpaired t-test, depending on whether the same experimental animals were available for the drug-treated and associated control (vehicle) experiments. At every post-administration time point, the relative change was computed, and a separate statistical test was applied. As described earlier, the time-series from which the relative changes were computed was either the raw series of blood hormone samples, or a smoothed series derived from the raw series by averaging over a "window" of typically from two to four samples. Smoothing by means of windows of various sizes was merely a tool to enable drug effects to be seen which would otherwise be obscured by episodic fluctuations of hormone levels in the unsmoothed (window size = 1) data. A drug's effect on a hormone level can be expressed in terms of a "change difference", i.e., the difference in the average change of hormone level (relative to pre-administration between the drug-treated and control groups). The change difference represents the change of hormone level which is ascribable to drug treatment. The physiological significance of a change difference is better indicated by

the corresponding "change difference percentage", defined as the ratio of the change difference to the average control group value in the smoothing window which precedes drug administration in the drug-treated time series, i.e., the window which encompasses samples only up through time zero (drug administration time). Drug effects will therefore be expressed as change difference percentages; the maximal change in the drug-treated series relative to pre-treatment will also be given. Note that negative change differences of more than 100 percent (of initial control level) are possible; such a condition can only occur, however, if the control series exhibits a significant upward trend while the corresponding drug-treated series exhibits a downward trend.

1. Acute Drug Effects on Testosterone Levels

a) Studies of Drug Effects on Stimulated Leydig Cells. Direct Gonadal Studies (in vivo and in vitro).

In Vivo Studies. In order to assess possible direct effect of the opioids on testosterone production in vivo, morphine and DADLE were administered in separate experiments to HCG pretreated male monkeys (Figure 4). HCG ($t_{1/2}$=23 hours) was given in order to stimulate the testes exogenously and thus remove them from the control of endogenous LH. Drug or vehicle was administered four hours after HCG treatment. Blood was drawn just prior to drug treatment (zero hours) and one, two, and three hours post drug administration. No effects of these opioids on testosterone levels were observed one to three hours after adminstration. In this study, aminoglutethamide, a known inhibitor of steroid synthesis, was administered as a positive control (Haynes and Larner, 1975). Aminoglutethamide was observed to depress testosterone levels by 28% (change difference with respect to controls) at three hours post drug administration.

In Vitro Studies. To further evaluate the possible direct effect of morphine sulfate on testicular testosterone production, an in vitro study consisting of an isolated mouse Leydig cell preparation modified from the in vitro LH-bioassay of Van Damme et al., (1974) was used. Leydig cells from mice, instead of rhesus monkeys, were used in order to avoid castration of these expensive primates and in order to use an established in vitro method to examine direct drug effects. Cells were incubated for a three hour period in the presence of serum from ovariectomized rhesus monkeys (complete diagram of method in Appendix, Figure

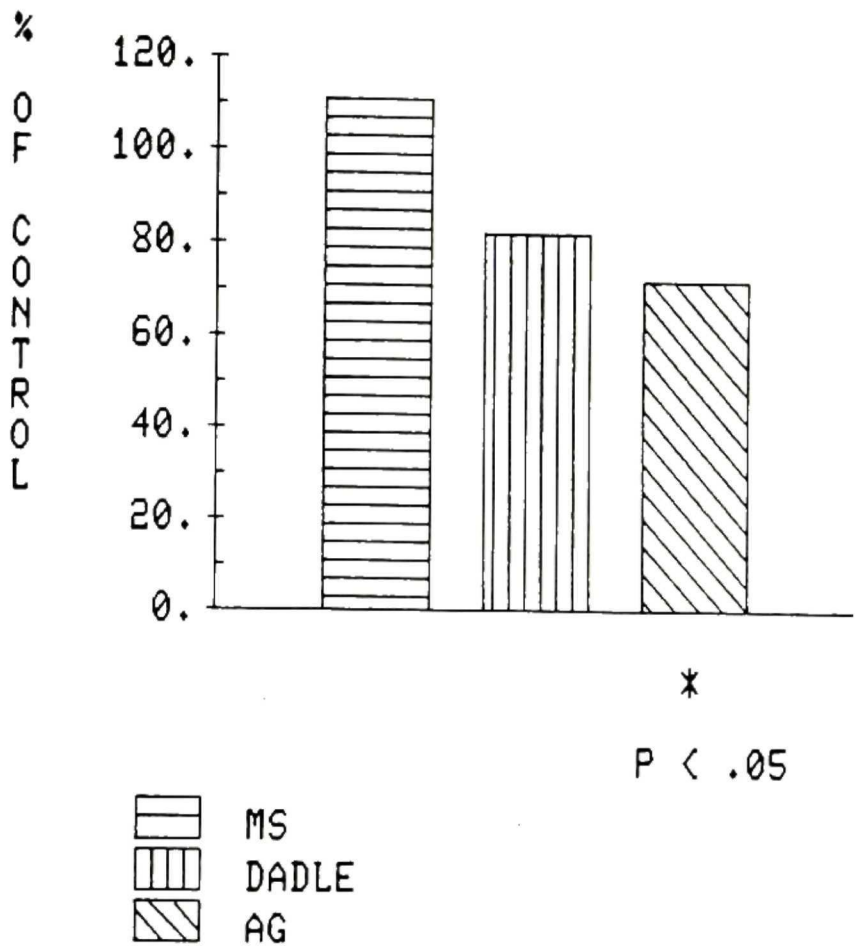

Figure 4. Effects of morphine sulfate (MS, 1.0 mg/kg), DADLE (20 µg/kg), and aminoglutethamide (AG, 75 mg) on testosterone production in HCG pretreated monkeys. The bars represent average percent of control for the drugs indicated. Only AG produced a level that differed significantly from control ($p < 0.05$).

1A). Serum from ovariectomized female monkeys was used as a source of rhesus LH since following ovariectomy high concentrations of LH and low concentrations of steroids are present in the blood. Morphine was added to this serum to yield concentrations which were within the range of typical blood levels after acute administration (150-1650 ng/dl) and 10-fold higher. When compared to controls (serum without drug), morphine had no direct effect on testosterone production. These results are illustrated in the Appendix (Table 1A).

b) Studies of Drug Effects on Normal Plasma Testosterone Levels.

To study the effect of opioid agonists and antagonist on plasma testosterone levels, drugs or vehicles were administered by an intravenous injection through catheters to adult male monkeys. Blood was drawn through the catheter at 20 minute intervals for over four hours inclusive of one hour before drug or vehicle and three hours after drug or vehicle administration. All blood drawings began at 0700. This time of day was chosen because preliminary work indicated that the diurnal fluctuations in testosterone levels were less pronounced in the 0700-1100 period than in most other four hour periods.

Morphine sulfate. A significant decrease in plasma testosterone levels was observed following the administration of morphine sulfate. The smoothed data for both drug-treated and control (vehicle) series are shown for three dose levels. After administering 1.0 mg/kg morphine sulfate (Figure 5), an approximate decrease of 200% (change difference with respect to vehicles) was observed at 80 - 100 minutes, and an absolute decrease of 70% from pretreatment levels was seen by three hours. Testosterone levels remained below vehicle levels for the full three-

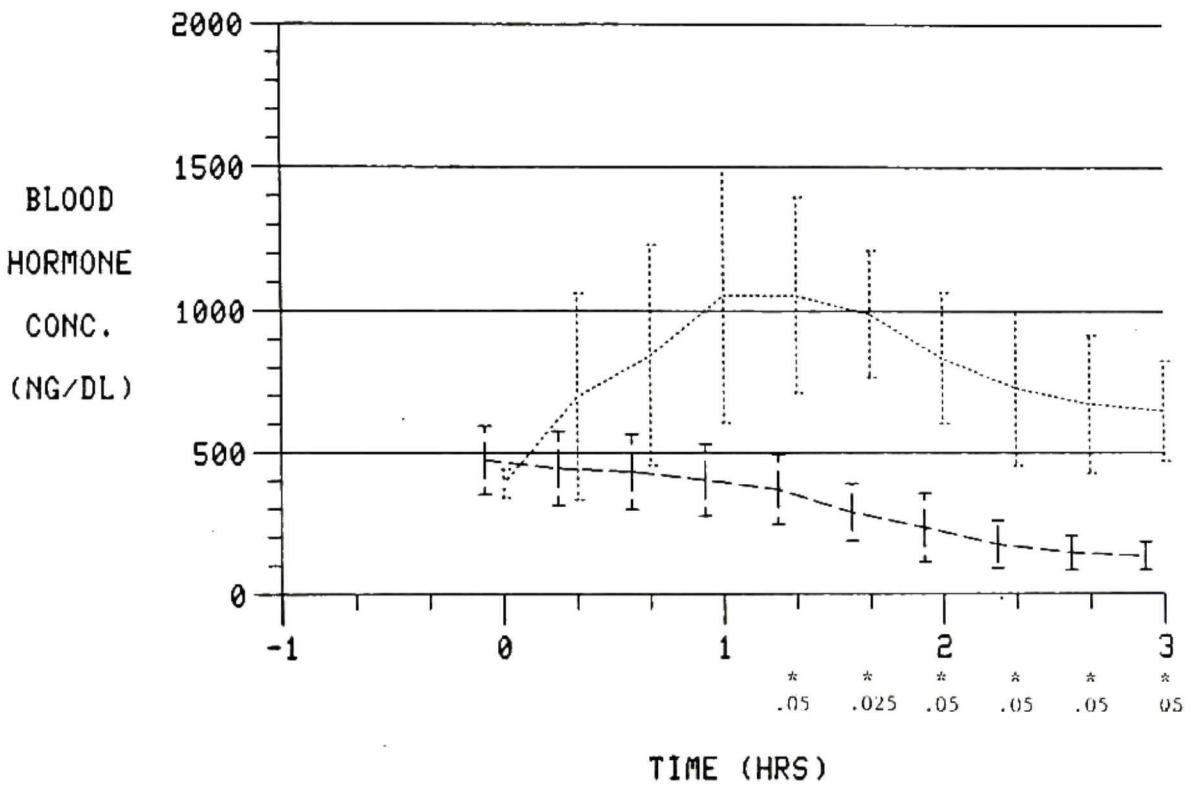

Figure 5. Effect of morphine sulfate (1.0 mg/kg) or vehicle on plasma testosterone levels. Morphine was administered at time zero hours. Points represent the moving averages (±SEM) for vehicle (.....) and treated (- - - -) groups. N = 5, averaging window size was four. Windows where significant inter-group differences in changes from pretreatment (first window) level occurred are denoted by asterisks and associated p-values.

hour observation period following drug administration. No significant effects were observed with the other dose levels studied, 0.50 mg/kg and 0.25 mg/kg morphine sulfate (Figures 6 and 7, respectively).

[D-Ala^2,D-Leu^5]-Enkephalin (DADLE). The effects of DADLE on plasma testosterone levels are shown in Figures 8 and 9. Administration of 10 µg/kg of DADLE produced a decrease in blood testosterone levels of approximately 34% (Figure 8). Levels were depressed 60-80 minutes after DADLE administration and remained depressed for three hours. Significant effects were not observed at the other dose levels studied (5.0 and 20 µg/kg). However, at the 20 µg/kg dose (Figure 9), a definite decreasing trend occurred with respect to pretreatment levels. The latter levels were themselves unusually low (cf. Figure 8, for example, as well as the set of controls) and probably limited further drug-induced decreases, thus preventing statistically significant negative change differences from being observed.

β-Endorphin. Administration of β-end (10.0 and 20.0 µg/kg) had no effect on testosterone levels (Figure 10). Although there was considerable variation in testosterone levels prior to endorphin administration, there was no significant change in levels with respect to vehicle.

Naloxone. The opioid antagonist naloxone caused a marked increase in testosterone levels (Figures 11, 12, and 13). Statistically significant increases were observed with naloxone dose levels of 30 µg/kg - 2.0 mg/kg. At the 2.0 mg/kg dose, naloxone was responsible for a 4.3-fold increase with respect to pre-treatment levels (540% change difference from controls), with peak testosterone levels occurring within 60 - 100 minutes after drug treatment. Administration of 1.0 mg/kg and 0.5 mg/kg

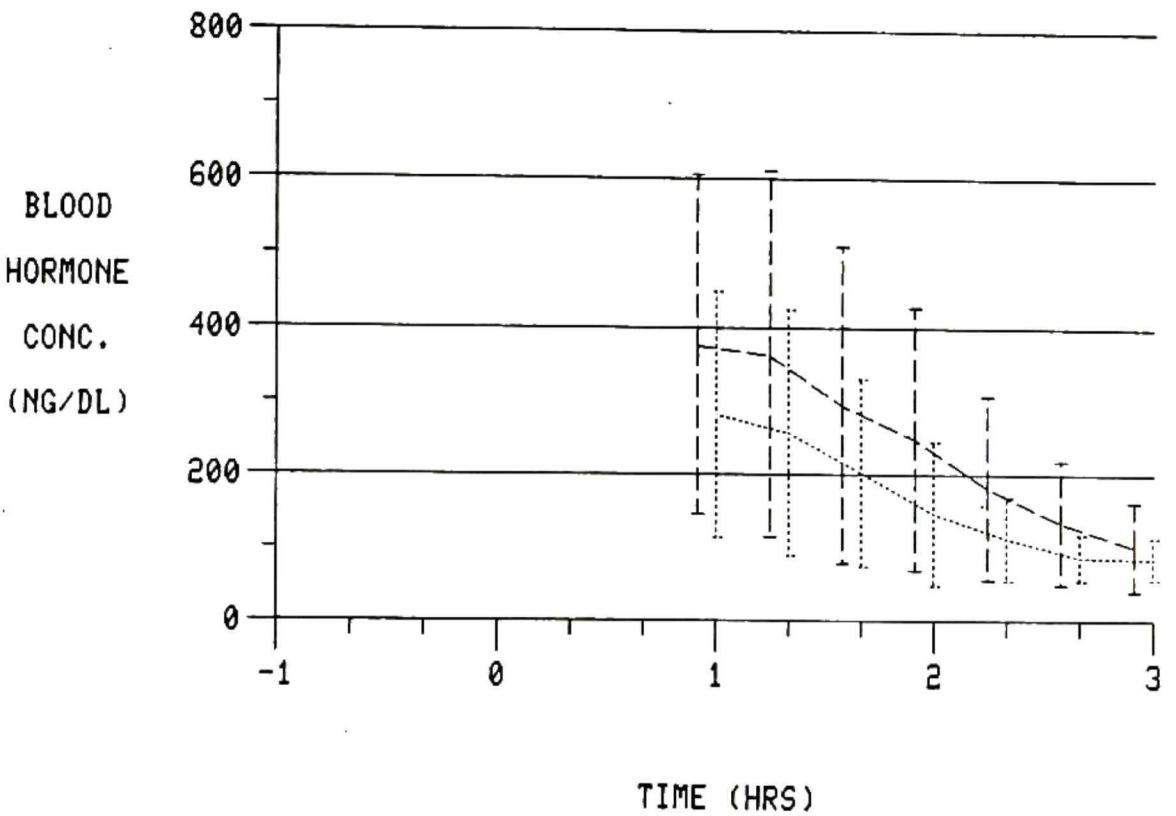

Figure 6. Effect of morphine sulfate (0.5 mg/kg) or vehicle on plasma testosterone levels. Morphine was administered at time zero hours. Points represent the moving averages (±SEM) for vehicle (.....) and treated (- - - -) groups. N = 4, averaging window size was four. No significant inter-group differences in changes from pretreatment (first window) level occurred. In this experiment the first samples were drawn at time zero.

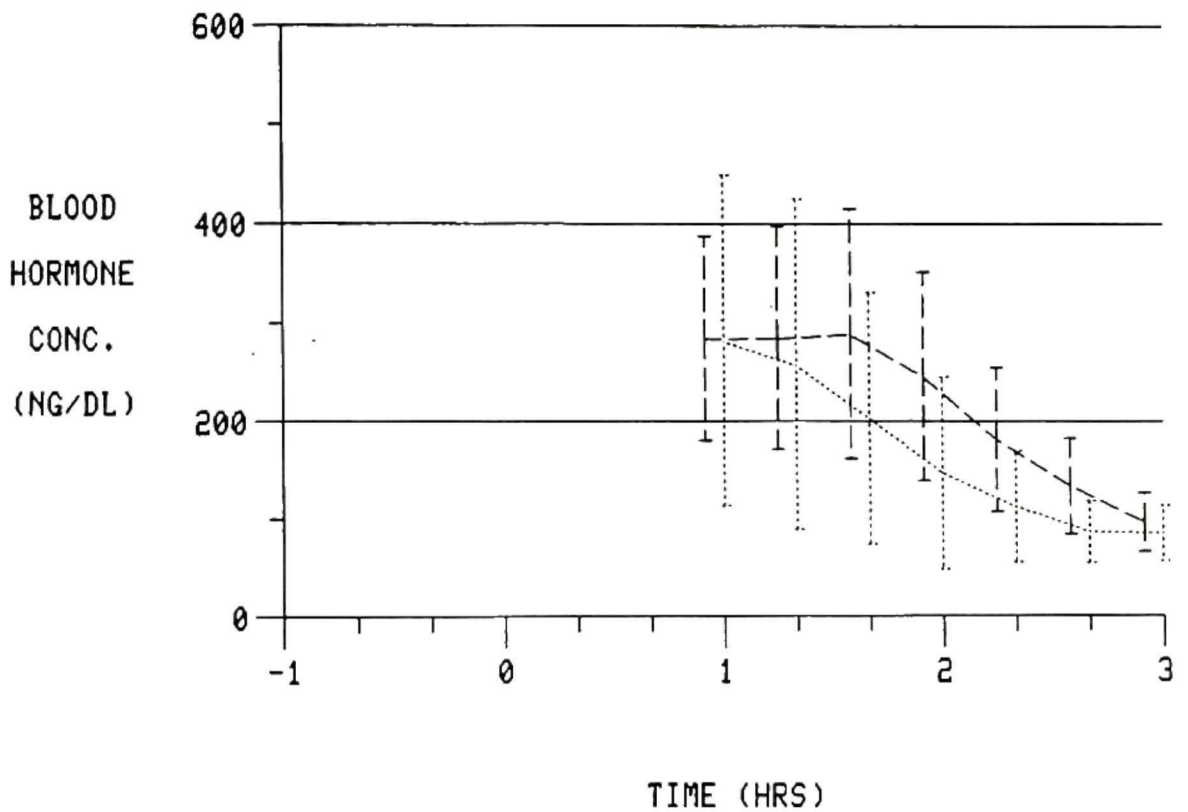

Figure 7. Effect of morphine sulfate (0.25 mg/kg) or vehicle on plasma testosterone levels. Morphine was administered at time zero hours. Points represent the moving averages (±SEM) for vehicle (.....) and treated (- - - -) groups. N = 4, averaging window size was four. No significant inter-group differences in changes from pretreatment (first window) level occurred. In this experiment the first samples were drawn at time zero.

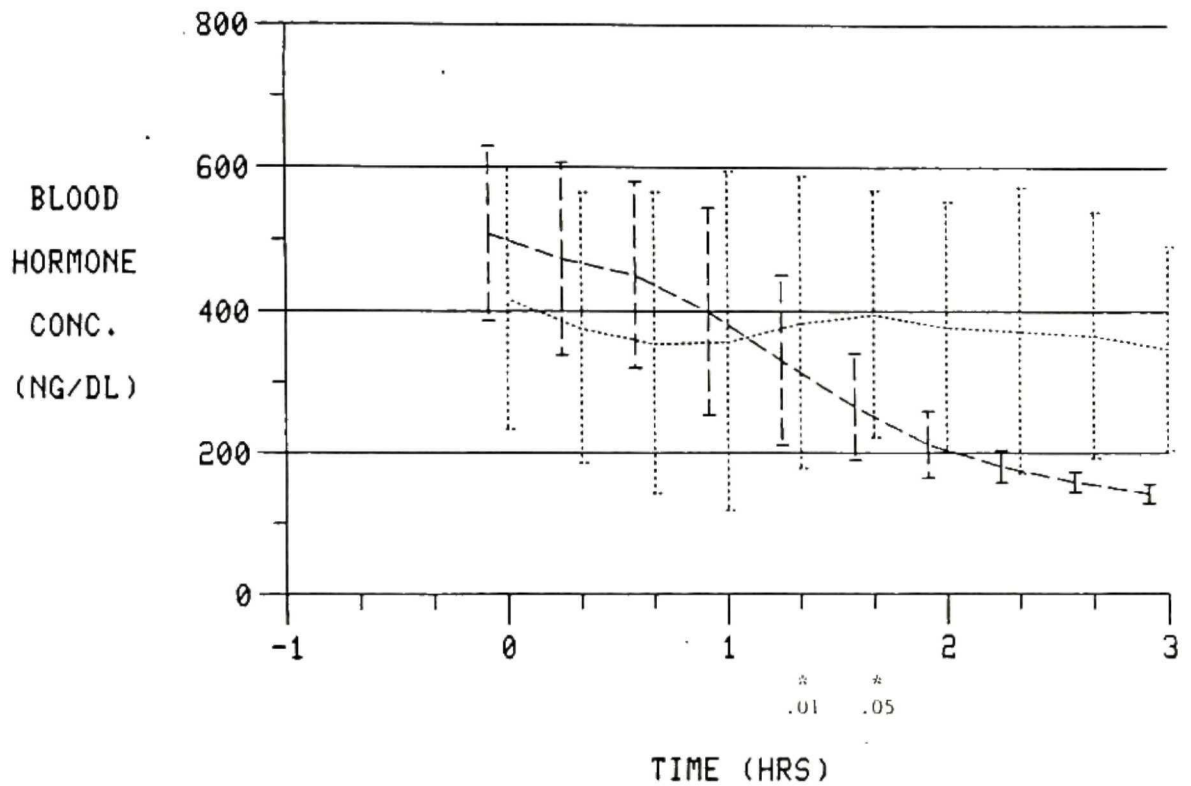

Figure 8. Effect of DADLE (10.0 µg/kg) or vehicle on plasma testosterone levels. DADLE was administered at time zero hours. Points represent the moving averages (±SEM) for vehicle (.....) and treated (- - - -) groups. N = 4, averaging window size was four. Windows where significant inter-group differences in changes from pretreatment (first window) level occurred are denoted by asterisks and associated p-values.

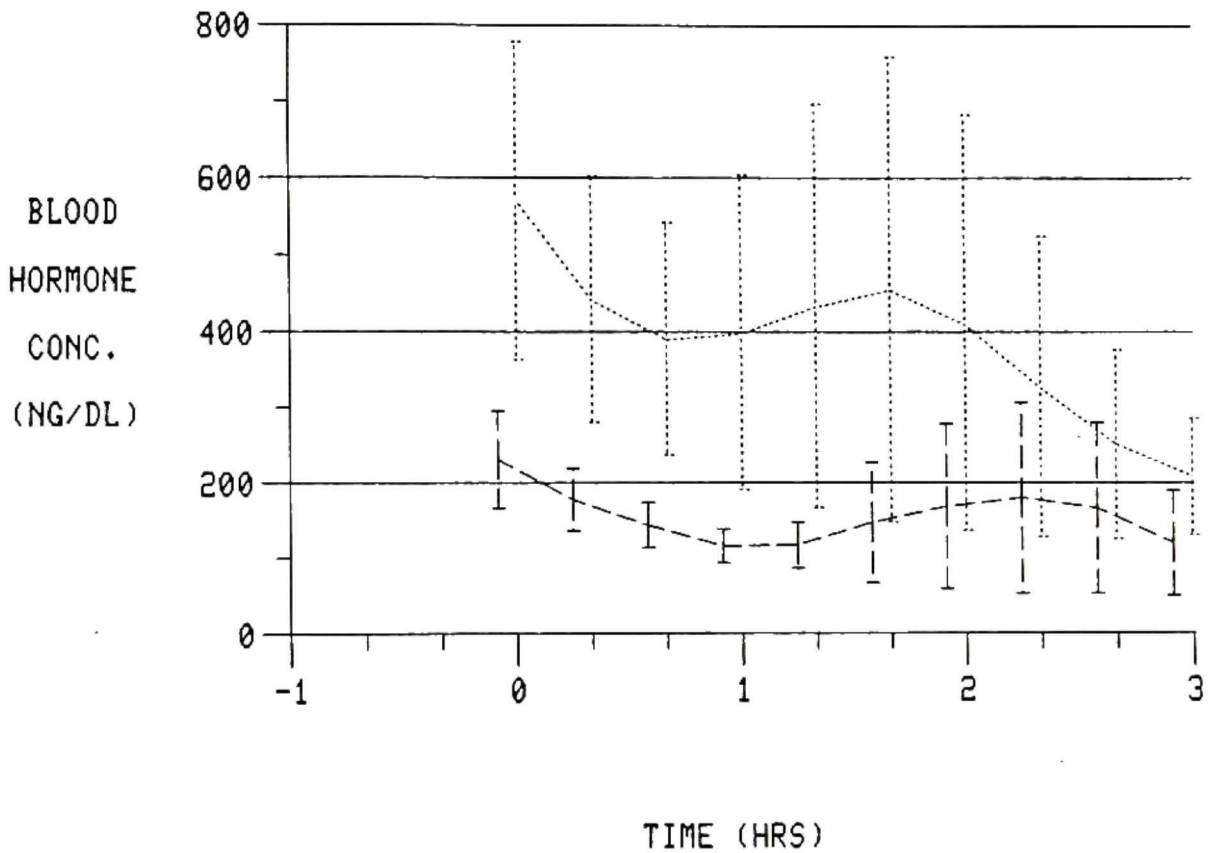

Figure 9. Effect of DADLE (20.0 µg/kg) or vehicle on plasma testosterone levels. DADLE was administered at time zero hours. Points represent the moving averages (±SEM) for vehicle (.....) and treated (- - - -) groups. N = 4, averaging window size was four. No significant inter-group differences in changes from pretreatment (first window) level occurred.

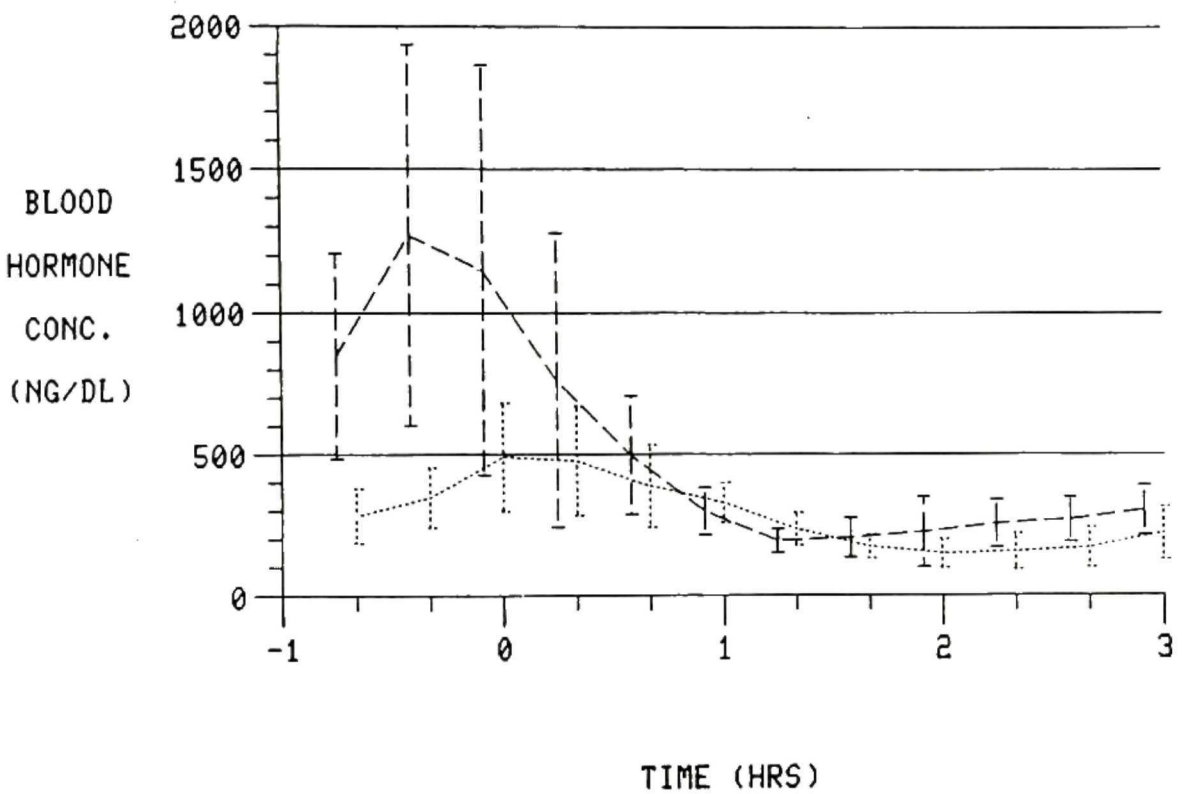

Figure 10. Effect of β-endorphin (10 μg/kg) or vehicle on plasma testosterone levels. β-endorphin was administered at time zero hours. Points represent the moving averages (±SEM) for vehicle (.....) and treated (- - - -) groups. N = 5, averaging window size was two. No significant inter-group differences in changes from pretreatment (first window) level occurred.

