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thesis entitled

I. ROLE OF ENDOGENOUS OPIOID PEPTIDE IN REGULATION OF PHASIC AND PULSATILE RELEASE OF GONADOTROPINS

II. RELATION OF HORMONES AND FOOD INTAKE TO DEVELOPMENT AND HORMONE DEPENDENCY OF CARCINOGEN-INDUCED MAMMARY TUMORS

presented by

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has been accepted towards fulfillment
of the requirements for

Ph.D. degree in Physiology

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Major professor

Date 10/20/82

O-7639

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Paul William Sylvester

A DISSERTATION

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

Department of Physiology

1982

ABSTRACT

I. ROLE OF ENDOGENOUS OPIOID PEPTIDES IN REGULATION OF PHASIC AND PULSATILE RELEASE OF GONADOTROPINS

II. RELATION OF HORMONES AND FOOD INTAKE TO DEVELOPMENT AND HORMONE DEPENDENCY OF CARCINOGEN-INDUCED MAMMARY TUMORS

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I. In ovariectomized (OVX) rats given estradiol benzoate (EB), morphine (MOR) prevented, whereas naloxone (NAL) enhanced the surge of LH and FSH on the day of treatment (day 1). On the next day (day 2), whereas NAL-treated rats showed no surge. In EB-progesterone (P) treated rats, MOR blocked, whereas NAL had no effect on the gonadotropin surge on day 1. On day 2, MOR-treated rats showed a large gonadotropin surge, whereas NAL-treated rats showed no surge. Ovx rats were given a subcutaneous (sc) injection of EB or EB-P 3 days prior to experimentation. The rats were then given injections of NAL, MOR or saline every hour for 3 hours. Pulsatile LH release was suppressed by EB or EB-P. Naloxone was able to counteract inhibition of pulsatile LH release by these steroids. These results suggest a possible role for the endogenous opioid peptides (EOP) in modulating steroid regulation of gonadotropin secretion.

II. Food restriction for 7 days before and either 7 or 30 days after 7,12-dimethylbenz(a)anthracene (DMBA) administration resulted in a significant reduction in average tumor number and size. Treatment for 8 days with EB produced a significant increase in mammary tumor incidence despite underfeeding, whereas underfed rats given haloperidol (HAL, an anti-dopaminergic drug), EB and GH showed development and growth of

mammary tumors equal to that of full-fed controls. These results indicate that reduced food intake just prior to and after DMBA administration can produce inhibition of mammary tumor development, and that EB or the combination of EB, HAL and GH can counteract the inhibition produced by underfeeding.

Sixteen weeks after DMBA administration, animals were OVX to determine hormone-dependency of mammary tumors. Tamoxifen (TAM, an anti-estrogen) given during the first week after DMBA injection resulted in a significant reduction of mammary tumor incidence, but in a 3-fold increase in the number of autonomous tumors in the total tumor population. Rats treated with the combination of TAM and CB-154 (a dopaminergic agonist) also showed suppressed mammary tumor incidence, but a 5-fold increase in the appearance of hormone-independent tumors. Rats given either EB, CB-154 or HAL showed the same tumor incidence and hormone-dependency as controls. These results indicate that suppression of estrogen, but not PRL at the time of tumor induction not only reduced the incidence and number of mammary tumors, but the tumors that developed show less hormone dependency.

This dissertation is dedicated to my mother and father.

ACKNOWLEDGEMENTS

I would like to thank Dr. Joseph Meites for always allowing me to pursue my interests. His example of unselfish and total dedication to teaching and research has left a lasting impression on me.

I will always be grateful to the members of the neuroendocrine research unit both past and present for the humor, inspiration, insight, and caring that was always there. Specifically, I would like to thank Drs. Charles Aylsworth, Warren H.T. Chen, Dipak Sarkar, Richard Steger, and Dean A. Van Vugt for their constant aid and friendship.

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αmpt	= alpha methyl para tyrosine
AP	= anterior pituitary
β-END	= beta endorphin
CB-154	= bromocriptine
CNS	= central nervous system
CRF	= corticotropin-releasing factor
DA	= dopamine
DDC	= diethyl-dithiocarbamate
DLF	= dorsal longitudinal fasciculus
DOPS	= dihydroxyphenylserine
EB	= estradiol benzoate
EOP	= endogenous opioid peptides
GIF	= growth hormone-inhibiting factor
GnRH	= gonadotropin-releasing hormone
GRF	= growth hormone-releasing factor
HAL	= haloperidol
LEU-ENK	= leucine enkephalin
MAO	= monoamine oxidase
MET-ENK	= methionine enkephalin
MFB	= medial forebrain bundle
MOR	= morphine
NAL	= naloxone
NE	= norepinephrine
OVX	= ovariectomized
P	= progesterone
PCA	= parachlorophenylalanine
PCPA	= parachloroamphetamine
PIF	= prolactin-inhibiting factor
PMS	= pregnant mare serum
PRF	= prolactin-releasing factor
PRL	= prolactin
RIA	= radioimmunoassay
SAL	= saline
TAM	= tamoxifen
TRH	= thyrotropin-releasing hormone
U-14,624	= 1-phenyl-4-(2-theazolyl)-thiourea
5-HTP	= 5-hydroxytryptophan
5-HT	= serotonin
6-OH-DA	= 6-hydroxydopamine

INTRODUCTION

I. Role of Brain Opiates in Regulating Pituitary Gonadotropic Function

In order for an individual to survive under natural condictions, he must be able to regulate his internal environment to adapt to the challenges and stresses from the external environment. This ability to adapt reflects the activity of complex regulatory mechanisms which integrate the many body systems to insure adequate homeostasis of the internal environment. These regulative and intergrative mechanisms are co-ordinated by both the endocrine and nervous systems. The fusion of endocrinology and neurobiology has occurred over the last half-century or so, and has created the hybrid field of neuroendocrinology.

The hypothalamus appears to serve as the center, whereby information from all parts of the central nervous system (CNS) is funnelled to regulate the secretion of anterior pituitary (AP) hormones. Hypophysiotrophic hormones synthesized in specialized neurosecretory cells of the hypothalamus and other brain regions are released into the hypophysial portal circulation and travel to the pituitary to affect hormone secretion.

Target organ hormones feed back to modulate the action of the hypothalamic-pituitary system by altering the magnitude of the CNS neural signal and/or the responsiveness of the pituitary to the releasing hormone. Negative feedback regulation of the neuroendocrine

system is mediated via hormones from target organs feeding back to inhibit the AP hormone secretion. This is demonstrated by the observation that removal of a target organ results in increased AP hormone secretion, such as the post-castration rise of gonadotropins. In contrast, the enhancement of pituitary hormone secretion by target organ hormones can occur in the form of positive feedback regulation, and is illustrated by the estrogen-induced preovulatory surge of gonadotropins.

In the past the modulatory role of the hypothalamic biogenic amines on the neuroendocrine system has been intensely investigated. With regard to the hypothalamic-pituitary-gonadal axis, the noradrenergic system has been found to be primarily stimulatory, whereas central dopaminergic and serotonergic systems have been reported to be either inhibitory and stimulatory to luteinizing hormone (LH) release (Krieg and Sawyer, 1976; Vijayan and McCann, 1978). Since the discovery of the endogenous opioid peptides (EOP) less than a decade ago, many investigators have been interested in the involvement these substances in the regulation of the neuroendocrine system. Acute administration of EOP or morphine (MOR) has been shown to inhibit, whereas naloxone (NAL), a specific opiate receptor antagonist, stimulates gonadotropin release (Bruni et al., 1977). These results suggest that the EOP tonically inhibit basal LH release. Because the opiates and NAL do not act directly on the pituitary to alter hormone secretion, their action appears to be mediated through a hypothalamic mechanism (Cicero et al., 1977).

Therefore, it was of interest to determine the involvement the EOP have in the regulation of gonadotropin secretion during dynamic

physiological states and their interactions with the ovarian steroids. To examine the involvement of EOP during positive feedback of ovarian steroids on LH, I administered MOR and NAL during the "critical period" for gonadotropin release in estrogen on estrogen-progesterone treated long-term ovariectomized rats. It has been demonstrated that LH in ovariectomized rats is released in a pulsatile manner, and ovarian steroids act to suppress this release. Since NAL has been shown to counteract the negative feedback action of gonadal steroids (Cicero et al., 1979; Van Vugt et al., 1982), it was of interest to examine the effects of MOR and NAL had on the pulsatile release of LH in ovariectomized rats treated or not treated with ovarian steroids.

II. Endocrine and Nutritional Relationships to Mammary Tumors

The complex neuroendocrine control systems, such as negative and positive feedback loops, depend on highly efficient cell to cell co-ordination within the tissues of the organism. The growth and differentiation of each cell is normally well-controlled to guarantee homeostasis of the individual. Occasionally however, genetic mutation occurs and cells can lose their vital communication with other cells, and populations of malignant cells develop. The endocrine environment of the rat has been shown to be critically important during the induction of mammary cancer. The majority of mammary carcinomas found in rats are dependent on hormones for growth. Estrogen and PRL are essential for mammary tumorigenesis in the rat, and in general, treatments which increase secretion of these hormones stimulate tumor growth, whereas treatment which inhibit secretion of these hormones inhibit growth (Meites, 1972).

Recently, there has been a growing awareness of the important association between nutrition and cancer, both as a means of prevention and treatment. Previously it has been demonstrated that underfeeding significantly inhibits the incidence of spontaneous mammary tumors in mice (Tannenbaum and Silverstone, 1950). Inhibition of mammary tumorigenesis by caloric restriction does not appear to be due to the lack of essential dietary components since underfed animals appear to be in good general health and live longer than full-fed controls. While the mechanism(s) by which underfeeding induces tumor suppression in rats is not completely understood. There is evidence to suggest that the endocrine system is involved. Severe food-restriction in animals results in pituitary insufficiency similar to that seen in hypophysectomized rats and leads to a condition referred to as "pseudohypophysectomy" (Mulinos et al., 1940). Recently, underfeeding was shown to decrease secretion of 5 AP hormones as measured by radioimmunoassay (RIA) (Campbell et al., 1977).

It has been established that the first week after carcinogen administration is critical for induction of mammary tumors in Sprague-Dawley rats (Dao, 1962). Since food-restriction results in decreased AP and ovarian function, and because PRL and estrogen are essential for mammary tumorigenesis, hormonal deficiencies during the first week after carcinogen administration may be responsible for the inhibition of mammary tumor development seen in underfed rats. It was of interest therefore to determine whether administration of PRL and estrogen during the critical first week after carcinogen administration could overcome the inhibition produced by restricted or chronic underfeeding on mammary

tumorigenesis. In addition, we also wanted to determine if food-restriction limited to the week before and the first critical week after carcinogen administration was as effective in inhibiting mammary tumor development as chronic underfeeding.

While the majority of carcinogen induced mammary tumors in rats are dependent on PRL and estrogen, a small percentage of these tumors display hormone-independency on autonomous growth. The mechanisms involved in establishment of autonomous tumors are not well understood. Since the hormonal milieu at the time of tumor induction greatly influences mammary tumor development, I was interested in determining whether the hormonal-dependency or independency that subsequently develops in carcinogen induced mammary tumors, was related to their initial hormonal dependency or independency during the critical first week after carcinogen administration.

LITERATURE REVIEW

I. The Hypothalamic-Hypophysial Axis

A. Classical Observations of Functional Relationship Between Hypothalamus and Adenohypophysis

The pituitary gland lies beneath the hypothalamus and is connected to the hypothalamus by a thin stalk. The functional interrelationship between the pituitary and hypothalamus has been firmly established over the last 60 years. The first evidence of pituitary control by the CNS was discovered by Erdheim (1909), when he noted that gonadal atrophy was correlated with lesions of the hypothalamus. Aschner (1912) later showed that gonadal atrophy could be induced by placing a lesion in the anterior hypothalamus, while leaving the pituitary intact. Subsequently, investigators have demonstrated that hypothalamic lesions induced atrophy of the thyroid (Cahane and Cahane, 1938), adrenal cortex (deGroot and Harris, 1950), and blocked stress induced hypertrophy of the adrenal glands (Ganong and Hume, 1954). In contrast, electrical stimulation of hypothalamic regions of the brain can induce ovulation (Harris, 1937), increased thyroid (Harris, 1948a), and adrenal cortex secretion (deGroot and Harris, 1950). The effects of hypothalamic stimulation is specific since direct electrical stimulation of the pituitary had no effect (Markee et al., 1946).

Popa and Fielding (1930) demonstrated that the hypothalamus and pituitary were connected by a group of coiled capillaries termed the hypothalamic portal vessels. They hypothesized that blood flowed in the direction of the hypothalamus from the pituitary. Wislocki and King (1936) later showed that blood actually flowed from the hypothalamus to the pituitary. This was later confirmed by Green and Harris (1947). These studies led Harris (1948) to propose that chemical regulatory substances from the hypothalamus are transported to the pituitary via the hypothalamic portal vessels.

Classical experiments by Harris further demonstrated the initimate association between the hypothalamic influence and pituitary function. Sectioning of the pituitary stalk generally produced only transient effects on pituitary function due to regeneration of the portal vessels (Harris, 1949). Removal of the pituitary gland from its original position in the sella turcica and transplanting it to the anterior chamber of the eye or underneath the kidney capsule resulted in atrophy of the gonads, adrenals and thyroid glands, but retention of corpora lutea function (Harris, 1948b; Harris and Jacobsohn, 1952; Everett, 1954). Pituitary dysfunction as a result of transplantating the same pituitary back to its original position underneath the median eminence, where regeneration of the portal vessels occurs (Nikitovitch-Winer and Everett, 1958). These studies demonstrated the importance of hypothalamic control over pituitary function.

B. Anatomy of the Hypothalamus

The hypothalamus is a complex network of neurons and neurosecretory cells lying in the most ventral portion of the diencephalon (Jenkens, 1972). The hypothalamus is bounded rostrally by the lamina

terminalis; caudally by the mammillary bodies; dorsally by the hypothalamic sulcus; ventrally by the tuber-cinereum and median eminence, and laterally in part by the internal capsule, basis pedunculi and subthalamus (Nauta and Haymaker, 1969).

The hypothalamus can be divided functionally and anatomically into medial and lateral regions. The medial region contains the majority of neuroendocrine activity, while the lateral region is part of an intricate neuronal system that connects the limbic forebrain with the mesencephalon (Martin et al., 1977). Nuclei are arranged and distributed in 3 major zones within the hypothalamus.

The periventricular zone contains the suprachiasmatic, paraventricular and arcuate nuclei. The median zone contains the medial preoptic, anterior hypothalamic, ventromedial, dorsomedial, premammillary and posterior hypothalamic nuclei. The lateral zone contains the lateral preoptic, supraoptic, lateral hypothalamic and mammillary nuclei. All hypothalamic nuclei, except the arcuate and the median eminence are located bilaterally on either side of the third ventricle (Martin et al., 1977).

Afferent fiber connections to the hypothalamus arise from the brain-stem reticular formation and limbic forebrain structures. These inputs include the mammillary peduncle, dorsal longitudinal fasciculus (DLF) and medial forebrain bundles (MFB) (Nauta and Haymaker, 1969). The mammillary peduncle and DLF originate in the central gray substance of the mesencephalon and enter the hypothalamus caudally. The mammillary peduncle enters the mammillary bodies where it then turns laterally and terminates in the lateral hypothalamic and preoptic

nuclei. The DLF terminates primarily in the posterior and dorsal areas of the hypothalamus.

The MFB is the major afferent and efferent conduction system between the hypothalamus and other brain regions. The ascending component of the MFB arises from the paraaqueductal gray matter of the brainstem and terminates in the olfactory-septal regions of the telencephalon. The descending components of the MFB have the opposite origin and termination sites. Throughout its course, the MFB receives input from laterally adjacent sources such as limbic and striatial structures. It traverses the hypothalamus through the dorsal aspects of the lateral preoptico-hypothalamic region (Nauta and Haymaker, 1969).

Afferents from the limbic system to the hypothalamus include the fornix, stria terminalis, ventral amygdalofugal pathway, and the descending branch of the MFB. The fornix takes origin from the hippocampus, and traverses the septal region where it splits into 2 columns. The columns then turn caudally and terminate in the mammillary bodies. The striae terminalis arises from the corticomedial amygdala and terminates in the septum, preoptic area, and the medial hypothalamus. The ventral amygdalofugal pathway originates in the basolateral amygdala and enters the lateral hypothalamic regions. The precise termination of this pathway with the hypothalamus is unknown (Martin et al., 1977). Evidence for the existence of the retinohypothalamic tract has also been reported (Riss et al., 1963).

C. Neurosecreton

In general, all neurons have the ability to synthesize and release specific substances. Impulses are transmitted from 1 cell to another at

synapses and transmission is primarily chemically mediated. The pre-synaptic axon liberates a chemical mediator which alters the permeability of the post-synaptic neuronal membrane. The chemical mediators in the process are called neurotransmitters. Neurosecretory cells are a population of specialized neurons, which along with the ability to conduct impulses, release specific hormonal substances (neurohormones) directly into the bloodstream to affect distant target organs. Neurosecretory cells in the hypothalamus contain proteinaceous material in axons and cell bodies and Scharrer and Scharrer (1940) first suggested they have endocrine functions.

Cells specializing in the production of neurohormones often occur within specific locations in the nervous systems of invertebrates and vertebrates. They display cytological signs of much more extensive glandular activity than those of ordinary neurons as evidenced by their prominent content of membrane-bound granules of varying electron opacity.

Neurosecretory cells showed the basic morphological features that were characteristic of the typical neuron with axons, dendrite neurofibrillae, developed endoplasmic reticulum, Golgi apparati, axoplasmic transport. The synthesis and transport of neurosecretory products were also similar to ordinary neuronal mechanisms, with synthesis of raw protein material in the endoplasmic reticulum, packaging of products into neurosecretory granules in the Golgi apparatus and movement of the neurosecretory material by axoplasmic flow from its area of production to the site of discharge (Bern and Knowles, 1966).

Based on the proposed concept of neurosecretion and the realization of the crucial role of the portal vascular system in controlling pitiutary function, Harris (1948b) proposed that the hypothalamus secretes specific substances into the portal capillaries of the median eminence. These substances are transported to the AP by the portal vessels to regulate the AP hormone secretion. This "portal vessel-chemotransmitter hypothesis" has continued to serve as a basic model for the study of neuroendocrinology. During the past few decades, the search to identify the hypophysiotropic hormones of the hypothalamus has been intensely pursued.

D. Hypophysiotrophic Hormones of the Hypothalamus

This area of research has had such a large impact on the scientific community in general that in 1977, Dr. Andrew V. Schally and Dr. Roger Guillemin shared the Nobel Prize in Physiology and Medicine for their pioneering work on the identificatiqn and structural analyses of a number of these hypothalamic hypophysiotrophic factors.

Saffran and Schally (1955) and Guillemin and Rosenberg (1955) were the first to demonstrate that the hypothalamus contained a substance that regulates AP activity. By using an in vitro system involving the incubation of hypothalamic and pituitary tissue, they demonstrated that after the addition of norepinephrine (NE), ACTH was released from the AP. They named this hypothalamic substance corticotropin releasing factor (CRF). CRF has eluded definitive structural analysis until just last year when Vale and his co-workers (1981), identified a 41-residue hypothalamic peptide that appears to be CRF.

Using a similar hypothalamic-pituitary coincubation system, others have demonstrated both releasing and inhibitory activity of the

hypothalamus on AP function. Shibusawa et al. (1956) reported hypothalamic releasing activity for TSH. Thyrotropin releasing hormone (TRH) was purified and synthesized independently 14 years later by the laboratories of Guillemin (Burgus et al., 1969) and Schally (Boller et al., 1969), and shown to be a simple cyclic tripeptide containing residues of glutamic acid, histidine and prolamine.

Releasing factors have also been found in the hypothalamus for LH (McCann et al., 1960) and FSH (Igarashi and McCann, 1964; Mittler and Meites, 1964). It was subsequently found that the same substance stimulates the release of both FSH and LH and is now called gonadotropin-releasing hormone (GnRH) and is identified and synthesized as a linear decapeptide (Matsuo et al., 1971a; 1971b).

Hypothalamic releasing activity for GH was first demonstrated by Deuben and Meites (1964). Later, it was demonstrated that GRF activity was localized in the ventromedial hypothalamic nucleus (Krulich et al., 1972). Purification and synthesis of GRF has not yet been demonstrated. Hypothalamic inhibitory activity for GH has also been demonstrated (Krulich et al., 1968). Growth hormone inhibitory hormone (GIF or somatostatin) was subsequently isolated, and structure was characterized as a tetradecapeptide containing a single disulfide bridge (Brazeau et al., 1973).

Meites et al. (1960) were the first to demonstrate a stimulatory influence of the hypothalamus on PRL release from the AP, but few attempts have been made to purify and isolate PRF thus far. An inhibitory influence of the hypothalamus on release of PRL has been demonstrated (Pasteel, 1961; Talwalker et al., 1961, 1963). The

structural sequence of a PRL-inhibiting factor (PIF) has eluded detection thus far.

With the advent of specific RIAs for LRH, TRH, and somatostatin, and the development of a micropunch technique for isolation of discrete brain areas, the regional distribution of these three hypothalamic peptides has been described. Within the hypothalamus, the arcuate nucleus and median eminence contained the bulk of TRH (Brownstein et al., 1974), and 5-HT (Brownstein et al., 1975). These 2 hypothalamic areas corresponded to the hypophysiotropic area described earlier by Halasz et al. (1962). GnRH is found in the preoptic-suprachiasmatic area and neurons in the preoptic area are believed to be the only source of median eminence GnRH in the rat (Baker et al., 1975). Both TRH and somatostatin have been localized in extra-hypothalamic brain regions (Brownstein et al., 1974; Brownstein et al., 1975). Somatostatin has also been isolated outside the CNS in pancreatic islet cells (Patel and Reichlin, 1978) and gastric and intestinal mucosa (Arimura et al.,1975).

Besides specific influences on their respective AP hormones, GnRH, TRH, and somatostatin were found to influence the release of other AP hormones. GnRH was reported to increase GH release in some patients with active acromegaly (Faglia et al., 1973). TRH stimulates both PRL (Jacobs et al., 1971) and GH (Kato et al.,1975) release. Somatostatin was found to inhibit TRH induced TSH release (Vale et al., 1974) and inhibits the secretion of both glucagon and insulin (Koerher et al.,1974).

II. Localization of Biogenic Amines and Opiates in the Brain and Hypothalamus

A. Norepinephrine (NE)

Norepinephrine terminals are derived from fibers originating in the pons and reticular tegmentum of the mesencephalon. The ascending NE fibers in the midbrain reticular area are separated into dorsal and ventral bundles. The dorsal NE bundle arises from cell bodies in the locus ceruleus and innervates the cortical brain regions. The ventral NE bundle innervates the hypothalamus and preoptic area via the medial forebrain bundle (Fuxe and Hokfelt, 1968).

Lesions of the MFB resulted in a decrease in hypothalamic NE content (Kobayashi et al., 1974). In addition, hypothalamic deafferentation resulted in a loss of both dopamine- -hydroxylase activity and decreased hypothalamic NE levels (Brownstein et al., 1974). This suggests that the cell bodies which produce NE lie outside the hypothalamus and that only their axons enter the hypothalamus.

Within the hypothalamus, NE is for the most part, uniformly distributed in all nuclei. The highest concentrations of NE were found in the retrochiasmatic area, dorsomedial nucleus, periventricular nucleus, and median eminence (Palkovits et al., 1974).

B. Dopamine (DA)

The majority of DA neurons in the hypothalamus originates from an intrahypothalamic system, known as the tuberoinfundibular DA pathway. In this system cell bodies located in the arcuate and periventricular nuclei send their axons to terminals in the external layer of the median eminence (Fuxe and Hökfelt, 1968), and possibly other areas (Renaud,

1976). Within the hypothalamus, DA is highly concentrated in the median eminence and arcuate nucleus (Palkovits et al., 1974).

Weiner et al. (1972) demonstrated that there was no significant decrease in hypothalamic DA content following total hypothalamic deafferententation. This suggests that extra-hypothalamic dopaminergic cell bodies provide little input to the hypothalamus. Brownstein et al. (1976) however, showed that lesions in the substantia nigra reduced medial hypothalamic DA levels. This, in contrast to the work of Weiner et al. (1972), indicates that the nigrastriatal dopamine pathway may provide significant input to the hypothalamus.

C. Serotonin (5-HT)

Serotoninergic neurons originate in the raphé complex in the mesencephalon. The cell bodies send their axons midline in the MFB in 2 main ascending bundles, called the medial and lateral ascending 5-HT pathways. These pathways terminate in the forebrain regions, including the hypothalamus (Palkovits et al., 1977). It is believed that both the dorsal and median raphe nuclei innervate the hypothalamus. The median raphe nucleus appear to be the primary source of 5-HT fibers innervating the suprachiasmatic nucleus, anterior hypothalamic area, and medial preoptic area. The arcuate nucleus appears to receive equal innervation from both the dorsal and median raphe nuclei (Van DeKar and Lorens, 1979). Decreased hypothalamic 5-HT levels were observed following lesioning of the raphé complex, lesioning the MFB, or total hypothalamic deafferentation (Weiner et al., 1972; Saavedra et al., 1974).

D. Opiates

Using receptor binding assays and bioassays with mouse vas deferens and guinea pig ileum, evidence suggesting the existence of opiate-like

substances in the brain were discovered by Terenius and Wahlström (1974; 1975 and Hughes (1975). Hughes and co-workers (1975) later isolated and characterized two opiate-like pentapeptides, called methionine-enkephalen (MET-ENK) and leucine-enkephalin (LEU-ENK). Li and Chung (1976) subsequently isolated a 31-amino acid peptide with opiate activity from the pituitary, called beta-endorphin (β- END). In the next few years, a number of other endogenous opiates have been isolated and characterized, including alpha-endorphin, gamma-endorphin, and dynorphin. β-END contains the amino acid sequence of MET-ENK in its N-terminus and it was initially thought that the enkephalin was a breakdown product of β-END. However, now this appears unlikely since both synthesis and release of enkephalins and endorphins occurs independently of 1 another and in different areas of the brain and pituitary.

It appears that β-lipotropin, β-END, and ACTH share a common precursor molecule called pro-opiocortin. Pro-opiocortin has been identified as the precursor for ACTH in the AP and β-END in the intermediate pituitary and -lipotropin is an intermediate step between the precursor and β-END (Fratta et al., 1979). MET-ENK appears to be cleaved from a larger hexapeptide molecule (Huang et al., 1979). Dynorphin contains within its amino acid sequence, LEU-ENK. Whether LEU-ENK is a breakdown product of this larger molecule is unknown, but because of the different location of these opiates in the brain and pituitary, this seems unlikely (Goldstein et al., 1979).

The regional distribution in brain of the opiate receptors and the endogenous enkephalins closely parallel each other, with the highest

concentrations of both MET-ENK and LEU-ENK (Smith et al., 1976; Adler, 1980). It appears that the enkephalin neurons do not send axons in large fiber tracts to distant brain areas, but rather function as local short-fiber interneurons. The enkephalins are in highest concentration in the striatum, anterior hypothalamus, mesencephalic central gray, amygdala, accumbens nucleus and medial hypothalamus. Moderate levels are found in the thalamus, cortex, and brainstem areas and low concentrations are found in the central white matter, cerebellum and spinal cord. Concentrations of enkephalins in the pituitary are minimal. MET-ENK in any given area of the brain is found in two to 8 times higher concentration than that of LEU-ENK (Smith et al., 1976; Adler, 1980).

The endorphins are primarily concentrated in the pituitary, and specifically in the intermediate lobe. The posterior pituitary has not been shown to contain endorphin (Bloom et al., 1977). Concentrations of the endorphins in the brain are small compared to those found in the pitiutary. In the brain, the highest concentration of β-END is found in the medial hypothalamus. Lesser amounts are found in the periventricular thalamus, substantia nigra, mesencephalic central gray, medial amygdaloid nucleus, locus ceruleus and zona incerta. β- END containing cells in the arcuate nucleus of the hypothalamus send axons in the form of a major fiber bundle to the locus ceruleus.

III Control of Gonadotropin Secretion

A. Profile of Serum Gonadotropin and Steroid Hormones During the Estrous Cycle

Estrous cyclicity in the female rat is dependent in part on environmental patterns of light and darkness. Everett (1961) found that

rats on a daily regime of 14 hours light /10 hours dark, with lights on at 0500 hours and off at 1900 hrs, showed regular 4-day vaginal cyclicity. However, a small percentage of rats display 5-day cyclicity with an additional day of diestrus. Under these conditions rats ovulate at approximately 0100-0300 hours on the day of vaginal estrus. The method of assessing changes in vaginal cytology consists of swabbing the vaginal lumen and examining the cells under a microscope (Stockard and Papanicolaou, 1917).

Arbitrarily, the first stage of the estrous cycle can be considered as estrus. Estrus lasts 36 hours and is characterized by the presence of cornified epithelial cells in the vaginal lumen. However, the time of heat and copulation (behavioral estrus) is not the same as vaginal estrus. Behavioral estrus begins and is most intense during late vaginal proestrus and ends during the period of vaginal estrus. The next stage of the estrous cycle is called metestrus and vaginal smears show progressively less and less cornified cells and the increased presence of leucocytes. Metestrus lasts approximately 10-14 hours and mating is usually not permitted. Diestrus is the third stage of the estrous cycle and lasts about 36 hours. Vaginal smears are characterized almost entirely by leukocytes. This is followed by the last stage called proestrus. Proestrous vaginal smears contain nucleated epithelial cells and this stage lasts approximately 12 hours. The patterns of hormone secretion by the ovaries, pituitary, and hypothalamus during the course of the estrous cycle will now be discussed.

On the day of estrus, 12 hours after ovulation, estrogen, P, LH, and PRL levels in the blood are low (Butcher *et al.*, 1974). At this time, serum FSH levels are declining but have not reached basal levels

yet. During the early afternoon of metestrus, blood estrogen, progesterone (P), PRL, LH, and FSH are at basal levels. At the this time, new follicles, under the stimulation of basal gonadotropin levels, begin to grow as does the theca interna of these follicles. The theca interna is believed to be the major source of ovarian estrogen production (Turner and Bagnara, 1976). Plasma P levels at this time are slightly elevated as a result of corpora lutea secretion. On the morning of diestrus, PRL, LH, and FSH levels in the blood are still low, but follicles continue to grow and estrogen production is increased. The pre-dominate estrogen secreted by the ovary is 17 -estradiol. It is believed that follicular production of estrogen at this time prevents follicle atresia (Harman et al., 1975) and sensitizes the ovary to the action of the gonadotropins by increasing LH receptors in the theca interna and FSH receptors in the granulosa cells (Richards and Midgley, 1976; Louvet and Vastukaities, 1976). At this time, P levels are low, as a result of corpora lutea lysis. If P levels, however, remain elevated, there is slower follicle development and estrogen production in the ovary (Schwartz, 1969). Elevated P levels are believed to be the principal reason why some rats have 5-day versus 4-day estrous cycles (Buffler and Rosen, 1974).

Estrogen levels in the blood continue to rise during the afternoon of diestrus. At this time, LH, FSH, PRL, and P levels are low. Estrogen levels peak on the morning of proestrus. This surge of estrogen is essential to bring about the subsequent surges of PRL, LH, and FSH on the afternoon of proestrus. If ovariectomy (Schwartz, 1964), injections of P (Brown-Grant, 1967), anti-estrogen drugs (Callantine et al., 1966) or antisera to estrogen (Neill et al., 1971) are administered

to rats before the surge of estrogen occurs, no surge of LH, FSH, or PRL results. However, if similar treatments are administered to rats after the surge of estrogen occurs, there is no effect on the surge of gonadotropins. The surge of estrogen also increases the sensitivity of the pitiutary to the action of GnRH (Turgeon and Barraclough, 1977). Estrogen levels decline during the early afternoon of proestrus.

Between 1400 and 1800 hours of proestrus, there occurs a sharp surge of LH that lasts approximately 30 min, and the exact timing of this surge is variable between individual rats. This surge of LH is induced by a surge of GnRH in the hypophysial portal blood (Sarkar et al., 1976). FSH levels in the blood also surge at the same time as LH, but in constrast to LH, FSH continues to rise until it peaks during the morning of estrus. There is a surge of PRL at the same time as the gonadotropins, but the physiological significance is not entirely clear. Prolactin is not needed to induce ovulation (Barraclough et al., 1971) and if the PRL surge is blocked, there is little effect on the estrous cycle (Neill and Smith, 1974). The surge of PRL acts to induce regression of corpora lutea from previous cycles (Wuttke and Meites, 1971) and increases preovulatory P secretion (Gelato et al., 1976).

By 2100 hours of proestrus serum levels of LH, PRL, and estrogen have reached basal levels, as does GnRH in the portal blood. Only FSH and P levels at this time are elevated. On the morning of estrus between 0100 and 0300 hours, ovulation occurs. Serum LH, PRL, estrogen and P are low, but FSH is still high.

B. Negative Feedback

Ovariectomy in female rats results in the removal of the target organs for gonadotropins and results in increased release of

gonadotropins (Ramirez and McCann, 1963; Gay and Midgley, 1969). Similar results occur if female rats are exposed to long-term administration of an anti-estrogen (Döcke, 1969). Administration of gonadal steroids to ovariectomized rats causes a decrease in gonadotropin levels in the blood (Ramirez and McCann, 1963; Ramirez et al., 1964). Ovariectomy in rats also results in enlarged gonadotrophs in the pituitary. However, if the pituitary is removed and transplanted to other sites in the body not adjacent to the hypothalamus, these enlarged cells do not develop (Hohlweg and Junkmann, 1932). These results indicated that regulation of gonadotropins is under tonic inhibition by the ovaries in female rats and is dependent upon central input, from the hypothalamus. This hypothalamo-pituitary-gonadal feedback loop, can be both inhibitory and stimulatory in nature. Positive feedback will be discussed in the following section.

Secretion of gonadotropins, following castration differs in male and female rats. In the male, a significant increase in LH levels can be detected within 8 hours after orchidectomy (Gay and Midgley, 1969). In the female rat, however, a significant rise in LH levels in not seen until 2-3 days following ovariectomy. Estrogen is the most potent steroid for the inhibition of LH, and decreases in serum LH levels can be detected within 2 hours after estrogen administration (Blake, 1977a). Progesterone (except in very large doses) has no effect in suppressing elevated gonadotropin levels after castration (McCann, 1962; Chen et al., 1977).

Estrogen demonstrates a biphasic effect on LH secretion at the level of the pitutiary. Pituitary responsivness to GnRH is suppressed initially after estrogen administration (Libertun et al., 1974), then

after a period of 8-12 hours there is a facilitated responsiveness (Henderson et al., 1977). Estrogen also has a negative feedback action on gonadotropin release at the level of the hypothalamus. This inhibitory action is restricted to the MBH, since surgical isolation of the MBH does not abolish the negative feedback action of estrogen (Blake, 1977b). Progesterone has also been shown to exert negative feedback control on LH release at both the pituitary and hypothalamic level. In the hypothalamus, progesterone acts directly to inhibit release of GnRH into the portal blood (Sarkar and Fink, 1979), and synergizes with estrogen to acutely suppress pituitary responsiveness to GnRH (Chen et al., 1977).

C. Positive Feedback

Unlike males, female rats display a spontaneous preovulatory surge of gonadotropins on the afternoon of proestrus, which is dependent on a preceeding surge of estrogen. This positive feedback effect on LH secretion was first demonstrated by Hohlweg (1944) when he induced ovulation in prepubertal rats by administration of gonadal steroids. Estrogen, when injected during diestrus also can advance the time of ovulation (Everett, 1948). No surge of LH occurs if the preceeding surge of estrogen is blocked by surgical or pharmacological methods (Schwartz, 1964; Brown-Grant, 1967; Callantine et al., 1966; Neill et al., 1971).

Various models have been developed to simulate the changes in plasma steroid concentrations that occur before and during the spontaneous LH surge, in order to investigate the mechanism of positive feedback. The first model involves giving multiple injections of EB or implanting EB containing silastic capsules s.c. in long-term

ovariectomized rats (Caligaris et al., 1971). In this experimental model, elevated levels of estrogen in the blood induce a daily proestrous-like surge of LH between 1700 and 1800 hours. This diurnal rhythm of LH release will persist indefinitely as long as elevated blood estrogen levels are maintained (Legan et al., 1975). A second model used to study the stimulatory effects of ovarian steroids on LH secretion, consists of injecting P 72 hours after EB administration in long-term ovariectomized rats (Caligaris et al., 1968). Unlike the LH surge which results every afternoon in ovariectomized rats treated only with EB, EB-P primed animals display an LH surge about 5 hrs after injection of P, and no surge occurs the next day.

While the EB or EB-P primed ovariectomized rat may be physiologically less relevant than the normal cycling rat, these experimental models provide a convenient method for studying positive feedback by providing a large, predictable, synchronized surge of LH, and eliminates the need of obtaining daily vaginal smears in rats. The LH surge in the normal cycling rat on the afternoon of proestrus displays great variation between individual animals in the timing and magnitude of the surge, but is generally between 200 and 400 ng/ml. In contrast, the LH surge is 3-4 times larger in the EB-primed and up to 10 times larger in the EB-primed ovariectomized rat models when compared to the intact rat on proestrus (Fink, 1979).

Sarkar et al. (1976) demonstrated that GnRH levels in the hypophysial portal blood remain low throughout the estrous cycle until a surge occurs on the afternoon of proestrus at the same time as the spontaneous surge of LH. This was the first demonstration that the afternoon surge of LH on proestrus results from an increased release of

GnRH from the brain. Because of the large surge of LH induced in EB and EB-P primed ovariectomized rats, it was suspected that these steroids stimulate greater release of GnRH into the portal blood than during the estrous cycle. It was discovered, however, that portal blood at the time of the LH surge of EB-primed ovariectomized rats, showed only a small increase of GnRH and this increase was slight compared to the GnRH surge found in the proestrus rat (Sarkar and Fink, 1979). In addition, GnRH levels in the portal blood of EB-P primed ovariectomized rats during the surge of LH was not significantly different from that of oil treated controls which did not show a surge of LH (Sarkar and Fink, 1979). The paradox as to why little or no increase of GnRH is seen in the portal blood of steroid- primed ovariectomized rats, even though these animals displayed a massive surge of LH, was shown to result from the effects these steroids have on pituitary responsiveness to GnRH.

It was shown that on the afternoon of proestrus between 1700 and 1800 hours, pituitary responsiveness in the rat increased 50 times over that seen at the same time on diestrus and this was dependent on estrogen (Aiyer et al., 1974). These investigators also found that pituitary responsiveness to GnRH at 1700 hours is 2-3 times greater in EB-primed and 7 times greater (4 hours after P injection) in EB-P primed ovariectomized rats as compared to the proestrus rat at 1700 hours.

The above studies demonstrated that ovarian steroids increased pituitary sensitivity to GnRH released into the portal blood. Other studies indicate that the positive feedback action of estrogen also is mediated by centrally stimulated GnRH release from the brain. Deafferentiation of the preoptic area from the MBH blocked the proestrus surge of LH (Blake et al., 1972) and ovulation (Halasz and Gorski, 1967).

Similar deafferentiation or anterior hypothalamic lesions also blocked the steroid-induced LH surge in ovariectomized rats (Neill, 1972; Blake, 1977b). Most evidence now suggests that the preoptic area is the same site at which estrogen enhances GnRH release. It has also been shown that GnRH release, as a result of electrical stimulation of the preoptic area, is enhanced in the presence of estrogen, but estrogen has no effect on GnRH release when electrical stimulation is applied to the MBH (Sherwood et al., 1976). Lesion studies have also shown that the suprachiasmatic nucleus is important for maintenance of regular estrous cycles (Clemens et al., 1976). Lesions of the suprachiasmatic nucleus result in persistent failure of ovulation and produces abnormalities in estrogen cyclicity associated with the light-dark patterns.

The surge of GnRH and subsequently of LH as a result of estrogen stimulation supports the idea of the daily neuronal signal for LH release (Everett and Sawyer, 1949). Estrogen is believed to "turn on" this daily surge signal as evidenced by the fact that estrogen increases the firing rate of hypothalamic and preoptic area neurons (Fink and Geffen, 1978). Progesterone is believed to "shut off" this estrogen-induced daily surge signal, because P reduced the firing rate of hypothalamic and preoptic neurons (Fink and Geffen, 1978). This suggestion is supported by the finding that in EB-primed ovariectomized rats, a daily surge of LH continues until estrogen levels in the blood are reduced (Legan et al. 1975). Estradiol-benzoate primed ovariectomized rats show an enhanced LH surge on the day of P injection, but subsequent surges of LH do not occur (Freeman et al., 1976; Legan and Karsch, 1975). Similarly, in the normal cycling rat, a surge of P follows the afternoon surge of LH on proestrus (Barraclough et al.,

1971). It is believed that this rise in blood P is responsible for preventing a subsequent surge of LH the following day (Freeman et al., 1976; Blake, 1977a).

D. Concept of the "Critical Period" of Gonadotropin Release

The "critical period" of LH release defines a time period before and during which the administration of a variety of neuropharmacological drugs (barbiturates, atropine, MOR, chlorpromazine, etc.) inhibits the preovulatory release of gonadotropins (Everett, 1964). The existence of a "critical period" was first demonstrated by Everett et al. (1949). Rats maintained under a 14 hour light /10 hour dark schedule, with lights on at 0500 hours and off at 1900 hours, show the "critical period" between 1400 and 1600 hours on the day of proestrus. Blake (1974) has since demonstrated that the length of the "critical period" is actually much longer and lasts approximately 7 hours (1400-2200 hours on proestrus). Blake suggested that administration of central acting drugs during the "critical period" interferes with the expression of the estrogen induced daily surge signal which is responsible for the initiation of the proestrous surge of LH, thus blocking the LH surge and ovulation.