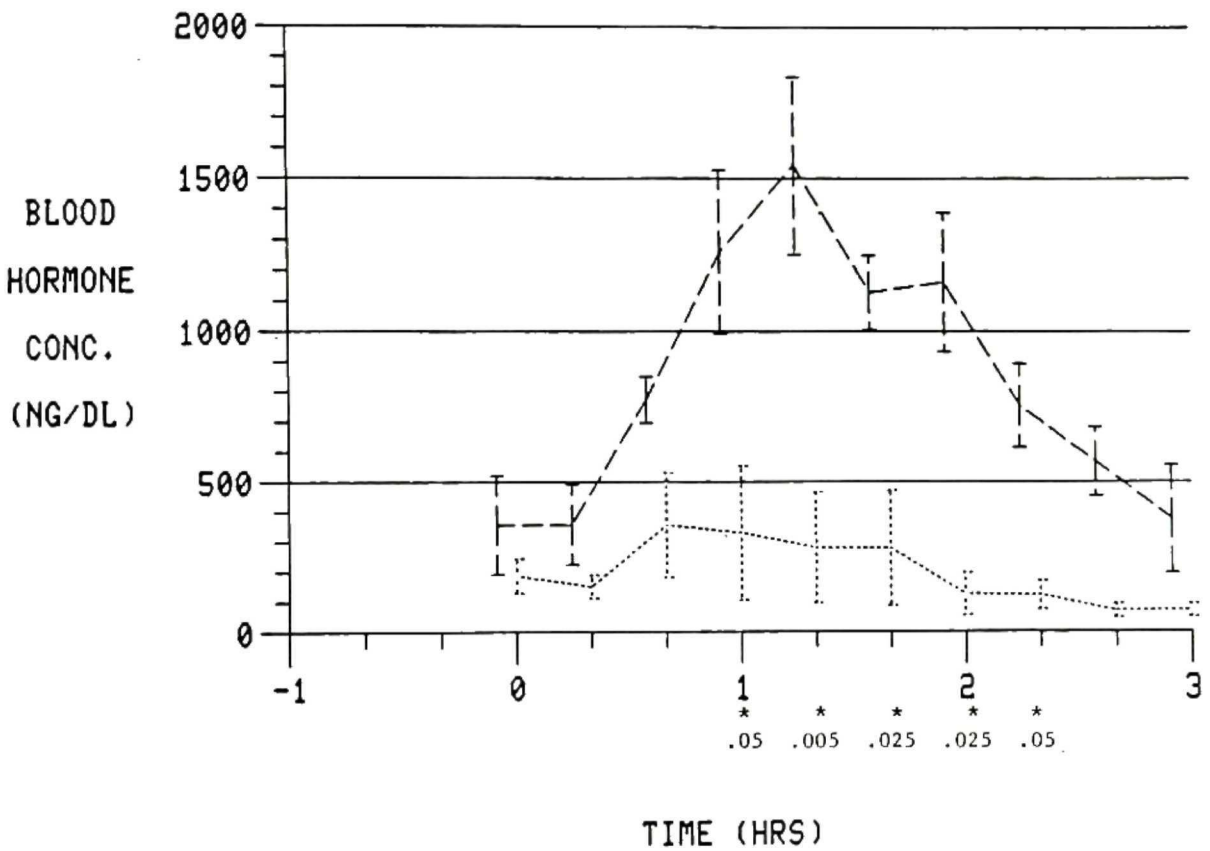

Figure 11. Effect of naloxone (2.0 mg/kg) or vehicle on plasma testosterone levels. Naloxone was administered at time zero hours. Points represent the moving averages (±SEM) for vehicle (.....) and treated (- - - -) groups. N = 4, averaging window size was one. Windows where significant inter-group differences in changes from pretreatment (first window) level occurred are denoted by asterisks and associated p-values. In this experiment the first samples were drawn at time zero.

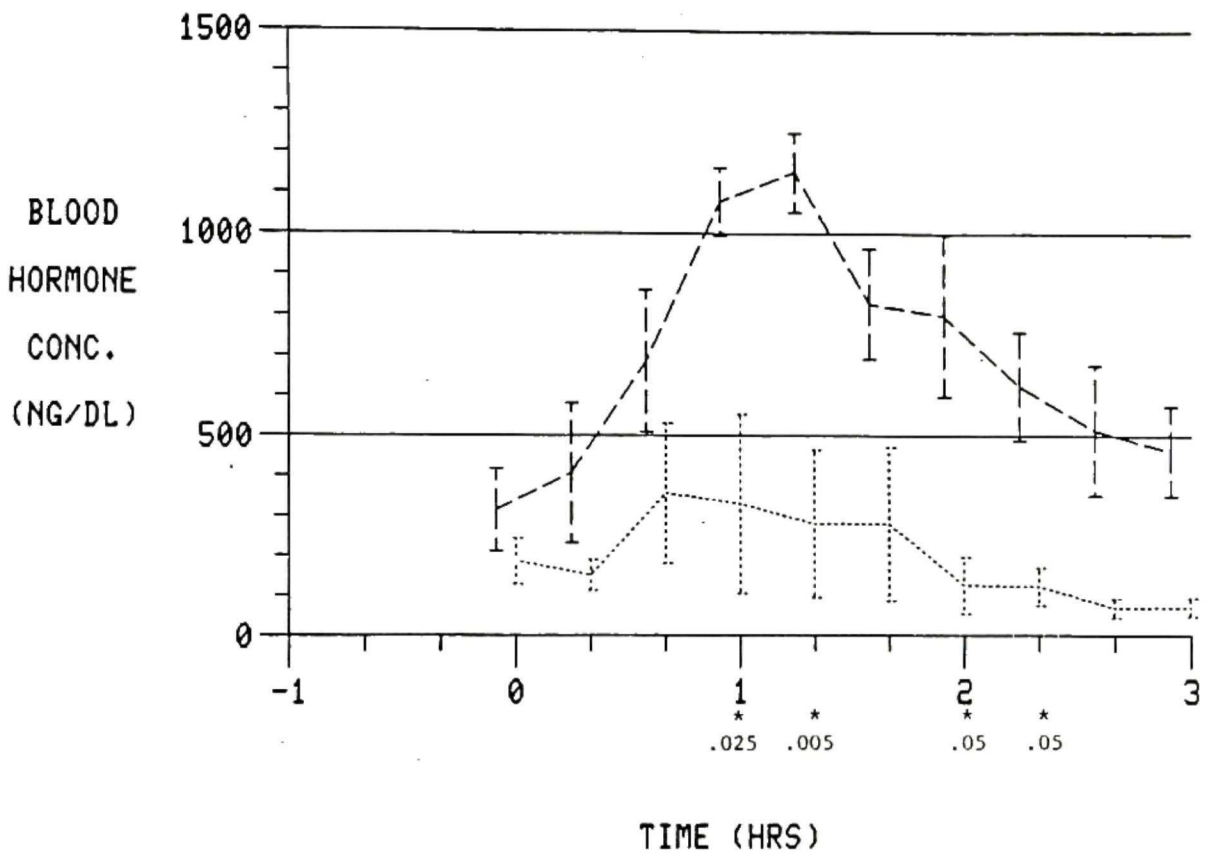

Figure 12. Effect of naloxone (1.0 mg/kg) or vehicle on plasma testosterone levels. Naloxone was administered at time zero hours. Points represent the moving averages (±SEM) for vehicle (.....) and treated (- - - -) groups. N = 5, averaging window size was one. Windows where significant inter-group differences in changes from pretreatment (first window) level occurred are denoted by asterisks and associated p-values. In this experiment the first samples were drawn at time zero.

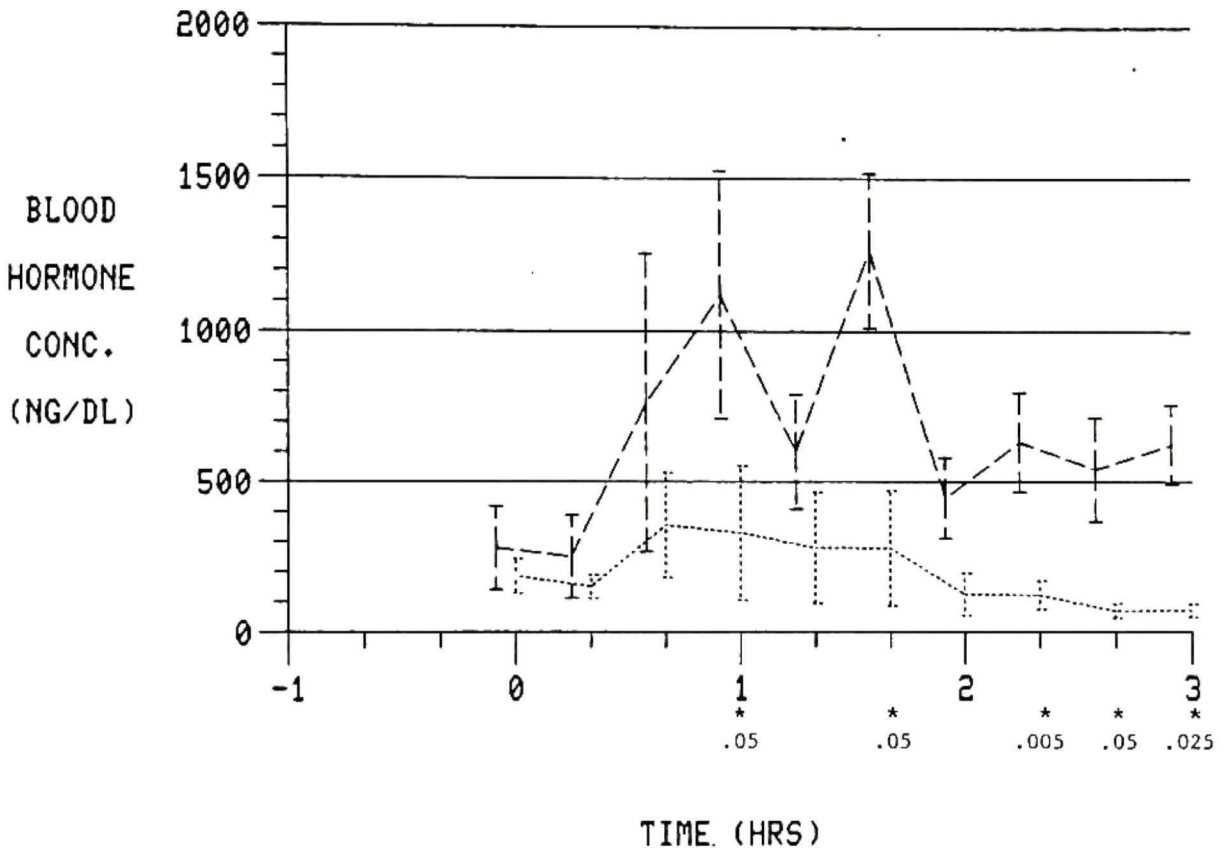

Figure 13. Effect of naloxone (0.5 mg/kg) or vehicle on plasma testosterone levels. Naloxone was administered at time zero hours. Points represent the moving averages (±SEM) for vehicle (.....) and treated (- - - -) groups. N = 5, averaging window size was one. Windows where significant inter-group differences in changes from pretreatment (first window) level occurred are denoted by asterisks and associated p-values. In this experiment the first samples were drawn at time zero.

naloxone produced 3.7- and 4.5-fold average increases from time zero minutes (400% and 480% change differences) in plasma testosterone levels, respectively. Testosterone levels remained elevated up to 140 - 180 minutes following naloxone administration.

2. Acute Drug Effects on LH and Prolactin Levels

The effects of opioid agonists and antagonist on plasma LH and PRL levels were studied by measuring plasma concentrations of these hormones at 20 minute intervals for four hour time periods. As determined from our previous studies, this time period allowed for the observation of opioid drug effects on LH and PRL levels.

Morphine Sulfate. The effect of morphine sulfate (1.0, 0.5, and 0.25 mg/kg) on LH levels is shown in Figures 14, 15, and 16. Administration of 1.0 mg/kg of morphine sulfate resulted in a depression of blood LH levels. Significant depressions (-64% change difference) occurred within 40 minutes after drug administration, and LH levels remained depressed for at least one hour. No significant drug effects were demonstrated at the lower dose levels.

Prolactin blood levels were noticeably increased after these three doses of morphine sulfate administration (Figures 17, 18, and 19). This increase began within 20 minutes after drug treatment and was observed at all dose levels studied. Prolactin levels remained significantly elevated above pretreatment levels for up to three hours. Net PRL levels increased by factors of 1.8, 2.6, and 11.6 (change differences of 49%, 48%, and 637%) at 0.25, 0.5, and 1.0 mg/kg dose levels, respectively.

[D-Ala^2,D-Leu^5]-Enkephalin. As shown in Figures 20 and 21,

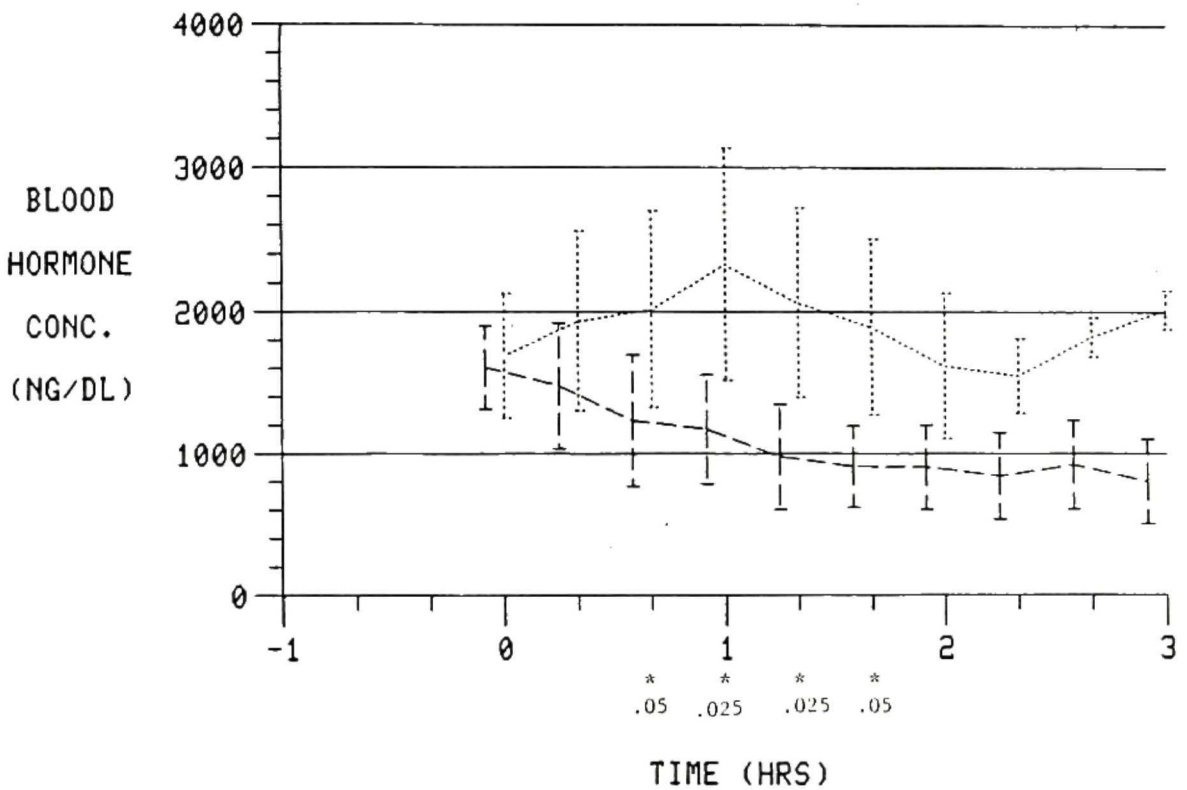

Figure 14. Effect of morphine sulfate (1.0 mg/kg) or vehicle on plasma LH levels. Naloxone was administered at time zero hours. Points represent the moving averages (±SEM) for vehicle (.....) and treated (- - - -) groups. N = 5, averaging window size is four. Windows where significant inter-group differences in changes from pretreatment (first window) level occurred are denoted by asterisks and associated p-values. In this experiment the first samples were drawn at time zero.

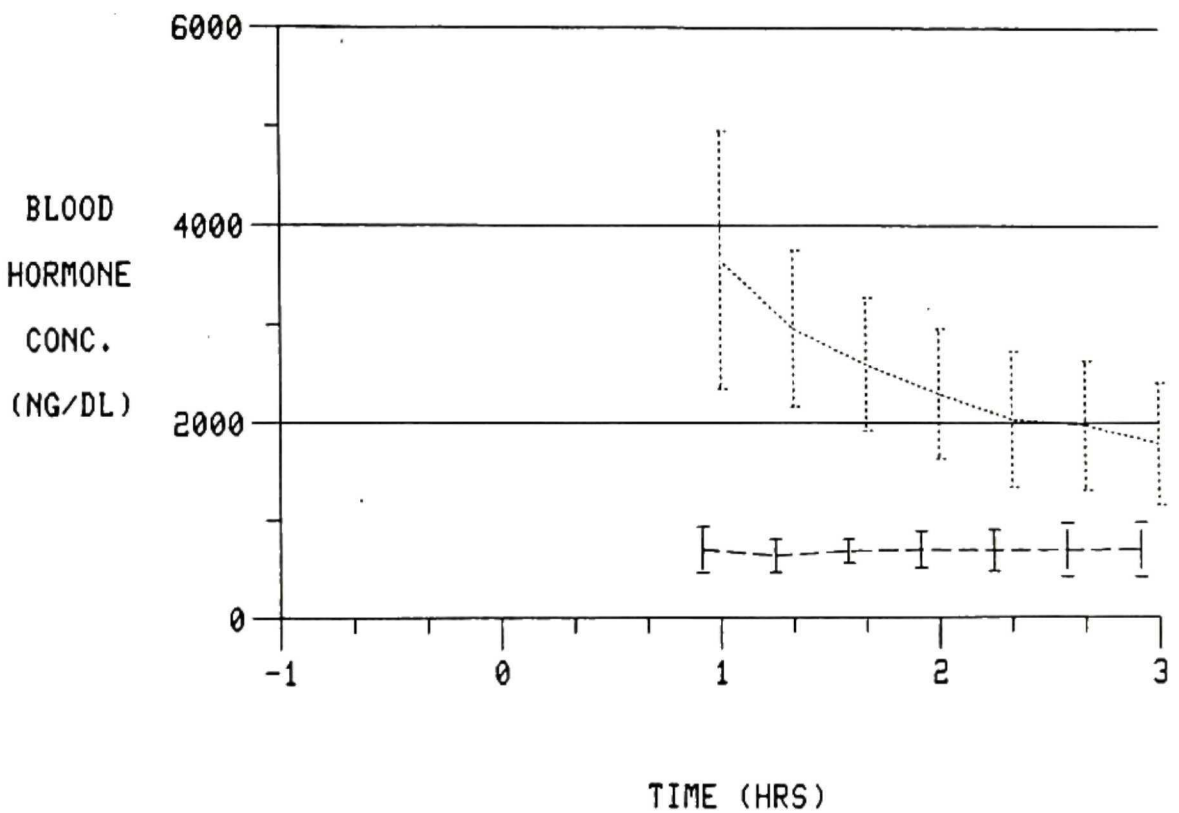

Figure 15. Effect of morphine sulfate (0.5 mg/kg) or vehicle on plasma LH levels. Morphine was administered at time zero hours. Points represent the moving averages (±SEM) for vehicle (.....) and treated (- - - -) groups. N = 4, averaging window size was four. No significant inter-group differences in changes from pretreatment (first window) level occurred. In this experiment the first samples were drawn at time zero.

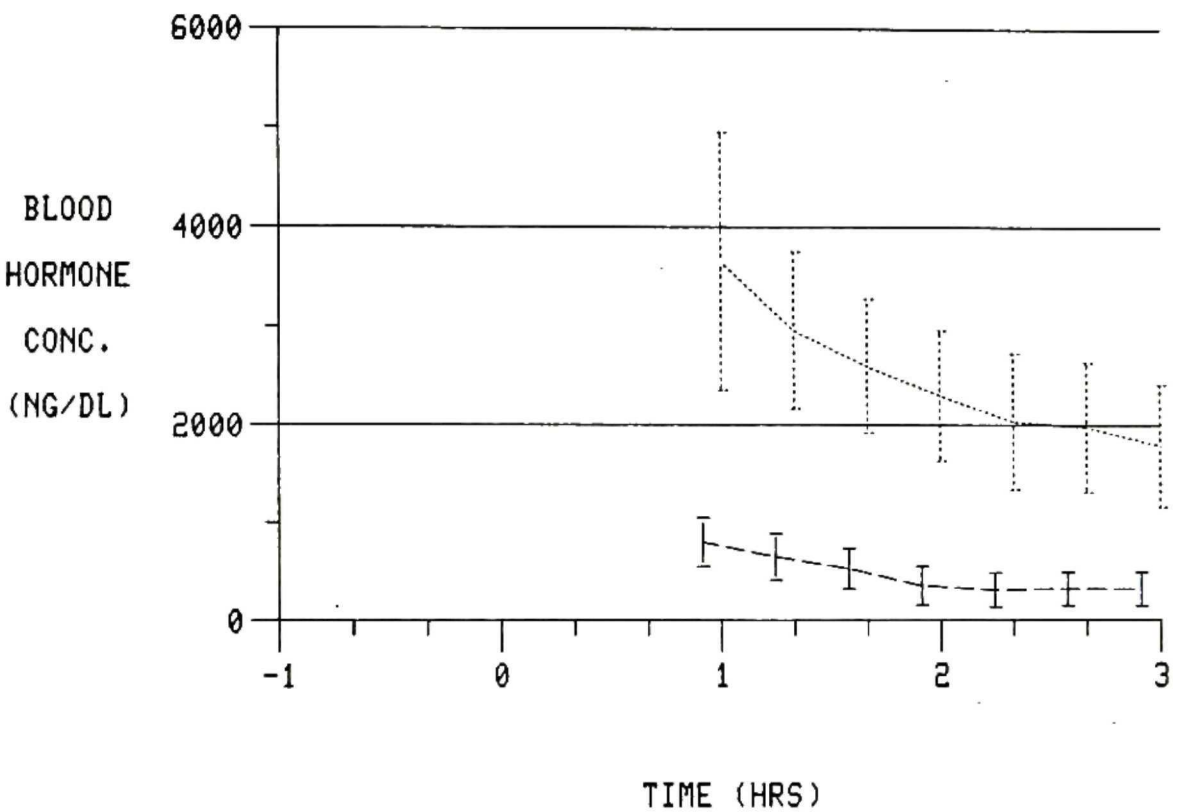

Figure 16. Effect of morphine sulfate (0.25 mg/kg) or vehicle on plasma LH levels. Morphine was administered at time zero hours. Points represent the moving averages (±SEM) for vehicle (.....) and treated (- - - -) groups. N = 4, averaging window size was four. No significant inter-group differences in changes from pretreatment (first window) level occurred. In this experiment the first samples were drawn at time zero.

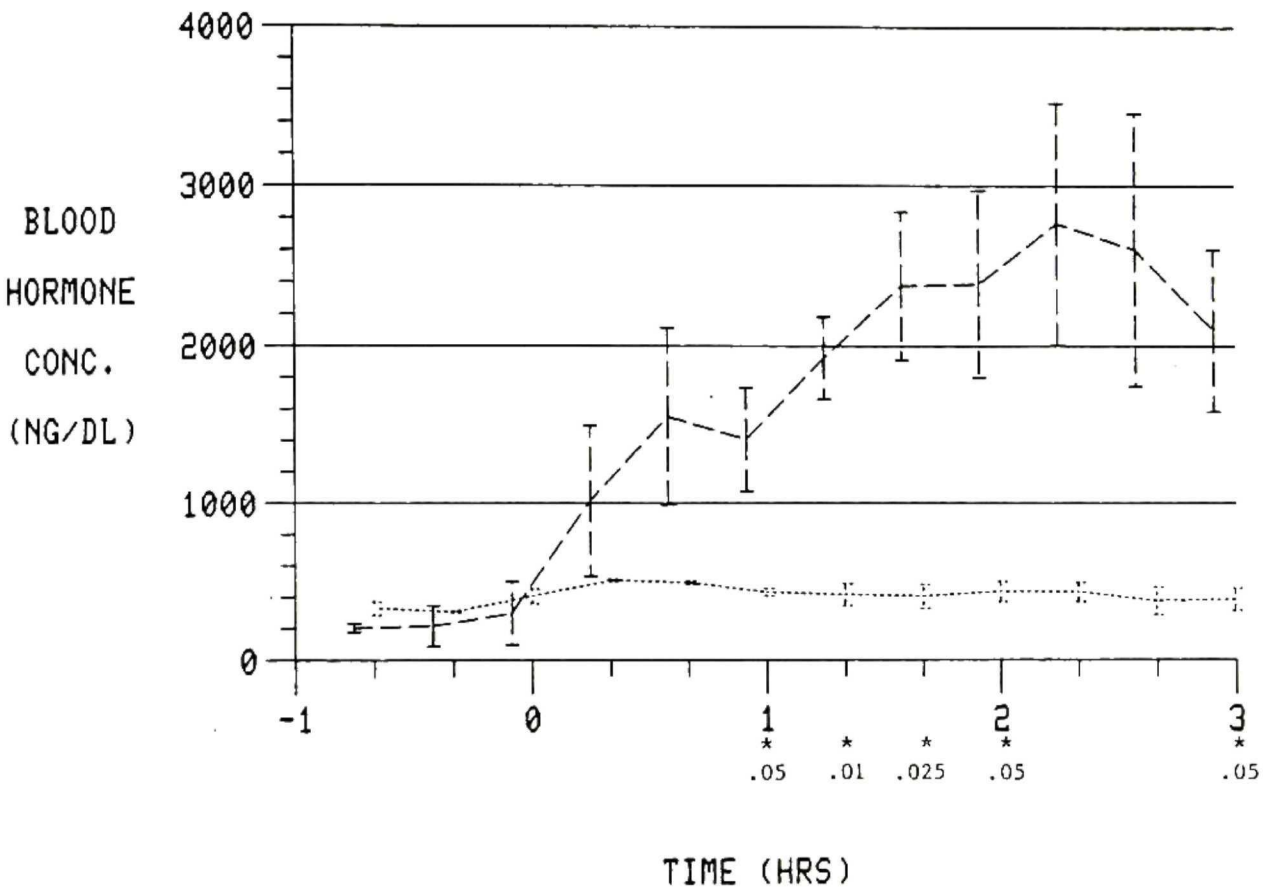

Figure 17. Effect of morphine sulfate (1.0 mg/kg) or vehicle on plasma PRL levels. Morphine was administered at time zero hours. Points represent the moving averages (±SEM) for vehicle (.....) and treated (- - - -) groups. N = 5, averaging window size was two. Windows where significant inter-group differences in changes from pretreatment (first window)level level occurred are denoted by asterisks and associated p-values.

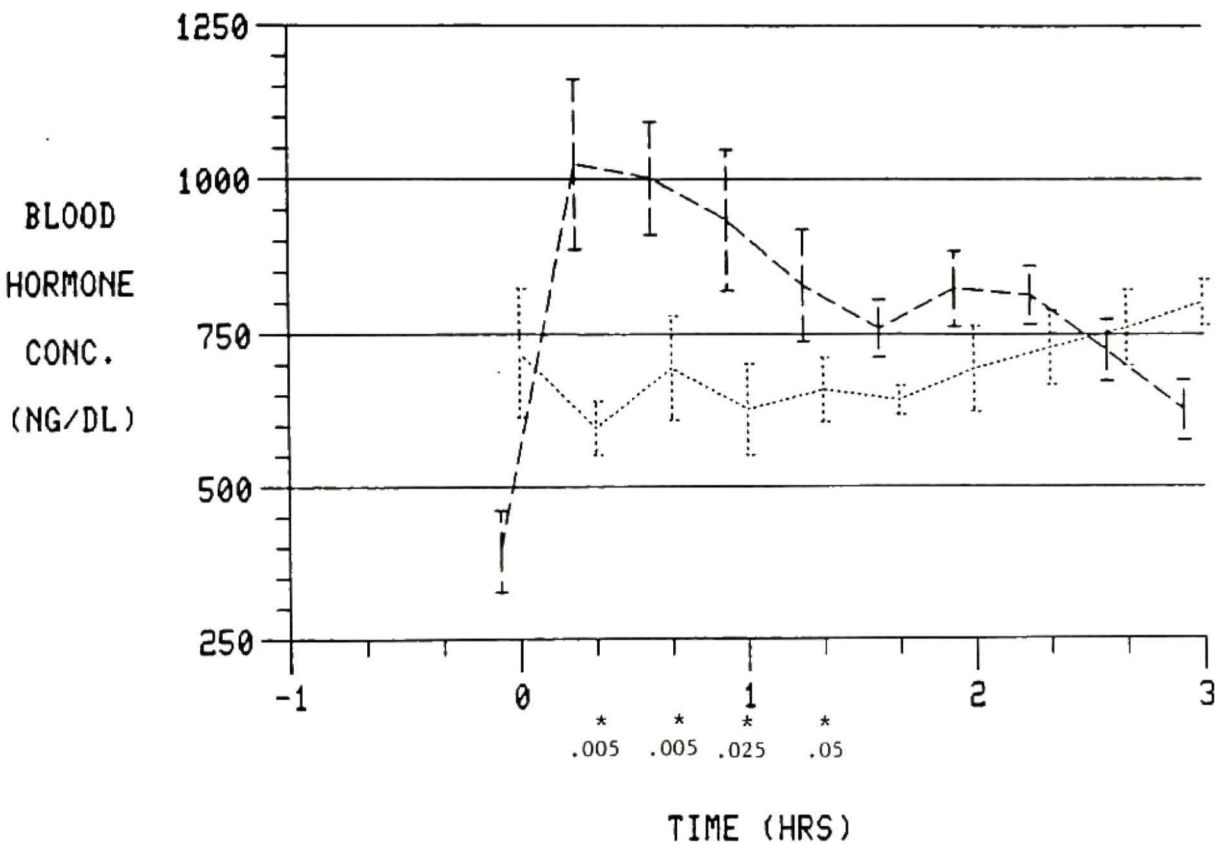

Figure 18. Effect of morphine sulfate (0.5 mg/kg) or vehicle on plasma PRL levels. Morphine was administered at time zero hours. Points represent the moving averages (±SEM) for vehicle (.....) and treated (- - - -) groups. N = 4, averaging window size was one. Windows where significant inter-group differences in changes from pretreatment (first window) level occurred are denoted by asterisks and associated p-values. In this experiment the first samples were drawn at time zero.

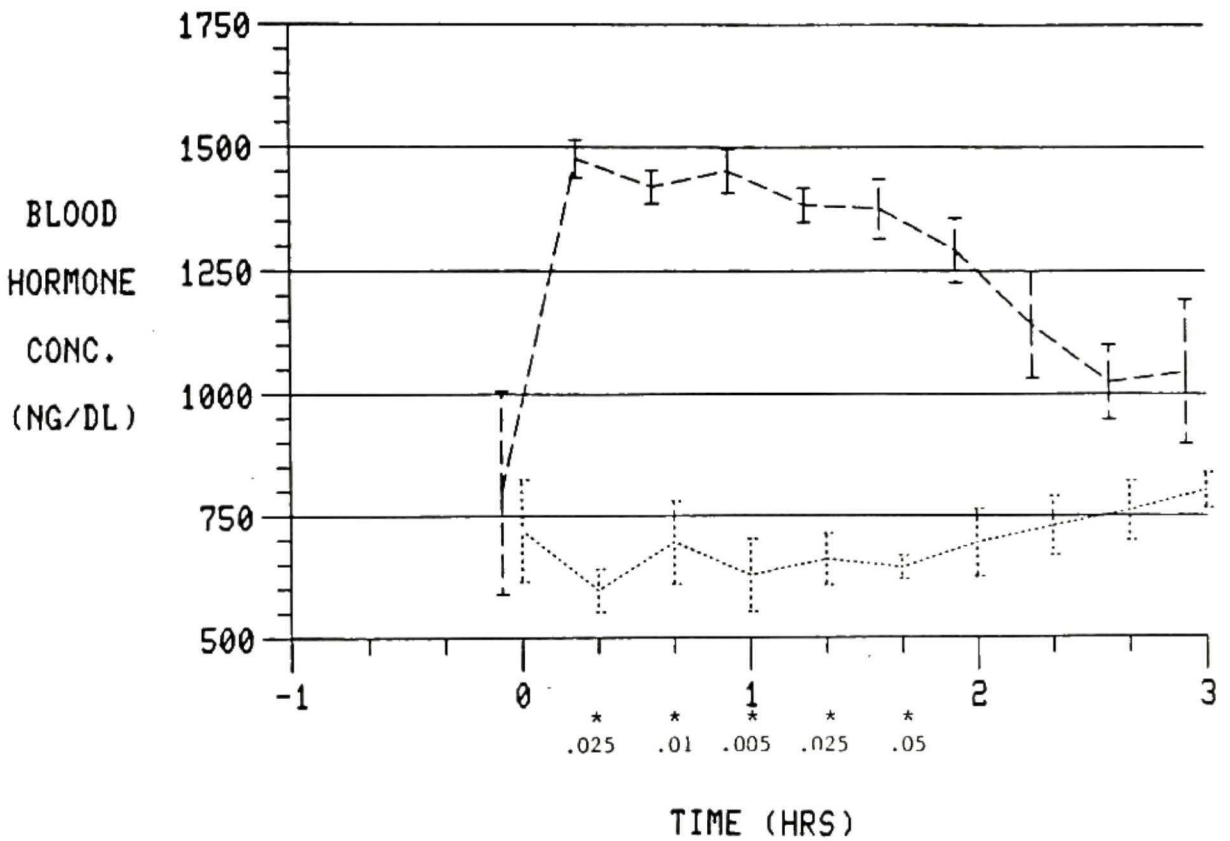

Figure 19. Effect of morphine sulfate (0.25 mg/kg) or vehicle on plasma PRL levels. Morphine was administered at time zero hours. Points represent the moving averages (±SEM) for vehicle (.....) and treated (- - - -) groups. N = 4, averaging window size was one. Windows where significant inter-group differences in changes from pretreatment (first window) level occurred are denoted by asterisks and associated p-values. In this experiment the first samples were drawn at time zero.

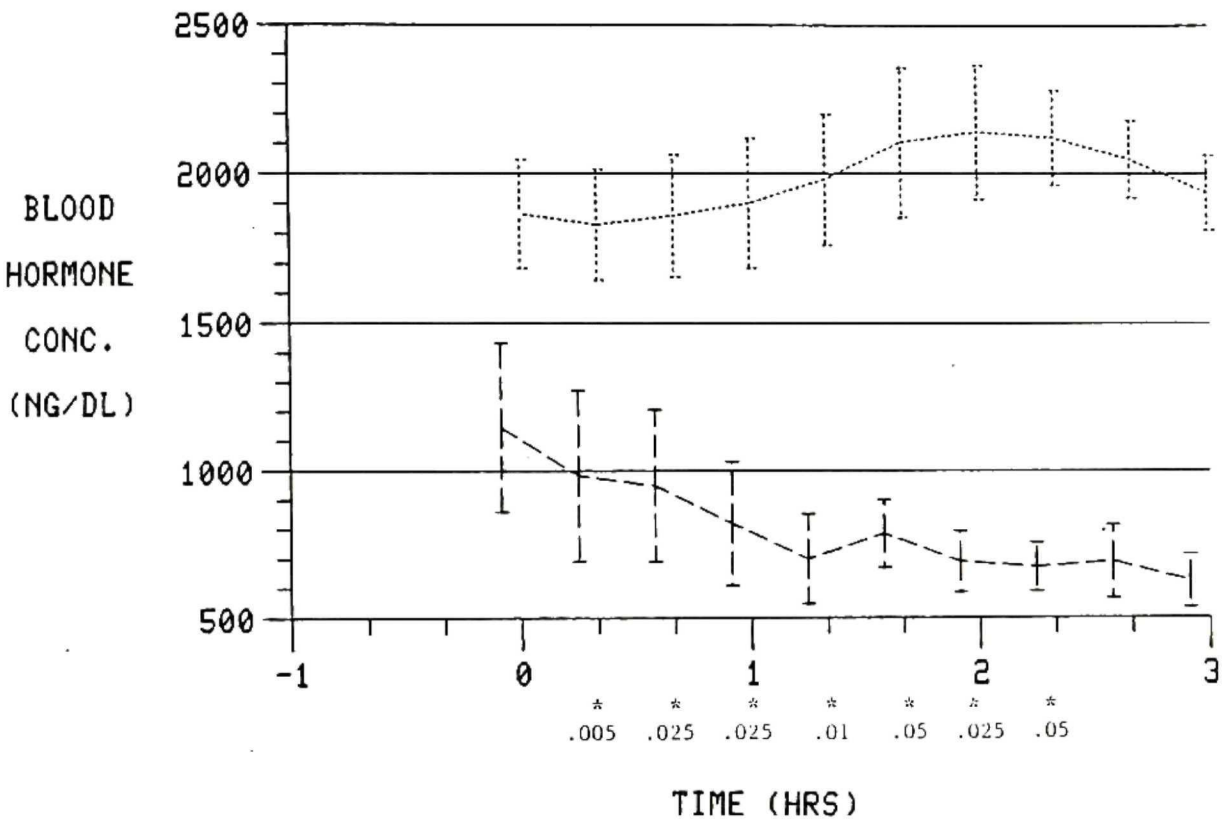

Figure 20. Effect of DADLE (10.0 µg/kg) or vehicle on plasma LHL levels. DADLE was administered at time zero hours. Points represent the moving averages (±SEM) for vehicle (.....) and treated (- - - -) groups. N = 4, averaging window size was four. Windows where significant inter-group differences in changes from pretreatment (first window) level occurred are denoted by asterisks and associated p-values.

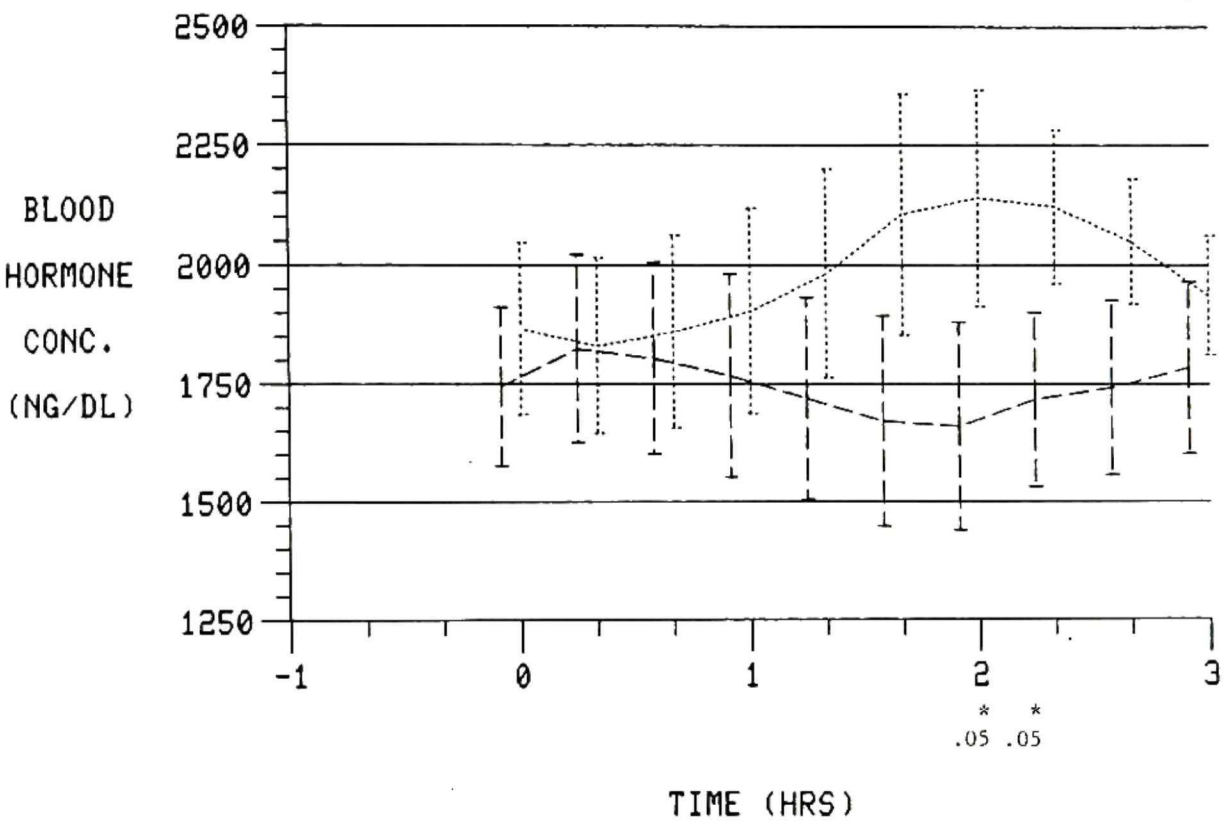

Figure 21. Effect of DADLE (5.0 µg/kg) or vehicle on plasma LHL levels. DADLE was administered at time zero hours. Points represent the moving averages (±SEM) for vehicle (.....) and treated (- - - -) groups. N = 5, averaging window size was four. Windows where significant inter-group differences in changes from pretreatment (first window) level occurred are denoted by asterisks and associated p-values.

significant depressions in LH levels were observed after treatment with DADLE. Maximum decreases of 30 - 40 percent (change differences of -30% to -40%) began within 40 minutes after administration of 10.0 µg/kg DADLE and were sustained for approximately 140 minutes. A change difference of of -19% was produced by a 5.0 µg/kg dose of the drug (Figure 21). However, no statistically significant change difference was found with the 20 µg/kg dose. As in the case of testosterone at the same dose, the LH pretreatment levels measured were unusually low (600 ng/dl vs. typical pretreatment values in the 1200 - 2000 ng/dl range).

Prolactin blood levels were not affected by DADLE administration within two hours after drug administration (Figure 22). However, PRL levels were depressed 30 - 46 percent (negative change differences) two to three hours after administration of 10.0 mg/kg DADLE.

β-Endorphin. No significant alterations in the levels of LH were observed following β-end administration (Figure 23).

Increases in PRL levels were observed immediately following administration of β-end 10.0 µg/kg (Figure 24) and 20.0 µg/kg (data not shown). Prolactin levels remained elevated above vehicle for up to three hours. Maximum change differences of 74% and 30%, and increases over pretreatment PRL levels of 64% and 19%, were obtained with the 10.0 µg/kg and 20.0 µg/kg doses, respectively.

Naloxone. A marked stimulation in LH levels was observed after naloxone administration (Figures 25, 26, and 27). The 2.0 mg/kg dose brought about a eight-fold increase with respect to pre-treatment levels (237% change difference) of LH. This increase occurred within 40 minutes after drug treatment and lasted from 60 to 180 minutes, depending on the animal. Administration of 0.5 and 1.0 mg/kg naloxone resulted in LH

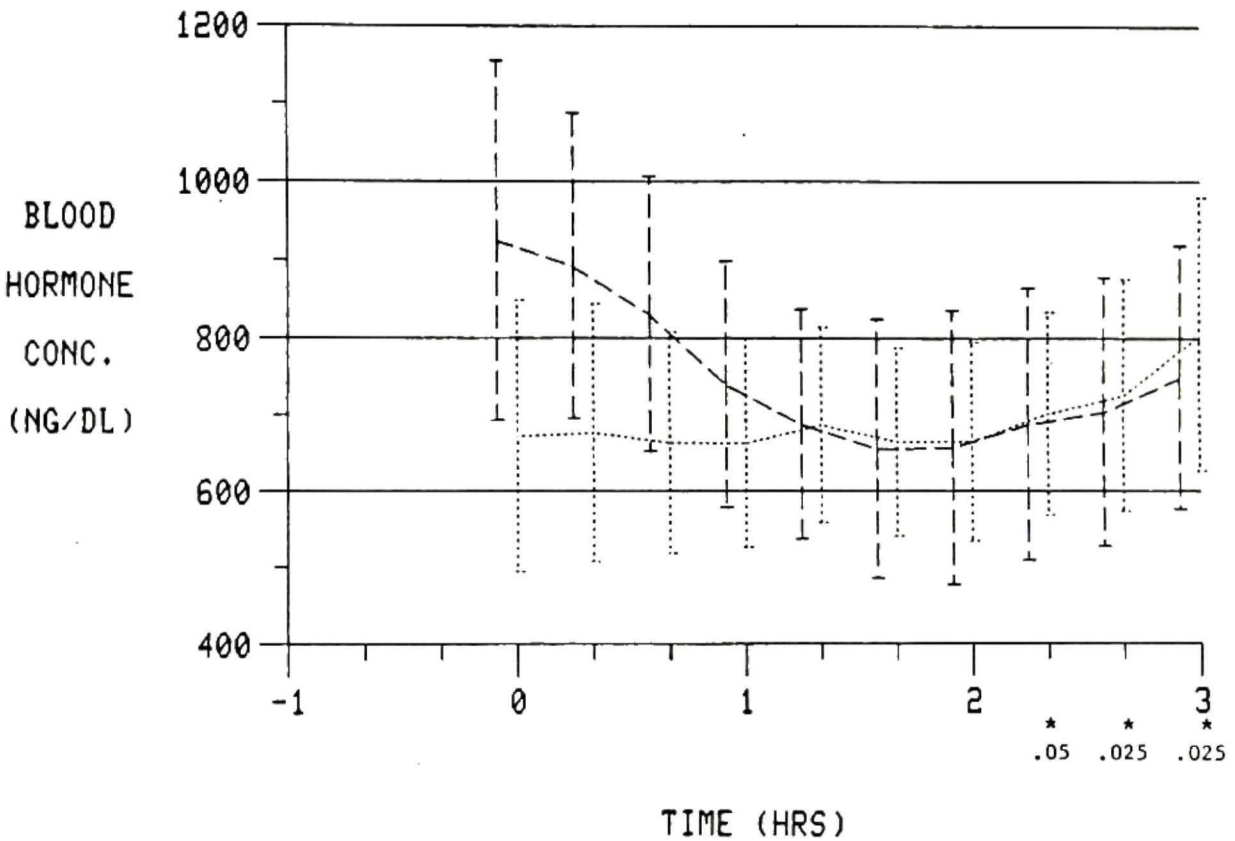

Figure 22. Effect of DADLE (10.0 µg/kg) or vehicle on plasma PRL levels. DADLE was administered at time zero hours. Points represent the moving averages (±SEM) for vehicle (.....) and treated (- - - -) groups. N = 4, averaging window size was four. Windows where significant inter-group differences in changes from pretreatment (first window) level occurred are denoted by asterisks and associated p-values.

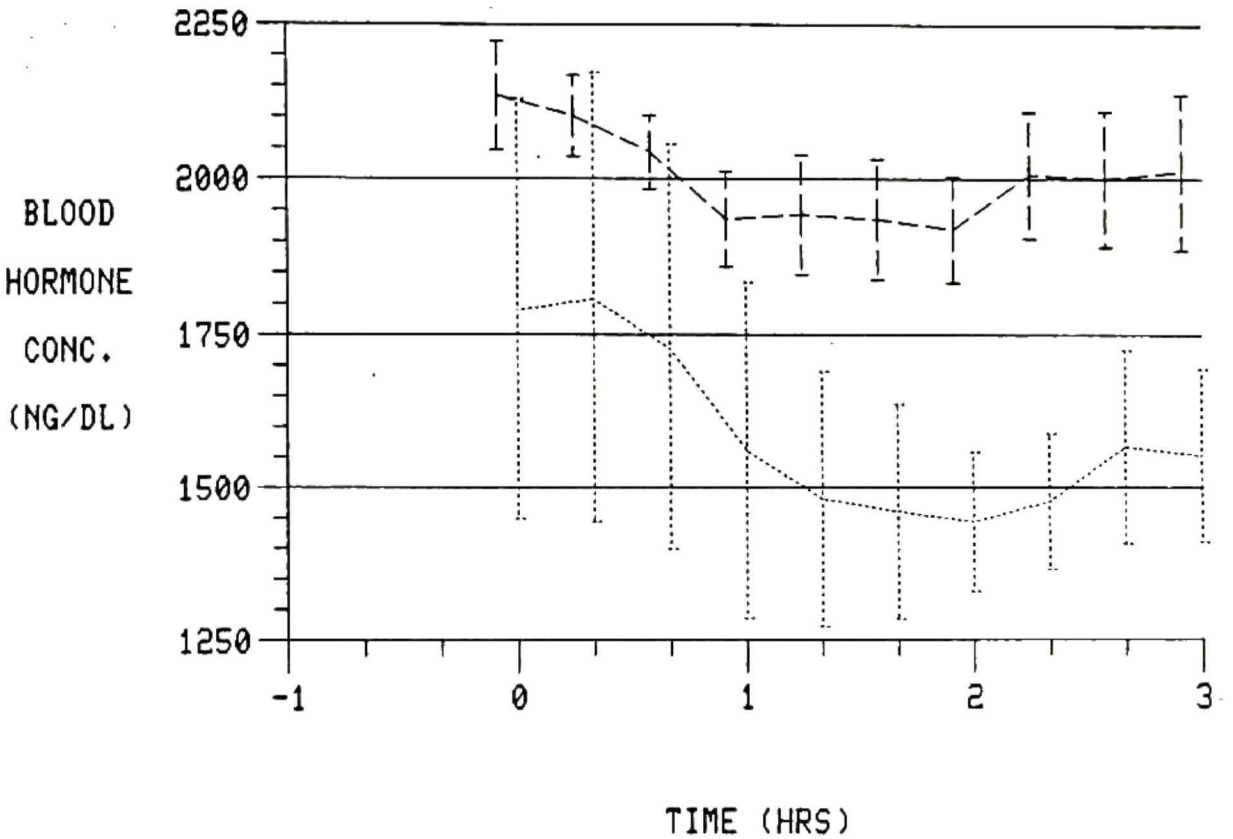

Figure 23. Effect of β-endorphin (20.0 µg/kg) or vehicle on plasma LH levels. β-endorphin was administered at time zero hours. Points represent the moving averages (±SEM) for vehicle (.....) and treated (- - - -) groups. N = 5, averaging window size was four. No significant inter-group differences in changes from pretreatment (first window) level occurred.

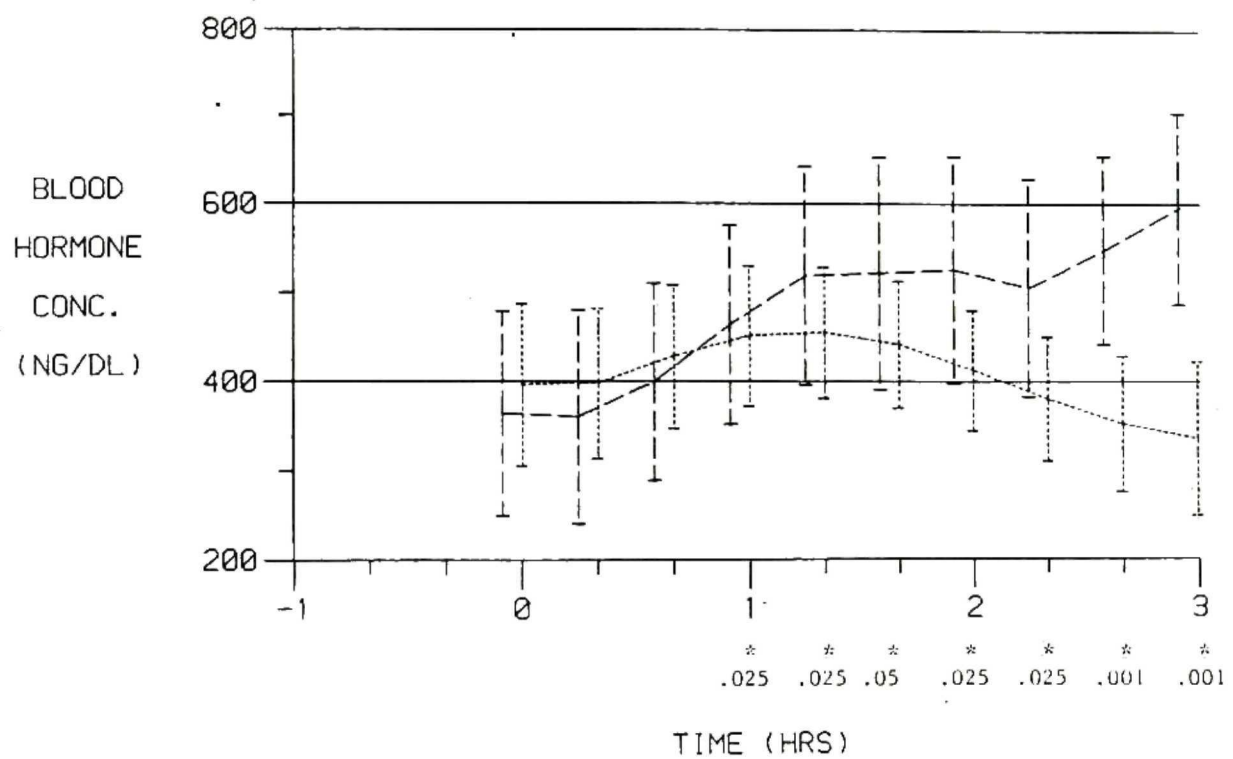

Figure 24. Effect of β-endorphin (10.0 µg/kg) or vehicle on plasma PRL levels. β-endorphin was administered at time zero hours. Points represent the moving averages (±SEM) for vehicle (.....) and treated (- - - -) groups. N = 5, averaging window size was four. Windows where significant inter-group differences in changes from pretreatment (first window) level occurred are denoted by asterisks and associated p-values.

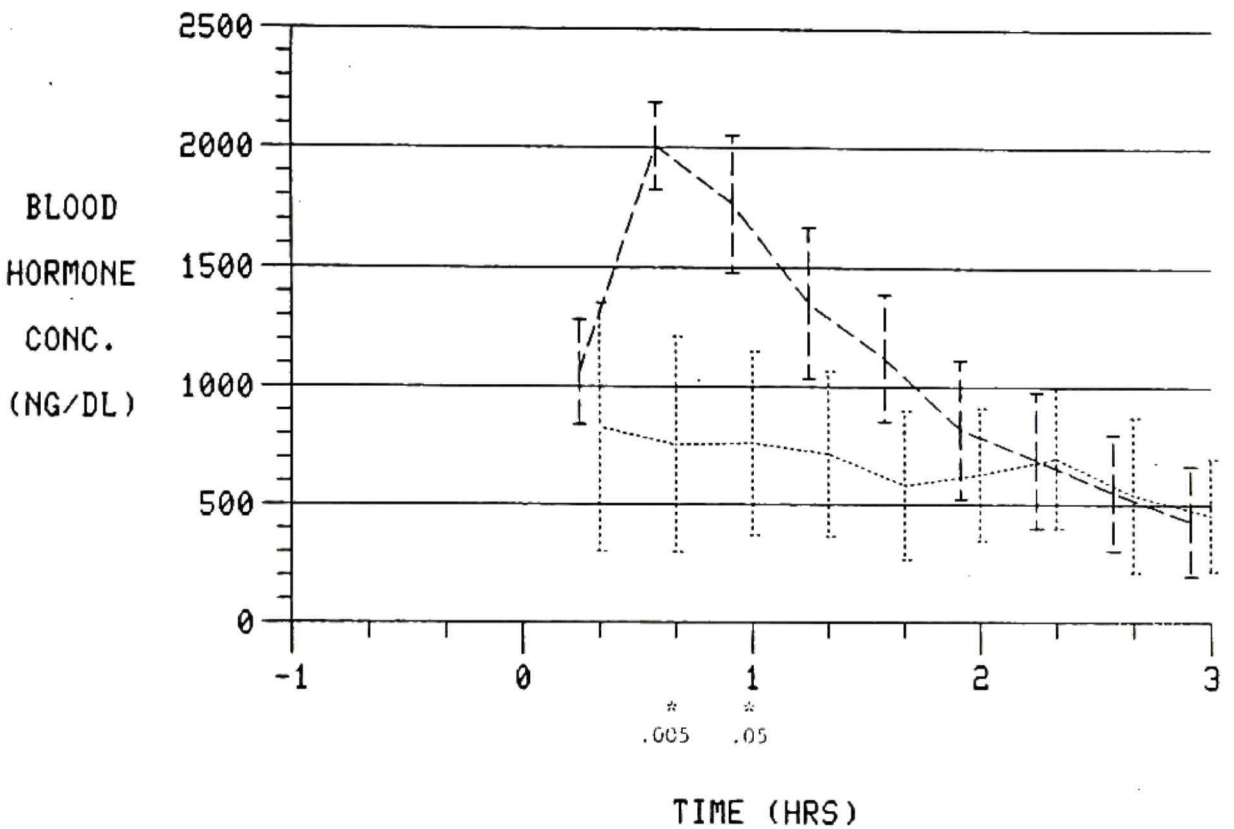

Figure 25. Effect of naloxone (2.0 mg/kg) or vehicle on plasma LH levels. Naloxone was administered at time zero hours. Points represent the moving averages (±SEM) for vehicle (.....) and treated (- - - -) groups. N = 4, averaging window size is two. Windows where significant inter-group differences in changes from pretreatment (first window) level occurred are denoted by asterisks and associated p-values. In this experiment the first samples were drawn at time zero.