E. Biogenic Amines and Opiate Involvement in Gonadotropin Release

A role of central neurotransmitters in the regulation of AP hormone secretion was first postulated by Tabrenhaus and Sosken (1941). These investigators demonstrated that application of acetylcholine to the AP gland resulted in pseudopregnancy. Sawyer et al. (1947) later demonstrated that administration of α-adrenergic blockers is able to prevent the reflex release of LH in rabbits, and injection of NE induces

ovulation (Sawyer, 1952). Similar results were also shown in rats (Markee et al., 1952; Everett, 1961). The localization and mapping of aminergic, peptidergic and opioid neuronal systems of the brain further illustrates the close association of these neurotransmitters with GnRH containing neurons in the preoptic-suprachiasmatic area and their terminals in the median eminence (Elde and Hokfelt, 1978).

Numerous reports in the literature demonstrate that the central catecholamines are an important activator of LH secretion. Brain monoamine depletors, such as reserpine, block LH release induced by pregnant mare serum (PMS) in immature rats (Barraclough and Sawyer, 1957). This inhibitory effect of reserpine on ovulation was prevented when animals were pretreated with the monoamine oxidase (MAO) inhibitor, pargyline, presumably by blocking the metabolism of the catecholamines in the synapse. Likewise, α-methyl-p-tyrosine (α-mpt) which depletes central catecholamine stores by competitively inhibiting the activity of the rate limiting enzyme in catecholamine synthesis, tyrosine hydroxylase, blocked PMS-induced ovulation in immature rats (Lippman et al.,1967) and the proestrus or estrogen-induced LH surge in adult rats (Kalra et al., 1972; Kalra and McCann, 1974). Administration of catecholaminergic neurotoxic drugs, such as 6-hydroxydopamine (6-OH-DA) into the lateral ventricle of rats, was also shown to block the proestrous or estrogen-induced surge of LH (Martinovic and McCann, 1977; Simpkins et al., 1979).

Central noradrenergic neurons have been shown facilitate the release of LH from the pituitary. Intraventricular injections of NE stimulate LH release (Krieg and Sawyer, 1976; Vijayan and McCann, 1978). Infusion of hypothalamic fragments in vitro with NE causes the release

of GnRH into the surrounding medium (Negro-Vilar and Ojeda, 1978). Evidence for the involvement of the NE system in the induction of the proestrous LH surge is also convincing. Pharmacological studies using drugs which lower NE levels in the hypothalamus, such as α-mpt or dopamine- -hydroxylase inhibitors, such as diethyldithiocarbamate (DDC) and 1-phelyl-4-(2-thiazolyl)-thiourea (U-14,624), when administered to rats, block the proestrus surge of LH (Kalra and McCann, 1974). The effect of these drugs was reversed by treatment with dihydroxyphenylserine (DOPS), which selectively increases NE. Treatment with L-dopa in these animals, which mainly increases dopamine (DA), was without effect.

Administration of 6-OH-DA in low doses, selectively depletes only NE and leaves hypothalamic DA stores unchanged (Breese and Traylor, 1971). A low dose of 6-OH-DA was shown to block the LH surge during proestrus (Simpkins et al., 1979). The PMS-induced surge of LH was inhibited by phenybenzamine administration, but was not affected by phentolamine, yohimbine, DL- or D-propranolol or clonidine (Sarkar and Fink, 1981). Phenoxybenzamine also inhibited the PMS-induced surge of GnRH in the portal blood (Sarkar and Fink, 1981). These results suggest that the GnRH, and subsequently the LH surge, depends upon the functional integrity of central noradrenergic neurons, which facilitate the GnRH release through α-adrenergic receptors. This is supported by the finding that NE turnover and NE concentrations in the median eminence, increase prior to the LH surge (Lokstrom, 1977). Barraclough and co-workers (Raune et al., 1981) have also demonstrated that at the time of the LH surge, median eminence GnRH content declines and median eminence NE turnover rates greatly increase. The decline of median

eminence GnRH levels in this study was interpeted to represent the relase of GnRH into the portal circulation. Conversely, the proestrous surge of LH is abolished by electrical lesioning of the central NE pathway (Martinovic and McCann, 1977).

In contrast of the central NE system, the role of the dopaminergic system in the regulation of LH secretion is controversial. Intraventricular injections of DA in ovariectomized EB-P-primed rats stimulated LH secretion (Kamberi et al., 1969; Vijayan and McCann, 1978), and DA also was shown to stimulate GnRH release from hypothalamic fragments in vitro (Rotszstein et al., 1976), which is blocked by pimozide, a specific DA receptor blocker.

There is considerable evidence however, that the central DA system also inhibits GnRH secretion. Fuxe and co-workers (1967) demonstrated that DA turnover in the median eminence is negatively correlated with gonadotropin release. Likewise, infusion of DA, DA agonists, or L-dopa, reduced LH levels in intact or ovariectomized rats (Drouva and Gallo, 1977; Mueller et al., 1976). It also has been shown that intraventricular infusion of DA does not stimulate LH release, but actually blocks LH secretion induced by NE (Sawyer et al., 1974). In contrast to the finding of Rotszstein et al. (1976), others have demonstrated that DA inhibits GnRH release from rat hypothalamus incubations (Mizachi et al., 1973). Implantation of pituitaries under the kidney capsule, leads to a lasting elevation of serum PRL levels which in turn stimulates activity of the tuberoinfundibular DA system (Gudelsky et al., 1976), and prevents the post-castration rise of LH (Grandison et al., 1977). Similar pituitary transplants decrease LH levels in castrated rats (Beck et al., 1977; Vijayan and McCann, 1978). Blockade of DA receptors

either had no effect on LH levels or caused further elevation of blood LH (Gnodde and Schuiling, 1976; Drouva and Gallo, 1976).

Dopamine receptor agonists have also been shown to inhibit the PMS-induced surge of LH in premature rats, while chlorpromazine, a DA receptor blocker has the same effect (Agnati et al., 1977). Sarkar and Fink (1981) showed that the surge of GnRH in the portal blood is inhibited by DA acting on receptors that are blocked by pimozide and domperidone, but facilitated by DA acting on receptors that are blocked by haloperidol. The existence of 2 different types of dopaminergic receptors may explain the conflicting reports as to the role of the central DA system in LH release. The stimulatory effect of DA on LH release may be due to an action on DA receptors that are blocked by haloperidol, while the inhibitory effect of DA on LH release is mediated by its action on receptors blocked by pimozide or domperidone. These 2 types of DA receptors are probably influenced by te steroid millieu of the rats since systemic injection of DA stimulates LH release in steroid-primed ovariectomized rats, whereas high doses of DA suppressed serum LH in ovariectomized unprimed rats (Vijayan and McCann, 1978).

In general, the serotonergic system is inhibitory to LH secretion. Intraventricular administration of 5-HT suppressed the release of gonadotropins in intact and castrated rats (Kamberi et al., 1971; Schneider and McCann, 1970). Pulsatile release of LH was inhibited in ovariectomized rats when the mid-brain dorsal raphe nucleus was electrically stimulated (Arendash and Gallo, 1978). Systemic injection of 5-hydroxytryptophan (5-HTP), the immediate precursor of 5-HT, has been shown to block ovulation (Kordon et al., 1968; Kamberi, 1973). Administration of p-chlorophenylalamine (PCPA) during the "critical

period" of LH release, enhances ovulation in PMS treated immature rats (Kordon et al., 1968). Electrical stimulation of the raphe nuclei increased 5-HT turnover and blocked ovulation in rats (Carrer and Taleisnik, 1970). The inhibitory action of 5-HT on LH release appears to be mediated by the medial basal hypothalamus, since microinjection of serotonergic drugs blocked ovulation only when administered in this brain region (Kordon, 1969; Domanski et al., 1975). Fuxe et al. (1974) showed that estrogen increased 5-HT turnover in ovariectomized rats whereas P reduced 5-HT turnover back to basal levels. It has also been shown that the increased turnover of 5-HT during suckling may be responsible for the suppression of LH release in to the blood (Mena et al., 1976).

The serotonergic system also appears to have a stimulatory role on LH release. The afternoon surge of LH in ovariectomized estrogen-primed rats is abolished by pretreatment with PCPA and restored by subsequent administration of 5-HTP (Hery et al., 1976). Destruction of 5-HT terminals with 5,7-dihydroxytryptamine reduced serum LH in male rats and the return of LH to normal values coincided with the regrowth of the serotonergic nerve fibers (Wuttke et al., 1977). Chen et al. (1981) demonstrated that administration of PCPA or parachloroamphetamine (PCA) blocked the LH surge in ovariectomized steroid primed rats, and 5-HTP not only reversed this effect, but greatly potentiated the LH surge. These results indicate that 5-HT has a stimulating role on LH release during the estrogen-induced preovulatory surge.

Since the discovery of the EOPs a few years ago, many reports have indicated that the EOP play a role in the regulation of gonadotropin release. Morphine and EOP have been shown to inhibits basal LH release,

while NAL, a specific opiate receptor antagonist, increases LH secretion (Bruni et al., 1977). These results indicate that the EOP tonically inhibits the basal release of LH. This effect of the opiates and NAL on basal LH release has been shown to be both sex and age-related, since in prepubertal females, NAL increased LH levels, but is ineffective in males until approximately 30 days of age (Ieiri et al., 1979; Blank et al., 1979).

Recent evidence also indicates that the EOP are involved in the negative feedback action of gonadal steroids on LH release. Naloxone has been shown to block the inhibitory effect of testosterone on LH release in castrated male rats (Cicero et al., 1979; 1980). Likewise, NAL blocked the feedback inhibition of estrogen or the combination of estrogen and P in ovariectomized female rats (Van Vugt et al., 1982). Thus, the EOP are involved in gonadal steroid inhibition of LH secretion in male and female rats.

Early studies also indicated that the EOP play a role during the proestrus surge of LH. Morphine blocked ovulation (Barraclough and Sawyer, 1955) and the LH surge during proestrus (Pang et al., 1977) when administered during the "critical period" for LH release. This action of MOR was reversed by NAL (Pang et al., 1977). The effects of MOR or NAL administered on the afternoon of proestrus also alters the surges of LH, FSH and PRL (Ieiri et al., 1980). These investigators showed that MOR delayed and suppressed the surge of LH and this effect was reversed by NAL. Administration of NAL alone did not alter the magnitude of the LH surge on the afternoon of proestrus, but significantly extended the duration of the surge. Hypothalamic and pituitary content of MET-ENK is very high on the morning of proestrus, but decreases significantly on

the afternoon of proestrus (Kumar et al., 1979). These investigators speculated that decreased MET-ENK levels on the afternoon of proestrus may contribute to the surge of gonadotropins, whereas the high levels during the morning could be involved in the surge of PRL.

The mechanism(s) by which NAL and the opiates exert their effects on LH release are not entirely clear, but appear to be mediated via the hypothalamus since opiates do not exert their effects directly on the pituitary (Cicero et al., 1977; 1979). Morphine does not block the effect of GnRH on secretion of LH by the pituitary and has no effect on LH release from pituitary explants. In addition, hypothalamic content of GnRH is increased by acute administration of MOR, and this was interpreted as indicating a decrease of LHRH release into the portal blood (Muraki et al.,1978).

Considerable evidence indicates that hypothalamic norepinephrine is involved in mediating the inibitory effect of EOP on LH release. Opiates depress hypothalamic DA turnover in the median eminence (Ferland et al., 1977; Van Vugt et al., 1979), and reduce DA concentrations in the portal blood (Gudelsky et al., 1979). Morphine also has been shown to decrease NE concentration in the hypothalamus (deWied et al., 1974; Kalra and Simpkins, 1981). Also, depletion of hypothalamic NE with either α-mpt or DDC, or blocking the action of NE with the α-adrenergic receptor blocker, phenoxybenzamine, inhibited NAL-induced LH release (Van Vugt et al., 1981). It appears therefore, that hypothalamic NE is involved in mediating the stimulatory effects of NAL on LH release.

Hypothalamic 5-HT activity is increased by MOR and the opiates (Ieiri et al., 1980; Van Loon and DeSouza, 1978). Since 5-HT generally acts to inhibit LH release, the inhibitory effects of the opiates and

the stimulatory action of NAL on LH release may involve a serotonergic mechanism. Other brain neurotransmitters also may be influenced by the opiates to alter LH release. These data strongly suggest that the brain opiates are important intermediaries in the regulation of LH release by interacting with biogenic amines or directly on GnRH hypothalamic neurons.

F. Control of Pulsatile LH Release

Luteinizing hormone in ovariectomized rats is released in a pulsatile manner (Gay and Sheth, 1972). The mechanism which generates this episodic secretion appears to be mediated by the hypothalamus and not the AP gland itself. Incubations of pituitaries have shown that release of LH occurs in a non-pulsatile manner when the medium was perfused with constant levels of GnRH (Osland et al., 1975). However, when GnRH was administered in a pulsatile manner, LH release also was also pulsatile. Sarkar and Fink (1980) recently demonstrated that GnRH is released in a pulsatile fashion in ovariectomized rats, and these pulses of GnRH correlated with the LH pulses seen in systemic blood. In addition, central acting barbiturates, such as pentobarbital were shown to inhibit pulsatile LH release (Arendash and Gallo, 1978b).

Deafferentation of the MBH in rats resulted in non-pulsatile LH release in ovariectomized rats (Blake and Sawyer, 1974; Arendash and Gallo, 1978). Thus, it appears that different input to the MBH is required for stimulation of pulsatile LH secretion. Brain neurotransmitter involvement in the regulation of pulsatile LH release has recently undergone active investigation.

Hypothalamic NE has been shown to stimulate pulsatile LH release in ovariectomized rats. Drugs which block NE synthesis (Drouva and Gallo,

1976; Grodde and Schuiling, 1976) or block α-adrenergic receptors (Weick, 1977), inhibit pulsatile LH release. The effect of NE infusion into the third ventricle of the brain, however, appears to have a biphasic effect on pulsatile LH release. Prolonged infusion inhibits, while slow acute infusion of NE into the third ventricle of ovariectomized rats stimulates pulsatile LH release (Gallo and Drouva, 1979). The explanation for these findings is unknown, but high NE levels in the third ventricle may activate other inhibitory neuronal systems that can influence pulsatile LH release.

Third ventricle infusion of DA (Gallo and Drouva, 1979) or administration of dopaminergic agonists (Grodde and Schuiling, 1976) have been shown to inhibit pulsatile LH release. However, administration of dopaminergic antagonists do not alter episodic release of LH (Drouva and Gallo, 1976). These results indicate that hypothalamic DA activity is not involved in the tonic inhibition of pulsatile LH release in ovariectomized rats.

Evidence also indicates that brain 5-HT is involved in suppression of pulsatile LH release in ovariectomized rats. Electrical stimulation of the arcuate nucleus suppresses pulsatile LH release and this effect is blocked if animals are pretreated with 5-HT synthesis inhibitors (Gallo and Moberg, 1977). Electrical stimulation of the midbrain dorsal raphe nucleus also results in suppression of episodic LH secretion (Arendash and Gallo, 1978). When rats are pretreated with 5-HT synthesis inhibitors or 5-HT receptor blocks, stimulation of the dorsal raphe nucleus had no effect (Arendash and Gallo, 1978c). Administration of β-END has also been demonstrated to inhibit pulsatile secretion of LH in castrated rats (Kinoshita et al., 1980).

The ovarian steroid environment of an animal has also been shown to be critically important in determining the magnitude and direction of pulsatile LH release in response to neurotransmitter stimulation. Estrogen exerts negative feedback effects on pulsatile LH release in ovariectomized rats (Blake et al., 1974), as does P administration alone in ovariectomized rats (Blake et al., 1974). Injection of NE into the third ventricle at a dose which inhibited or had no effect on pulsatile LH release in unprimed ovariectomized rats, significantly stimulated episodic LH release in EB-P-primed ovariectomized rats (Gallo and Drouva, 1979). Injection of DA into the third ventricle had no effect on steroid suppression of pulsatile LH release in these animals (Gallo and Drouva, 1979).

IV. Hypothalamic Control of Prolactin Release

Many reports on regulation of PRL secretion have appeared since development of the first RIA for this hormone. Prolactin is essential for lactation and mammary growth, and is involved in mammary and pituitary tumors, reproduction and many other physiological functions.

The control of PRL secretion differs from that of most other AP hormones, in that it has no negative feedback inhibition from any target tissue it stimulates. The primary control of PRL comes from the communication between the hypothalamus and pituitary via the hypothalamo-hypophysial portal blood vasculature. This hypothalamic regulation of PRL is both stimulatory and inhibitory, with the latter predominating under basal conditions. Hypothalamic control is mediated by peptidergic hormones and neurotransmitters. Estrogens and adrenal cortical steroids can act directly on the pituitary to regulate PRL secretion.

A. Inhibition of Prolactin Release

Prolactin release is increased after removal of hypothalamic influences. Destruction of the median eminence, the final common neural pathway to the pituitary or transplantation of the pituitary underneaththe kidney capsule, result in continuous PRL secretion but decreased secretion of all other AP hormones (Everett, 1954). Hypothalamic extract, when added to pituitary incubations (Meites et al., 1981; 1963; Talwalker, 1963), cultures (Pasteels, 1961), or injected into rats (Grosvenor et al., 1964), produce a decrease in PRL release. The chemical identity of this PRL-release-inhibiting-factor (PIF) is unknown, but it may be mainly dopamine. Many hypothalamic substances have been shown to have PIF activity.

Most evidence indicates that hypothalamic biogenic amines are the primary regulators of PRL secretion. Removal of catecholamines from hypothalamic extracts results in loss of PIF activity (Shaar and Clemens, 1974). Hypothalamic extracts contain high concentrations of catecholamines (Schally et al., 1976). Agents which increase catecholamine activity in the brain, such as L-dopa, the (immediate precursor of catecholamines), or monamine oxidase inhibitors which interfere with the degradation of catecholamines, (Lu and Meites, 1971), decrease PRL secretion.

Dopamine appears to be the primary substance in the hypothalamus which inhibits PRL release. Dopamine injected into the third ventricle of rats (Kamberi et al., 1970), or added directly to pituitary incubations (MacLeod, 1969), inhibits release of PRL. Dopaminergic agonists such as apomorphine or piribedil (Mueller et al., 1973) or various ergot alkaloids (Nicoll et al., 1970; Wuttke et al., 1971), also

decrease PRL secretion. Conversely, blood PRL levels are increased by the DA antagonists, pimozide (Clemens et al., 1971), sulpiride (Meites et al., 1972), haloperidol (Dickerman et al., 1972), and reserpine and chlorpromazine (Lu et al., 1970).

Anatomical evidence indicates that an intimate association exists between hypothalamic dopamine and pituitary PRL function. Dopaminergic neurons originate in the arcuate nucleus, and terminals of these tuberoinfundibular neurons in the median eminence are in close association with the hypophysial portal vasculature (Fuxe et al., 1975). Dopamine receptors have been localized on pituitary membranes (Brown et al., 1976). Disruption of this pathway by median eminence lesions (Meites et al., 1963; Welsch et al., 1971) elevates PRL levels.

Tuberoinfundibular DA exerts tonic inhibition on the release of PRL from the AP. This is supported by the finding that hypophysial portal blood contains DA in concentrations sufficient to inhibit PRL secretion (Ben-Jonathan et al., 1977; Plotsky et al., 1978). The release of DA from the tuberoinfundibular neurons into the hypophysial portal blood is dependent upon the continued synthesis of DA. Inhibition of DA synthesis by α-methyltyrosine, which blocks the rate limiting enzyme in DA synthesis, tyrosine hydroxylase, causes a marked reduction in DA concentration in pituitary stalk blood and increases systemic blood PRL levels (Gudelsky and Porter, 1979).

The evidence that DA accounts for most of the hypothalamic PIF is very strong, but there is some evidence that DA does not account for all PIF activity in the hypothalamus. After incubation of rat pituitaries with haloperidol (Quijada et al., 1974) or pimozide (Vale et al., 1976), to block DA receptors, addition of rat hypothalamic extracts can still

inhibit PRL release. In addition, when all catecholamines are removed from hypothalamic extracts, they still contain PIF activity (Takahara et al., 1974).

The involvement of norepinephrine in regulation of PRL secretion is controversial. Norepinephrine and epinephrine, have been shown to inhibit PRL release in vitro (MacLeod, 1969). When smaller doses of these neurotransmitters were used however, PRL release was shown to be enhanced (Koch et al., 1970). Administration of norepinephrine synthesis inhibitors (Clemens and Meites, 1977) decreases, while addition of DOPS, a precursor of norepinephrine, increases PRL release in vivo (Donoso et al., 1971). Injection of norepinephrine into the third ventricle causes release of PRL in ovariectomized or ovariectomized steroid-primed rats (Vijayan and McCann, 1978). Clonidine, an α-receptor agonist, was found to inhibit the proestrus surge of PRL (Vijayan and McCann, 1978). Thus, the physiological significance of norepinephrine on PRL release is difficult to assess at this time.

Intraventricular injections of acetylcholine or systemic administration of cholinergic drugs have been shown to inhibit PRL release (Grandison et al., 1974; Grandison and Meites, 1976). Cholinergic agonists and cholinesterase inhibitors also have been shown to block the surge of PRL on the afternoon of proestrus (Libertum and McCann, 1974; Blake et al., 1973) and the estrogen induced afternoon PRL surge in ovariectomized rats (Subramarian et al., 1976). Low doses of cholinergic antagonists, such as atropine and scopolamine, have not been shown to increase PRL levels, but block cholinergic inhibition (Ruiz de Galarreta et al., 1981). Cholinergic inhibition of PRL can also be

blocked if animals receive prior treatment with a dopaminergic antagonist (Grandison and Meites, 1976). It appears therefore, that cholinergic inhibition of PRL is mediated by increasing hypothalamic dopaminergic activity.

Adrenal glucocorticoids also have been found to be important in the regulation of PRL secretion. When glucocorticoids were added to pituitary incubations, PRL release was reduced (Clemens and Meites, 1977). Low doses of corticosterone were also found to inhibit PRL release in hypophysectomized rats carrying a pituitary graft under the kidney capsule (Leung et al., 1979) and to inhibit the stress-induced release of PRL (Euker et al., 1975). Adrenalectomy increased whereas administration of glucocorticoids inhibited PRL release (Chen et al., 1976). It appears that adrenal glucocorticoid inhibition of PRL release is mediated by direct action on the AP.

B. Stimulation of Prolactin Secretion

Injection of crude hypothalamic extracts in estrogen-primed female rats initiated mammary secretion and indicated that the hypothalamus contains a PRF (Meites et al., 1980). Many substances found in the hypothalamus have since been found to contain PRF activity. It has been shown that TRH can stimulate release of both TSH and PRL in humans (Bower et al., 1971) and rats (Tashijan et al., 1971; Lu et al., 1972). It seems unlikely, however, that TRH is a physiological PRF. TRH can be separated chromatographically from hypothalamic PRF extracts (Szabo and Frohman, 1976). There are also many physiological conditions in which TSH and PRL secretion do not coincide. Rats placed in cold temperature show an increased TSH release, but a marked reduction in serum PRL levels, and the opposite is seen when animals are placed in a warm

environment (Mueller et al., 1974). Restraint stress results in increased PRL but decreased TSH levels in the blood (Mueller et al., 1976).

Serotonin has been shown to be a powerful agent for stimulating PRL release. Administration of 5-HT to estrogen-primed female rats stimulated milk secretion (Meites et al., 1967). Intraventricular (Kamberi, 1971) and systemic (Lawson and Gala, 1975) injections of 5-HT increased PRL levels in the blood of rats. Systemic injections of 5-hydroxytryptophan (5-HTP), the precursor of 5-HT caused a significant increase in blood PRL levels in estrogen primed rats (Caligaris and Taleisnik, 1974), and this was blocked by pre-treatment with para-chlorophenylalanine (PCPA), a serotonergic neurotoxin. Intravenous infusion of L-tryptophan, the substrate for 5-HT synthesis, increased human PRL release (MacIndoe and Turkington, 1973). Treatment with PCPA and methysergide (a 5-HT receptor antagonist) blocked the suckling induced rise of PRL. The serotonergic agonist, quipazine, also stimulated PRL release (Krulich et al., 1975; Clemens et al., 1976).

Restraint stress was shown to be associated with increased turnover of 5-HT in the hypothalamus (Mueller et al., 1976) and is believed to be responsible for elevated PRL levels under this condition. Stimulation of serotoninergic neurons in the raphé complex of the midbrain, the ultimate source of hypothalamic 5-HT, increased, whereas destruction of this brain area decreased serum PRL levels (Advis et al., 1979). The mechanism by which 5-HT stimulates PRL is not known, but evidence suggests that its action is mediated indirectly by other hypothalamic agents. Intraventricular administration of 5-HT produced a reduction of DA concentration in the hypophysial portal blood, but co-infusion of

dopamine did not block 5-HT stimulation of PRL (Pilotte and Porter, 1981). It appears that 5-HT stimulation of PRL release may not only be mediated by decreasing dopamine activity, but also by stimulating PRF activity as well.

Recently, the EOP have been shown to increase PRL release (Bruni et al., 1977). Morphine had previously been shown to initiate lactation in estrogen primed rats (Meites, 1962) and stimulate PRL release (McCann et al., 1974). Injection of NAL, a specific opiate antagonist, inhibited basal PRL release and blocked opiate-stimulated secretion (Bruni et al., 1977). Opiates do not appear to act directly on the AP to stimulate PRL release. Pituitaries incubated with MOR, EOP, or NAL, did not alter the release of PRL (Grandison and Guidotti, 1977; Shaar et al., 1977). This suggests that the EOP act via a hypothalamic mechanism to stimulate PRL release. The EOP have been shown to decrease median eminence DA turnover (Ferland et al., 1977; Van Vugt et al., 1979), and to decrease DA concentrations in the hypophysial portal blood (Gudelsky and Porter, 1979). If animals are pretreated with drugs which inhibit 5-HT activity, opiate stimulation of PRL release is blocked (Demarest and Moore, 1981). These results, together with the findings of Pilotte and Porter (1981), indicate that the EOP stimulates 5-HT neurons and inhibits hypothalamic DA activity, resulting in an increase in serum PRL levels.

Other hypothalamic substances, such as gamma-aminobutyric acid, substance P, neurotensin, prostaglandins and histamine all have been shown to stimulate PRL release (Meites, 1979). However, the physiological significance of these neuropeptides has not been fully evaluated.

Estrogen has also been found to be an important regulator of PRL synthesis and release. Estrogen was shown to increase pituitary PRL content and induce lactation in rats (Reece and Turner, 1937). This was later confirmed in rabbits (Meites and Turner, 1942), and in vitro studies (Meites and Nicoll, 1966) by bioassay of PRL. A dose-dependent increase in PRL release by estrogen in ovariectomized rats was first demonstrated by RIA a few years later (Chen and Meites, 1970). Hypophysectomized rats with pituitary implants underneath the kidney capsyle, also showed elevated PRL levels in response to estrogen administration (Meites et al., 1972). These studies demonstrate a direct stimulatory effect of estrogen on the pituitary.

Prolactin levels are found to be higher in females than male rats, and this is believed to be due to the influence of estrogen. In general, estrogen always stimulates and never inhibits PRL release. The proestrus afternoon surge of PRL is preceded by a surge of estrogen (Meites and Clemens, 1972). Removal of estrogen by ovariectomy (Meites et al., 1972), or by administration of estrogen antiserum (Neill et al., 1971) on the morning of proestrus, blocked the afternoon surge of PRL. Rats that contain PRL secreting pituitary tumor implants, responded to estrogen treatment with increased PRL release (Mizuno et al., 1964).

Estrogen also appears to influence hypothalamic activity in its regulation of PRL secretion. Estrogen-primed rats showed decreased hypothalamic PIF activity as compared to non-estrogen primed controls (Ratner and Meites, 1964). Implantation of estrogen into the median eminence increased serum PRL levels (Nagasawa et al., 1969). The exact mechanism by which estrogen acts centrally to stimulate PRL secretion is not known. However, it has been shown that acute injections of estrogen

decrease DA concentrations in the hypophysial portal blood, and this was associated with elevated PRL levels in the circulation (Cramer et al., 1979).

It is well understood that dopamine inhibits PRL release, but PRL also has been shown to feed back and influence DA activity. Elevated PRL levels have been shown to decrease endogenous pituitary PRL release (Meites and Clemens, 1972; Advis et al., 1977). Rats bearing PRL secreting tumors have higher concentrations of DA in the hypophysial portal blood than non-tumor bearing rats (Cramer et al., 1979). Prolactin and PRL-releasing drugs produced increased activity of tuberoinfundibular dopaminergic neurons (Fuxe and Hökfelt, 1970). This autoregulatory mechanism for PRL release is termed "short-loop-feedback," but a physiological role for this mechanism has not been established.

Acute exposure to estrogen decreased DA in the portal blood, whereas chronic exposure increased DA concentrations (Gudelsky and Porter, 1979). It has also been demonstrated that females have higher DA levels in the portal blood than males, and female levels vary throughout the estrous cycle (Ben-Jonathan, 1977). It thus appears that estrogen acts acutely on the tuberoinfundibular neurons to alter secretion of DA into the hypophyseal portal blood directly. Chronic estrogen stimulation of PRL secretion elicits elevated PRL levels increases DA activity. The reason serum PRL remains elevated in the presence of increased DA activity is that estrogen inhibits DA action on the pituitary (Lu and Meites, 1972).

V. Role of Hormones in Murine Mammary Tumorigenesis

The endocrine environment of the rat mammary gland is of critical importance in the induction of mammary cancer. When intact female rats were fed 3-methylcholanthrene (MC), all animals developed mammary cancer, but no tumors developed in their hypophysectomized litter-mates (Huggins et al., 1959a). When hypophysectomized rats fed MC were treated with estrogen, P and growth hormone (GH), and exposed to a carcinogen, mammmary tumor development occurred (Young et al., 1961). Hormonal status has little or no influence on hormone-independent mammary tumors, but the majority of mammary carcinomas found in the rat are hormone-dependent. Changes in hormone levels can either accelerate or retard mammary tumor growth, depending on the magnitude and direction of the change.

Reduction or even complete extinction of mammary tumor growth occurred after ovariectomy (Huggins et al., 1959b). Stimulation of mammary cancer growth was seen in pregnancy and pseudopregnancy when steroid lactogenic hormone levels are high (Dao and Sunderland, 1959). It is clear that AP and gonadal steroid hormones are of primary importance in the initiation and growth of mammary carcinomas.

A. Prolactin

Prolactin alone is not considered tumorigenic, but its presence favors the development of mammary tumors in rats. Physiological conditions and treatments that increase serum PRL levels, stimulate mammary tumorigenesis, whereas conditions and treatments which reduce serum PRL inhibit mammary tumorigenesis (Welsch and Nagasawa, 1977). However, an excess of PRL can inhibit development of carcinogen-induced mammary tumors.

Injections of PRL into intact female rats stimulated mammary tumor growth (Kelly et al., 1974). Neuroleptic drugs increased endogenous PRL secretion (Welsch and Meites, 1970). Haloperidol (Quadri et al., 1973), perphenazine (Bodger et al., 1974), and sulpiride (Pass and Meites, 1976) and increased mammary tumorigenesis. Prolactin secretion stimulated by TRH, estrogen or adrenalectomy, also increased the number and size of 7,12-dimethylbenz(a)anthracene (DMBA) induced mammary tumors (Chen et al., 1977; Meites et al., 1972; Chen et al., 1976). Pituitary grafts underneath the kidney capsule increased blood PRL levels and stimulated mammary tumor growth (Welsch et al., 1968). Lesions in the median eminence, which disrupted tuberoinfundibular DA influence on the AP (Clemens et al., 1968), or estrogen implants in the median eminence (Nagasawa and Meites, 1970), stimulated PRL release and mammary tumorigenesis.

Reduction of PRL levels in the blood by hypophysectomy reduced hormone-dependent mammary tumor growth (Clifton and Sudharan, 1975) and PRL replacement reinitiated growth of these tumors. Dopaminergic receptor agonists or drugs which increased hypothalamic DA activity, such as ergot alkaloids, L-dopa, parayline, piribedil, alpha-methyl-p-tyrosine, decreased serum PRL and inhibited mammary tumor growth (Cassell et al., 1971; Quadri et al., 1973; Hodson et al., 1978). Administration of anti-PRL antiserum also caused regression of mammary tumors (Butler and Pearson, 1971). Naltrexone and NAL, specific opioid receptor antagonists, recently were shown to inhibit growth of mammary carcinomas in rats, and this was attributed to their suppression of PRL release (Aylsworth et al., 1979).

B. Estrogen

Besides PRL, estrogen has been established to be important for growth of hormone-dependent mammary tumors. Chronic administration of estrogen in rats has been shown to result in development of mammary tumors (Noble and Collip, 1941). Estrogen in small doses also has been shown to stimulate growth of established mammary tumors (Huggins, 1962). In contrast, removal of estrogen influence by ovariectomy (Dao, 1962) or by TAM, an anti-estrogenic drug (Jordan, 1976), prior to or shortly after carcinogen administration, significantly inhibited mammary tumorigenesis. These treatments produced similar effects on established mammary tumors (Huggins et al., 1959; Jordan and Jaspar, 1976). Mammary tumor regression as a result of ovariectomy was reversed by estrogen administration (Huggins et al., 1962).

Estrogen stimulates mammary tumors directly, and indirectly by stimulating PRL release (Meites and Nicoll, 1966). Estrogen had no effect on mammary tumorigenesis in the absence of the pituitary (Sterental et al., 1963). Evidence that estrogen plays more than an indirect role in tumor growth is demonstrated by the fact that tumor regression in ovariectomized-adrenalectomized rats was only temporarily reversed by PRL (Nagasawa and Yanai, 1970). In addition lesions of the median eminence stimulated PRL release and mammary tumor growth in rats (Sinha et al., 1973). However, when the ovaries were removed from these rats, mammary tumor regression occurred. Others have reported that PRL only slightly stimulates mammary tumors in ovariectomized adrenalectomized rats, but if PRL was administered in combination with only 0.01 ug of estradiol, the tumors responded with significant growth

increases (Leung and Sasaki, 1975). These results suggest that both estrogen and PRL are necessary for mammary tumor growth and development.

C. Progesterone

It has been reported that progesterone administration to rats prior to or shortly after carcinogen administration, significantly inhibits mammary tumorigenesis (Jabara and Harcourt, 1970; Welsch et al., 1968; Kledzik et al., 1974). Progesterone treatment after carcinogen, however, shortened the latency of mammary tumor appearance and increased tumor yield (Huggins et al., 1962). In the same study, these investigators also showed that induced pregnancy 15 days after DMBA administration significantly increased mammary tumorigenesis (Huggins et al., 1962). They concluded that elevated secretion of progesterone during pregnancy was responsible for stimulated mammary tumor growth. Thus, P treatment given prior to carcinogen administration inhibits, whereas, P treatment after carcinogen administration, stimulates mammary tumorigenesis in rats.

Studies with established mammary tumors have shown that P alone does not support tumor growth (Jabara, 1967; Horwitz and McGuire, 1977). It has been shown that P shortens the latency period and stimulates growth of DMBA-induced mammary carcinomas in androgenized female rats in the presence, but not in the absence of ovaries (Yoshida et al., 1980). Since estrogen is necessary for the synthesis of P receptors, these authors suggested that the lack of tumor stimulation by P in ovariectomized rats was due to the absence of P receptors. Their studies indicated that P, together with estrogen and PRL may be involved in supporting growth of hormone-dependent mammary tumors in rats.

D. Growth Hormone

The role, if any, of GH in mammary tumor development is uncertain. Long-term injections of GH were reported to result in increased incidence of mammary tumors in rats (Evans and Simpson, 1931; Moon et al., 1950). However, no increase in tumor incidence was seen in hypophysectomized rats given GH (Moon et al., 1951). Talwalker et al. (1964) reported that GH acted synergistically with PRL in promoting DMBA-induced mammary tumor development in ovariectomized rats. Growth hormone also was found to cause slight stimulation of mammary tumor tissue in vitro, but this stimulation was much less than that by PRL (Iturri and Welsch, 1976). These effects of GH should be viewed with caution, since the GH used was not completely pure and many have been contaminated with some PRL.

Administration of GH in rats with established mammary tumors had little effect on tumor growth (Nagasawa and Yanai, 1970; Li and Yang, 1974). Median eminence lesions have been shown to increase PRL and decrease GH secretion, but mammary tumor growth was markedly enhanced by such lesions (Clemens et al., 1968). The involvement of GH in rat mammary tumorigenesis does not appear to be critical and may only provide a supportive role.

E. Insulin

Insulin is known to be important for milk production in the lactating mammary gland. Injection of small doese of insulin to lactating rats can increase milk production (Kumareson and Turner, 1965), whereas induction of diabetes results in a sharp reduction of lactogenesis (Martin and Baldwin, 1971). Large doses of insulin can decrease lactation. Mammary gland explants in vitro have also been

shown to require insulin for maintenance and cell DNA synthesis in the normal mammary gland and it appears to be essential for mitosis to occur (Hallowes et al., 1973).

In the DMBA-induced mammary tumor, insulin stimulated DNA synthesis and tumor growth both in vivo and in vitro (Henson et al., 1967; Heuson and Legros, 1970). Insulin did not prevent mammary tumor regression as a result of ovariectomy (Heuson et al., 1972). However, diabetes-induced tumor regression was not prevented by injections of estrogen (Heuson and Legros, 1972). Insulin and PRL stimulated mammary tumor growth more than PRL treatment alone in hypophysectomized rats (Heuson et al., 1972). Induction of diabetes in tumor bearing rats resulted in significant reduction of tumor size (Heuson and Legros, 1972), and this was reversed by insulin replacement (Cohen and Hilf, 1974). Thus, insulin is of some importance for growth of mammary tumors and appears to play a facilitative or permissive role in the actions of other hormones.

F. Adrenal Glucocorticoids

Administration of adrenal glucocorticoids in rats inhibited the growth of mammary tumors (Hilf et al., 1965). Glucocorticoids inhibited mammary tumor growth directly, since breast tissue response depends on the presence of glucocorticoid receptors (Lippman et al., 1976). Adrenal glucocorticoid also inhibited mammary tumor growth indirectly by reducing AP secretion of PRL (Schwinn et al., 1976). Adrenalectomy in rats stimulated mammary tumor growth and increased serum PRL levels, and this was reversed by glucocorticoid replacement (Chen et al., 1976).

Aylsworth et al. (1979) demonstrated that elevated blood glucocorticoid levels were responsible for regression of DMBA-induced

mammary tumors in post-partum lactating rats. When tumor bearing rats were adrenalectomized immediately after parturition, regression did not occur and mammary tumor growth increased similarly to non-lactating intact controls (Aylsworth et al., 1979). Dexamethasone, a synthetic glucocorticoid, also has been shown to inhibit growth of established mammary tumors, even in the presence of elevated PRL levels (Aylsworth et al., 1980). These data strongly suggest that the mechanism by which glucocorticoids inhibit growth, is via direct action on the mammary tumor.

G. Concept of "Critical Period" After Carcinogen Administration

Acute changes in endogenous hormone levels in the rat play a critical role in initiation and development of carcinomas induced by aromatic hydrocarbons. Mammary tumors arise in undifferentiated, rapidly proliferating epithelial terminal end buds and terminal ducts, present in the mammary gland of young virgin females (Russo and Russo, 1978). The highest incidence of DMBA-induced mammary tumors arise at 50-55 days of age and are reduced markedly in younger and older animals (Huggins et al., 1961).

Dao (1962) established that there is a "critical period" for about 1 week after carcinogen administration in Sprague-Dawley rats. If the ovaries were removed prior to carcinogen treatment and then replaced by donor ovarian grafts 30 days later, no mammary tumors developed. If ovaries were removed 7 days after carcinogen treatment and 30 days later rats received ovarian grafts, a full complement of tumors developed. This experiment suggests that neoplastic transformation in the cells of the mammary gland cannot take place in the absence of ovarian hormones and PRL, since ovariectomy reduces PRL levels.

Estrogen and PRL induced mitosis in mammary epithelial cells (Huggins, 1965). By promoting cellular replication and DNA synthesis, these hormones sensitize the mammary gland to maximal carcinogen binding and subseqent tumor incidence at 55 days of age. Removal of estrogen and PRL by ovariectomy blocks hormone-dependent mitotic activity and renders the gland refractory to carcinogen action.

Numerous experimental studies have shown that increased or decreased estrogen and PRL levels prior to carcinogen administration result in significant inhibition of mammary tumorigenesis (Meites, 1972; Kledzik et al., 1974; Welsch et al., 1969; Dao, 1962; Clemens and Scharr, 1972; Cohen, 1981). Elevation of these hormones after carcinogen administration, however, results in a significant stimulation of mammary tumor growth. The decreased susceptibility of tumor induction by these hormones is attributed to acceleration (the state of elevated hormone levels) of mammary gland maturation during puberty. As stated earlier, mammary gland development before or after 55 days of age was refractory to tumor induction (Huggins et al. 1961). Thus, alterations of the hormonal millieu of the rat at the time of initiation establishes long-lasting and apparently permanent effects on mammary tumorigenesis, even when hormone levels return to normal shortly after this "critical period."

VI. Effects of Caloric Restriction

A. On Mammary Tumorigenesis

Early studies by Tannenbaum (1940) were the first to show that caloric-restriction decreased the incidence of spontaneous mammary tumors in mice. Animals subjected to chronic food-restriction not only had fewer mammary tumors, but tumor appearance also was later than in

animals fed ad libitum (Tannenbaum, 1940). Underfed animals appear to be in good general health, do not show signs of clinical nutritional deficiencies, and live longer than full-fed controls. Thus, the mechanism by which caloric-restriction inhibits mammary tumorigenesis does not appear to be due to the lack of some essential dietary component (Tannenbaum, 1942).