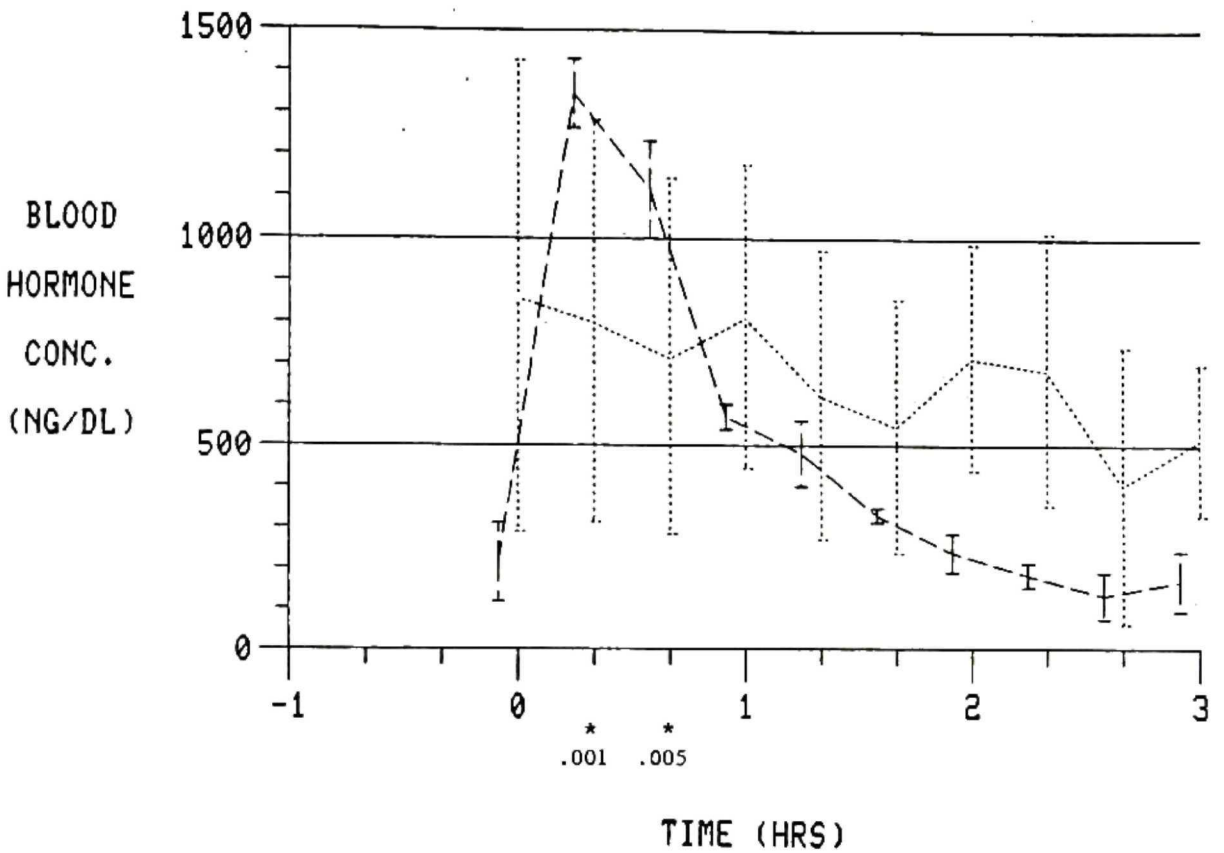

Figure 26. Effect of naloxone (1.0 mg/kg) or vehicle on plasma LH levels. Naloxone was administered at time zero hours. Points represent the moving averages (±SEM) for vehicle (.....) and treated (- - - -) groups. N = 4, averaging window size is one. Windows where significant inter-group differences in changes from pretreatment (first window) level occurred are denoted by asterisks and associated p-values. In this experiment the first samples were drawn at time zero.

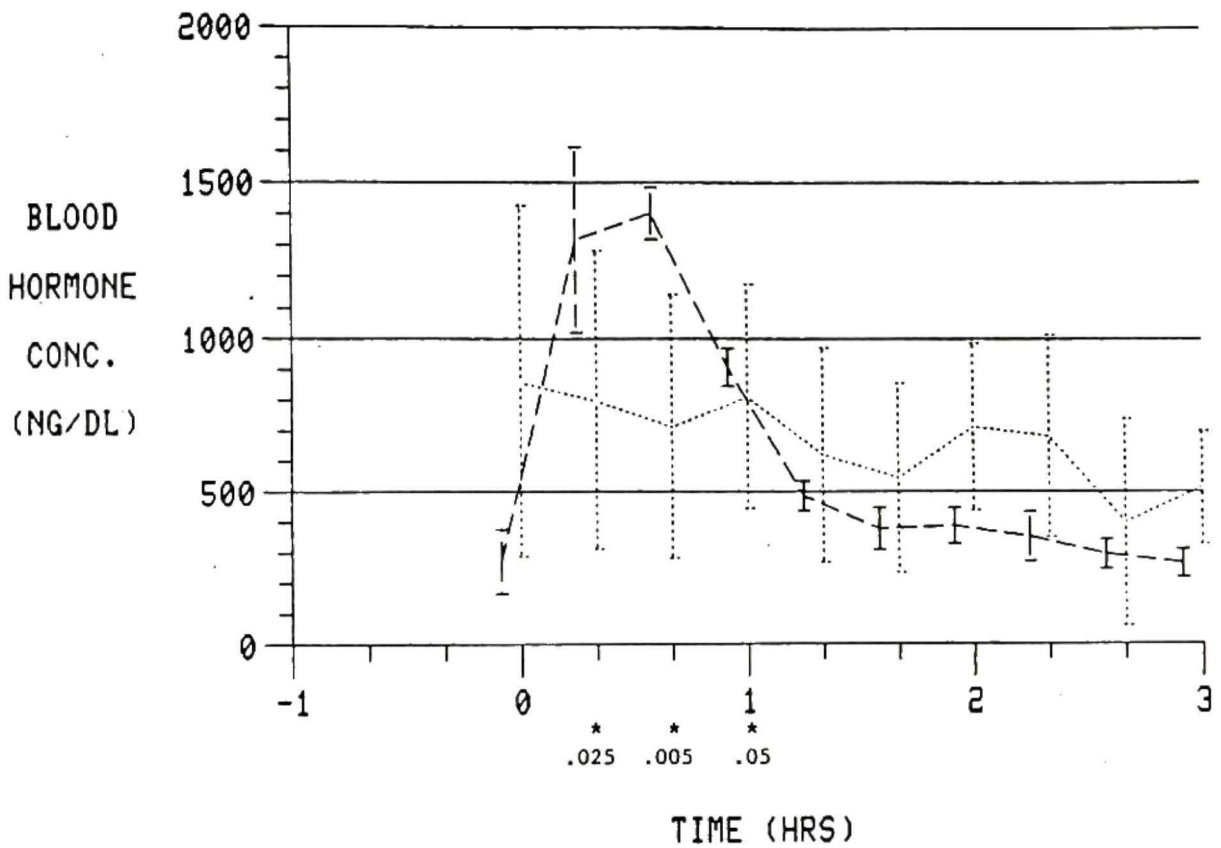

Figure 27. Effect of naloxone (0.5 mg/kg) or vehicle on plasma LH levels. Naloxone was administered at time zero hours. Points represent the moving averages (±SEM) for vehicle (.....) and treated (- - - -) groups. N = 4, averaging window size is one. Windows where significant inter-group differences in changes from pretreatment (first window) level occurred are denoted by asterisks and associated p-values. In this experiment the first samples were drawn at time zero.

increase factors of five- and six-fold over pretreatment levels and (change differences of 149% and 139%), respectively. These increases occurred in all cases within 40 minutes after drug administration. LH levels in all animals returned to normal within 100 minutes after drug treatment.

Administration of naloxone 0.5 mg/kg and 1.0 mg/kg elicited no significant effects on plasma PRL levels, although at the latter dose a change difference of -25% and an absolute decrease from pretreatment levels by 20% occurs during the 60 - 140 minute period (Figure 28). With 2.0 mg/kg dose naloxone, statistically significant decreases in PRL levels, change difference of 43% or 9% from pretreatment, were observed at approximately two hours post drug administration (Figure 29).

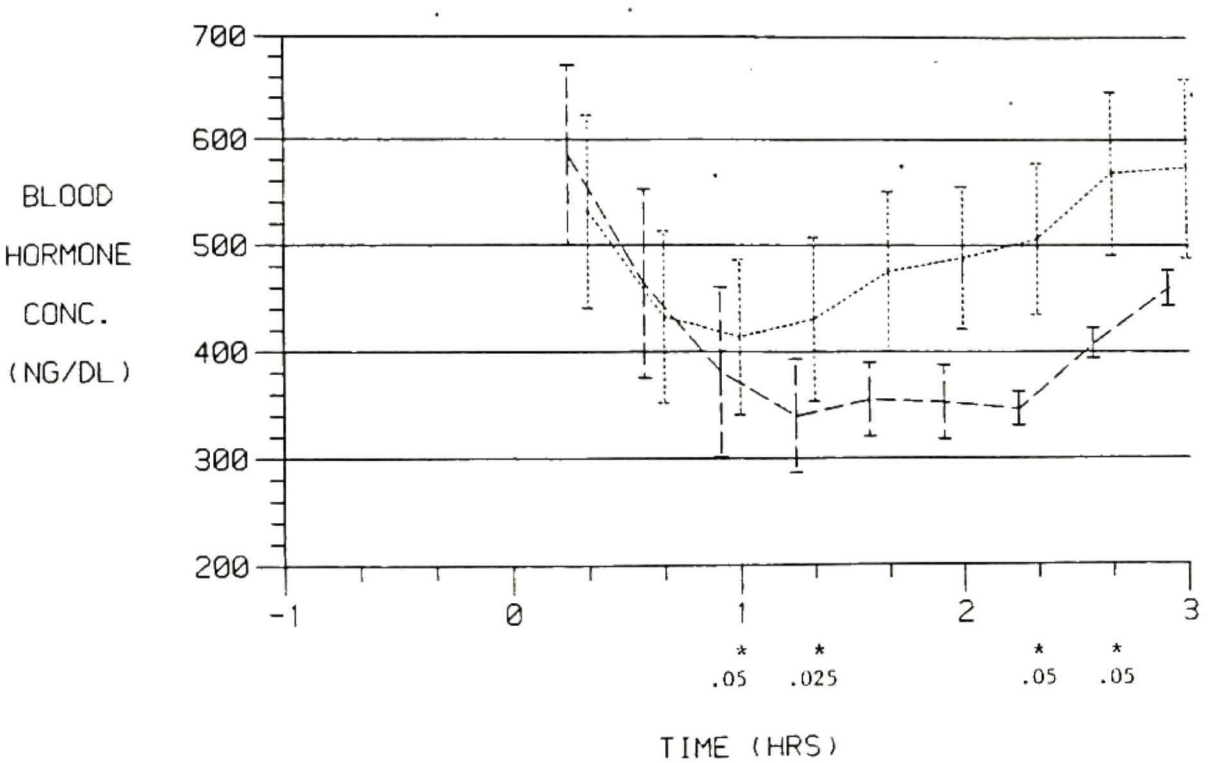

Figure 28. Effect of naloxone (1.0 mg/kg) or vehicle on plasma PRL levels. Naloxone was administered at time zero hours. Points represent the moving averages (±SEM) for vehicle (.....) and treated (- - - -) groups. N = 5, averaging window size was four. No significant inter-group differences in changes from pretreatment (first window) level occurred. In this experiment the first samples were drawn at time zero.

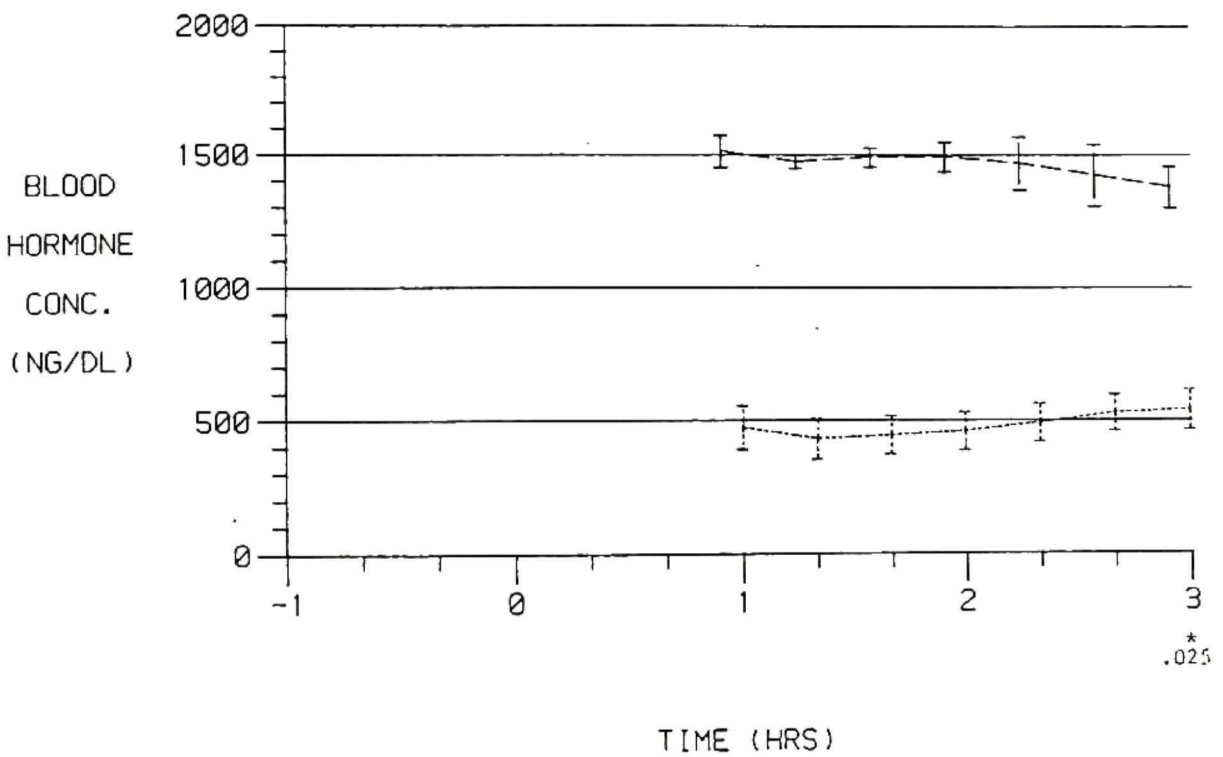

Figure 29. Effect of naloxone (2.0 mg/kg) or vehicle on plasma PRL levels. Naloxone was administered at time zero hours. Points represent the moving averages (±SEM) for vehicle (.....) and treated (- - - -) groups. N = 5, averaging window size is four. Windows where significant inter-group differences in changes from pretreatment (first window) level occurred are denoted by asterisks and associated p-values. In this experiment the first samples were drawn at time zero.

3. Mechanisms of Acute Drug Effects on Reproductive Hormones

To study the possible mechanisms of action of the opioid drugs, experiments were designed to examine the site of opioid action (pituitary, hypothalamic, or receptor) and the effect of opioids on episodic hormone release.

a) Site of Action Studies.

Two sets of studies were done in order to further define the site of opioid action. In the first of such studies, GnRH was administered in an attempt to reverse the opioid depression of LH levels. This study was designed to indicate whether or not a pituitary site of action was involved in the opioid action. In these experiments, blood was drawn at 20 minute intervals for a period of four hours (one hour before and three hours after opioid or vehicle treatment). At 80 minutes after morphine or DADLE administration, GnRH was given intravenously. The time of GnRH administration was determined from previous studies to be within the period of maximal opioid effect on plasma LH levels.

The second study was designed to determine whether opioid effects were elicited through opiate receptors. In these studies, naloxone was administered 100 minutes after morphine or DADLE treatment. The time of naloxone administration was chosen from our previous studies in which it was determined to be within the period of opioid effect on LH, PRL, and testosterone levels. Various dose levels of naloxone were studied, but two dose levels 0.03 and 1.0 mg/kg were used since these dose levels are thought to represent effects on different opiate receptor populations.

Naloxone antagonizes both μand δopiate receptors at high doses (Kosterlitz, 1980). To ensure antagonism of both μ-and δ-receptors, the 1.0 mg/kg dose of naloxone was chosen. This dose is approximately 3-fold

higher than the maximum pA_2 value reported for naloxone (Yaksh and Howe, 1982). The pA_2 value is a measure of antagonism and in vivo is equal to the negative logarithm of the molar concentration of the dose of antagonist required to reduce agonistic potency by one-half. Due to differences in affinity, naloxone at low dose levels primarily antagonizes μ-opiate receptors. The 0.03 mg/kg dose of naloxone is at the lower limit of concentrations indicated by the range of pA_2 values for morphine-naloxone interaction. This dose is approximately one-tenth of that required to give the pA_2 value for DADLE-naloxone interactions (Yaksh and Howe, 1982), thus favoring a μ-receptor antagonism.

Morphine Sulfate - Reversal by GnRH. Figures 30 and 31 illustrate the effect of GnRH on vehicle and morphine treated monkeys. The first phase of this experimental series, opioid treatment, produced the expected depression in LH. (This depression may not be apparent in the figures as the y-axis is expanded to incorporate the response to GnRH). Administration of 100 μg of GnRH 80 minutes after morphine treatment resulted in a prompt stimulation of LH levels. These LH responses to GnRH in morphine-treated animals were indistinguishable in magnitude and duration to the GnRH responses in vehicle pretreated monkeys. This indicates that morphine does not depress LH levels via the pituitary but at a higher site such as the hypothalamus.

[D-Ala^2,D-Leu^5]-Enkephalin - Reversal by GnRH. Administration of DADLE (20 μg/kg) produced the expected decrease in LH level (Figure 32). When GnRH (100μg) was administered 80 minutes later, it caused a rapid and sizeable increase in the plasma levels of LH. These LH responses to GnRH did not differ significantly from the GnRH-stimulated increase seen in the vehicle pretreated group. As seen for morphine, these results

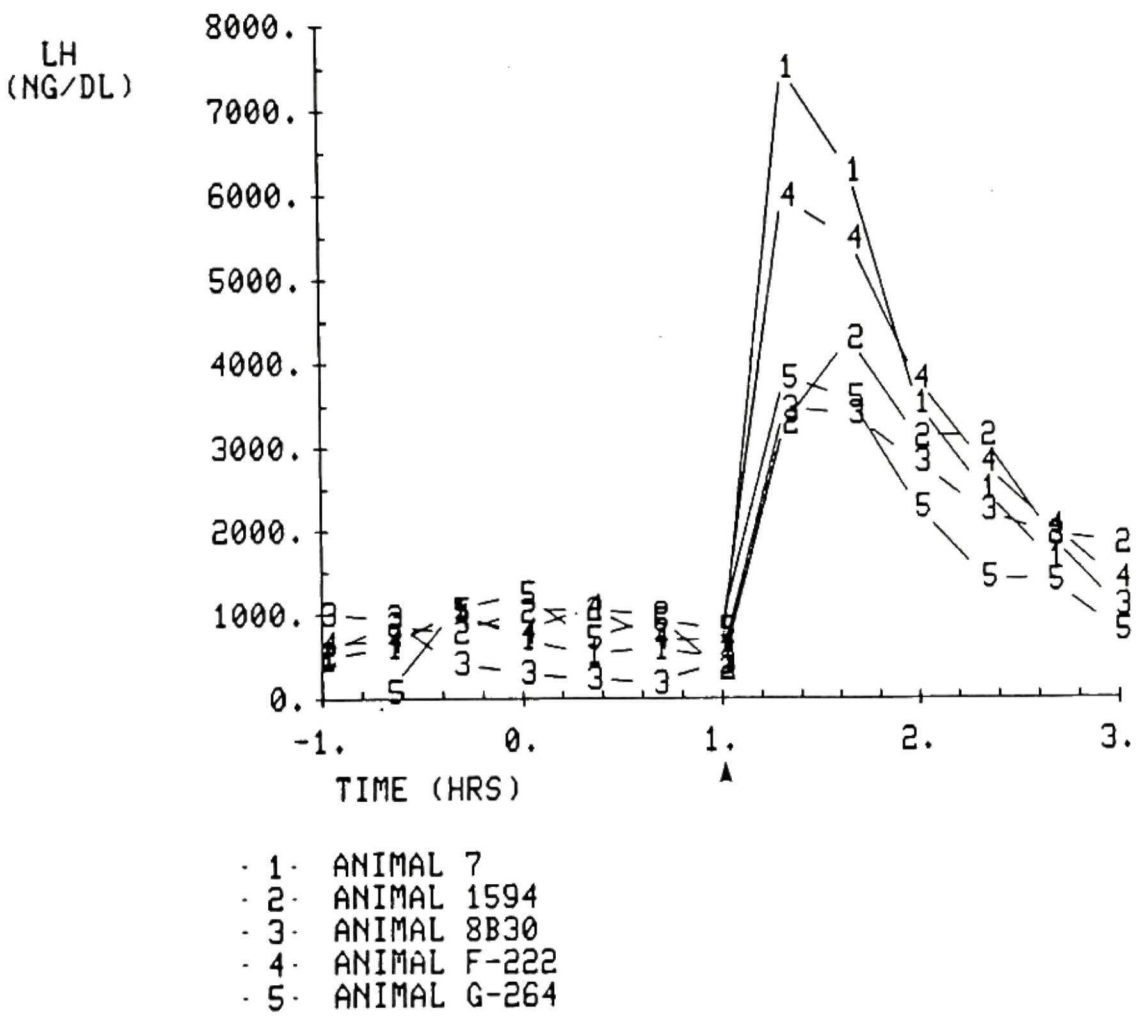

Figure 30. Effect of GnRH (100 µg) on plasma LH levels after pretreatment with vehicle. Vehicle was administered at time zero hours. GnRH was administered at 60 minutes as indicated by the arrowhead. Shown are the plasma concentrations of the hormone for five monkeys at the times indicated.

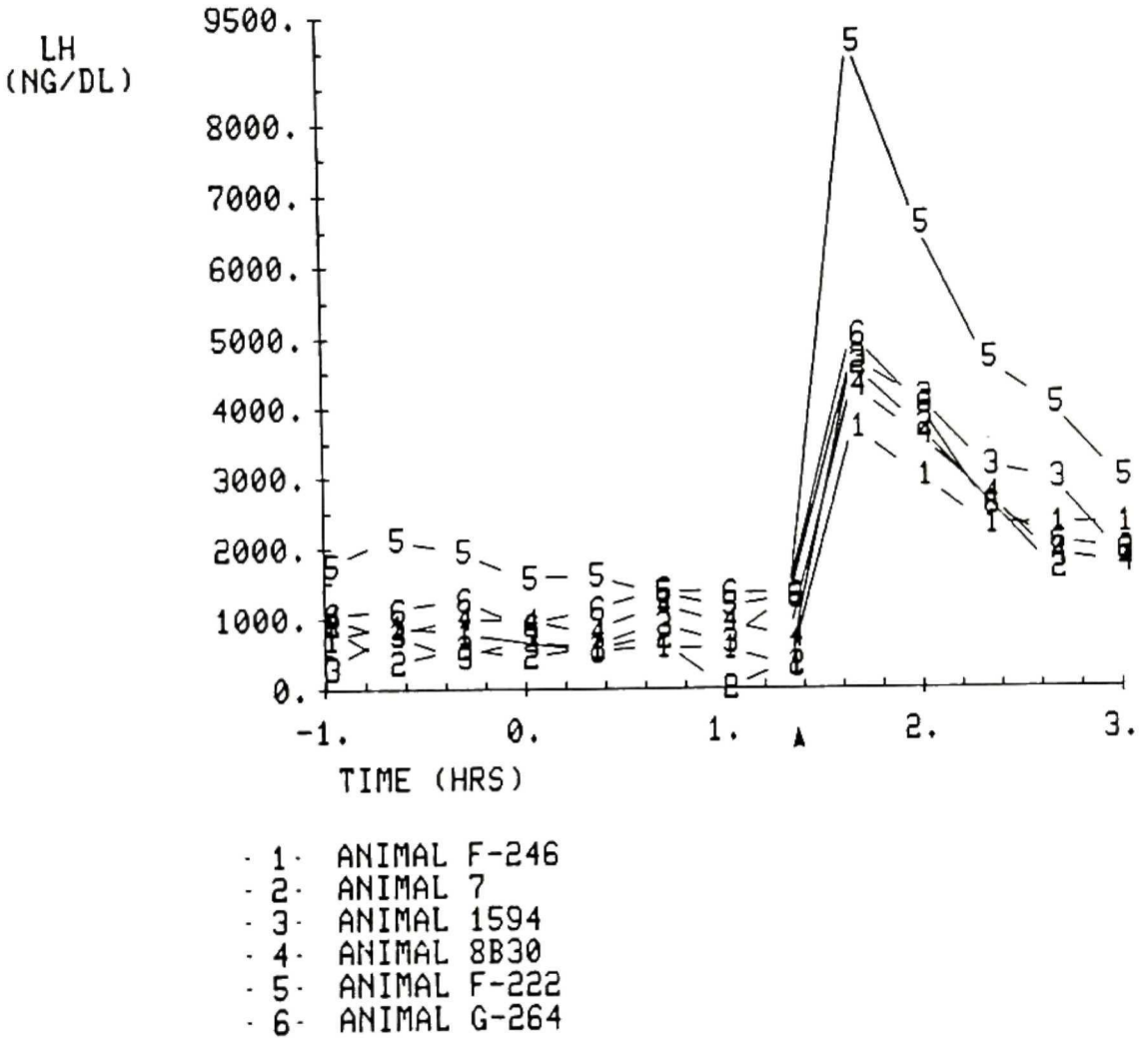

Figure 31. Effect of GnRH (100 µg) on plasma LH levels after pretreatment with morphine. Morphine (1.0 mg/kg) was administered at time zero hours. GnRH was administered at 80 minutes as indicated by the arrowhead. Shown are the plasma concentrations of the hormone for six monkeys at the times indicated.

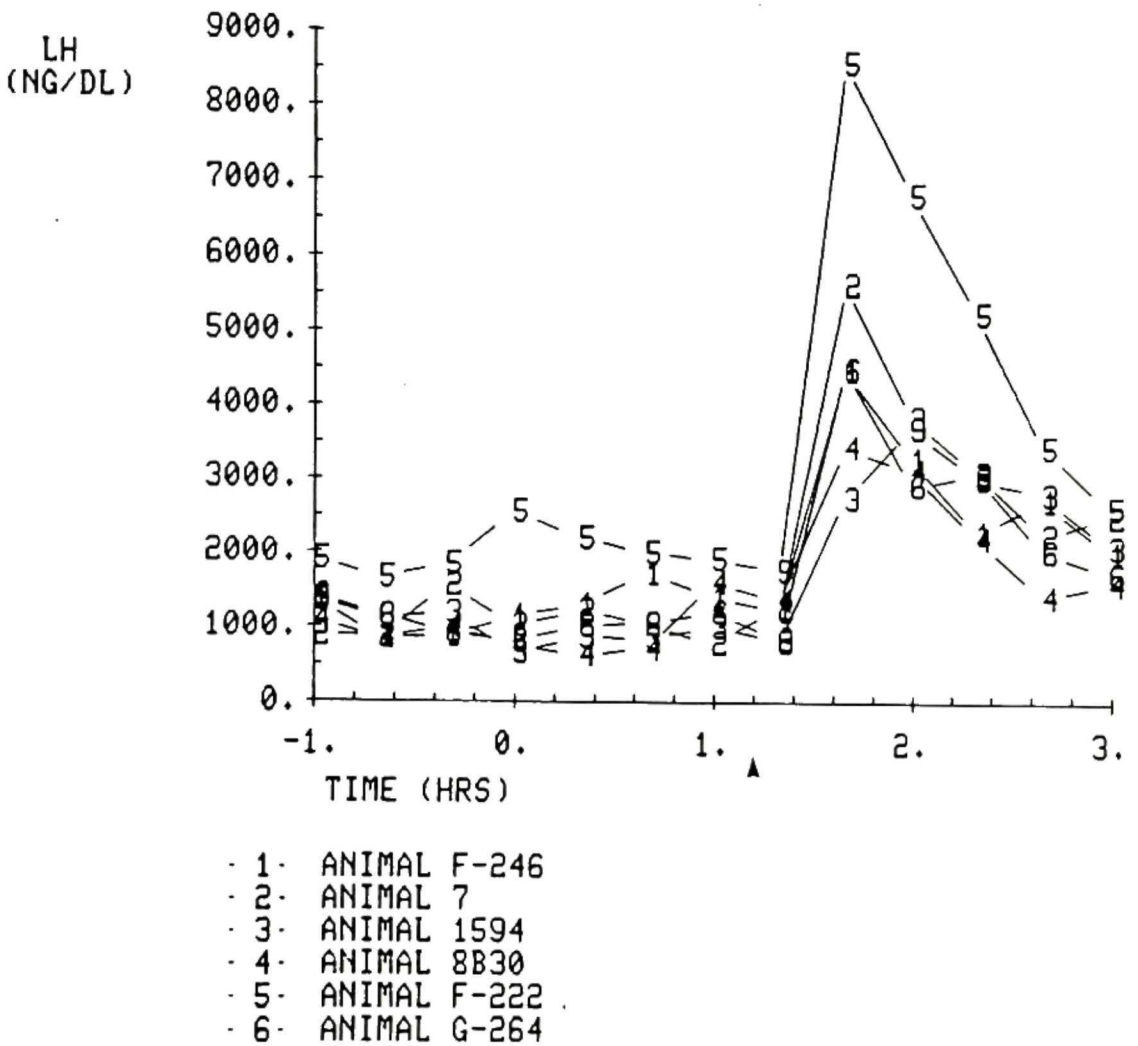

Figure 32. Effect of GnRH (100 µg) on plasma LH levels after pretreatment with DADLE. DADLE (20 µg/kg) was administered at time zero hours. GnRH was administered at 80 minutes as indicated by the arrowhead. Shown are the plasma concentrations of the hormone for six monkeys at the times indicated.

support a site of action above the pituitary for DADLE's effect on LH levels.

Morphine Sulfate - Competition by Naloxone. Figures 33, 34, and 35 show the effect of naloxone (1.0 mg/kg) on testosterone, LH, and PRL plasma levels following pretreatment with vehicle at time zero hours. Naloxone was administered at 100 minutes after vehicle treatment. LH levels were increased two-fold, although not significant. Testosterone levels were significantly increased 12-fold, while PRL levels were decreased 69% following naloxone administration. Figures 36, 37, and 38 illustrate the effects of a lower dose naloxone (0.03 mg/kg) on these sex hormones. Naloxone was administered 100 minutes after vehicle pretreatment at time zero. Testosterone and LH were increased an average of 12- and 2-fold, respectively, and PRL was decreased (67%). Figures 35, 37 and 38 are presented as moving averages instead of raw data since in these studies naloxone's effect is not obvious.

After pretreatment with morphine sulfate (time zero), testosterone levels were depressed approximately 60% and LH levels were decreased 12% from vehicle. Figures 39 and 40 show that the subsequent administration of naloxone (1.0 mg/kg) at 100 minutes in these morphine pretreated monkeys completely reversed the morphine effect and produced rises in testosterone and LH which did not differ significantly from those seen with naloxone alone. Pretreatment with morphine produced increases in PRL levels (120% over controls). Figure 41 shows that subsequent administration of 1.0 mg/kg of naloxone 100 minutes after morphine pretreatment caused this increase to be reversed. The effect of naloxone on PRL levels in morphine pretreated monkeys did not differ significantly from that observed with vehicle pretreatment.

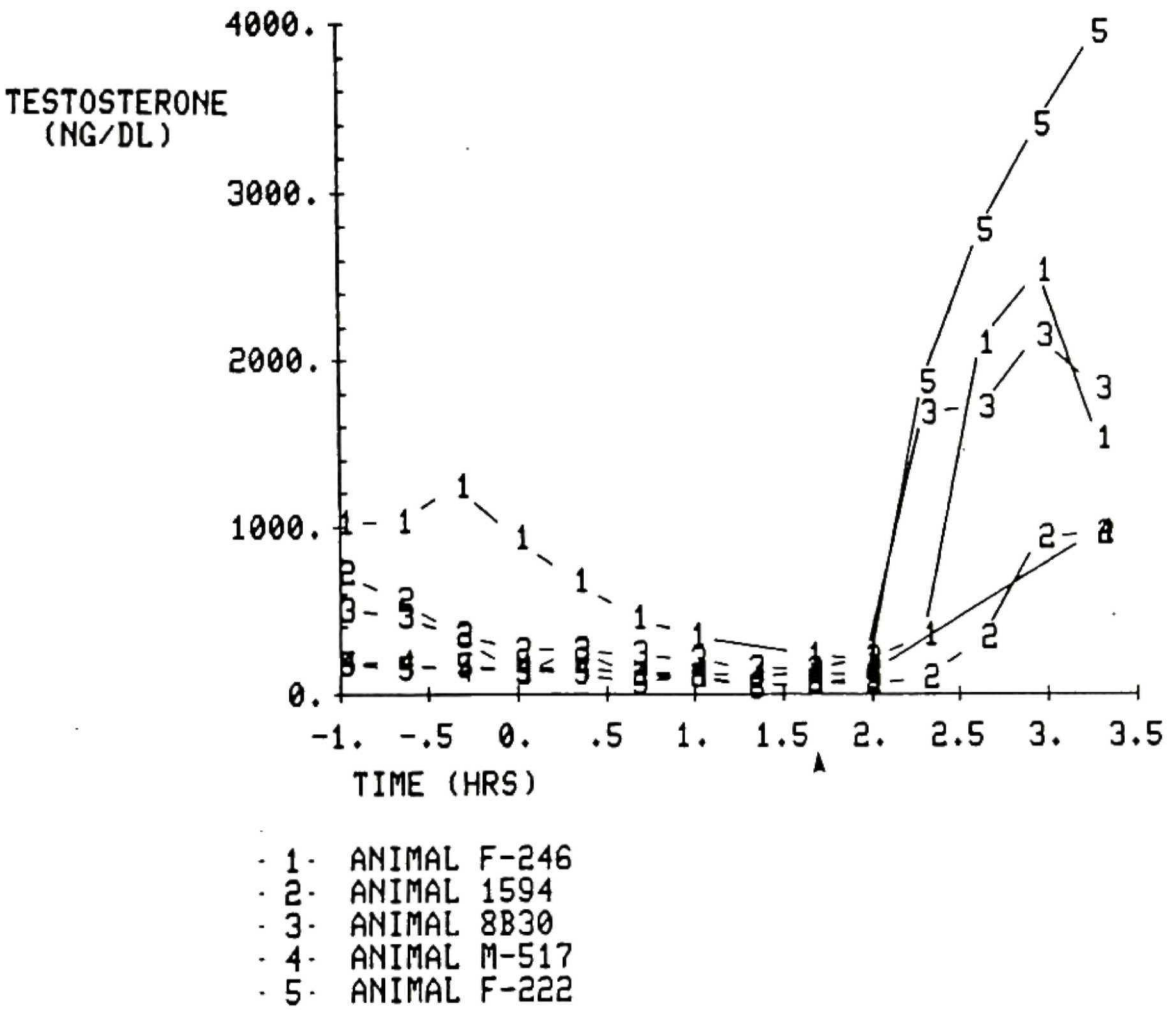

Figure 33. Effect of naloxone (1.0 mg/kg) on plasma testosterone levels after pretreatment with vehicle. Vehicle was administered at time zero hours. Naloxone was administered at 100 minutes as indicated by the arrowhead. Shown are the plasma concentrations of the hormone for five monkeys at the times indicated.

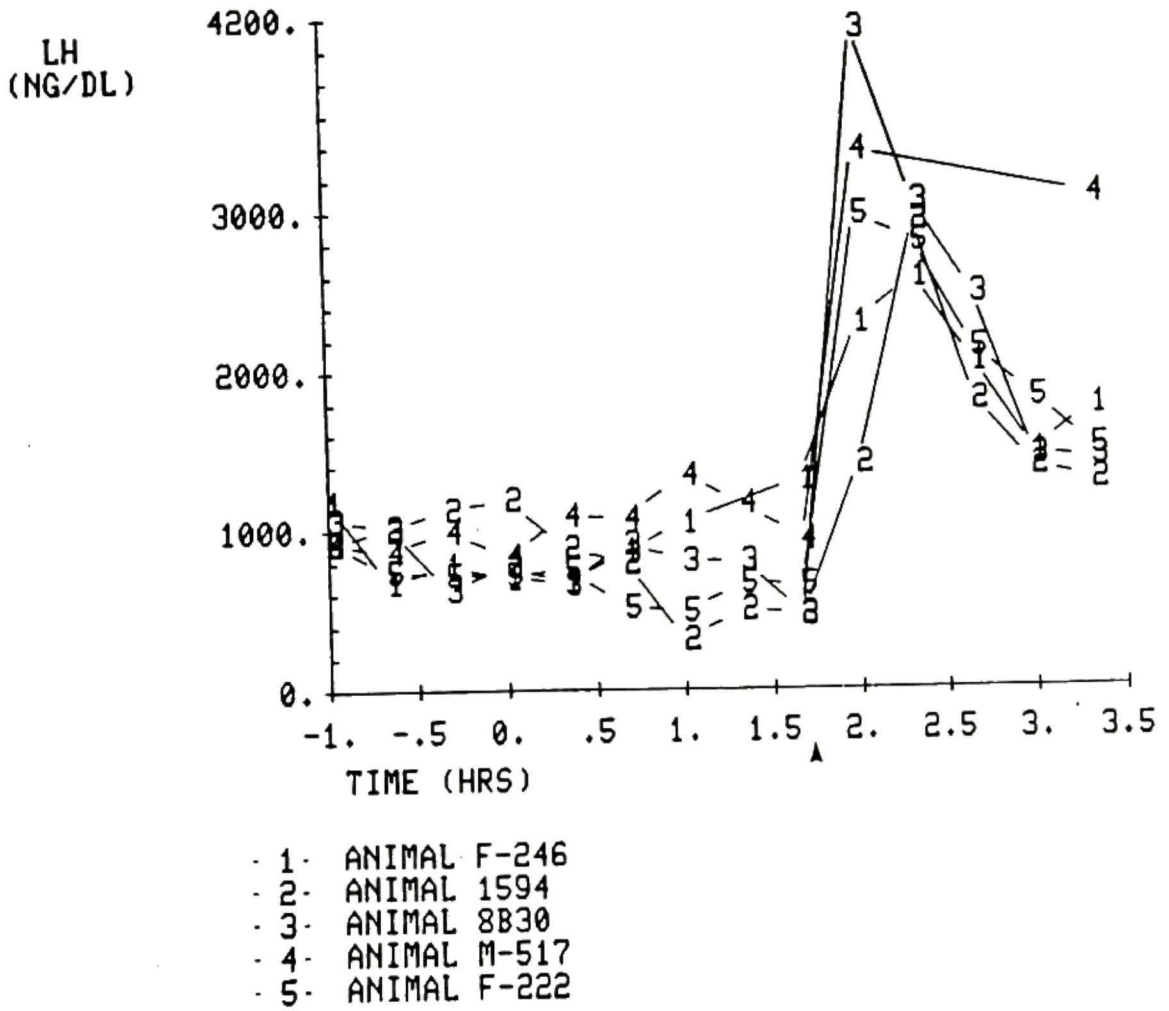

Figure 34. Effect of naloxone (1.0 mg/kg) on plasma LH levels after pretreatment with vehicle. Vehicle was administered at time zero hours. Naloxone was administered at 100 minutes as indicated by the arrowhead. Shown are the plasma concentrations of the hormone for five monkeys at the times indicated.

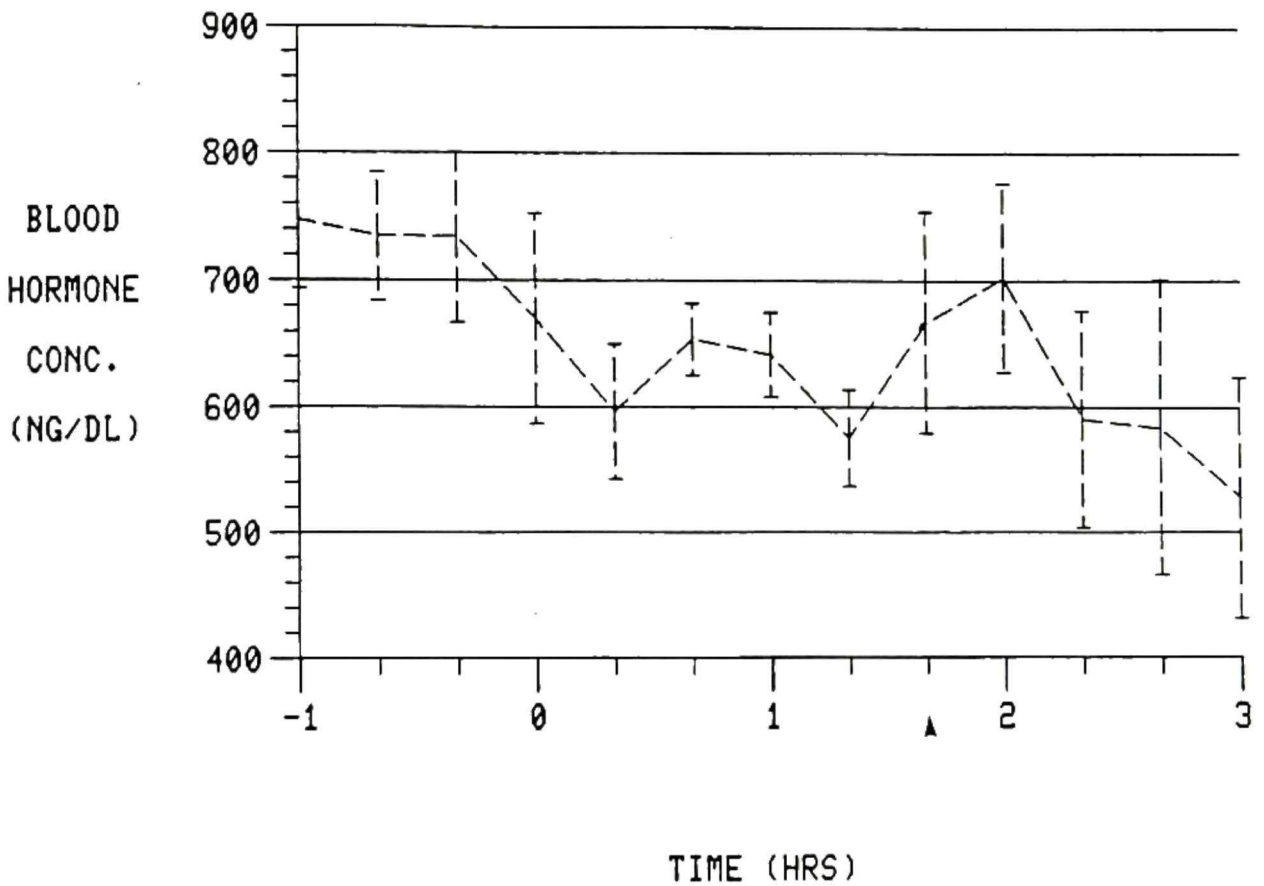

Figure 35. Effect of naloxone (1.0 mg/kg) on plasma PRL levels after pretreatment with vehicle. Vehicle was administered at time zero hours. Naloxone was administered at 100 minutes as indicated by the arrowhead. Points represent the unsmoothed (window size = 1) average sample values (±SEM) for N = 5.

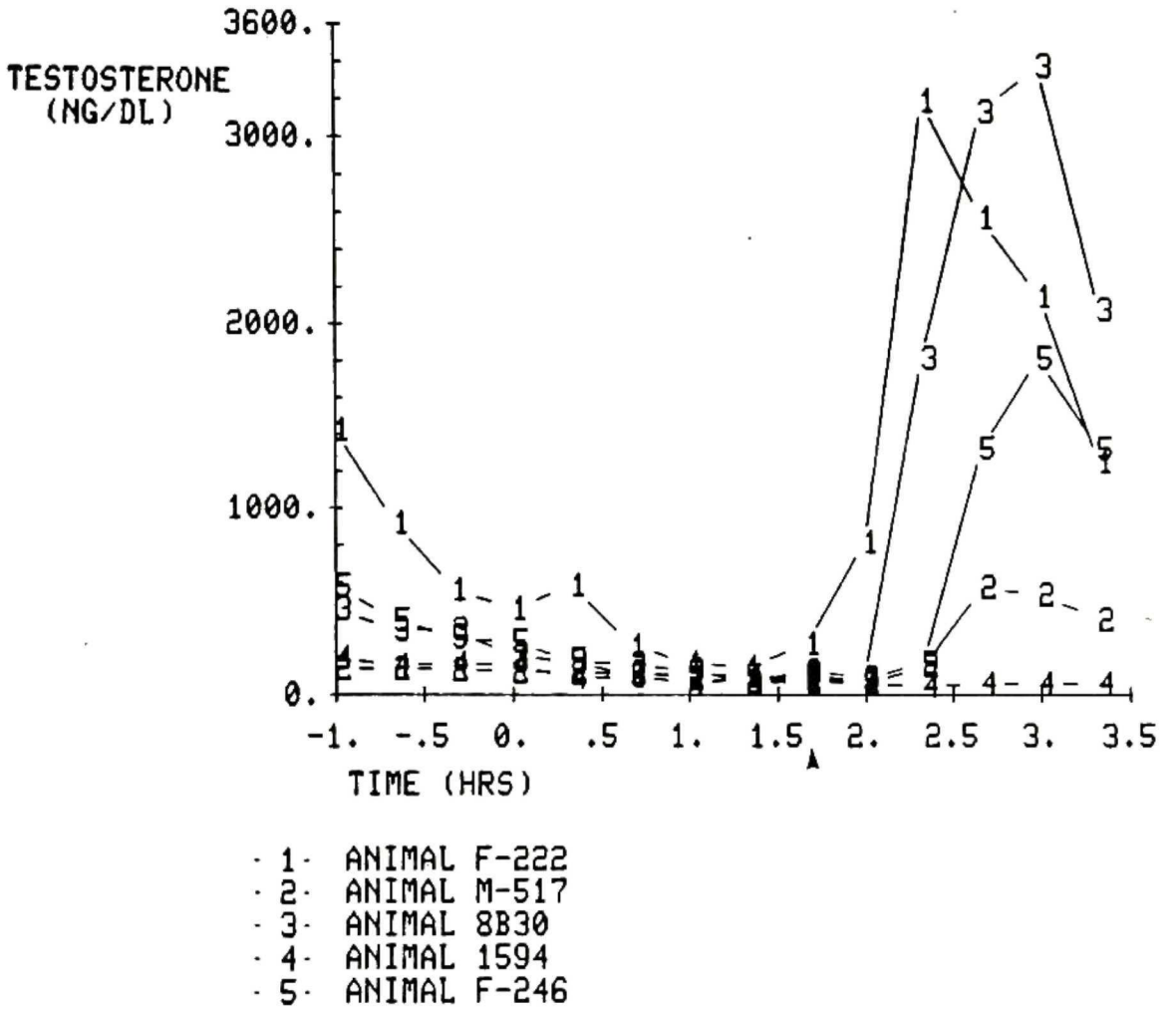

Figure 36. Effect of naloxone (0.03 mg/kg) on plasma testosterone levels after pretreatment with vehicle. Vehicle was administered at time zero hours. Naloxone was administered at 100 minutes as indicated by the arrowhead. Shown are the plasma concentrations of the hormone for five monkeys at the times indicated.

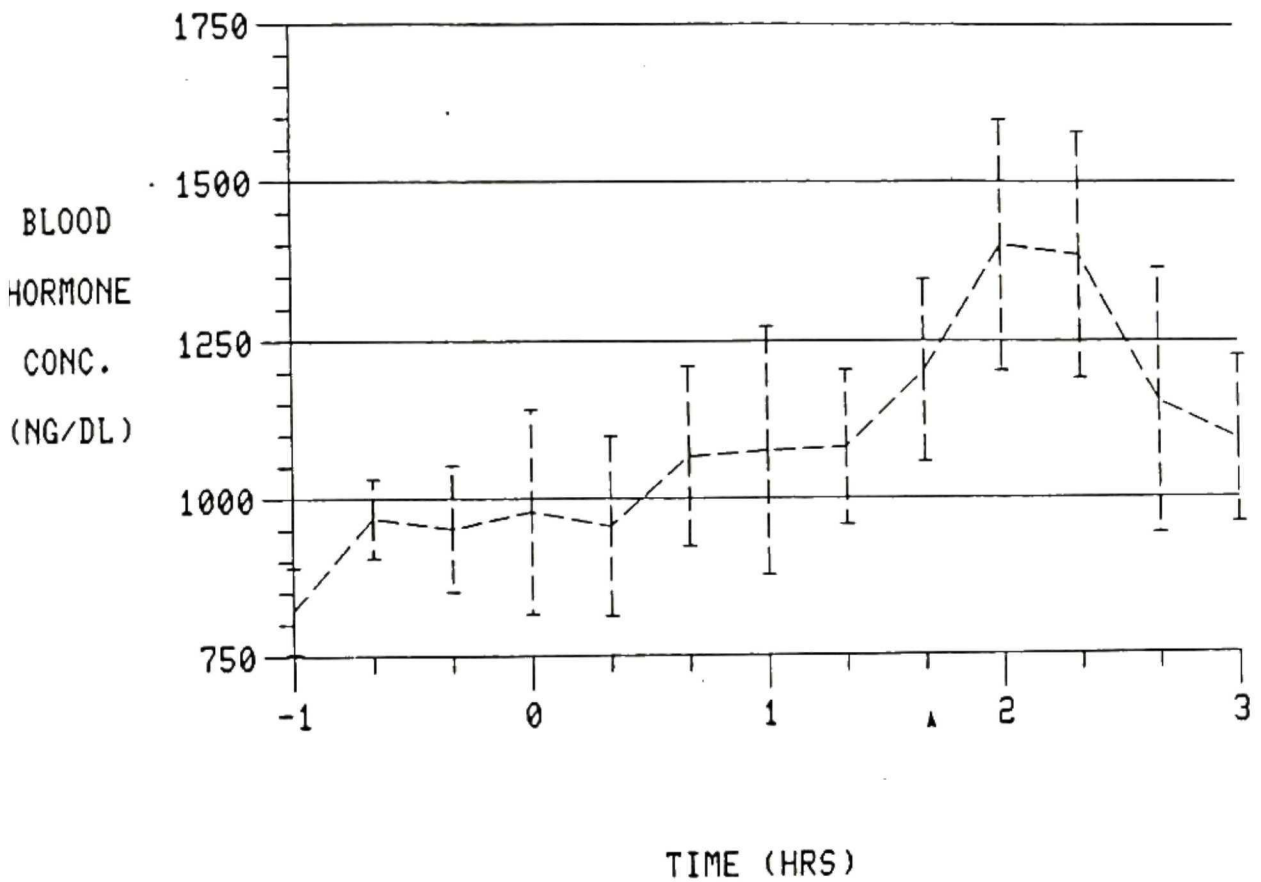

Figure 37. Effect of naloxone (0.03 mg/kg) on plasma LH levels after pretreatment with vehicle. Vehicle was administered at time zero hours. Naloxone was administered at 100 minutes as indicated by the arrowhead. Points represent the unsmoothed (window size = 1) average sample values (±SEM) for N = 5.

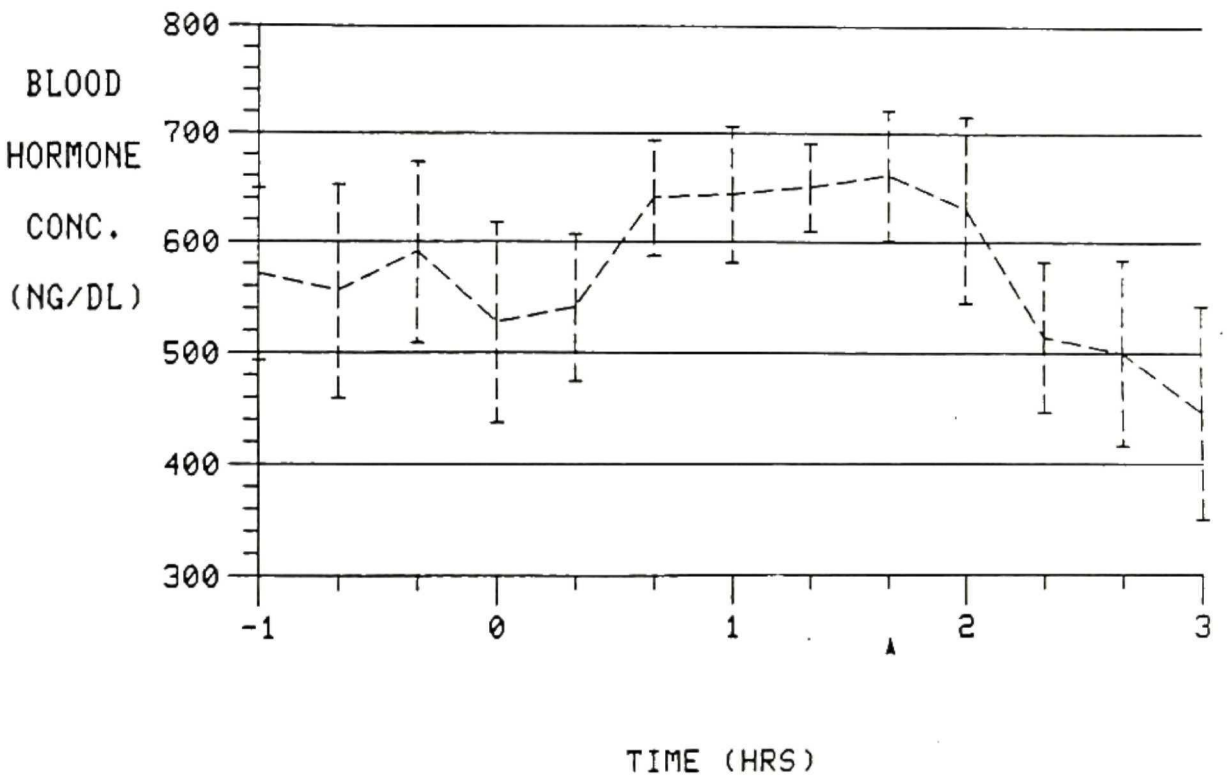

Figure 38. Effect of naloxone (0.03 mg/kg) on plasma PRL levels after pretreatment with vehicle. Vehicle was administered at time zero hours. Naloxone was administered at 100 minutes as indicated by the arrowhead. Points represent the unsmoothed (window size = 1) average sample values (±SEM) for N = 5.