The relationship of the inhibition of tumor formation to the degree of caloric restriction is not linear. As caloric intake is reduced, there is only gradual inhibition of tumor formation. However, when dietary restriction reaches a critical level, there is a sharp inhibition of tumor formation (Tannenbaum, 1945a). Inhibition of mammary tumorigenesis by underfeeding was greatest when underfeeding was begun prior to tumor appearance (Tannenbaum, 1944). Ross and Bras (1971) demonstrated that early caloric-restriction at the time of tumor induction resulted in long-term inhibition of mammary carcinoma in rats. Thus, the timing, as well as the severity of underfeeding, are important determinants for the inhibition of mammary tumorigenesis. Recently, it was shown that as little as a 20% reduction in food intake over a 2 year period can significantly inhibit the development of spontaneous mammary tumors in rats and mice (Tucker, 1979).

Intermittent caloric-restriction, as in animals that are fasted twice a week for 24 hours, followed by ad libitum feeding between fasting, does not inhibit the incidence or growth of spontaneous mammary carcinoma, even though these animals consumed significantly less food over time, as compared to full-fed controls (Tannenbaum and Silverstone, 1950). These investigators also demonstrated a correlation between body weight and tumorigenesis in mice with varying degrees of caloric

restriction. They showed that larger animals are more susceptable to spontaneous mammary tumors than smaller animals, and food restriction was more effective for the inhibition of tumorigeneous in the large mice (Tannenbaum and Silverstone, 1953).

Caloric-restriction also inhibits the development of carcinogen induced mammary tumors. Underfeeding inhibits diethylstilbesterol (Dunning et al., 1949) and DMBA (Welsch and Meites, 1978) induced mammary tumors. Food-restriction also inhibited the growth of established mammary tumors, regardless of whether the tumors were chemically induced (Welsch and Meites, 1978; Leung et al., 1980), spontaneous (Tannenbaum, 1942), or transplanted (Tarnowski et al., 1955). Whereas restricted caloric intake decreased the incidence of spontaneous and carcinogen-induced mammary tumors, administration of large doses of carcinogen overcame this inhibition, regardless of the level of underfeeding (Tannenbaum and Silverstone, 1957).

Moderate chronic underfeeding has been shown to increae the cell-mediated immune response in rodents (Good et al., 1976). This heightened immune response in underfed animals may partially explain their lower tumor incidence. However, when food-restriction is severe, T- and B-cell function also is depressed (Good et al., 1976).

B. On Endocrine Function

The mechanisms by which underfeeding induces tumor suppression is not completely known, but it appears to involve the endocrine system. In studies with mice, normal mammary gland development was inhibited by food-restriction, and some investigators concluded this was the result of suppressed hormone stimulation (Huseby et al., 1945). Pituitary insufficiency as a result of underfeeding, leads to decreased estrogen

production as indicated by regression in size of the ovaries, uterus, and mammary gland, similar to that seen in hypophysectomized animals (Mulinos et al., 1940). This condition has been referred to as "pseudohypophysectomy" (Mulinos et al., 1940).

Additional evidence that the underfeeding causes a suppression of AP function was reported by Campbell et al. (1976). These investigators demonstrated that food-restriction resulted in decreased secretion of AP hormones, including PRL and gonadotropins, as measured by RIA. Underfeeding also reduced secretion of ovarian steroids, as indicated by initial irregularities and final loss of estrous cycles in rats (Piacsek and Meites, 1967).

Estrogen and PRL stimulated mitotic activity in mammary epithelial cells (Huggins, 1965), whereas underfeeding suppressed mitosis of this tissue, reflecting decreased secretion of these hormones (Bullough, 1950). A 50% reduction in food intake reduced the growth of established mammary tumors in rats, and this reduction was reversed by injections of estrogen and, haloperidol (the latter, to increase serum PRL levels), or the combination of estrogen and haloperidol (Welsch and Meites, 1978; Leung et al., 1980).

In addition to suppression of pituitary hormones, severe food-restriction can increase adrenocorticoid activity (Boutwell et al., 1948; Tannenbaum and Silverstone, 1957). Adrenal hyperfunction in food-restricted rats may also inhibit mammary tumorigenesis, since glucocorticoids have been shown to directly inhibit mammary tumor growth (Hilf et al., 1965).

VII. Development of Hormone-Dependent Versus Hormone-Independent Mammary Tumors

A. Hormonal Responses of Mammary Tumors

Most mammary tumors found in rats are hormone-dependent and regress after removal of the pituitary or ovaries. Hormone-dependent tumors may undergo histological changes characterized by carcinoma cell death, flattening of epithelium and large acumin lumina (Young et al., 1963). A small percentage of mammary tumors in rats are hormone-independent and are not influenced by changes in hormone levels.

Regression of hormone-dependent tumors as a result of ovariectomy or hypophysectomy can be reversed by estrogen and PRL replacement (Pearson et al., 1969). However, the degree and duration of mammary tumor growth, stimulated by hormone replacement, show great variability. Initially, it was suggested that PRL was the primary hormone needed to maintain mammary tumor growth (Pearson et al., 1969). Later, it was demonstrated that tumor growth maintained by PRL alone was only temporary in ovariectomized-adrenalectomized rats (Nagawawa and Yanai, 1970). Prolactin was found to maintain tumor growth in ovariectomized rats in only a few cells and estrogen was required for long-term stimulation of tumor growth (Sinha et al., 1973).

Studies which attempted to classify DMBA-induced mammary tumors as either estrogen or PRL dependent, indicated that few tumors can be placed in either category. Rather, hormone-dependent DMBA tumors are dependent on both estrogen and PRL (Bradley et al., 1976; Leung et al., 1975). Progesterone has recently been shown to have a primary role in maintaining growth of hormone-dependent mammary tumors. Progesterone alone can maintain static tumor growth in ovariectomized-

adrenalectomized rats (Minasian-Batmanian and Jabara, 1981). When perphenazine (which stimulates PRL release), at a dose low enough to only maintain static tumor growth, combined with P, significantly stimulated tumor growth (Minasian-Batmanian and Jabara,1981). These investigators also showed that in the absence of perphenazine, P was able to maintain static growth in the presence of low PRL levels. Thus, the mechanism by which P maintains static tumor growth is not dependent on the presence of PRL.

Hormone-dependency in DMBA-induced mammary tumors was shown to decline with increased age and increased size of tumors (Griswald and Green, 1970; Bradley et al., 1976). It was also shown by Griswald and Green (1970), that approximately 94% of the mammary tumors found in rats 3 months after DMBA administration can be classified as adenocarcinomas. At 5-6 months, this percentage drops to 80% and by 9 months only 40% of the tumors were adenocarcinomas. The majority of tumors found in rats 9 months after DMBA administration were classified as adenomatous hyperplasia, fibroadenomatous hyperplasia, or "mixed." Dao (1964) reported that while most DMBA induced tumors initially regress following hormone ablation, many of these tumors show growth after 2 months. These tumors showing renewed growth, however, were found to be entirely different tumor types from the original adenocarcinomas that regressed after ovariectomy.

It appears that DMBA-induced tumors are heterogeneous in cell population hence respond to hormonal manipulation differently. Hormone-dependent and hormone-independent tumors are found on the same animal. The growth rate of a tumor in response to a particular hormone may reflect the number of hormone-dependent versus hormone independent cells

within the tumor. A tumor which contains only hormone-dependent cells responds to hormone stimulation or ablation to a greater extent than a tumor with a small population of hormone-dependent cells. Tumor dependency on hormones changes with time and may indicate that cell populations are in a dynamic state and undergo differentiation with time.

B. Hormone Receptor Involvement

Hormone action is mediated by interactions with specific receptors found on or within the cell. Thus hormone receptors on mammary tumors, as in normal mammary tissue, may reflect the hormonal responsiveness of a tumor. This relationship of hormone receptors to hormone responsiveness has been the subject of extensive investigation. Estrogen and PRL binding is generally lower in hormone-independent than in hormone-dependent mammary tumors (McGuire et al., 1971; Turkington, 1974). However, because of the large variability of receptor concentration found in both types of tumors, it is impossible to make an accurate prediction of hormonal responsiveness based solely on estrogen or PRL binding sites (DeSombre et al., 1976; Holdaway and Friesen, 1976).

Tumor regression in ovariectomized rats results in a sharp decline of estrogen receptors, and estrogen or PRL replacement can only partially reverse this loss of estrogen receptors (Vignon and Rochefort, 1976). Estrogen replacement, however, does not restore receptor levels if serum PRL levels are suppressed in these rats. Prolactin has been shown to restore estrogen receptor levels in ovariectomized rats (Leung and Sasaki, 1975). By contrast, large doses of estrogen result in marked reduction of PRL binding in DMBA-induced mammary tumors in rats

(Kledzik et al., 1976). Prolatin binding is 3 times higher in DMBA-induced mammary tumors than in normal mammary tissue (Smith et al., 1976). Administration of PRL decreases PRL receptor number, but increases growth of the tumor, whereas decreasing serum PRL levels, result in reduced tumor growth but have no effect on receptor content (Smith et al., 1976). These investigators also found that low doses of estrogen increase tumor growth, but do not alter PRL receptor number. Others have shown that PRL administration will increases its own receptor number, but this results from an increase in the number of tumor cells (Lesneak and Roth, 1976).

When DMBA-induced mammary tumors were ranked according to their growth response to PRL administration, PRL receptor levels were highest in tumors that responded the most to PRL treatment (Kelly et al., 1974). Costlow and McGuire (1977) identified PRL receptor sites in DMBA-induced mammary tumors by using autoradiography. They found that in some tumors all cells contained PRL receptors, whereas in other tumors up to 50% of the cells remained unlabelled. These results demonstrate that mammary tumors contain heterogeneous cell populations, and tumor hormonal responsiveness may be directly related to the number of cells within that tumor that contains hormone receptors.

While it is impossible to predict hormone-dependency of mammary tumors on the basis of estrogen on PRL binding sites, when both hormone receptors are taken into account, a 90% accuracy of prediction is possible (DeSombre et al., 1976). In addition, c-AMP binding within a tumor has been shown to be inversely proportional to tumor growth (Bodwin et al., 1978). A very accurate prediction of hormone-dependency is demonstrated if estrogen, PRL, and c-AMP binding are all assessed

(Bodwin et al., 1980). Why certain mammary tumors that contain PRL and estrogen receptors continue to grow after ovariectomy remains unknown. Further understanding of hormonal involvement in the biochemical events that control mammmary tumor cell growth is needed.

MATERIALS AND METHODS

I. Research Animals

Animals used in studies described here were female Sprague-Dawley rats obtained from Harlan Research Facilities (Indianapolis, IN), and were housed in a temperature ($25\pm0.5^{o}C$) and light controlled (14 hr light, 0500-1900 hr /10 hr dark) room. All rats were provided Ralston Purina Rat Chow (Ralston Purina Co., St. Louis, MO) and water *ad libitum*, unless specified otherwise, throughout the periods of acclimation and experimentation.

II. Blood Sampling

Blood was collected by decapitation, by orbital sinus puncture under light ether anesthesia, or by a chronically implanted right atrial cannula. Saline (0.87% NaCl) filled intracardiac venous cannulae were implanted under ether anesthesia. Each silastic cannula (Dow Corning, Midland, MI, 0.025 in ID, 0.047 in OD) was inserted into the right external jugular vein 32 mm from the right atrium. The free end was brought underneath the skin to the back of the neck and exited 2 cm posterior to the base of the skull. Upon securing the cannula in place, it was flushed with 0.5 ml sterile saline and the free end was closed with a smooth wire plug. Immediately after surgery the animals were injected with 0.2 ml penicillin (30,000 U) and transferred to individual cages. On the day of experimentation, the wire plug was removed and a

silastic tubing extension 30 cm long, filled with sterile saline was attached to each cannula and placed outside each animal's cage. At this time, the cannula was again flushed with 0.5 ml saline. At no time was heparin used. The cannula extension was attached 2 hrs prior to the experiment so the animals could adapt to this new condition without removing the rats from the animal room. Blood was stored overnight at 4°C and serum was separated and frozen at -20°C until assayed for hormones.

III. Drug and Endocrine Manipulations

Drugs and hormones were administered by several different routes. The diluent, concentration and route of administration are stated in the Materials and Methods section of each experiment.

IV. Tumor Induction

Mammary tumors were reduced in animals by the method of Huggins et al. (1965). Virgin female rats, 55-60 days of age, were given a 1 ml lipid emulsion containing 5 mg of 7,12-dimethylbenz(a)-anthracene (DMBA) by tail vein injection under light ether anesthesia. The DMBA emulsion was kindly provided by the Upjohn Co., Kalamazoo, MI. Most tumors became palpable 1-3 mo after DMBA injection.

V. Tumor Measurements

Tumors were palpated and measured at weekly intervals, beginning 1 month after administration of DMBA. Palpable mammary tumors were measured with a vernier caliper and the 2 largest perpendicular diameters were recorded and averaged. Weekly tumor measurements were totaled for each rat, and expressed as "summation of average tumor diameter per rat" for each treatment group. Average period of tumor appearance was calculated for all tumors in each treatment group. A

tumor which had decreased by 5 mm or more in average tumor diameter was classified as regressing. A tumor that had increased by more than 5 mm in average tumor diameter was classified as growing, and a tumor that had changed less than 5 mm in average tumor diameter was considered stable. To determine hormonal-dependency of mammary tumors, tumor-bearing rats were bilaterally ovariectomized and tumor growth was followed during the subsequent weeks.

VI. Radioimmunoassay (RIA) of Hormones

Serum PRL, LH and FSH were measured by a double antibody technique of Niswender et al. (1968; 1969) or as described in the NIAMDD RIA kits. These are non-equillibrium assays which used a specific antibody to rat-PRL, rat-LH, and rat-FSH. Rat-PRL, LH and FSH were iodinated using chloramine-T, followed by separation on a P-60 bio-gel column (Bio-Rad Laboratories, Richmond, CA). Antibody-antigen complexes were precipitated by addition of rabbit gamma globulin antiserum produced in sheep. Serum samples were run in either duplicate or triplicate. Only serum volumes which gave hormone values which corresponded to the linear portion of the standard curve were used. Hormone concentrations were expressed as the mean $\pm$ standard error of the mean (S.E.).

VII. Statistical Analysis

Statistical differences between group means for serum LH, FSH and PRL levels, average number of LH pulses per 3 hr period, mean pulse amplitude, average latency period in tumor appearance, number of tumors per rat, average tumor diameter, summation of average tumor diameter and body weight were determined by one-way analysis of variance and Student-Newman-Keuls' tests for multiple comparisons among groups. Statistical differences in tumor incidence between treatment groups were determined

by X^2 with Yates' correction. The results were considered to be significant if $p<0.05$ when compared to controls.

EXPERIMENTAL

I. Effects of Morphine and Naloxone on Phasic Release of Luteinizing Hormone (LH) and Follicle-Stimulating-Hormone (FSH)

A. Objectives

Several recent studies have indicated that the EOP can influence the secretion of gonadotropic and other pituitary hormones. Acute injections of MOR or MET-ENK were reported to inhibit, whereas NAL, a specific opiate receptor antagonist, stimulated LH and FSH release (Bruni et al., 1977; Cicero et al., 1976). The EOP also have been implicated in the regulation of the proestrus LH surge in cycling female rats (Muraki et al., 1979) and in LH release in prepubertal rats (Ieiri et al., 1979). Morphine and EOP were shown to block ovulation and the preovulatory gonadotropin surge on the afternoon of proestrus (Barraclough and Sawyer, 1955; Pang et al., 1977) and these effects were reversed by NAL (Packman and Rothchild, 1976). The rise in LH produced by castration of male rats was partially prevented by MOR and enhanced by NAL (Van Vugt et al., 1982).

It previously was demonstrated that ovariectomized rats treated with EB showed a daily proestrous-like surge of LH, and that estrogen is the stimulus which "turn-on" the daily neural signal for LH release (Caligaris et al., 1971; Legan et al., 1975). It also was shown that injection of P to ovariectomized EB-primed rats enhanced the LH surge on

the day of P injection, but abolished subsequent LH surges (Legan and Karsch, 1975; Freeman et al., 1976). It is believed that P "turns-off" the expression of the daily LH surge induced by estrogen. The purpose of the present investigation was to examine the effects of MOR and NAL on the EB-induced daily surge signal for LH and FSH release in ovariectomized rats, and their effects on the ability of P to block expression of these daily neural signals.

B. Materials and Methods

Animals: Female rats, weighing 250-300 g, were ovariectomized for at least 4 weeks before treatment with ovarian steroids to induce daily gonadotropin surges.

Drugs: Morphine sulfate (MOR, Mallinkrodt Laboratories, St. Louis, MO), and naloxone hydrochloride (NAL, Endo Laboratories, Garden City, NY), were dissolved in 0.87% NaCl solution (SAL). Estradiol benzoate (EB) and progesterone (P, Sigma Chemical Co., St. Louis, MO), were dissolved in corn oil. Synthetic gonadotropin releasing hormone (GnRH) was kindly provided by Dr. K. Folkers (Inst. for Biomedical Research, University of Texas, Austin, TX), and was dissolved in saline. All injections were given subcutaneously (sc).

Experiments: In Experiment 1, 24 ovariectomized rats were given two injections of 20 ug EB at 1000 hours with an interval of 72 hours. On the day following the second injection, the animals were divided into 3 groups and given 4 injections to insure effective drug levels during the entire critical period for phasic gonadotropin release, of either MOR (5 mg/kg), NAL (0.2 mg/kg), or SAL, at 1300, 1500, 1700, and 1900 hours). Blood was collected via orbital sinus puncture under light

ether anesthesia at 1000, 1700, and 2000 hours on the day of drug treatment (day 1) and the following day (day 2).

In Experiment 2, 24 rats were treated with EB and drugs in the same manner as in Experiment 1. However, starting at 1500 hours on day 1, the animals were given 6 consecutive injections of either SAL or GnRH (50 ng /100 g B.W.) sc every 30 min. Blood was collected at 1000, 1800 and 2000 hours for 2 days, as in Experiment 1.

In Experiment 3, 24 rats were injected first with EB, followed 72 hours later by a 2.5 mg P injection at 1100 hours. On the day of P injection, drug treatments and blood sampling were performed in a manner similar to Experiment 1.

Experiment 4 was conducted in the same manner as Experiment 3, except that a 10 mg dose of P was used. Blood was taken at 1000 and 1700 hours on days 1 and 2.

Hormone Assays and Statistical Analysis

Serum levels of LH and FSH were assayed by standard RIA procedures with NIAMDD kits, kindly provided by Dr. A.F. Parlow. The LH results were expressed as ug/ ml in Figures 1 and 2 and ng/ml in Table 1, in terms of the respective reference preparation. Analysis of variance and Student-Newman-Keuls' test for multiple comparison among groups were used to analyze the data for the significance of differences among means.

C. Results

Effects of MOR and NAL on the LH and FSH Surges in EB- EB-Treated Ovariectomized Rats

EB treatment of control (SAL) rats induced an afternoon surge of LH on day 1 and 2 (Figure 1). The LH surge was blocked by MOR on day 1,

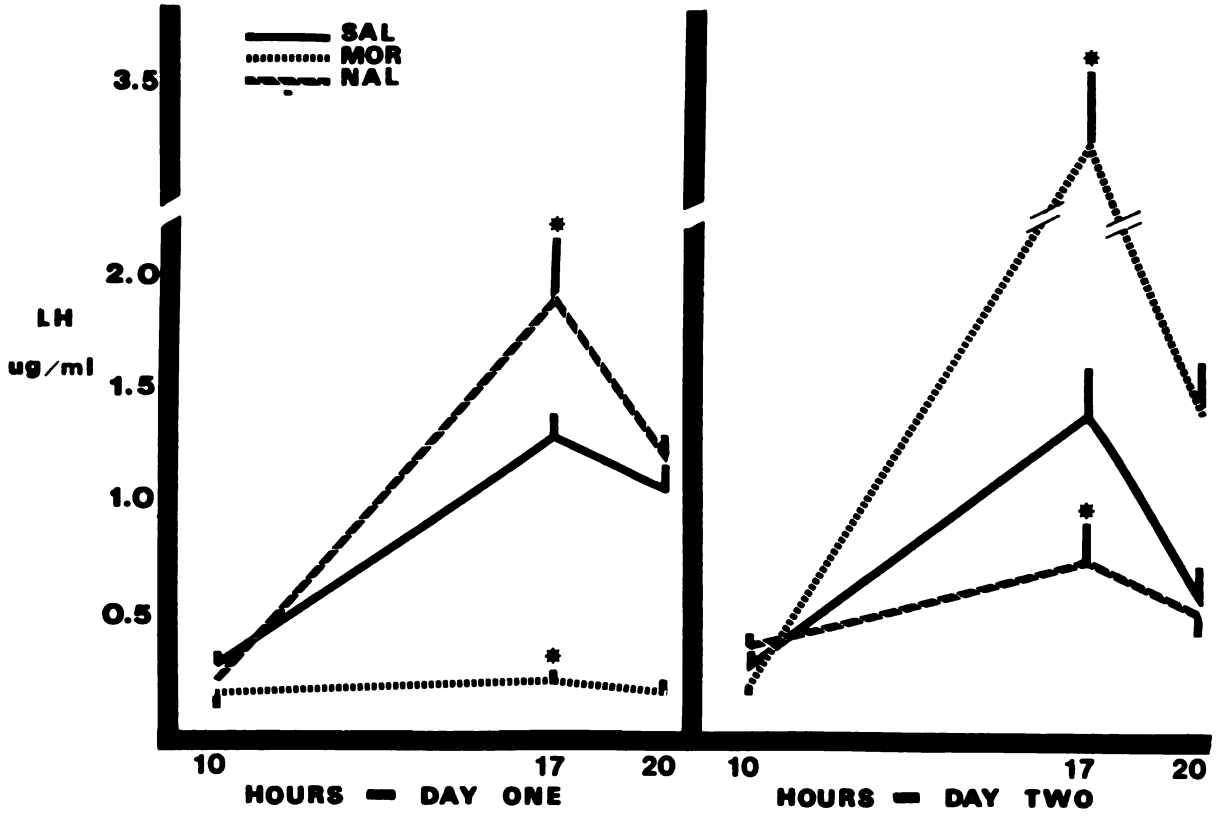


FIGURE 1.

Serum LH concentrations in estradiol benzoate (EB, 20 ug) primed ovariectomized rats on day 1 and day 2. Rats were given 4 sc injections of morphine (MOR, 5 mg/ kg), naloxone (NAL, 0.2 mg/kg), or saline (SAL, 0.87% NaCl) at 1300, 1500, 1700, and 1900 hours on day 1. Each point represents the mean and vertical bars represent the S.E. *$p<0.05$, as compared to SAL controls at 1700 h.

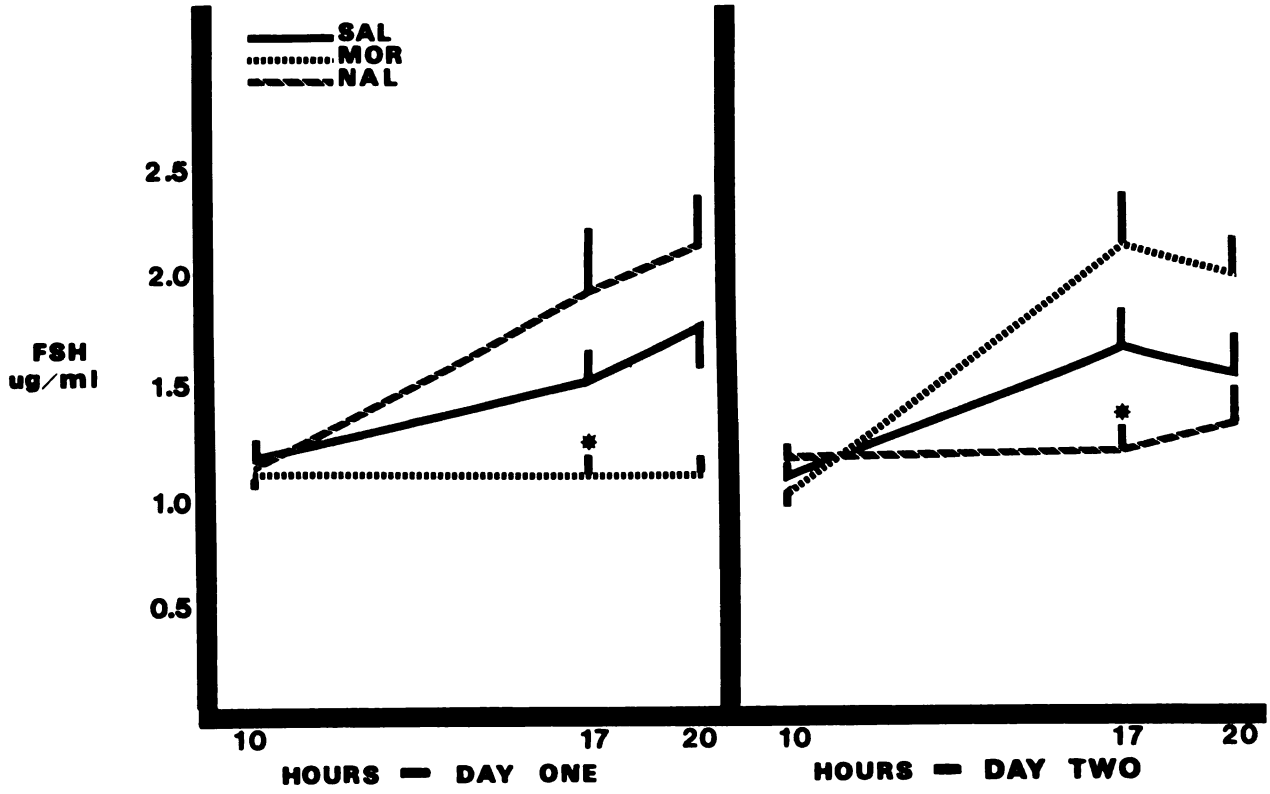


FIGURE 2.

Serum FSH concentrations in estradiol benzoate (EB, 20 ug) primed ovariectomized rats on day 1 and day 2. Rats were given 4 sc injections of morphine (MOR, 5 mg/ kg), naloxone (NAL, 0.2 mg/kg), or saline (SAL, 0.87% NaCl), at 1300, 1500, 1700, and 1900 hours on day 1. Each point represents the mean and vertical bars represent the S.E. *$p<0.05$, as compared to SAL controls at 1700 h.

whereas the NAL-treated group showed a significantly greater LH surge than the SAL-treated controls on day 1. On the next day, the trend was reversed, with the MOR group showing a large rebound LH surge and the NAL group showing no significant surge. Serum FSH showed a surge in the control rats similar to that of LH. MOR blocked, but NAL had no significant effect on the FSH surge on day 1 (Figure 2). On day 2, MOR-treated rats showed no effect, whereas NAL treatment suppressed the FSH surge.

Effects of GnRH on the LH and FSH Release in MOR and NAL-Treated Ovariectomized Rats Given EB-EB

Table 1 shows that the SAL and SAL controls displayed a surge of LH on days 1 and 2. The MOR and SAL group showed a block of the surge on Day 1 and a large rebound surge on day 2. The rats given MOR and GnRH showed a large LH surge on day 1 equal to that of SAL and GnRH-treated group and also showed a large surge on day 2. The SAL and GnRH group showed a very large LH surge on day 1 and day 2 surge equal to that of SAL and SAL controls. The NAL and SAL treated rats showed a characteristic potentiated LH surge on day 1 and on day 2, again showed a loss of the LH surge. The FSH response to the different treatments produced similar trends to that of LH. However, the effects of the treatments on serum FSH were of lesser magnitude than on LH (Table 2).

Effects of MOR and NAL on the LH and FSH Surges in EB-P Treated Ovariectomized Rats

The LH surge on day 1 in EB-P treated controls reached a peak about 3 times as high as in the EB-EB treated rats (Figure 3). MOR blocked the LH surge on day 1, but NAL had no effect on the LH surge. On day 2,

Table 1.
Effect of Morphine (MOR) and Naloxone (NAL) on Release of LH in Response to GnRH in EB-Treated Ovariectomized Rats

Treatment	Day One 1000 h	Day One 1800 h	Day One 2000 h	Day Two 1000 h	Day Two 1800 h	Day Two 2000 h
Saline + Saline (0.87%NaCl)(0.87% NaCl)	201±44[a]	1277±160	1112±80	261±24	1170±188	440±77
Morphine + Saline (5 mg/kg) (0.87% NaCl)	156±32	281±66*	319±77	264±34	3242±477*	1367±337
Morphine + GnRH (5 mg/kg) (50ng/100BW)	129±25	6496±834*	1254±195	396±74	2735±379*	1271±276
Naloxone + Saline (0.2 mg/kg)(0.87% NaCl)	227±48	1677±129	1358±268	246±32	588±117*	336±61
Saline + GnRH (0.87%NaCl)(50ng/100BW)	203±40	6393±488*	2375±331	216±38	1210±280	503±100

Number of rats = 8 per group. * $p<0.05$ as compared to controls (saline+saline) at 1800 h.
[a]Values expressed as Mean ± S.E.M. (ng/ml).
Serum LH concentrations in estradiol benzoate (EB, 20 ug) primed ovariectomized rats given 6 consecutive injections of either saline or GnRH (50 mg/100 BW) every 30 min starting at 1500 h.

Table 2.
Effect of Morphine (MOR) and Naloxone (NAL) on Release of FSH in Response to GnRH in EB-Treated Ovariectomized Rats

Treatment	Day One 1000 h	Day One 1800 h	Day One 2000 h	Day Two 1000 h	Day Two 1800 h	Day Two 2000 h
Saline + Saline (0.87%NaCl)(0.87%NaCl)	1080±89[a]	1375±112	1557±90	950±54	1320±62	1408±66
Morphine + Saline (5.0 mg/kg)(0.87%NaCl)	1248±116	1098±106	1364±235	1273±48	1754±72*	1923±249
Morphine + GnRH (5.0 mg/kg)(50ng/100gBW)	1315±135	2944±410*	1934±187	1140±59	1643±180	1937±155
Naloxone + Saline (0.2 mg/kg)(0.87%NaCl)	1122±101	1440±231	1673±112	996±150	1189±141	2345±90
Saline + GnRH (0.87%NaCl)(50ng/100gBW)	1130±157	2975±334*	2265±178	956±97	1567±73	1373±71

Number of rats = 8 per group. *$p<0.05$ as compared to controls (saline + saline) at 1800 hrs.
[a]Values expressed as Mean ± S.E.M.
Serum FSH concentrations in estradiol benzoate (EB, 20 ug) primed ovariectomized rats given 6 consecutive injections of either saline or GnRH (50 ng/100 BW) every 30 min starting at 1500 h.

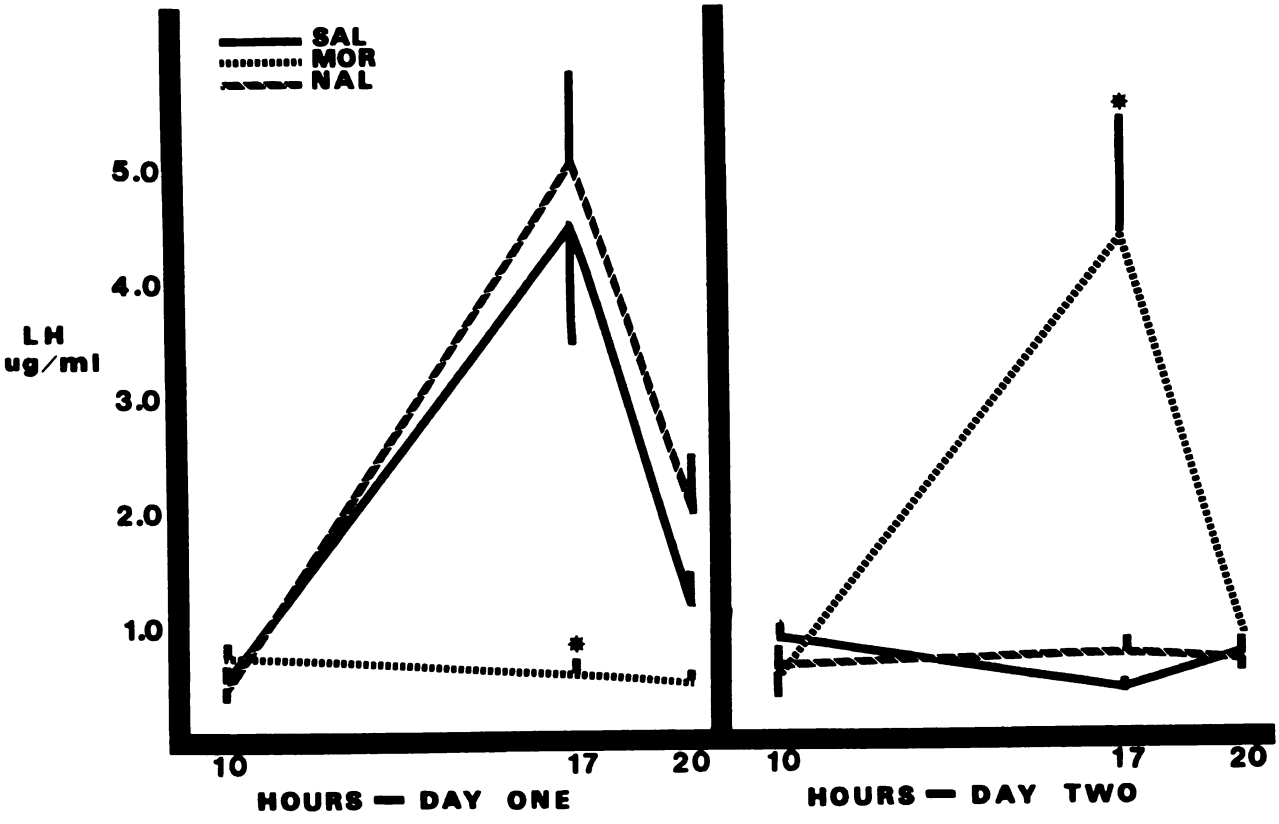


FIGURE 3.

Serum LH concentrations in estradiol benzoate (EB, 20 ug)-progesterone (2.5 mg) primed ovariectomized rats on day 1 and day 2. Rats were given 4 sc injections of morphine (MOR, 5 mg/kg), naloxone (NAL, 2 mg/kg), or saline (SAL, 0.87% NaCl), at 1300, 1500, 1700, and 1900 hours on day 1. Each point represents the mean and vertical bars represent the S.E. *$p<0.05$, as compared to SAL controls at 1700 h.

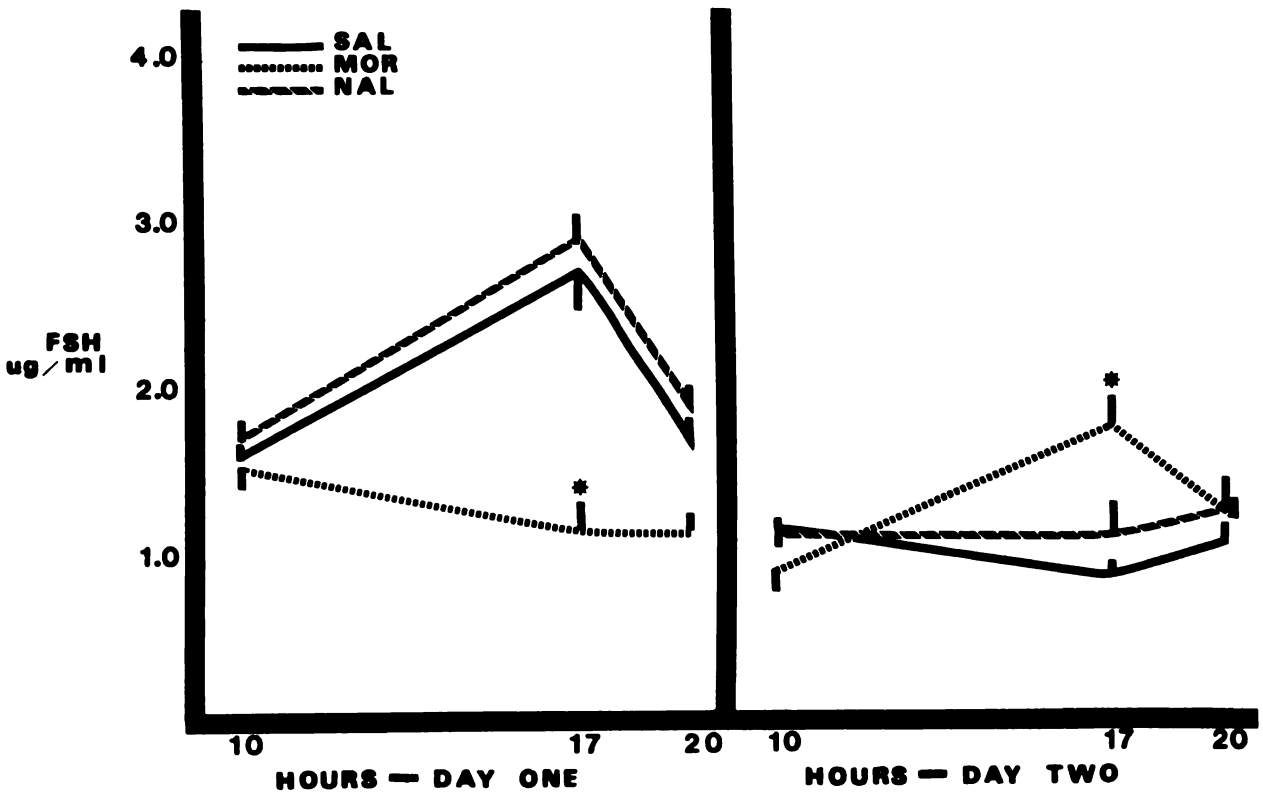


FIGURE 4.

Serum FSH concentrations in estradiol benzoate (EB, 20 ug)-progesterone (2.5 mg) primed ovariectomized rats on day 1 and day 2. Rats were given 4 sc injections of morphine (MOR, 5 mg/kg), naloxone (NAL, 0.2 mg/kg), or saline (SAL, 0.87% NaCl), at 1300, 1500, 1700, and 1900 hours on day 1. Each point represents the mean and vertical bars represent the S.E. *$p<0.05$, compared to SAL controls at 1700 h.

the NAL and SAL treated groups showed no LH surges in the EB-P treated rats. Surprisingly, however, the MOR treated group showed a large LH surge on day 2. Similar trends were observed on serum FSH after treatment with MOR or NAL (Figure 4).

To determine if the LH surge on Day 2 in MOR treated rats could be blocked by a higher dose of P, 10 mg/rat was given in Experiment 4. The NAL and SAL treated groups showed similar surges on LH on day 1, whereas the LH surge in the MOR group was blocked (Figure 5). On day 2, the LH surges were blocked in all groups. FSH responded similarly to LH (Figure 6).

D. Discussion

These observations show that MOR and NAL can alter expression of EB-induced daily surge signal in ovariectomized rats, not only on the day of drug treatment, but also on the next day. Previous observations demonstrated that MOR could inhibit the preovulatory surge of LH and ovulation in cycling rats (Barraclough and Sawyer, 1955; Pang et al., 1977), but subsequent events were not followed. In EB-EB treated rats, MOR blockade of the gonadotropin surge on day 1 resulted in a large rebound surge of LH on the afternoon of day 2. In contrast, NAL treatment potentiated the gonadotropin surge on day 1, but inhibited expression of the daily surge on day 2.

It seems unlikely that the unique effects of MOR and NAL on LH release on day 2 were due simply to the amount of hormones available for release by the pitutiary. MOR blockade of the LH surge on day 1 could have permitted a buildup of gonadotropin stores so that a rebound surge was seen on day 2. However, since rats given MOR and GnRH showed large

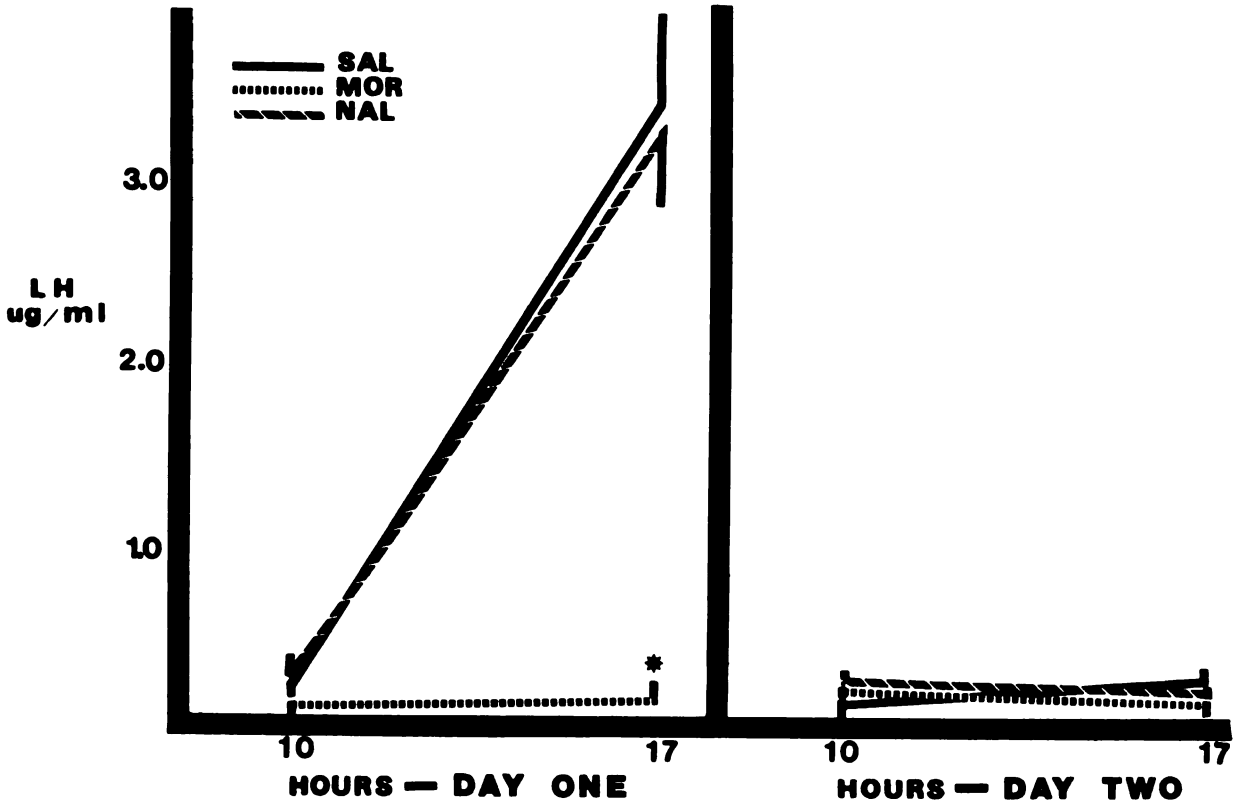


FIGURE 5.