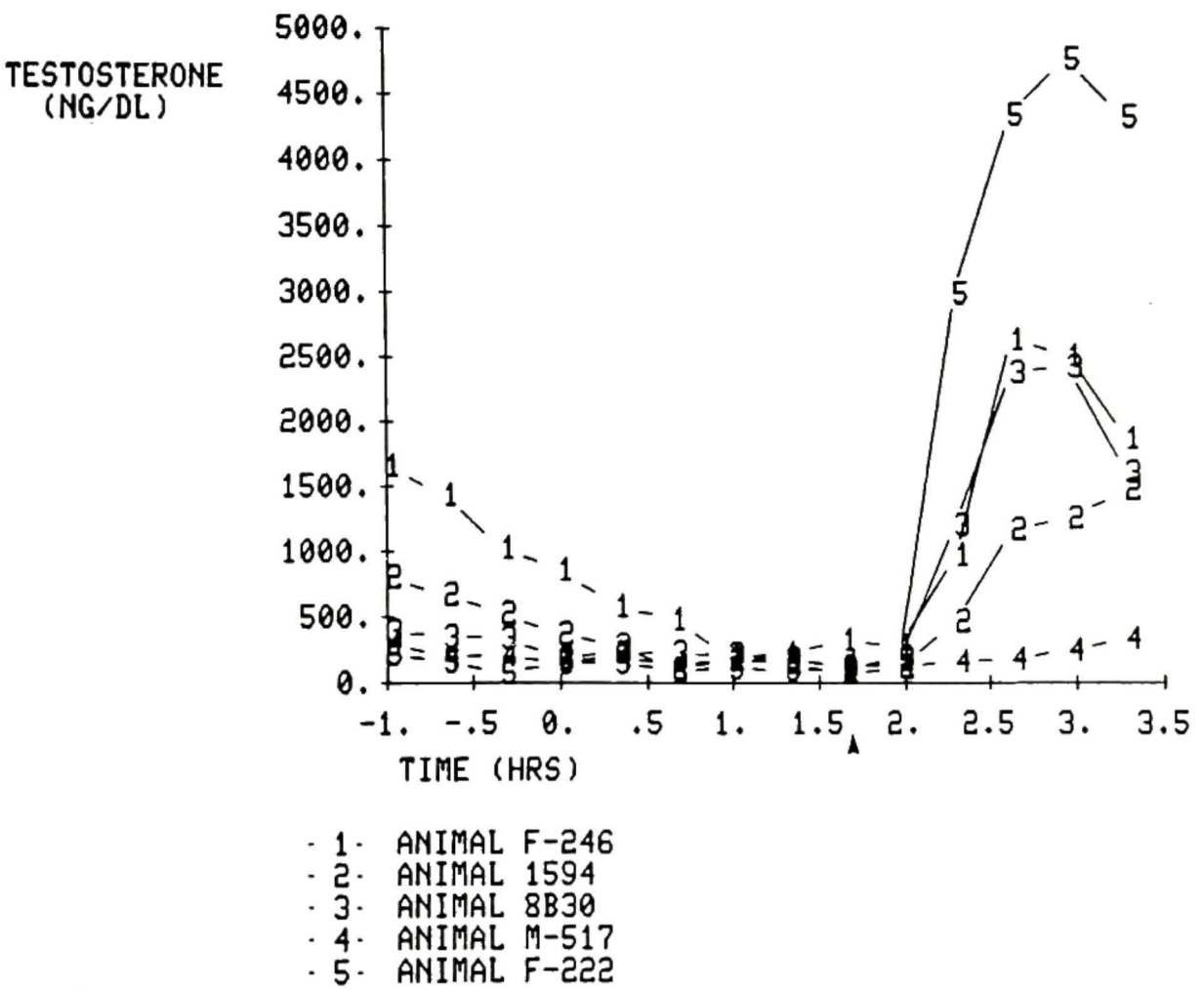

Figure 39. Effect of naloxone (1.0 mg/kg) on plasma testosterone levels after pretreatment with morphine. Morphine (1.0 mg/kg) was administered at time zero hours. Naloxone was administered at 100 minutes as indicated by the arrowhead. Shown are the plasma concentrations of the hormone for five monkeys at the times indicated.

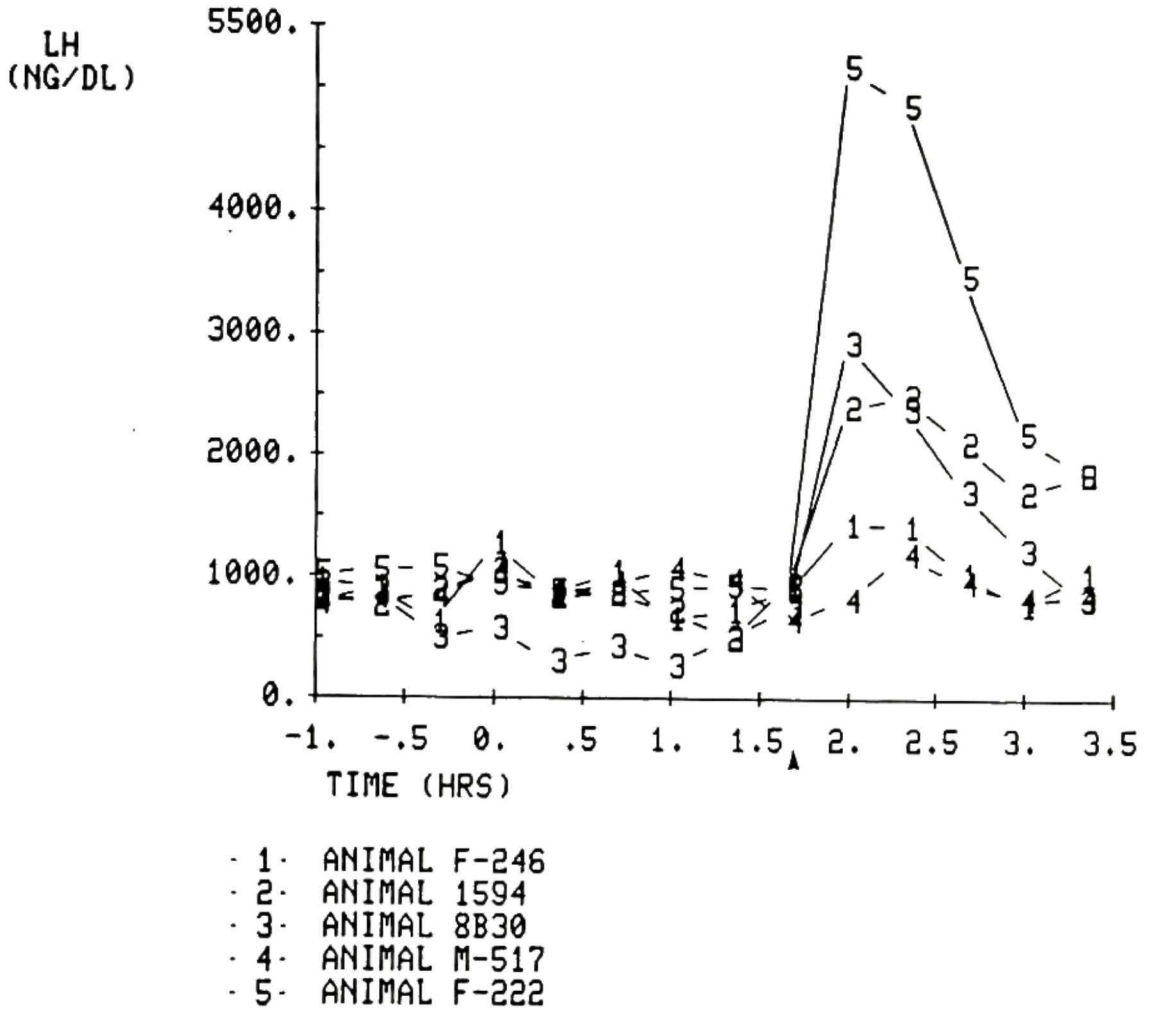

Figure 40. Effect of naloxone (1.0 mg/kg) on plasma LH levels after pretreatment with morphine. Morphine (1.0 mg/kg) was administered at time zero hours. Naloxone was administered at 100 minutes as indicated by the arrowhead. Shown are the plasma concentrations of the hormone for five monkeys at the times indicated.

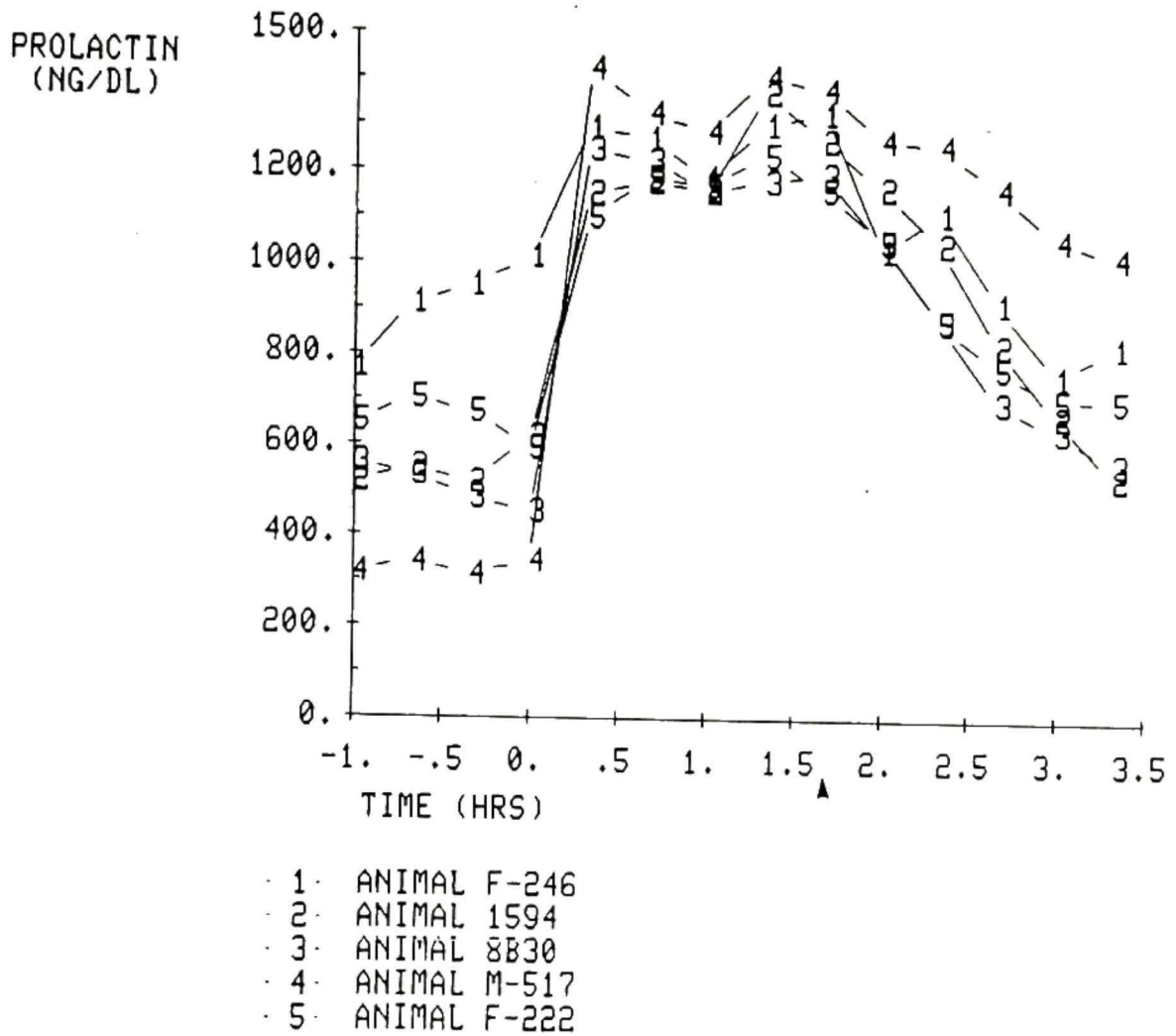

Figure 41. Effect of naloxone (1.0 mg/kg) on plasma PRL levels after pretreatment with morphine. Morphine (1.0 mg/kg) was administered at time zero hours. Naloxone was administered at 100 minutes as indicated by the arrowhead. Shown are the plasma concentrations of the hormone for five monkeys at the times indicated.

Administration of the low dose of naloxone (0.03 mg/kg) 100 minutes after pretreatment with morphine did not reverse the depressant effect of morphine on testosterone levels in four out of five monkeys (Figure 42). LH levels were briefly and slightly increased 20 minutes after administration of the 0.03 mg/kg dose of naloxone; however, this increase was significantly less than that elicited from the vehicle pretreated monkeys (Figure 43). As shown in Figure 44, the increase in PRL plasma levels due to morphine pretreatment was not reversed by the administration of 0.03 mg/kg naloxone.

DADLE - Competition by Naloxone. Pretreatment with DADLE at time zero hours caused a significant depression of testosterone levels 60% of vehicle). LH plasma levels were slightly decreased and DADLE pretreatment had no significant effect on PRL levels. As illustrated in Figures 45, 46, and 47, subsequent administration of 1.0 mg/kg naloxone at 100 minutes after drug administration reversed the effects of DADLE and resulted in increases in the plasma levels of testosterone and LH and a decrease in PRL levels. These effects following naloxone administration (1.0 mg/kg) were not significantly different than those observed subsequent to vehicle treatment (Figures 33,34, and 35.)

Pretreatment with DADLE resulted in decreased testosterone and LH plasma levels (to 40% and 70% of vehicle, respectively) and no effect on PRL levels. As shown in Figures 48 and 49, administration of low dose naloxone (0.03 mg/kg) at 100 minutes reversed the effects of DADLE on testosterone or LH levels. A decrease in PRL levels was observed following the 0.03 mg/kg of naloxone administration after DADLE pretreatment (Figure 50). This depression was not significantly different than that observed following vehicle pretreatment (Figure 38).

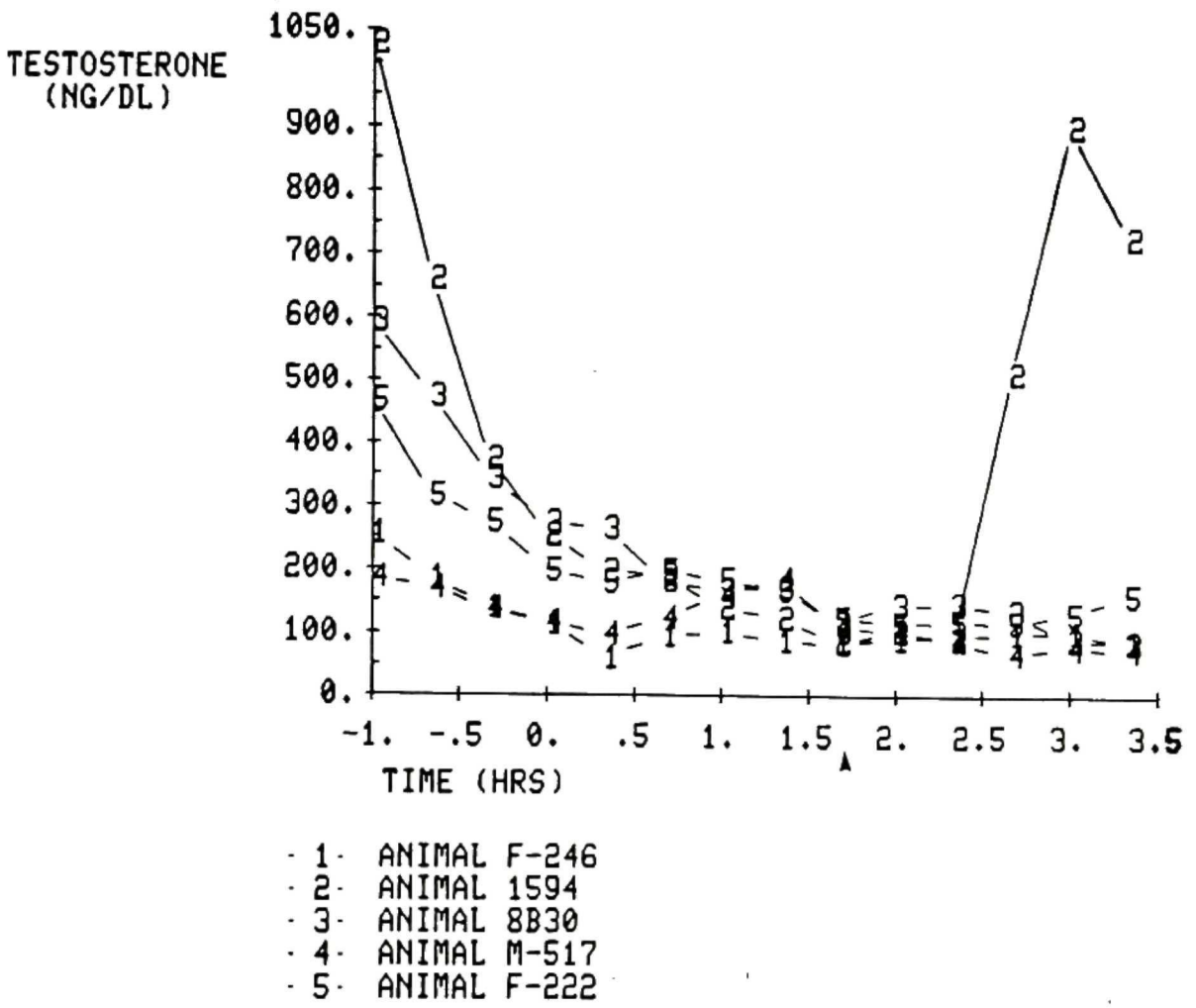

Figure 42. Effect of naloxone (0.03 mg/kg) on plasma testosterone levels after pretreatment with morphine. Morphine (1.0 mg/kg) was administered at time zero hours. Naloxone was administered at 100 minutes as indicated by the arrowhead. Shown are the plasma concentrations of the hormone for five monkeys at the times indicated.

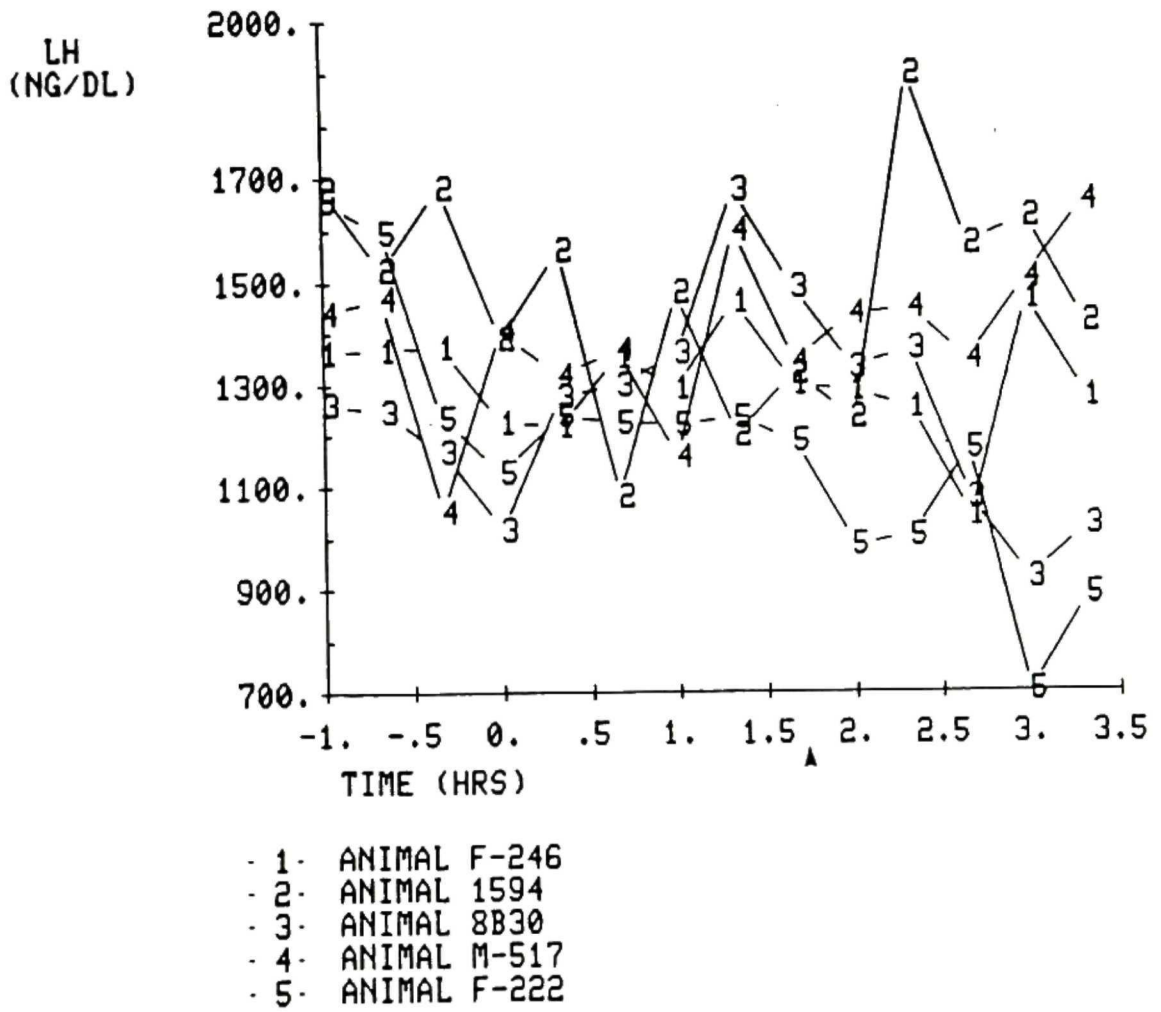

Figure 43. Effect of naloxone (0.03 mg/kg) on plasma LH levels after pretreatment with morphine. Morphine (1.0 mg/kg) was administered at time zero hours. Naloxone was administered at 100 minutes as indicated by the arrowhead. Shown are the plasma concentrations of the hormone for five monkeys at the times indicated.

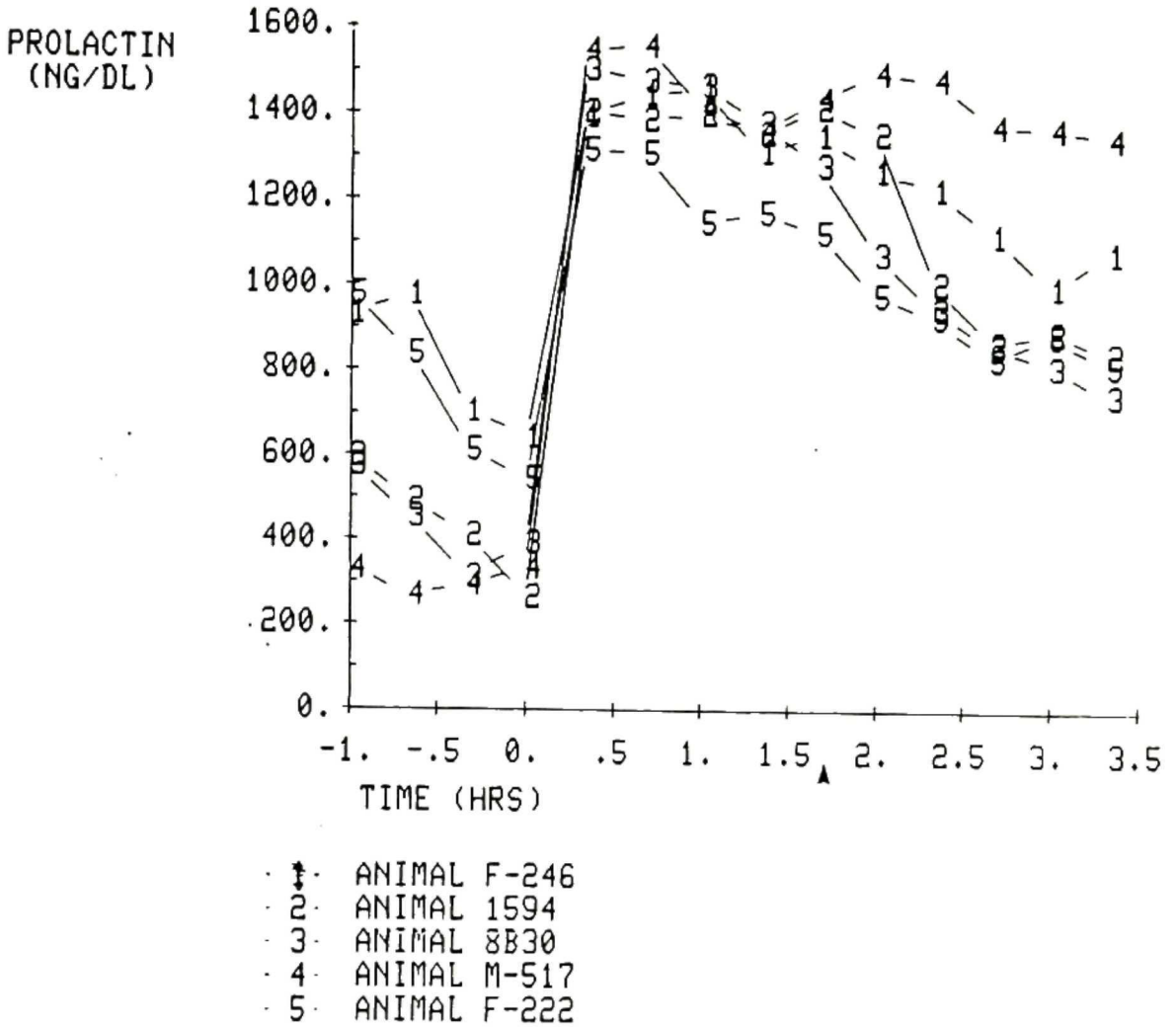

Figure 44. Effect of naloxone (0.03 mg/kg) on plasma PRL levels after pretreatment with morphine. Morphine (1.0 mg/kg) was administered at time zero hours. Naloxone was administered at 100 minutes as indicated by the arrowhead. Shown are the plasma concentrations of the hormone for five monkeys at the times indicated.

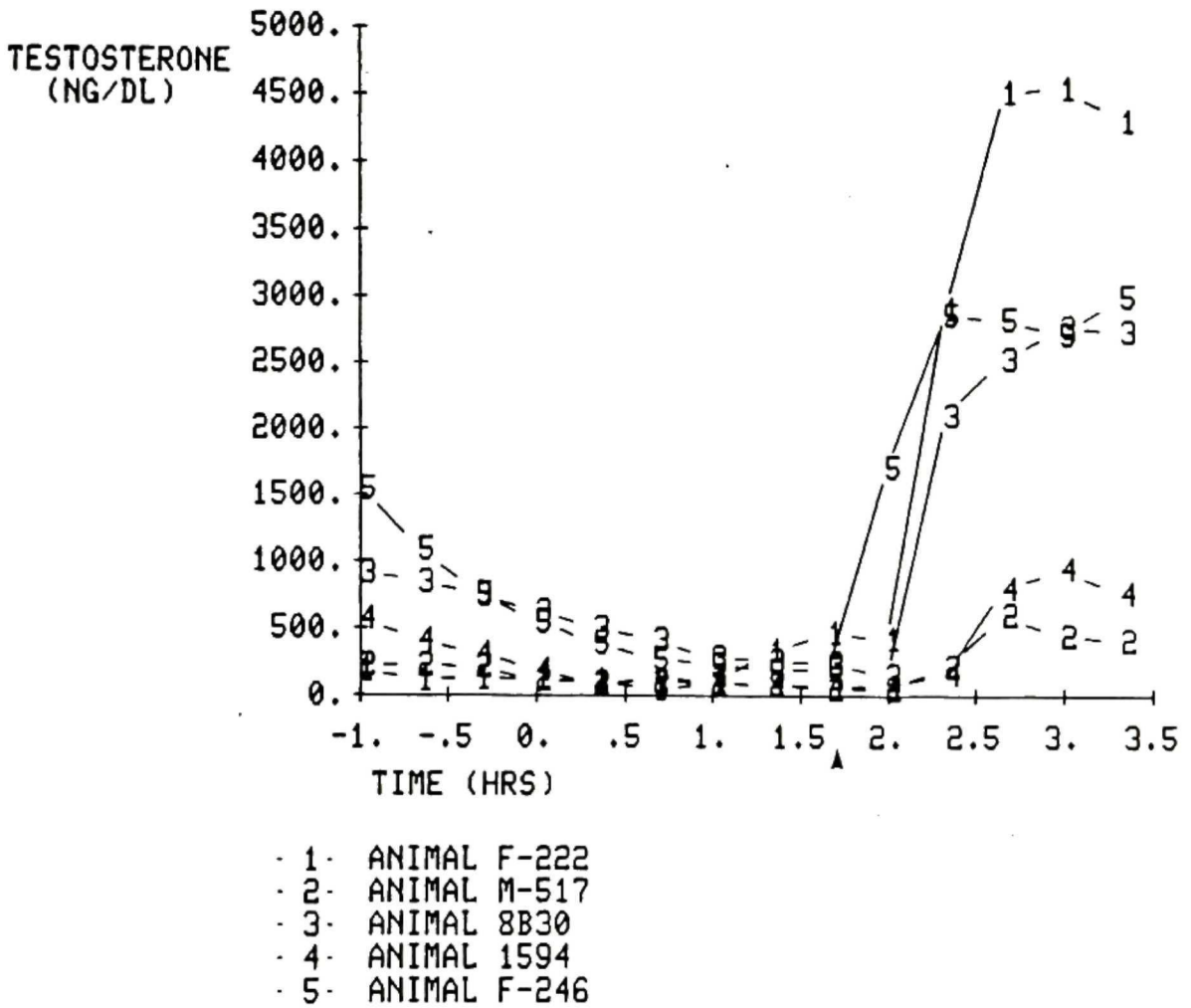

Figure 45. Effect of naloxone (1.0 mg/kg) on plasma testosterone levels after pretreatment with DADLE. DADLE (20 µg/kg) was administered at time zero hours. Naloxone was administered at 100 minutes as indicated by the arrowhead. Shown are the plasma concentrations of the hormone for five monkeys at the times indicated.