Serum LH concentrations in estradiol benzoate (EB, 20 ug)-progesterone (10 mg) primed ovariectomized rats on day 1 and day 2. Rats were given 4 sc injections of morphine (MOR, 5 mg/kg), naloxone (NAL, 0.2 mg/kg), or saline (SAL, 0.87% NaCl), at 1300, 1500, 1700, and 1900 hours on day 1. Each point represents the mean and vertical bars represent the S.E. *$p<0.05$, compared to SAL controls at 1700 h.

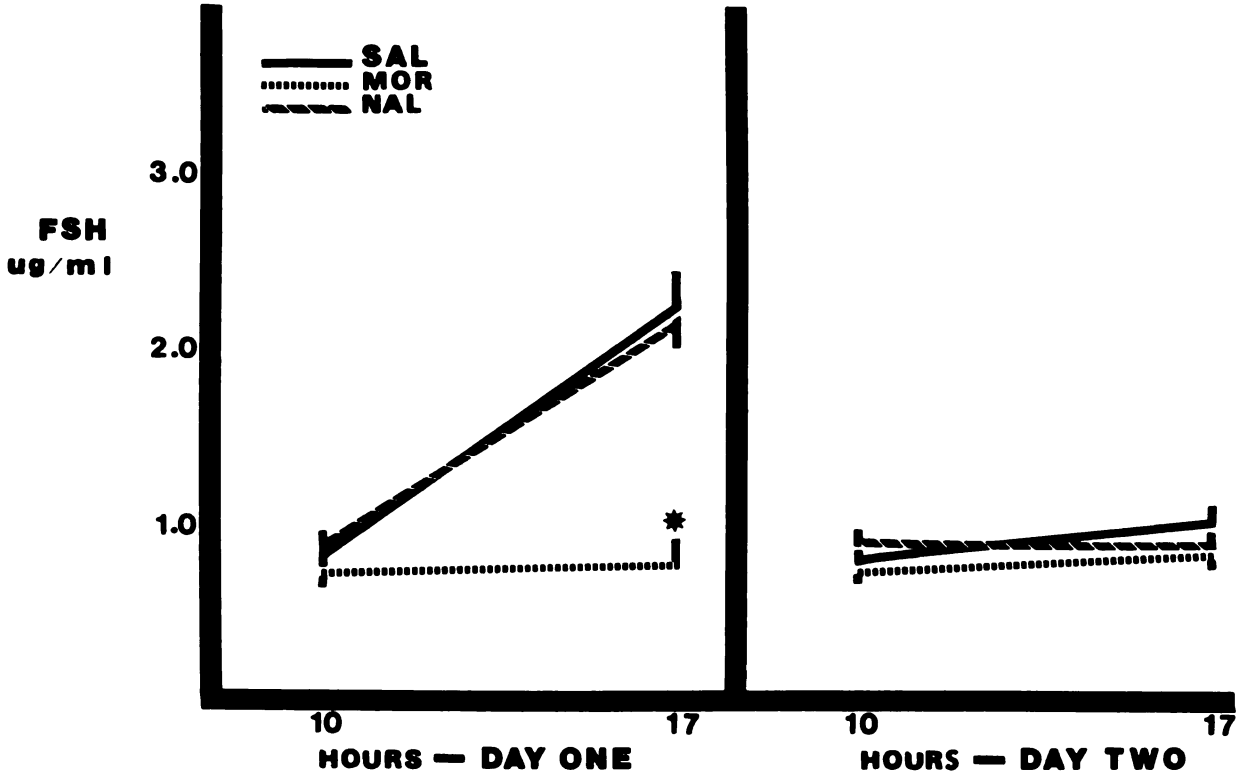


FIGURE 6.

Serum FSH concentrations in estradiol benzoate (EB, 20 ug)-progesterone (10 mg) primed ovariectomized rats on day 1 and day 2. Rats were given 4 sc injections of morphine (MOR, 5 mg/kg), naloxone (NAL, 0.2 mg/kg), or saline (SAL, 0.87% NaCl), at 1300, 1500, 1700, and 1900 hours on day 1. Each point represents the mean and vertical bars represent the S.E. *$p<0.05$, compared to SAL controls at 1700 h.

increases of LH on day 1 and demonstrated the same rebound surge of LH on day 2, it is reasonable to conclude that these surges seen on day 2 in MOR treated rats were not due merely to an increase in releasable gonadotropin stores on day 1. Likewise, the NAL potentiated surge on day 1 could have depleted the stores of gonadotropin and rendered the pituitary unable to respond with a normal gonadotropic surge on day 2. However, rats given SAL and GnRH did not show a suppression of the LH surge on day 2, as in the NAL and SAL-treated rats, even though the day 1 surge in the SAL and GnRH-treated rats was many times larger than that of the NAL and SAL-treated rats. Thus, the action of MOR and NAL on the gonadotropin surges are not believed to be due to alterations in capacity of the pituitary to secrete hormones, but rather to their central effects on the daily surge signal.

The EB-P induced LH surge in ovariectomized rats differs from that of EB-EB-treated animals, as previously reported, in that the surge was many times greater, and a subsequent LH surge did not occur. Estrogen is believed to turn on the daily surge signal, whereas P potentiates the LH surge on day 1, but turns it off subsequently (Freeman et al., 1976). Evidence also suggests that P may not inhibit the neural signal for the estrogen-induced LH surge, but rather render the hypothalamus unable to respond to the signal with sufficient GnRH release to induce a gonadotropin surge (dePaolo and Barraclough, 1979). NAL had no effect on the high LH surge in the EB-P-treated animals on day 1 or on the blockade of the gonadotropin surge on day 2. However, MOR blocked the EB-P-induced LH surges on day 1, but a large surge of LH was seen on the afternoon of day 2. This LH surge on day 2 in MOR-treated rats is in direct contrast to control rats in which P exposure inhibited the LH

surge on day 2. Nembutal blockage of the day 1 LH surge has also recently been shown to result in a large LH surge on day 2 in EB-P-treated ovariectomized rats (dePaolo and Barraclough, 1979). These results show that MOR injection on day 1 can antagonize the inhibitory effect of P on the gonadotropin surge induced by EB on day 2. This antagonism was found to be dose-related, since a higher dose of P (10 mg/rat) overcame the central action of MOR and prevented an LH surge on day 2.

The neural signal for the preovulatory gonadotropin surge in rats originates in the preoptic-anterior hypothalamic area (Goodman, 1978), where GnRH-containing neurons have been found (Flerkó et al., 1978). Under the appropriate estrogenic conditions, this signal results in the discharge of GnRH into the portal circulation (Fink et al., 1977; Sarkar and Fink, 1979). Estrogen also enhances the preoptic area stimulated release of GnRH (Sherwood et al., 1976) and increases the firing rate of the preoptic neurons (Fink and Geffen, 1978). Stimulation of the preoptic area by estrogen is believed to "turn-on" the daily preovulatory surge signal, whereas P decreases the firing rate of these neurons (Fink and Geffin, 1978) to possibly "turn-off" the surge signal.

Localization of ENK-containing neurons has been investigated immunohistochemically, and the distribution of these terminals was found to be adjacent to the cell bodies of the steroid-concentrating neurons in the preoptic and other areas of the hypothalamus (Sar et al., 1977). These observations suggest that the action of MOR and NAL occur at the preoptic-anterior hypothalamic areas of the brain, and that the opiates modulate steroid regulation of GnRH release. An alternate explanation

is that MOR and NAL act via other neurotransmitters in the brain to regulate GnRH release.

II. Effects of Morphine (MOR) and Naloxone (NAL) on Inhibition by Ovarian Hormones of Pulsatile Release of LH in Ovariectomized Rats

A. Objectives

Endogenous opioid peptides have been shown to inhibit secretion of LH, whereas NAL, a specific opioid antagonist, stimulates gonadotropin release (Bruni et al., 1977). The opiates also may participate in regulating the negative feedback of testosterone (Cicero et al., 1979) and estrogen (Van Vugt et al., 1982) on LH release, since NAL was able to counteract the inhibitory feedback by these steroids on LH release. The opiates and NAL have been shown not to alter GnRH-stimulated release of LH by the pituitary in vivo or in vitro (Cicero et al., 1977; 1979), indicating that their effects are mediated via hypothalamic mechanisms.

Luteinizing hormone in ovariectomized rats is released in a pulsatile manner (Gay and Sheth, 1972). Several hypothalamic neurotransmitters, as well as electrical stimulation of hypothalamic and other brain regions (Drouva and Gallo, 1976; Gallo and Osland, 1976; Gallo and Drouva, 1979; Gnodde and Schuiling, 1976; Weick, 1978) have been shown to greatly alter pulsatile LH release in ovariectomized rats. The ovarian steroid environment was shown to be of critical importance in determining the magnitude and direction of the LH response to these stimuli (Vijayan and McCann, 1978). The purpose of the present investigation was to examine the effects of MOR and NAL on the pulsatile release of LH in ovariectomized rats, with or without treatment with ovarian steroids.

B. Materials and Methods

Animals: Adult female Sprague-Dawley rats ovariectomized under ether anesthesia 4-5 weeks prior to use and weighed 350-400 g at the time of experimentation.

Blood Collection: Saline (0.87% NaCl) filled intracardiac venous cannulae were implanted under ether anesthesia. Drugs were injected via cannula every hour for the entire 3-hour experiment, starting 15 min prior to the first blood sampling. Blood samples in all experiments were taken at 15 min intervals for 3 hours (1000-1300 hours). An 0.1 ml sample of blood was first removed via a syringe. A second syringe was then used to withdraw 0.3 ml of blood, after which the contents of the first syringe were injected into the animal, followed by an injection of 0.3 ml of sterile saline. During the 3-hour period hematocrits decreased not more than 20%.

Drugs: Morphine sulfate (MOR, Mallinckrodt Laboratories, St. Louis, MO) and naloxone hydrochloride (NAL, Endo Laboratories, Garden City, NY) were dissolved in sterile saline (0.87% NaCl, USD, Cutter Laboratories, Berkeley, CA) and injected iv via an intra-atrial cannula. Treatment doses of MOR and NAL were selected based on their ability to effect changes in serum LH levels, as shown by time course and dose-response studies of these drugs in our laboratory (Bruni et al., 1977). EB and P (Sigma Chemicals, St. Louis, MO) were dissolved in corn oil and injected sc. A modification of the method of Ramirez and McCann (1963) was used in our study to examine the LH-releasing activity of MOR and NAL in steroid-primed ovariectomized rats.

Experiments: Each of the 3 experiments (1-3) contained 7 rats per treatment group. In Experiment 1, the day after cannula implantation, ovariectomized rats were given iv injections of MOR (5 mg/kg), NAL (2 mg/kg), MOR and NAL together, or saline, once every hour, starting at 1000 hours.

In Experiment 2, ovariectomized rats were given a sc injection of 20 ug EB at 1000 hours 3 days prior to drug treatment. Drug treatments and blood samplings were performed in a manner similar to Experiment 1 on the day following cannula implantation.

In Experiment 3, ovariectomized rats were given a sc injection of 20 ug EB and 10 mg P at 1000 hours days prior to drug treatment. Drug treatment and blood samplings were performed in the same manner as in Experiment 1 on the day after cannula implantation.

Hormone Assays and Statistical Analysis

Serum was separated by centrifugation and stored at -20°C until assayed for LH. Serum levels of LH were assayed by a standard RIA procedure with an NIAMDD kit, The serum LH values were expressed as ng/ml in terms of the NIAMDD rat LH-RP-1. Each experiment was assayed for LH separately and unknown serum samples were assayed in triplicates of 20 ul. An LH pulse was defined as occurring when serum LH concentrations rose in successive 15-min samples by at least 200 ng/ml. A pulse was considered to have terminated at a point before serum LH levels fell by at least 200 ng/ml. Analysis of variance and Student-Newman-Keuls' test for multiple comparisons among groups were used to determine if differences among means were significant.

C. Results

Experiment 1.

Effects of MOR and NAL on Pulsatile Release of LH in Ovariectomized Rats

SAL-treated ovariectomized rats showed episodic LH release, and amplitude and frequency of the pulses remained stable for the entire 3-hour sampling period (Figure 7). MOR treatment resulted in a significant decrease in LH pulse frequency, but did not alter the amplitude of pulsatile LH release when it did occur (Table 3). MOR treatment significantly reduced mean serum LH values when compared to SAL-treated controls. NAL treatment significantly increased the mean amplitude of LH pulses and slightly, but not significantly, increased the frequency of pulsatile LH release. NAL treatment also significantly increased mean serum LH levels. Rats treated with the combination of MOR and NAL displayed pulse frequency, amplitude and mean serum LH levels similar to values of SAL-treated controls.

Experiment 2.

Effects of MOR and NAL on the Pulsatile Release of LH in Ovariectomized-Estrogen-Treated Rats

Pretreatment with 20 ug of EB 3 days prior to drug treatment resulted in elimination of episodic LH release (Figure 8). MOR did not alter estrogen inhibition of pulsatile LH release, whereas NAL treatment reversed the inhibitory effects of estrogen on pulsatile LH release and the pulses were restored in all 7 animals tested. The average number of LH pulses for the 3-hour sampling period in NAL-treated rats was 4.14 ± 0.26, while the mean pulse amplitude was 445 ± 33 ng/ml. NAL treat-

FIGURE 7.

Effects of morphine (MOR) and naloxone (NAL) on pulsatile LH release in 2 representative animals in each treatment group of ovariectomized rats.

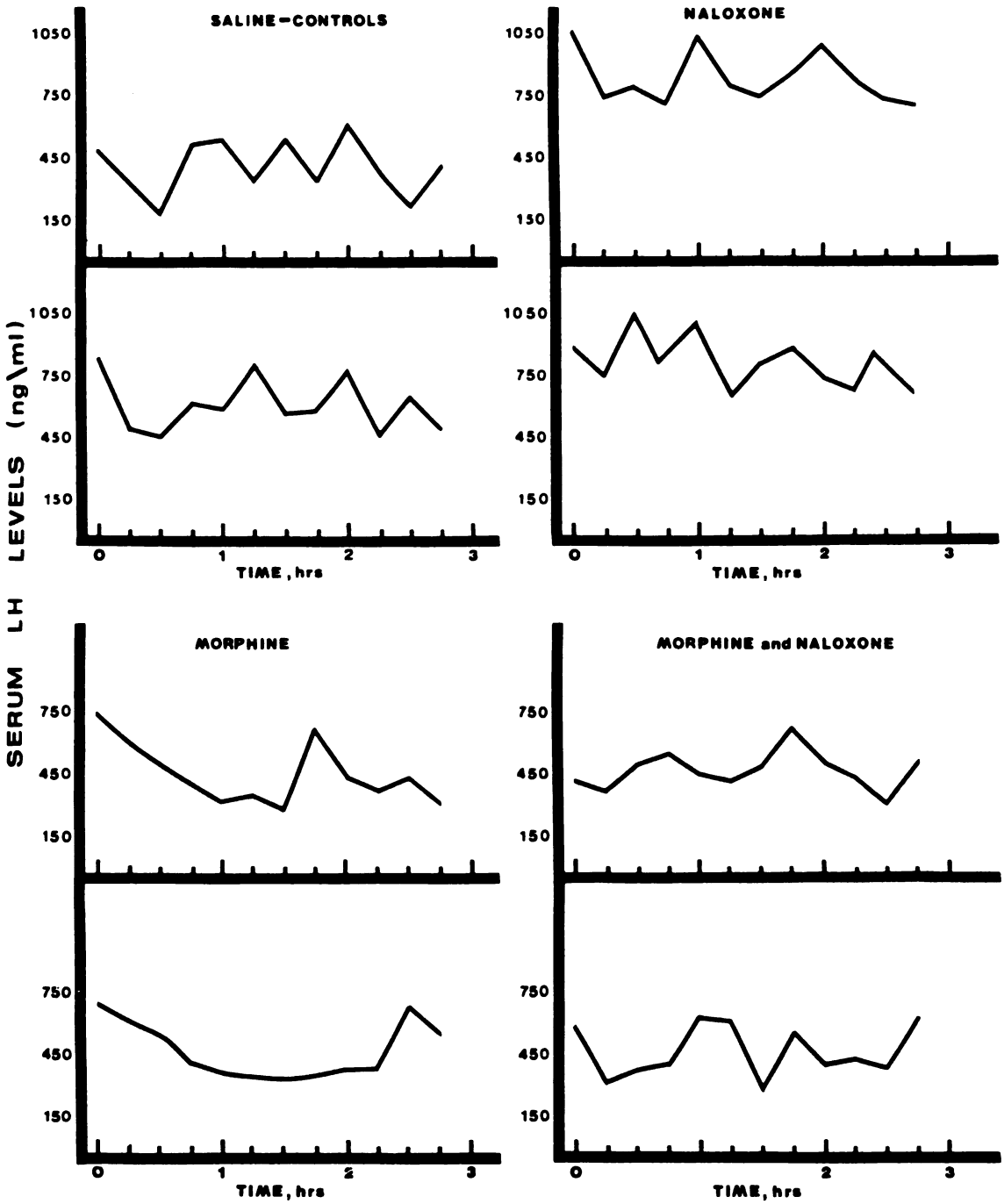

SALINE-CONTROLS
NALOXONE
MORPHINE
MORPHINE and NALOXONE
SERUM LH LEVELS (ng\ml)
1050
750
450
150
0
1
2
3
TIME, hrs

Table 3.

Effects of Morphine (MOR) and Naloxone (NAL) on Mean Number and Amplitude of LH Pulses

Treatment	Total Number of LH Pulses / All Rats	Average Number of LH Pulses / 3 h ± S.E.	Mean Pulse Amplitude ng/ml±S.E.	Mean Serum LH Levels ng/ml±S.E.
Control (0.87% NaCl)	33	4.71 ± 0.18	223 ± 30.0	582 ± 22
Morphine (5 mg/kg)	12	1.72 ± 0.14*	211 ± 38.1	496 ± 21*
Naloxone (2 mg/kg)	37	5.29 ± 0.18	288 ± 21.1*	742 ± 13*
Morphine (5 mg/kg)+ Naloxone (2 mg/kg)	30	4.29 ± 0.36	220 ± 33.3	567 ± 13

*$p<0.05$ as compared to saline-treated controls. Number of animals per group = 7.

ment almost tripled mean serum LH levels when compared to serum LH levels in controls (Table 4).

Experiment 3.

Effect of MOR and NAL on the Pulsatile Release of LH in Ovariectomized-Estrogen-Progesterone-Treated Rats

Pretreatment with 20 ug EB and 10 mg P 3 days prior to blood sampling resulted in the elimination of pulsatile release of LH (Figure 9). NAL treatment blocked the inhibitory effect of the steroids and pulsatile LH release was observed in 6 of 7 animals. The average number of LH pulses for the 3-hour sampling period in NAL-treated rats which showed pulses, was 3.42 ± 0.48, and mean pulse amplitude was 306 ± 46 ng/ml. NAL treatment more than doubled mean serum LH levels when compared to serum values in the steroid-treated ovariectomized rats (Table 5). Morphine had no effect on steroid inhibition of pulsatile LH release.

D. Discussion

These results demonstrate that in estrogen and estrogen-progesterone-treated ovariectomized rats, NAL blocked the inhibitory effects of these steroids on pulsatile LH release. Previously, NAL was shown to block testosterone inhibiton of LH release in castrated male rats (Cicero et al., 1980) and to block estrogen inhibition of LH release in ovariectomized rats (Van Vugt et al., 1982). Naloxone treatment has also been found to enhance pulsatile LH release in women during both the late follicular phase (Quigley and Yen, 1980) and luteal phase (Ropert et al., 1981) of the menstrual cycle. These reports, together with the present findings, are believed to indicate that the EOPs participate in mediating the negative feedback exerted by gonadal steroids on the hypothalamic-hypophysial-LH system.

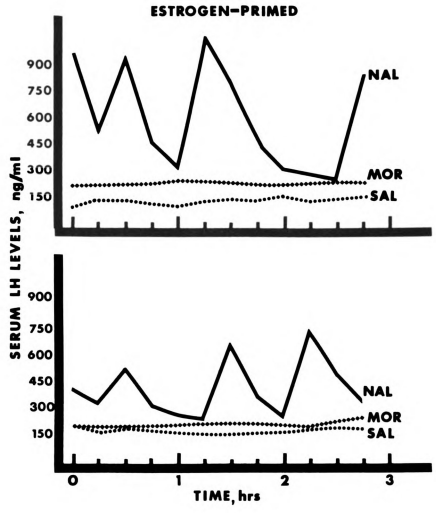


FIGURE 8.