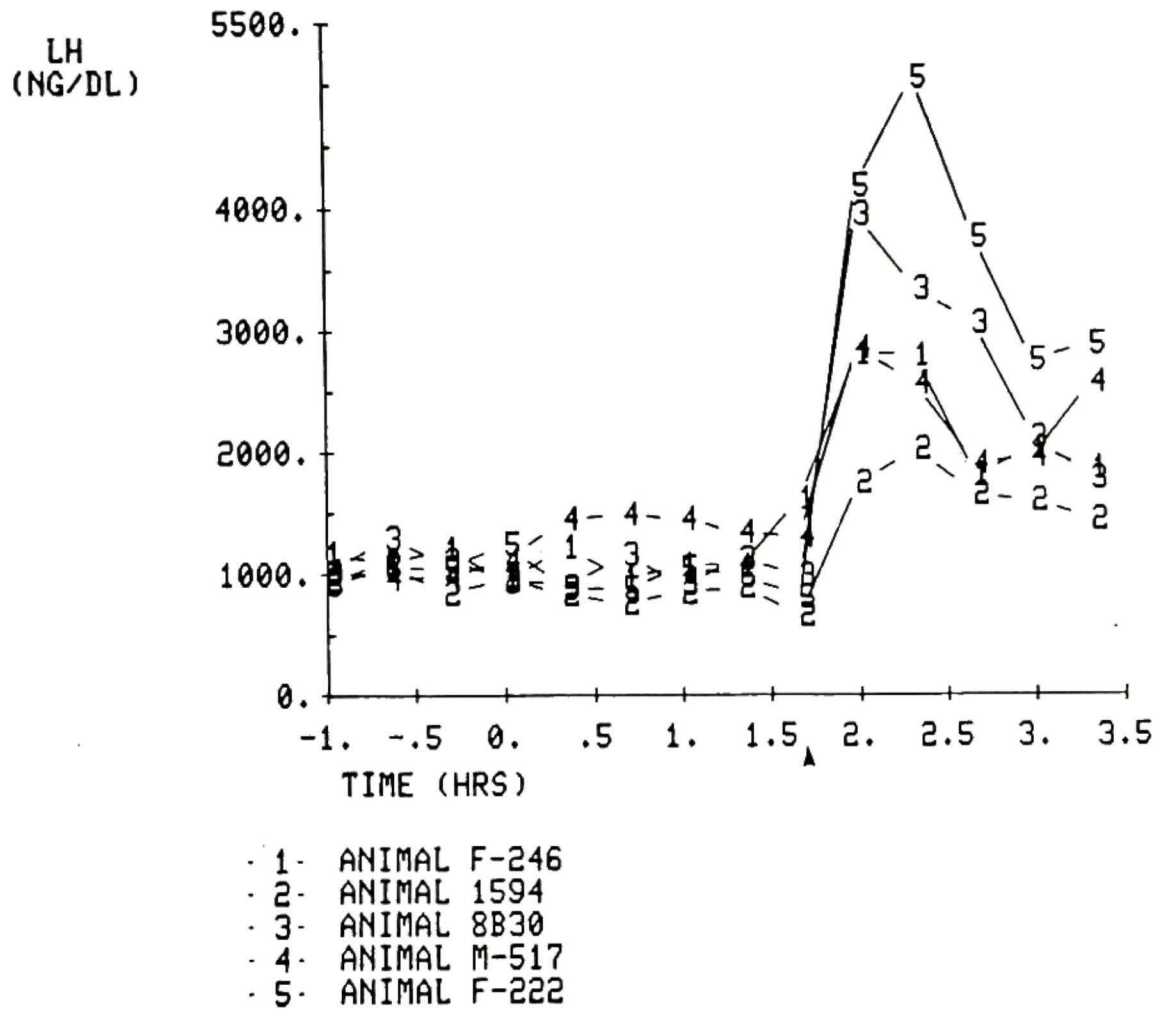

Figure 46. Effect of naloxone (1.0 mg/kg) on plasma LH levels after pretreatment with DADLE. DADLE (20 µg/kg) was administered at time zero hours. Naloxone was administered at 100 minutes as indicated by the arrowhead. Shown are the plasma concentrations of the hormone for five monkeys at the times indicated.

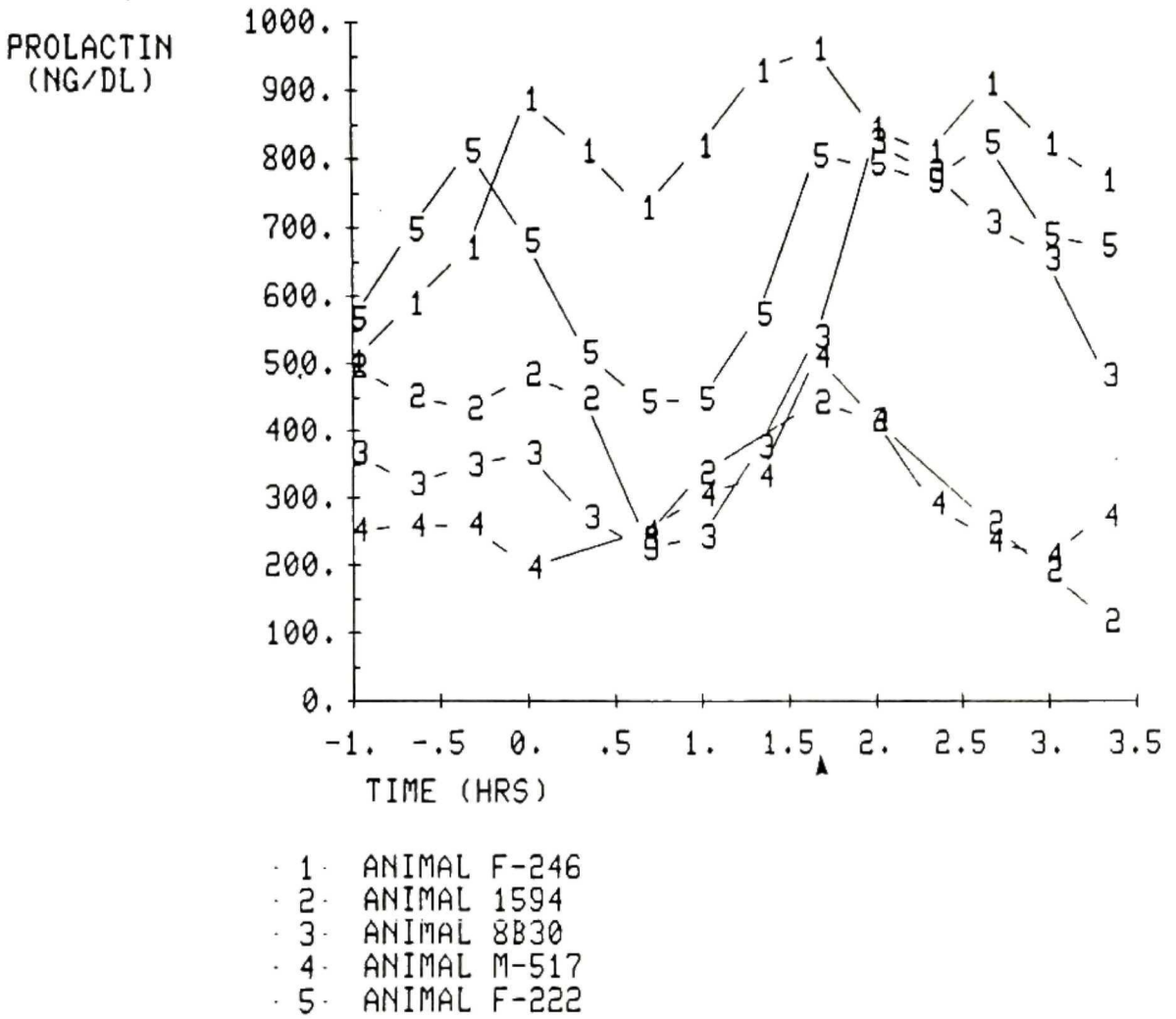

Figure 47. Effect of naloxone (1.0 mg/kg) on plasma PRL levels after pretreatment with DADLE. DADLE (20 µg/kg) was administered at time zero hours. Naloxone was administered at 100 minutes as indicated by the arrowhead. Shown are the plasma concentrations of the hormone for five monkeys at the times indicated.

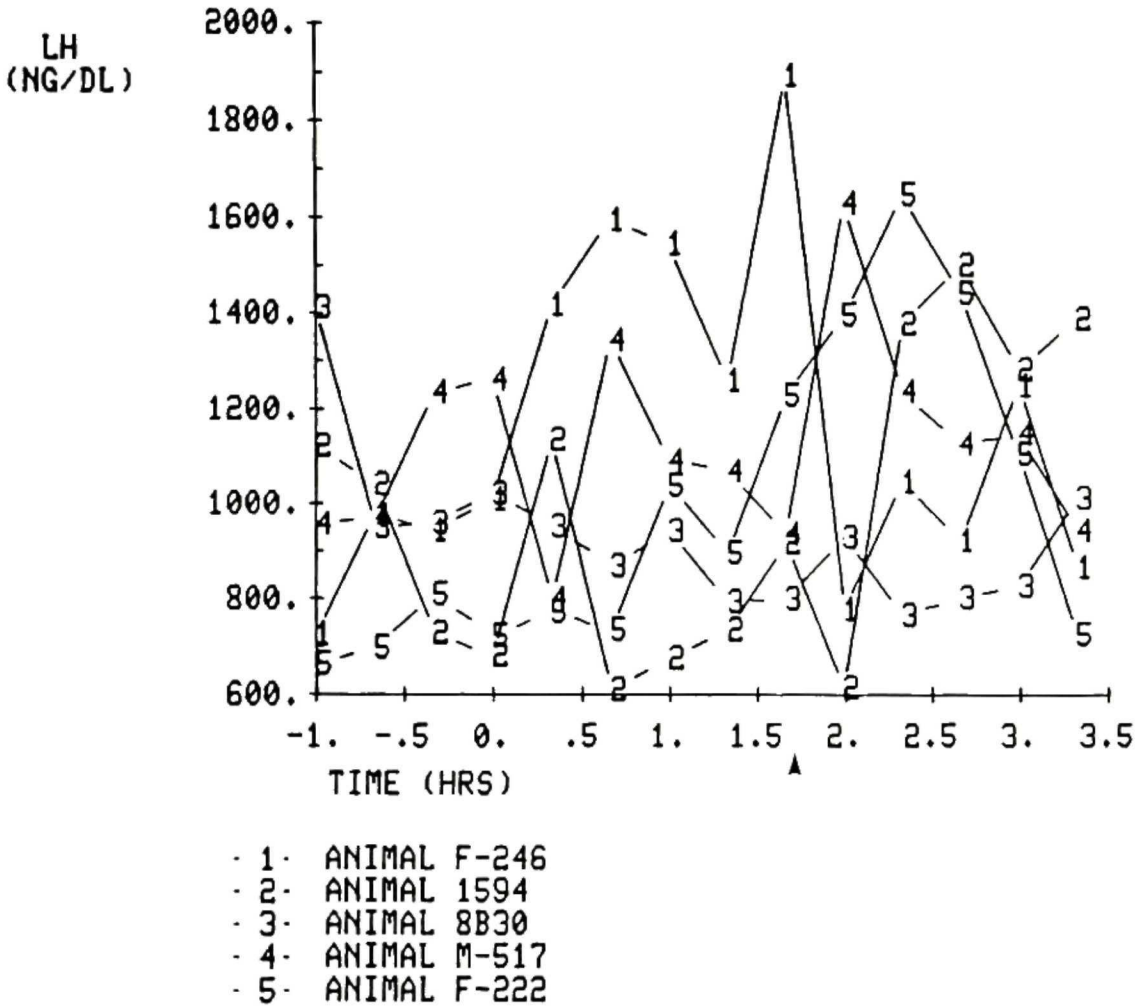

Figure 49. Effect of naloxone (0.03 mg/kg) on plasma LH levels after pretreatment with DADLE. DADLE (20 µg/kg) was administered at time zero hours. Naloxone was administered at 100 minutes as indicated by the arrowhead. Shown are the plasma concentrations of the hormone for five monkeys at the times indicated.

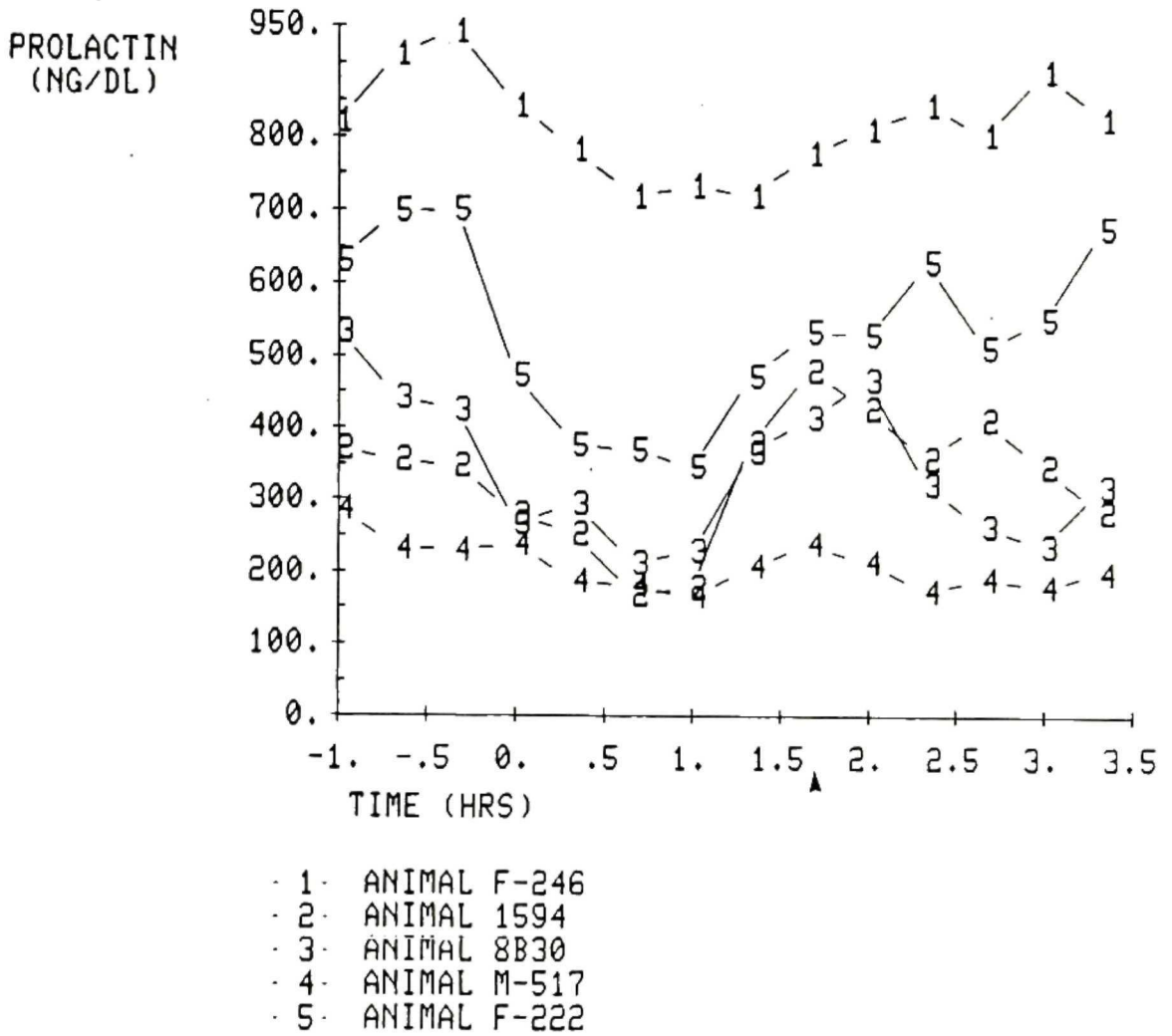

Figure 50. Effect of naloxone (0.03 mg/kg) on plasma PRL levels after pretreatment with DADLE. DADLE (20 µg/kg) was administered at time zero hours. Naloxone was administered at 100 minutes as indicated by the arrowhead. Shown are the plasma concentrations of the hormone for five monkeys at the times indicated.

b) Episodic Hormone Release - Normal Patterns. In order to determine whether the opioids are involved in the neuroendocrine mechanisms responsible for modulating the frequency and amplitude of sex hormone release (pulses), morphine sulfate or DADLE was administered intravenously and blood was drawn through the catheters every 20 minutes for three hours. First, the normal episodic release patterns of testosterone, LH, and PRL were determined. Then, as described in Chapter 3, Methods, the hormone level time courses were then determined and opioid-treated groups compared to the appropriate vehicle-treated group. The hormone level time courses for groups of vehicle-treated animals were analyzed to determine dominant frequency and amplitude of episodic fluctuations. Grand averages of the dominant frequency and amplitude were found, taking into account all vehicle-treated groups. The overall average normal values found are shown in Table 1.

Table 1. Normal Episodic Release Characteristics

	Dominant Frequency (cycles/hr)	Dominant Period (minutes)	Amplitude (ng/dl)
Testosterone	0.78	77	91
LH	0.78	77	295
Prolactin	0.81	74	62

In each case the dominant frequency was converted to a period between pulses (in minutes) by dividing 60 by the dominant frequency. Testosterone is seen to exhibit a dominant episodic variation at a period of about 77 minutes and an average (root mean square, or rms) amplitude of 91 ng/dl. LH episodic variations are seen to occur with the same

dominant frequency as testosterone and to have an average amplitude of about 295 ng/dl. Normal prolactin episodic variations appear to exhibit a dominant frequency which is only slightly higher. The amplitude of PRL episodes is only about 15% of the average normal PRL level, however, and may not be large enough to indicate the presence of conspicuous "pulses". In contrast, the sizes of the amplitudes associated with the testosterone and LH episodes, when considered as fractions of typical average levels, strongly suggest that these dominant frequencies correspond to average frequencies of pulsatile release.

Episodic Hormone Release - Effects of Morphine. Table 2 summarizes the results of analyzing episodic release patterns after morphine administration and comparing to vehicle patterns. For both the fluctuation amplitude and the dominant frequency several relevant statistics are as follows: the means of the treated and control groups, the signficance level (0.1 or below only) of the difference between the values for the treated and control groups, and the difference itself when significant. (Where differences were not statistically significant at $p < 0.1$ or below, the second and fifth columns contain "-", and difference values are omitted.)

Morphine had no effect on testosterone episodic amplitude; a lowering effect on frequency is suggested by the result for the lowest dose level but was not consistently observed at the higher dose levels. Morphine caused no significant change in LH frequency but produced a definite reduction in LH episodic amplitude. The effect on prolactin episodes was to increase their amplitude in a dose-dependent fashion (67 ng/dl to 143 ng/dl at 0.5 mg/kg dose; 67 ng/dl to 171 ng/dl at 1.0 mg/kg dose) while not significantly affecting their frequency.

Table 2. Morphine Effects on Episodic Hormone Release

Hormone/ Drug Dose	Fluctuation Amplitude (ng/dl rms)			Dominant Frequency (cycles/hr)		
	Sig. Level	Treated Mean	Control Mean	Sig. Level	Treated Mean	Control Mean
		Change			Change	
Testosterone						
0.25 mg/kg	-	55.21	52.15	0.025	.7102	.9104
						-.2002
0.5 mg/kg	-	78.88	52.15	-	.8875	.9104
1.0 mg/kg	-	48.71	52.15	0.1	.7162	.9104
						-.1942
LH						
0.25 mg/kg	0.05	187.4	501.7	-	.7764	.7892
		-314.3				
0.5 mg/kg	-	252.3	501.7	-	.8134	.7892
1.0 mg/kg	0.1	191.8	501.7	-	.8812	.7892
		-309.9				
Prolactin						
0.25 mg/kg	0.1	128.8	67.3	0.1	.8325	.7315
		61.54				.1009
0.5 mg/kg	0.1	143.1	67.3	-	.7577	.7315
		75.8				
1.0 mg/kg	0.05	170.8	67.3	0.1	.9141	.7315
		103.5				.1826

Episodic Hormone Release - Effects of DADLE. The results of the analysis are shown in Table 3. A consistent pattern of decrease in testosterone episodic amplitude due to DADLE was seen, although only at 5 µg/kg dose was the decrease (134 ng/dl to 28 ng/dl, or 79%) significant at $p < 0.05$. A smaller decrease in LH episodic amplitude is suggested by the data, but it is not definitive (at 5 µg/kg dose $p < 0.1$ only). At the 10 µg/kg dose, while there was no amplitude effect, LH episodic frequency appeared to increase ($p < 0.05$). A parallel increase in testosterone dominant frequency may be noted from the table, although it was not significant at $p < 0.1$.

On the other hand, no frequency increase is suggested at the highest dose level (20 µg/kg), so it is questionable whether DADLE has any real effect on episodic frequency. DADLE did not affect PRL episodic frequency appreciably. It appears to be responsible for depressing PRL episodic amplitude by about 25% at the 10 µg/kg dose ($p < 0.025$). Apparent decreases and increases at the other two dose levels were significant only at $0.05 < p < 0.1$.

Episodic Hormone Release - Effects of ß-endorphin. Table 4 gives the results of the analysis. At the 10 µg/kg dose, ß-end appeared to increase episodic amplitudes of all three hormones (testosterone, +177%; LH, +153%; PRL, +122%), all of which were significant at $p < 0.05$ or below. At the 20 µg/kg dose, only a comparatively small 38% increase in PRL episodic amplitude was seen, and no significant effects on testosterone or LH amplitude were seen. There did appear to be a significant lowering of testosterone episodic frequency (0.87 to 0.66 cycles/hr) at the higher dose, but no appreciable effects on episodic frequencies of the other hormones.

Table 3. DADLE Effects on Episodic Hormone Release

Hormone/ Drug Dose	Fluctuation Amplitude (ng/dl rms)			Dominant Frequency (cycles/hr)		
	Sig. Level	Treated Mean	Control Mean	Sig. Level	Treated Mean	Control Mean
		Change			Change	
Testosterone						
5 µg/kg	-	75.65	134.7	-	.8335	.7114
10 µg/kg	0.05	27.62	134.7	-	.8718	.7114
		-107.08				
20 µg/kg	-	61.88	84.25	-	.7782	.7075
LH						
5 µg/kg	0.1	105.3	197.4	-	.8398	.8163
		-92.09				
10 µg/kg	-	197.5	197.4	0.05	.9881	.8163
					.1718	
20 µg/kg	-	172.4	206.3	-	.8088	.8986
Prolactin						
5 µg/kg	0.1	54.56	82.78	-	.8208	.8278
		-28.22				
10 µg/kg	0.025	62.22	82.78	-	.7321	.8278
		-20.56				
20 µg/kg	0.1	91.18	71.94	-	.6743	.7764
		19.24				

Table 4. β-Endorphin Effects on Episodic Hormone Release

Hormone/ Drug Dose	Fluctuation Amplitude (ng/dl rms)			Dominant Frequency (cycles/hr)		
	Sig. Level	Treated Mean	Control Mean	Sig. Level	Treated Mean	Control Mean
		Change			Change	
Testosterone						
10 µg/kg	0.05	183.8	66.47	-	.8064	.8655
		117.4				
20 µg/kg	-	61.78	66.47	.025	.6569	.8655
					-.2086	
LH						
10 µg/kg	0.01	364.5	155.0	-	.8816	.7872
		209.6				
20 µg/kg	-	141.9	155.0	-	.8591	.7872
Prolactin						
10 µg/kg	0.025	109.0	48.74	-	.7669	.8077
		60.27				
20 µg/kg	0.05	66.91	48.74	-	.7710	.8077
		18.16				

Episodic Hormone Release - Effects of Naloxone. Results of the analysis are presented in Table 5. No naloxone effects on episodic frequency were observed at any dose levels for any of the hormones. Naloxone consistently increased testosterone episodic amplitude by about 100% or more. The effects seen on LH episodic amplitude were similar. The LH amplitude was increased by averages of 88% and 67% at the two lower doses and was sharply increased (173%) at the highest dose. PRL episodic amplitude was not convincingly affected at the lower doses, but registered nearly a 400% increase at the 2 mg/kg naloxone dose.

Table 5. Naloxone Effects on Episodic Hormone Release

Hormone/ Drug Dose	Fluctuation Amplitude (ng/dl rms)			Dominant Frequency (cycles/hr)		
	Sig. Level	Treated Mean	Control Mean Change	Sig. Level	Treated Mean	Control Mean Change
Testosterone						
0.5 mg/kg	0.01	336.6	93.26 243.3	-	.8860	.7721
1.0 mg/kg	0.1	173.0	93.26 80.28	-	.7595	.7721
2.0 mg/kg	0.1	230.7	93.26 137.4	-	.7515	.7721
LH						
0.5 mg/kg	0.01	346.0	183.4 162.6	-	.7446	.7738
1.0 mg/kg	.001	306.8	183.4 123.3	-	.8402	.7738
2.0 mg/kg	.025	501.2	183.4 317.8	-	.8181	.7738
Prolactin						
0.5 mg/kg	0.1	36.96	49.51 -12.55	-	.8003	.8532
1.0 mg/kg	-	41.57	49.51	-	.8019	.8532
2.0 mg/kg	0.001	246.0	49.5 196.5	-	.7887	.8532

CHAPTER 5

DISCUSSION

Our observations that administration of a specific opiate antagonist, naloxone, leads to a marked increase in LH levels and decrease in PRL levels in rhesus monkeys suggests that the role of the EOP in primate reproductive neuroendocrinology is a tonic inhibition of LH and a tonic stimulation of PRL. The EOP may be involved in reproductive hormone feedback regulation in the hypothalamus. They may mediate the negative feedback of testosterone and estradiol on the secretion of GnRH and subsequent secretion of LH. Support for such a role is the observation that naloxone has been found to block testosterone's negative feedback control of the hypothalamic-pituitary-gonadal axis in rodents (Cicero et al., 1979). Additionally, feedback influence of estradiol on hypothalamic opioid activity has been suggested by Ropert et al., (1981). The exact role of PRL in male primate reproductive function is yet to be discerned. If PRL possesses gonadotropic activity in man as in rodents, the marked increases in PRL levels and decreases in LH levels caused by morphine should counteract each other in the regulation of testosterone release. Although our results do not illustrate a total counteraction, a partial counteractive effect cannot be disregarded. The present studies do not provide enough information to assess the possibility of a partial effect. These endogenous system must be considered in the interpretation of the present studies. All the drugs studied, whether agonists or antagonists, exert their pharmacological actions as the consequence of the perturbation of these endogenous systems.

Drug Effects on Testosterone, LH, and PRL -

Opioid Agonists and Antagonists

The present studies clearly demonstrate that the opioid drugs interact with the neuroendocrine system of the adult male rhesus monkey. A summary of the observed effects is shown in Table 6.

Morphine Sulfate. To depress LH and testosterone levels, a 1.0 mg/kg dose of morphine sulfate was used. Below this dose, no effects on these hormones were observed. The depression in testosterone levels occurred 80 - 100 minutes after morphine administration while decreases in LH levels were observed within 40 minutes after drug treatment. This difference in the time of effect (40 - 60 minutes) corresponds to the lag period observed between LH change and the subsequent testosterone change. The testosterone changes being preceded by LH changes indicates an effect of morphine at the level of LH control. Similar depressions in LH and testosterone were observed in rodents; however, doses of morphine were three- to 16-fold higher (Bruni et al., 1977).

The lack of a direct effect on testosterone production is also supported by the in vivo and in vitro studies on direct gonadal effects. In these studies, morphine did not alter testosterone production in HCG treated monkeys, nor in isolated mouse Leydig cells. These results suggest that morphine's effect is exerted at the hypothalamic-pituitary level.

It is well established that GnRH elicits its effects at the level of the pituitary. Stimulation of LH release by GnRH is immediate but not dose-related in primates. Previous studies in our laboratory have shown that dose levels of GnRH below 50 µg produce minimal responses, whereas those greater than 50 µg produce maximal LH release. The exact

Table 6. Effects of Opioid Agonists and Antagonist on Hormone Blood Levels

Drug/ Dose/ Hormone	Max. Change Due to Drug (% of Basal)	Time of Max. Effect (min. post admin.)	Significance Level
MORPHINE			
0.25 mg/kg			
Testosterone	-	-	-
LH	-	-	-
Prolactin	+49	20 - 40	0.005
0.5 mg/kg			
Testosterone	-	-	-
LH	-	-	-
Prolactin	+48	20 - 60	0.005
1.0 mg/kg			
Testosterone	-200	40 - 100	0.025
LH	-63	0 - 60	0.025
Prolactin	+637	80 - 100	0.025
DADLE			
5 µg/kg			
Testosterone	-	-	-
LH	-19	60 - 120	0.05
Prolactin	-	-	-
10 µg/kg			
Testosterone	-34	60 - 120	0.01
LH	-39	20 - 80	0.025
Prolactin	-46	120 - 180	0.025
20 µg/kg			
Testosterone	-	-	-
LH	-	-	-
Prolactin	-	-	-

Table 6 (Cont.) Effects of Opioid Agonists and Antagonist on Hormone Blood Levels

Drug/ Dose/ Hormone	Max. Change Due to Drug (% of Basal)	Time of Max. Effect (min. post admin.)	Significance Level
DADLE			
10 µg/kg			
Testosterone	-	-	-
LH	-	-	-
Prolactin	+74	120 - 140	0.001
20 µg/kg			
Testosterone	-	-	-
LH	-	-	-
Prolactin	+30	140 - 180	0.05
NALOXONE			
0.5 mg/kg			
Testosterone	+480	60 - 100	0.05
LH	+149	40	0.005
Prolactin	-	-	-
1.0 mg/kg			
Testosterone	+400	60 - 100	0.005
LH	+139	20	0.001
Prolactin	-	-	-
2.0 mg/kg			
Testosterone	+540	60 - 100	0.005
LH	+237	40	0.005
Prolactin	-43	120 - 180	0.025

dose level above 50 µg will to differ for each animal. The dose level of 100 µg of HCG was chosen since it had been shown to produce a maximal effect in a majority of monkeys.

Reversal of morphine's depressant effect on LH by GnRH suggests that this opioid acts at the level of the hypothalamus. The observation that the GnRH release of LH with morphine pretreatment does not differ from that seen with vehicle pretreatment further suggests a site of action above the pituitary. This observation in primates of a lack of pituitary effect supports like findings in rodents (Meites et al., 1979). Thus, it is likely that in man, as in rodents and monkeys, that narcotics inhibit GnRH release through a hypothalamic mechanism. In the competition study between morphine and naloxone (1.0 mg/kg), naloxone's complete reversal of the morphine effect on LH and subsequently on testosterone levels indicates that morphine is acting on these sex hormones through opiate receptors. Lowering the dose of naloxone to 0.03 mg/kg resulted in only a partial reversal of the morphine effect on LH, and no reversal of morphine's effect on testosterone. Simultaneous administration of morphine (2.0 and 10.0 mg/kg) and naloxone (0.2 mg/kg) did not alter LH levels in rats (Meites et al., 1979), thus indicating a morphine-naloxone competition similar to that observed in our studies.

In contrast to LH and testosterone, morphine sulfate administration caused increases in plasma prolactin levels at all doses of morphine studied. The PRL increases appeared to be dose-dependent. Administration of 0.25, 0.5, and 1.0 mg/kg morphine sulfate resulted in change differences in PRL of 450, 480, and 1150 percent, respectively. These results are in agreement with studies in rodents (Meites et al., 1979) and humans (Kley et al., 1977) on PRL levels after narcotic treatment. As seen with the effects on LH levels, administration of morphine to rodents

in concentrations of 10- to 16-fold greater than in the present studies was necessary to produce responses of similar magnitude. Increases in PRL levels also appears to be mediated through opiate receptors. Administration of 1.0 mg/kg naloxone reversed the increase in PRL induced by morphine treatment; low dose naloxone (0.03 mg/kg) did not cause a reversal. This lack of reversal, in addition to the low dose levels of morphine increasing PRL levels, suggests that PRL may be more sensitive than LH to modulation by morphine.

[D-Ala^2, D-Leu^5]-Enkephalin. Administration of the Leu-enk analogue DADLE (10 µg/kg) decreased LH and testosterone plasma levels. The depression in monkey LH plasma levels produced by DADLE is consistent with decreases observed in normal human males administered DAMME (Stubbs et al., 1978). Initial decreases of testosterone were observed 60 - 80 minutes following DADLE administration. The decreases in LH preceded those in testosterone by approximately 40 minutes. As described with morphine sulfate, the time lag between LH and testosterone indicates a pituitary-hypothalamic site of action for DADLE. This is supported by the in vivo study using HCG pretreated monkeys. In this study, DADLE was unable to reduce testosterone levels. Further information regarding the level of action of DADLE was obtained from the GnRH reversal and naloxone competition studies. Administration of GnRH following pretreatment with DADLE resulted in a release of LH equal in magnitude to that seen without opioid pretreatment. This result indicates that DADLE is not acting at the pituitary to depress LH levels, but at a higher site in the hypothalamus. Unlike the effect of morphine sulfate, DADLE did not increase plasma PRL levels. Treatment with a Met-enk analogue, DAMME, produced marked increases in PRL levels (Stubbs, et al., 1978) in humans. This variation

in PRL effect may represent a distinction between the action of Met- and Leu-enk analogues. Administration of Met- and Leu-enkephalin had no effect on human PRL levels (Golstein, et al., 1981), but the question of their rapid proteolysis in blood must be addressed.

DADLE appears to elicit its effects through interaction with the opiate receptors. Administration of naloxone (1.0 mg/kg) completely reversed the effects of DADLE on testosterone and LH. A similar reversal by naloxone was observed after pretreatment with DAMME in humans (Grossman et al., 1981). This competition was not observed at the lower dose of naloxone (0.03 mg/kg). The absence of a competitive effect of naloxone at 0.03 mg/kg may be attributed to a selective antagonism of μ- over δ-receptors. This possibility is discussed in the following section regarding receptors.

β-Endorphin. No alteration in the plasma levels of LH or testosterone was observed after administration of 10 or 20 μg/kg of β-end. This failure to respond may be the result of too low of doses of this expensive peptide. The 35% increase (change difference) in plasma PRL levels observed with low doses of β-end (10 and 20 μg/kg) suggest that prolactin mediation by opioids is more sensitive than that of LH or testosterone.

In a limited number of samples provided by Holaday et al., administration of 700 μg/kg of β-endorphin produced a 50% decrease in LH plasma levels in adult male rhesus monkeys 2 hours after administration. This same dose elicited an increase in PRL plasma levels 5 minutes after administration. The rise in PRL lasted for at least 60 minutes with a maximum increase of 7-fold over pretreatment. Intravenous administration of 2.5 mg (approx. 40 μg/kg) human β-end to human males resulted in a 28% decrease in LH levels and a 182% increase in PRL levels (Reid, et al.

1981). In male rats, intraventricular administration of 0.5, 1.0, and 10.0 µg (approx. 2.0, 4.0, and 40.0 µg/kg, respectively) human β-end elicited increases in PRL levels of 800%, 1250%, and 700%, respectively (Dupont et al., 1980; Van Vugt et al., 1981). The variations in magnitude of these responses may represent differences in route of administration or species specificity. The primate results support the suggestion that the control of LH release is not as sensitive to β-end as is that of prolactin.

Naloxone. To evaluate the effects of endogenous effects of opioids on sex hormone levels, the opioid antagonist naloxone was used. Administration of naloxone increased plasma LH and testosterone levels. The increase in plasma LH levels preceded that of testosterone by 40 to 60 minutes indicating that naloxone alters testosterone levels through LH. Statistically significant increases in testosterone were observed at all dose levels. Similar increases in LH were observed in rats and human males. Increases of 200 - 300% were seen in humans administered 10 to 20 mg (≈ 0.2 mg/kg) naloxone, and in rats given 0.2 to 5.0 mg/kg naloxone (Grossman et al., 1981; Lightman et al., 1981; Meites et al., 1981). Administration of 0.25 mg/kg naloxone to our cathetered monkeys (data not presented here) produced LH increases of similar magnitude.

The interaction of naloxone with endogenous opioid receptors to produce increases in plasma LH levels supports the postulate that the EOP may be involved in the neuroendocrine mechanism responsible for the control of GnRH, and subsequently LH release. Naloxone apparently competes with the EOP and thereby alters the tonic inhibitory control exerted by the EOP on GnRH. It is interesting that the increases in LH elicited by GnRH (100 µg) and naloxone (2.0 mg/kg) are equal in time of onset

and magnitude (approximately 8-fold from pretreatment levels). This may indicate that this 2.0 mg/kg dose of naloxone causes a release of GnRH which in turn produces a maximal LH release from the pituitary. The effect of naloxone on PRL plasma levels provided further evidence that the EOP influence basal secretion of sex hormones in the primate. At all dose levels studied, naloxone depressed PRL levels. As postulated for LH, the EOP may exert a tonic stimulatory control over PRL release. It is the disruption of this stimulatory control through which naloxone may be eliciting its effects.

Dose-Response Relationships. While not explained in the literature, many reproductive endocrine studies with opioids have observed a lack of strict dose-response relationships (Cushman 1973; Bruni et al., 1977; Van Vugt et al., 1977). This observation may be attributed to a number of factors. One factor is the status of the endocrine system under consideration at time of drug administration. The reproductive system is not static, but constantly fluctuating due to internal factors (e.g., hormone feedback, illness) and psychogenic factors (e.g., stress, sensory stimuli) (Horowitz and Goble, 1979). With these variations in the system, it is difficult to determine the exact endocrine status at time of drug administration.

In order to minimize psychogenic effects induced by venipuncture or pharmacological effects induced by anesthetic or tranquilizing drugs, an indwelling jugular catheter system was instituted. Use of this system reduced neuroendocrine disturbance, while still allowing for data collection. In our previous studies, we have observed that testosterone and LH levels in chronically catheterized monkeys are lower than levels in monkeys stressed by venipuncture (a difference of approximately 3-fold).

We have also observed that the magnitude of inhibitory drug effects on these hormones is somewhat lower than those observed in stressed monkeys, but the findings may be relevant to the drug effects under physiological conditions. This difference may be due to the higher pretreatment hormone levels observed in the stressed monkeys.

Comparisons between pretreatment hormone levels and the magnitude of drug response have revealed additional information on drug-response relationships. In our studies, we observed that if a drug with inhibitory effects is administered to a monkey at a time of low hormone levels (i.e. during an episodic trough or due to psychogenic stimuli), the magnitude of the depressent effect is lessened. Conversely, stimulatory effects of drugs are reduced if the drug is administered during a period of high hormone levels. (High and low hormone levels being defined from each monkey's mean hormonal range). Dose-response relationships of the drugs used in the present studies must be considered in light of the above observations.

Dose-response relationships between morphine sulfate and plasma levels of testosterone or LH are difficult to assess, since at the two lowest of the three doses administered, no effects were observed. The minimum dose of morphine necessary to produce a significant decrease in LH or testosterone levels is probably between 0.5 and 1.0 mg/kg. No dose levels larger than 1.0 mg/kg were used due to the possibility of respiratory depression. Mean plasma testosterone levels in methadone-treated men showed no direct methadone dose-testosterone level relationship (Cushman, 1973). Prolactin levels responded in a dose-related manner. Morphine, at doses of 0.25 and 0.5 mg/kg, produced approximately equal increases in PRL levels; 1.0 mg/kg dose produced an increase in PRL approximately 12-fold higher than that obtained with the lower doses.

DADLE administration elicited dose-response relationships in testosterone, LH, and PRL at doses of 5.0 and 10.0 µg/kg. DADLE caused a slight decrease in LH plasma levels at the 5 µg/kg dose. Decreases in LH levels doubled with increasing dose. At this dose level no effect was observed in testosterone levels. The lack of testosterone effect may be attributed to the relatively small decrease in LH levels. This decrease may be too small or too short-lived to trigger a testosterone effect. The incremental changes in LH necessary to evoke changes in testosterone release and those of GnRH necessary to produce changes in LH release are as of yet unknown and may depend on the sensitivity of the pituitary and the gonads at time of drug administration. DADLE, at the 20 µg/kg dose, caused decreases in LH and testosterone levels when compared to pretreatment values, but not when compared to the vehicles (see Figure 8). This lack of statistical significance was due to an unexpected depression in vehicle hormone levels which occured at the approximate time of expected DADLE effect. At 20 µg/kg, DADLE produced no hormonal effects. Examination of the data shows that the levels of LH were low from the start of the series, thus, DADLE could cause no further decrease. Due to abnormally high pretreatment testosterone values, the vehicle series for 20 µg/kg DADLE decreased significantly at the time of expected drug effect, thus eliminating significant differences in the changes from pretreatment between the treated and vehicle groups.

Administration of β-end at 10 and 20 µg/kg dose levels did not effect testosterone nor LH plasma levels. As presented in the Results, substantially higher dose levels (at least 700 µg/kg) are necessary to produce a response. Both low doses of β-end significantly increased plasma PRL levels by approximately the same factor. In male rats, admin-

istration of one or 10 µg β-end produced significant increases in serum PRL levels, withthe higher dose being less effective than the lower dose (Van Vugt et al., 1981).

Naloxone produced consistant increases in LH and testosterone levels at all dose levels (0.5, 1.0, and 2.0 mg/kg). Maximal change in these hormone levels was larger at 0.5 mg/kg than at 1.0 mg/kg. This finding probably occurred because the hormone pretreatment values, at time of 0.5 mg/kg naloxone administration, were depressed. Prolactin levels were depressed in a dose-related manner, similar to that observed in male rats (Meites et al., 1979).

Relative Agonist Potencies. Comparisons of the concentrations of drugs in terms of grams/kg and moles/kg administered are shown in the following table.

Table 7. Drug Concentrations

Morphine Sulfate	1.00 mg/kg	1.50 nmoles/kg
	0.50	0.75
	0.25	0.38
DADLE	20.0 µg/kg	35.1 nmoles/kg
	10.0	17.5
	5.0	8.8
β-Endorphin	700.0 µg/kg	120.0 nmoles/kg
	20.0	5.8
	10.0	2.9
Naloxone - HCl	2.00 mg/kg	5.50 nmoles/kg
	1.00	2.80
	0.50	1.40
	0.03	0.08

To evaluate the relative potencies of any drugs, several factors must be considered. These include tissue penetration, distribution, and plasma half-life of the drugs. In the present studies, opioid agonist and naloxone penetration of the central nervous system is not dependent on blood-brain barrier (B-BB) permeability. Drugs which act at the pituitary or hypothalamus may enter via the paraventricular area where the B-BB is incomplete. For example, the Met-enk analogue DAMME does not penetrate the B-BB; however, this opioid has marked hypothalamic effects which produce changes in plasma LH and PRL levels (Grossman et al., 1981). Half-life ($t_{1/2}$) data for the EOP is not well known. Where information is reported, usually a single animal model is used. Half-lives change with animal model, and thus must not be considered as absolute for another animal. After intravenous administration of morphine sulfate to humans, a plasma $t_{1/2}$ of two to three hours has been determined by RIA, but the RIA technique does not distinguish between the free and conjugated forms of the drug (Foltz et al., 1980). Naloxone has a plasma $t_{1/2}$ of about one hour in humans (Jaffe and Martin, 1980). Synthetic human β-end disappears from human plasma in a triple exponential curve, the components of which yield half-lives of approximately 4, 13, and 46 minutes, respectively (Reid et al., 1981). The $t_{1/2}$ of Leu-enk in human plasma is less than one minute. The exact plasma $t_{1/2}$ of DADLE has not been determined, but in tissue preparations DADLE is inactivated within five minutes (Chrusciet, 1980).

When compared in terms of moles of drug administered per kilogram of monkey body weight, it was necessary to administer morphine sulfate in concentrations up to 100 times that of DADLE to elicit effects on LH. Similarly, a concentration of morphine sulfate 10 times that of β-end was needed to achieve equal effects on plasma LH levels. Equal increases in

PRL levels observed after morphine or β-end administration were produced with β-end concentrations one thirtieth that of morphine. This result agrees with the findings of Tseng et al., (1976), in which it was shown that β-end was 18 - 33 times more potent than morphine in regards to its analgesic effect in mice.

The disparity in dose and effect between the EOP and morphine indicates a higher potency for the EOP. Specifically, GnRH release appears to be sensitive to perturbation by DADLE > β-endorphin > morphine sulfate. Modulation of prolactin levels by the opioids studied is apparently most sensitive to the effects of β-end followed by morphine sulfate. The lack of any effect of DADLE on PRL levels further suggests that the EOP are acting through different receptor types.

Opiate Receptor Types. As cited previously, the opioids used in these investigations are known to interact with specific sub-types of opiate receptors. Morphine sulfate is a μ-receptor agonist. DADLE elicits its actions primarily through the δ-opiate receptor, while β-end exerts its pharmacologic activities by interacting with both the μ- and δ-receptors. Naloxone antagonizes the opioids at the μ- and δ-receptors, with a higher affinity for the μ-receptor (Kosterlitz, 1980).

That LH levels are affected by morphine and DADLE indicate that GnRH modulation by opiates involves both μ- and δ-receptors. Further support for multiple receptor control of GnRH is obtained from the competition studies involving the opioids and naloxone. High dose naloxone (1.0 mg/kg) antagonizes both μ-and δ-receptors. Administration of this dose of naloxone after morphine or DADLE treatment completely reversed the effects of the opioids. Alternatively, low dose naloxone (0.03 mg/kg) specifically antagonizes the μ-receptor subtype. Administration of low

dose naloxone after morphine treatment did not reverse the morphine effect on sex hormone levels. DADLE's effect on LH and testosterone levels were incompletely reversed by low dose naloxone.

The relative higher potency of DADLE over morphine in regards to LH and testosterone effects suggests a greater δ-receptor selectivity by opioids in modulation of GnRH release. In contrast, the ability of low dose naloxone to partially reverse DADLE's effects on LH and testosterone levels, while not reversing those of morphine, indicates a greater selectivity for μ-receptors. This discrepency in opiate receptor-type selectivity based on the sensitivity of responses to agonists and antagonists suggests a physiological reason for evoking both μ- and δ-receptors on the modulation of GnRH release.

The effects of these opioids on PRL plasma levels indicates a tonic stimulatory influence which appears to be mediated through the opiate receptor. Both high and low doses of naloxone antagonized this tonic stimulatory effect to produce depressions in PRL levels. The fact that low dose naloxone (0.03 mg/kg) elicited a depression in PRL suggests that the μ-receptor is involved. This observation supports studies in humans where low dose naloxone depressed serum PRL levels (Ruhin et al., 1974). Increases in the plasma PRL levels by β-end (μ and δ) and morphine (μ), along with the lack of an effect due to DADLE (μ), further supports the contention that μ-receptors are involved in PRL regulation.

Comparison of Episodic Effects on LH and Testosterone. The known stimulatory effect of LH on testosterone leads one to suspect that changes in LH episodic amplitudes would be accompanied by testosterone amplitude changes in the same direction. Tables 2 through 5 give indications of

such a connection between LH and testosterone amplitudes. For example, β-endorphin (10 μg/kg) appeared to increase the amplitudes of both hormones (Table 4). Naloxone at all three dose levels studied increased both LH and testosterone episodic amplitudes (Table 5). DADLE generally appeared to diminish the amplitudes of both hormones slightly, but only at the level of marginal statistical significance in these experiments (Table 3). The major exception found to this pattern of similar changes in amplitude is found in the results for morphine (Table 2), where decreases of 50% and more in LH amplitude have no parallel whatsoever in testosterone amplitude changes. These results, if not to be dismissed as statistical quirks, might be explained in terms of maximal hormone responsiveness. This possibility is considered below.

It may be noted from Table 2 that the LH amplitude of the single group of vehicle (control) series is a relatively large value, in fact, 70% above the grand average LH vehicle amplitude that was computed (Table 1). By contrast, average testosterone amplitude in both the vehicle group and the morphine treated groups in Table 2 is somewhat lower than the grand average value. The disparity in effects on LH and testosterone amplitudes may indicate that the control of testosterone pulsatile release by LH pulses had reached maximal responsiveness at approximately 200 ng/dl or less. This would explain the inability of the much higher vehicle LH amplitudes (500 ng/dl average) to stimulate a larger amplitude of testosterone release by the testes, and thus the reason for observing no testosterone amplitude decrease concomitant with LH amplitude decrease caused by morphine. If such a maximal response is indeed present here, then the fact that the testosterone amplitudes are below their grand average value suggests that testosterone's maximal response amplitude, i.e., amplitude of pulses beyond which it cannot be driven by strong LH pulses,

may itself be variable with factors other than LH, which is not difficult to conceive.

Implications of Effects on Hormonal Episodes. The several opioid agonists and the antagonist considered in these studies can be distinguished in terms of their simultaneous effects on gross hormone levels and on episodic fluctuations (see tables 1 through 6). Considering testosterone and LH first, morphine was seen to depress both the overall level (or trend) of these hormones and also to depress amplitudes of episodic fluctuations of LH about its trend. Naloxone elicited effects which are essentially opposite to those of morphine, in that it raised both the overall levels of LH and testosterone and their episodic amplitudes. These increases in LH pulse amplitudes agree with studies in men administered naloxone (Grossman et al., 1981). Additional studies of naloxone's effect on LH episodes were done in women and found increases in both LH amplitude and frequency (Ropert et al., 1981). The effects of DADLE on these hormones' gross levels were similar to the effects of morphine. In addition, DADLE lowered LH episodic amplitude, as did morphine, and also lowered testosterone amplitude. DADLE's effects on gross hormone levels were greatest at the intermediate dose level (10 µg/kg). The observation that these drugs appear to shift gross hormone levels and fluctuation amplitudes in the same direction suggests a role for the EOP in regulation of LH, with the EOP controlling the "reference" or target level for sex hormone output, and the size of the fluctuations about the reference level being proportional to the reference level itself.

The effect of β-end is somewhat different from that of morphine or naloxone in that no change in gross levels due to β-end administration

was observed, but increases in episodic amplitude were elicited at the lower level given. This suggests that β-end may be having its effect on LH release by modulating the strength of LH feedback control rather than in controlling the hormone's target level. The observed increase in fluctuation amplitude may be interpreted as a weakening of the feedback control produced by β-end, resulting in larger oscillations in GnRH and subsequent LH output. The differences among the opioid agonists and antagonist with regard to joint effects on gross levels and episodes may be due to differences in their affinities for and efficacies with the various opiate receptor subtypes, as described in preceding sections.

As indicated in Chapter 4, only a few cases of drug-induced shifts in episodic frequency were found. These are: an apparent lowering of testosterone frequency by morphine; increases in testosterone and LH frequency by DADLE; and a lowering of testosterone frequency by β-end. These frequency changes, if real, are difficult to explain in terms of a single general principle. In the case of morphine, the direction of the frequency shift (downward) is the same as that of the gross level shift, while for β-end, a testosterone frequency increase is produced without any corresponding change in gross level (although accompanied by an increase in episodic amplitude). With DADLE, frequency increases in episodes of testosterone and LH are accompanied by decreases in gross level as well as in amplitude. Explaining these rather complicated patterns of episodic and gross level changes appears to require detailed pharmaco-kinetic modeling. Unfortunately, our knowledge regarding the precise mechanisms by which the EOP participate in sex hormone release is not yet sufficient to allow such detailed modeling.

Summary and Conclusions

The present studies clearly demonstrate that opioids affect gonadotropin secretion in the non-human primate. Morphine sulfate and DADLE decrease plasma testosterone and LH levels. Opioids exert their actions through a hypothalamic mechanism and not via a pituitary or testicular mechanism. These effects are most likely produced through an inhibition of GnRH secretion. Whether opioids directly inhibit GnRH neurons or modulate GnRH release via other neurotransmitters is presently unknown. The depressant effects of opioids on LH and testosterone levels are reversed by the opiate receptor antagonist naloxone. This indicates that the opioids elicit these effects by acting through opiate receptors. Evaluation of opiate receptor types involved in the modulation of LH secretion suggests that both μ and δ opiate receptors affect GnRH release, with the δ-receptor-mediated path possessing greater potency.

Prolactin plasma levels are elevated after administration of morphine sulfate or β-endorphin. The stimulatory effect of morphine is reversed by naloxone administration, thus indicating an opiate receptor mediated effect. Lack of effect due to DADLE, in light of morphine and β-endorphin effects on PRL levels, suggests that PRL release is mediated by μ opiate receptors. The affinity of these receptors for β-endorphin is much higher than that for morphine.

Naloxone administration produces marked increases in plasma testosterone and LH levels. This result supports the postulate that EOP are involved in the physiological regulation of neuroendocrine mechanisms controlling gonadotropin release. Participation of EOP in the endocrine events leading to episodic LH, testosterone, and PRL release is strongly suggested by effects on episodic hormonal fluctuations caused by the

opioids studied.

If the EOP are involved in the physiological regulation of neuroendocrine mechanisms, the most likely site of action for such a role would be at the level of the hypothalamic neurotransmitters. Preliminary observations indicate that the EOP may affect secretion of biogenic amines in the hypothalamus. Since hypothalamic hypophysiotropic hormones are controlled by hypothalamic biogenic amines, a disruption of the amine metabolism could cause the observed effects (Meites et al., 1979; Schneider et al., 1970).

Acute administration of narcotics to male rats results in a decrease in the rate of turnover and synthesis of dopamine in the median eminence (Alper et al., 1980). Morphine decreases the activity of norepinephrine neurons of the locus coeruleus which terminate in the hypothalamus and are the major source of hypothalamic norepinephrine (Korf et al., 1974). In addition, single doses of morphine have been reported to increase turnover of serotonin in whole brain and hypothalamus of rats (Yarbrough et al., 1971; 1973).

In 1977 Ferland et al. injected Met-enkephalin into the lateral ventricles of male rats and observed a decrease in dopamine turnover and norepinephrine concentration in the median eminence. β-endorphin has also been shown to decrease dopamine turnover in the median eminence of rats (Van Vugt et al., 1979). Hypothalamic and brain stem levels of serotonin and its metabolite 5-hydroxyindoleacetate increase in rats following acute administration of β-endorphin (Van Loon and De Souza, 1978). Further studies are required to elucidate the precise mechanism(s) by which EOP modulate reproductive neuroendocrine function.

APPENDIX A

The following diagram (Figure 1A) outlines the procedure used in the *in vitro* Leydig cell bioassay. Table 1A presents results obtained in studies of direct drug effects on stimulated Leydig cells *in vitro*.

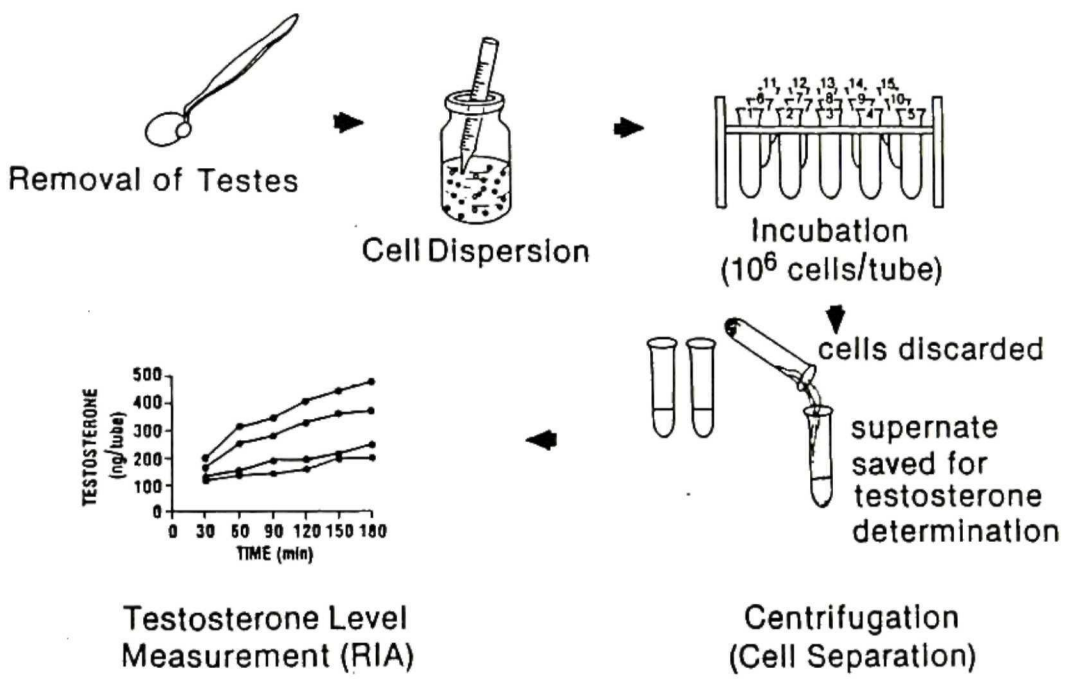

Figure 1A. Procedures for in vitro mouse Leydig cell assay. Diagrammed here are the in vitro procedures used to evaluate possible direct testicular effects of morphine sulfate on testosterone production.

Table 1A. Results For Morphine Sulfate (MS) and Acetaldehyde (AA) Relevant To Direct Testicular Effect On Isolated Mouse Leydig Cells

DRUG	DOSE (ng/ml)	PERCENT OF CONTROL	PERCENT CHANGE	STATISTICAL SIGNIFICANCE
MS	1.5	98	-2	Not Sig. at $p = 0.05$
MS	5.0	91	-9	Not Sig. at $p = 0.05$
MS	10.0	93	-7	Not Sig. at $p = 0.05$
MS	15.0	95	-5	Not Sig. at $p = 0.05$
MS	150.0	93	-7	Not Sig. at $p = 0.05$
AA*	2.0	45	-55	Sig. at $p < 0.01$

* Acetaldehyde was used as a positive control in these experiments.

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