Effects of morphine (MOR, 5 mg/kg), naloxone (NAL, 2 mg/kg), or saline (SAL, 0.87% NaCl) on pulsatile LH release in 2 representative animals (top and bottom) in each treatment group of ovariectomized rats treated 3 days earlier with 20 ug estradiol benzoate (EB).

Table 4.

Effects of Morphine (MOR) and Naloxone (NAL) on Mean Serum LH Levels in Ovariectomized Rats Treated 3 Days Earlier with 20 μg Estradiol Benzoate Per Rat

Treatment	N	ng LH / ml
Controls (0.87% NaCl)	7	159 ± 8*
Morphine (5 mg/kg)	7	190 ± 11
Naloxone (2 mg/kg)	7	459 ± 30**

* Mean ± S.E.
**$p<0.05$ as compared to saline treated controls.

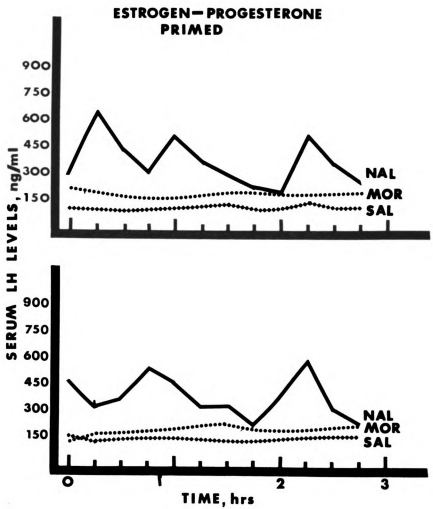


FIGURE 9.

Effects of morphine (MOR, 5 mg/kg), naloxone (NAL, 2 mg/kg), and saline (SAL, 0.87% NaCl) on pulsatile release of LH in 2 representative animals (top and bottom) in each treatment group of ovariectomized rats treated 3 days earlier with 20 ug estradiol benzoate (EB) and 10 mg progesterone (P).

Table 5.

Effects of Morphine (MOR) and Naloxone (NAL) on Mean Serum LH Levels in Ovariectomized Rats Treated 3 Days Earlier With 20 µg EB and 10 mg Progesterone per Rat

Treatment	N	ng LH / ml
Control (0.87% NaCl)	7	144 ± 5*
Morphine (5 mg/kg)	7	189 ± 7
Naloxone (2 mg/kg)	7	357 ± 18**

* Mean ± S.E.
**$p<0.05$ as compared with saline treated controls.

After injection, estrogen acts directly on the pituitary to inhibit LH release in ovariectomized rats, followed 6-9 hrs by a facilitatory action (Henderson et al., 1977). It has also been shown that ovariectomized estrogen-P-treated rats demonstrate a high sensitivity for LH-releasing activity three days following steroid treatment (Ramirez and McCann, 1963). Thus, NAL was administered after the acute direct inhibitory effects of estrogen or EB-P treatment on serum LH levels had occurred in ovariectomized rats, permitting us to study the LH-releasing activity of NAL.

The mechanism by which MOR, the EOPs, and NAL exert their effects on LH release is not entirely clear. They do not appear to exert their effects directly on the pituitary (Cicero et al., 1977; 1978), suggesting that their actions are mediated via hypothalamic mechanisms. There is considerable evidence that the noradrenergic system is a major promoter of GnRH release. Intraventricular injections of NE have been shown to increase serum LH levels (Krieg and Sawyer, 1976; Van Vugt et al., 1980) and to stimulate pulsatile LH release (Gallo and Drouva, 1979) in steroid-treated ovariectomized rats. The opiates apparently inhibit hypothalamic NE activity, since our laboratory recently found that NAL-stimulated LH release was associated with an increase in hypothalamic NE turnover (Van Vugt et al., 1981), and increased GnRH release from the hypothalamus (Van Vugt et al., 1980). Shortly after injection, MOR increased GnRH concentration in the hypothalamus (Simpkins and Kalra, 1980), probably reflecting inhibition of GnRH release. MOR also was reported to block catecholamine-induced GnRH release from hypothalamic tissue in vitro (Rotsztejn et al., 1978). Intraventricular infusion of NE, however, suppressed or had no effect on

pulsatile LH release in ovariectomized rats not treated with ovarian steroids (Gallo and Grouva, 1979). NAL treatment in ovariectomized rats, however, significantly enhanced pulsatile LH release and therefore the stimulatory action of NAL on LH release in ovariectomized rats may not be due entirely to activation of a hypothalamic noradrenergic mechanism.

MOR and the brain opiates have been shown to reduce hypothalamic DA activity (Ferland et al., 1977; Van Vugt et al., 1979), and to increaes 5-HT activity (Ieiri et al., 1980; Van Loon and deSouza, 1978), whereas NAL was reported to decrease 5-HT activity (Ieiri et al., 1980) and may increase DA activity. Dopaminergic and serotonergic mechanisms also were reported to be involved in the regulation of pulsatile LH release (Arendash and Gallo, 1978; Drouva and Gallo, 1976; Gallo and Drouva, 1979; Guoddi and Schuiling, 1976). Opioid-containing neurons have been found to be located in high concentrations in the hypothalamus and median eminence, the terminals of these neurons to be intimately associated with steroid concentrating and GnRH-containing neurons (Sar et al., 1977; Tramus and Leonardelli, 1979). These observations suggest, therefore, that the actions of NAL and MOR on pulsatile LH release in ovariectomized rats, treated or not treated with ovarian steroids, are mediated via hypothalamic neurotransmitters that in turn alter GnRH release.

III. Relationship of Hormones to Inhibition of Mammary Tumor Development by Underfeeding During the "Critical Period" After Carcinogen Administration

A. Objectives

Chronic restriction of food intake inhibits development of mammary tumors in mice and rats (Dunning et al., 1949; Tannenbaum and Silverstone, 1950). Food-restricted animals not only showed fewer mammary tumors, but tumor appearance also was later than in animals fed ad libitum (Tannenbaum, 1942). The mechanisms by which food restriction influences mammary tumorigenesis are not entirely clear. However, it has been shown that food restriction results in decreased secretion of AP hormones, including PRL and gonadotropins (Campbell et al., 1977). Mammary tumors induced by DMBA have been shown to be mainly dependent on PRL and estrogen stimulation (Meites, 1979), although PRL may be somewhat more important than estrogen in the rat (Meites et al., 1971; Pearson et al., 1969). Estrogen acts directly on the mammary tissue, as well as indirectly by stimulating pituitary PRL release (Meites, 1979). No definite role for GH has been established on mammary tumor development in rats (Evans and Simpson, 1931; Moon et al., 1951).

Dao (1962) established that there is a "critical period" of about one week after carcinogen treatment of Sprague-Dawley rats for establishment of mammary tumors. He reported that, if the ovaries were removed immediately after carcinogen treatment, no mammary tumors developed; but, if the ovaries were removed seven days after carcinogen treatment, a full complement of mammary tumors developed. It was of interest, therefore, to determine whether administration of PRL, estrogen, GH, or all three together, given during the "critical period"

after carcinogen administration, could overcome the inhibition produced by underfeeding on development and growth of mammary tumors in rats.

B. Materials and Methods

Treatments: Seven days prior to DMBA administration, 50-day old virgin female rats were divided into 6 groups (A to F), with 17 to 18 rats /group. Group A was fed rat chow (Ralston Purina Co., St. Louis, MO) ad libitum and served as full-fed controls. They consumed an average of about 20 g daily. Food-restricted rats were given 10 g of food once a day between 1000 and 1200 hrs; and it was noted that the entire ration was quickly consumed by the hungry rats.

At 57 days of age, the rats were each given a single iv injection of 1 ml lipid emulsion containing 5 mg 7,12-dimethylbenz(a)anthracene (DMBA, Huggins et al., 1959). Starting 1 day prior to and continuing for 7 days after DMBA injection, animals were subjected to various drug and hormone treatments. Groups A and B received a daily 0.1 ml sc injection of each vehicle (1 injection of corn oil and 2 injections of 0.87% NaCl solution). Group C received a daily sc injection of haloperidol (HAL, McNeil Laboratories, Ft. Washington, PA), at a dose of 0.5 mg/kg, suspended in 0.1 ml 0.87% NaCl solution, plus a 0.1 ml injection of both corn oil and 0.89% NaCl solution. HAL, a DA receptor blocker, was administered to increase pituitary PRL release. Group D was given a daily s.c. injection of bovine GH at a dose of 0.5 mg, suspended in 0.1 ml 0.87% NaCl solution to increase serum GH levels, plus a 0.1 ml injection of both corn oil and 0.87% NaCl solution. Group E received a daily sc injetion of EB (Sigma Chemical Co., St. Louis, MO) at a dose of 1 ug dissolved in 0.1 ml corn oil, to raise serum estrogen levels, plus 2 injections of 0.1 ml 0.87% NaCl solution. Group F was given a daily

0.1 ml sc injection of HAL (0.5 mg/kg), EB (1 ug/kg), and GH (0.5 mg/kg).

After 8 days of treatment, injections were terminated, but restricted food intake was continued until 30 days after DMBA administration. At this time, the caloric-restricted groups B to F were returned to ad libitum feeding for the remainder of the experiment.

Tumor Measurements: Tumor measurements and body weights were recorded at weekly intervals from the beginning until termination of the experiment. Average tumor diameter for each palpable tumor was determined by using the mean of the two largest perpendicular diameters as measured with vernier calipers. Tumor size was expressed as the summation of average tumor diameter of all tumors found in a treatment group. Average latency period was calculated for all tumors in a group.

Blood Collection and Hormone Assays: Blood was collected under light ether anesthesia by orbital sinus puncture on the last day of drug and hormone administration (7 days after DMBA administration) and on the last day (37th) of food restriction. The final blood sample was collected upon termination of the experiment (26th week) by decapitation, and mammary tumors were examined by gross dissection. In all three sampling periods, blood was collected between 1000 and 1100 hours, when PRL levels in female rats are approximately equal throughout the estrous cycle. Serum was separated by centrifugation and stored at -20°C until assayed for PRL by a standard RIA method.

Statistical Analysis: Statistical differences in tumor incidence between treatment groups were determined by X^2 with Yates' correction (1934). Statistical differences in serum PRL levels, average latency

period in tumor appearance, number of tumors per rat, and average tumor diameter between groups were determined by analyses of variance and Student-Newman-Keuls' test, used for multiple comparisons among groups. The results were considered to be signifiant if $p<0.05$ when compared to food-restricted controls.

C. Results

The effects of the different treatments on mammary tumor incidence are shown in Table 6. Tumor incidence in the full-fed controls (Group A) was 75%, and the average number of tumors per rat was 2.69. Average tumor latency period was 106.6 ± 6.5 days. Food restriction for 7 days prior to the 30 days after DMBA administration (Group B) decreased the incidence of tumors to only 29%, and average latency was 140.8 ± 7.8 days. These values were significantly different from those in the full-fed controls. The food-restricted rats, which received daily injections of HAL for one day prior to and 7 days after DMBA administration (group C), showed a slight but nonsignificant increase in incidence of tumors (60%) and a decrease in average latency period to 125.5 ± 6.6 days, when compared with food-restricted controls not given HAL (group B). However, the differences between groups C and B were not statistically significant. The food-restricted rats, which received daily injections of GH one day prior to and 7 days after DMBA administration (group D), showed no differences in tumor development when compared with the food-restricted controls (group B). Only 1 tumor appeared early in this group which resulted in a reduced average latency of 105.9 ± 8.8, as compared with the food-restricted (group B), and this difference was not found to be significant. Underfed rats, which received daily injections of EB for one day prior to and 7 days after DMBA administration (group

Table 6.

Effects of Different Drug and Hormone Treatment During the "Critical Period" in Underfed Rats on Development of Mammary Tumors at the End of 26 Weeks

Group	Treatment	No. of Rats / Group Initial	No. of Rats / Group Survived	No. of Rats With Tumors	Total No. of Tumors	% of Rats With Tumors	No. of Tumors /Tumor Bearing Rat	Average Latency Period (Days)
A	Full-Fed	18	17	13	33	76*	2.54	106.6±6.5*
B	Underfed	18	17	5	11	29	2.20	140.8±7.8
C	Underfed+HAL	17	15	9	13	60	1.44	125.5±6.6
D	Underfed+GH	17	15	5	7	33	1.40	105.9±8.8
E	Underfed+EB	17	14	10	24	71*	2.40	126.2±3.0
F	Underfed+HAL + GH + EB	17	14	12	33	86*	2.75	131.3±3.9

* $p<0.05$, as compared to caloric restricted controls (Group B).

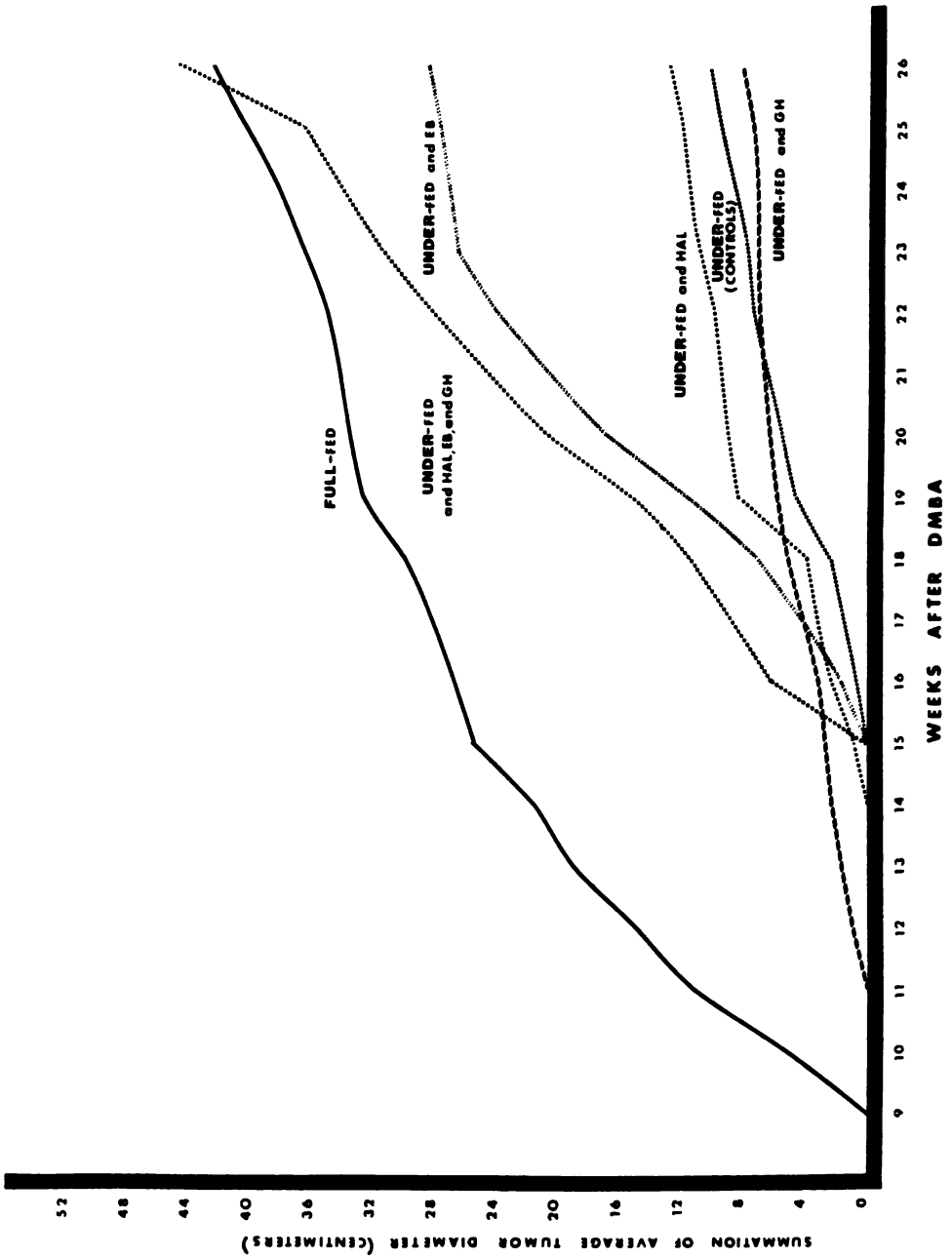
E), showed a significant increase in tumor incidence (71%). The decreased average latency period of 126.2±3.0 days was not found to be significantly different from that in the food-restricted controls (group B). Underfed rats, which received daily injections of HAL, GH, and EB 1 day prior to and 7 days after DMBA administration (group F), showed a significant increase in tumor incidence (86%) and in average number of tumors per rat (2.75). The average tumor latency period of 131.3±3.9 days was not significantly different from that in food-restricted controls (group B).

The effect of drug and hormone injections on mammary tumor size in the different treatment groups are shown in Figure 10. Tumors first appeared in the full-fed controls (group A) approximately 9 weeks after DMBA administration, and tumor size continued to increase for the duration of the experiment. Three tumors in the full-fed group were found to show regression after a period of growth. This regression was not complete. These tumors were included in determining average tumor diameter for the full-fed group (group A) in Figure 10. All the food-restricted groups, except the half-fed rats given GH (Group D), showed a delayed appearance of tumors which first appeared 13 to 14 weeks after DMBA administration.

The control rats restricted to underfeeding for 7 days prior to and 30 days after DMBA administration (group B) showed severe suppression of tumor size that persisted throughout the entire 26-week experiment (Fig. 9). Food-restricted animals in group C, which received daily injections of HAL one day prior to and 7 days after DMBA administration, showed a slightly earlier onset of mammary tumors, but no differences were seen in average tumor size, as compared with that of the food-restricted

FIGURE 10.

Summation of average tumor diameter per week after DMBA administration in under-fed rats with or without drug and hormone treatment during the "critical period." *$p<0.05$, as compared to under-fed controls.


SUMMATION OF AVERAGE TUMOR DIAMETER (CENTIMETERS)
0
4
8
12
16
20
24
28
32
36
40
44
48
52
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
WEEKS AFTER DMBA
FULL-FED
UNDER-FED and HAL, EB, and GH
UNDER-FED and EB
UNDER-FED and HAL
UNDER-FED (CONTROLS)
UNDER-FED and GH

controls (group B). The food-restricted animals in group D, which received daily injections of GH one day prior to and 7 days after DMBA administration, showed no differences in tumor size as compared to tumor size in the underfed controls (group B). Only 1 rat in this group showed early appearance of a single mammary tumor. Food restricted animals in group E, which received a daily injection of EB one day prior to and 7 days after DMBA administration, showed increased tumor size when compared to the food-restricted controls (group B), but tumor size was lower than in the full-fed controls (Group A). Food-restricted animals in Group F given daily injections of HAL, GH, and EB one day prior to and 7 days after DMBA administration showed significant increases in tumor incidence and size of tumor when compared with the food-restricted controls (Group B). These rats reached an average tumor size equal to that of the full-fed controls.

Serum PRL for each treatment group is shown in Table 7. The first blood sample was collected on the last day of treatment (7 days after DMBA administration) and showed that serum PRL levels were suppressed in the food-restricted controls (Group B), as compared with the full-fed controls (Group A). The food-restricted rats given HAL showed a significant increase in serum PRL, as did the food-restricted rats given EB. The food restricted rats given the combination of HAL, GH, and EB showed significantly greater serum PRL levels than any of the other groups. The serum PRL levels of all groups of half-feeding were significantly lower on the last day of food restriction (37th day) than full-fed controls (Group A). On the day the experiment was terminated (26th week) and when all animals had long since returned to ad libitum feeding, assays showed no differences in serum PRL levels amoung groups.

Table 7.

Serum Prolactin (PRL) Levels (ng/ml) at Different Periods of Drug, Hormone and Food-Restricted Treatments in Rats

Sampling	Full-Fed Controls Group A	Underfed Controls Group B	Underfed + HAL Group C	Underfed + GH Group D	Underfed + EB Group E	Underfed + HAL + GH + EB Group F
15th Day[a]	31.5±3.5[b]	9.8±1.2	63.2±8.8[b]	10.1±1.6	44.3±6.1[b]	134.5±18.2[c]
37th Day[d]	42.1±5.3[b]	5.1±1.0	4.8±1.2	6.5±1.4	9.2±3.2	5.9± 1.3
26th Week[e]	16.2±3.1	16.5±3.3	19.6±2.4	12.3±2.2	18.7±2.8	15.5± 2.9

[a]Blood collected 7 days after DMBA-administration. Blood removed by orbital sinus puncture.
[b]$p<0.05$, as compared to caloric-restricted controls (Group B).
[c]$p<0.05$, as compared to Groups B, C, and E.
[d]Blood collected on the last day of food-restricted diets. Blood removed by orbital sinus puncture.
[e]Blood collected after return to *ad libitum* feeding. Blood removed by decapitation.

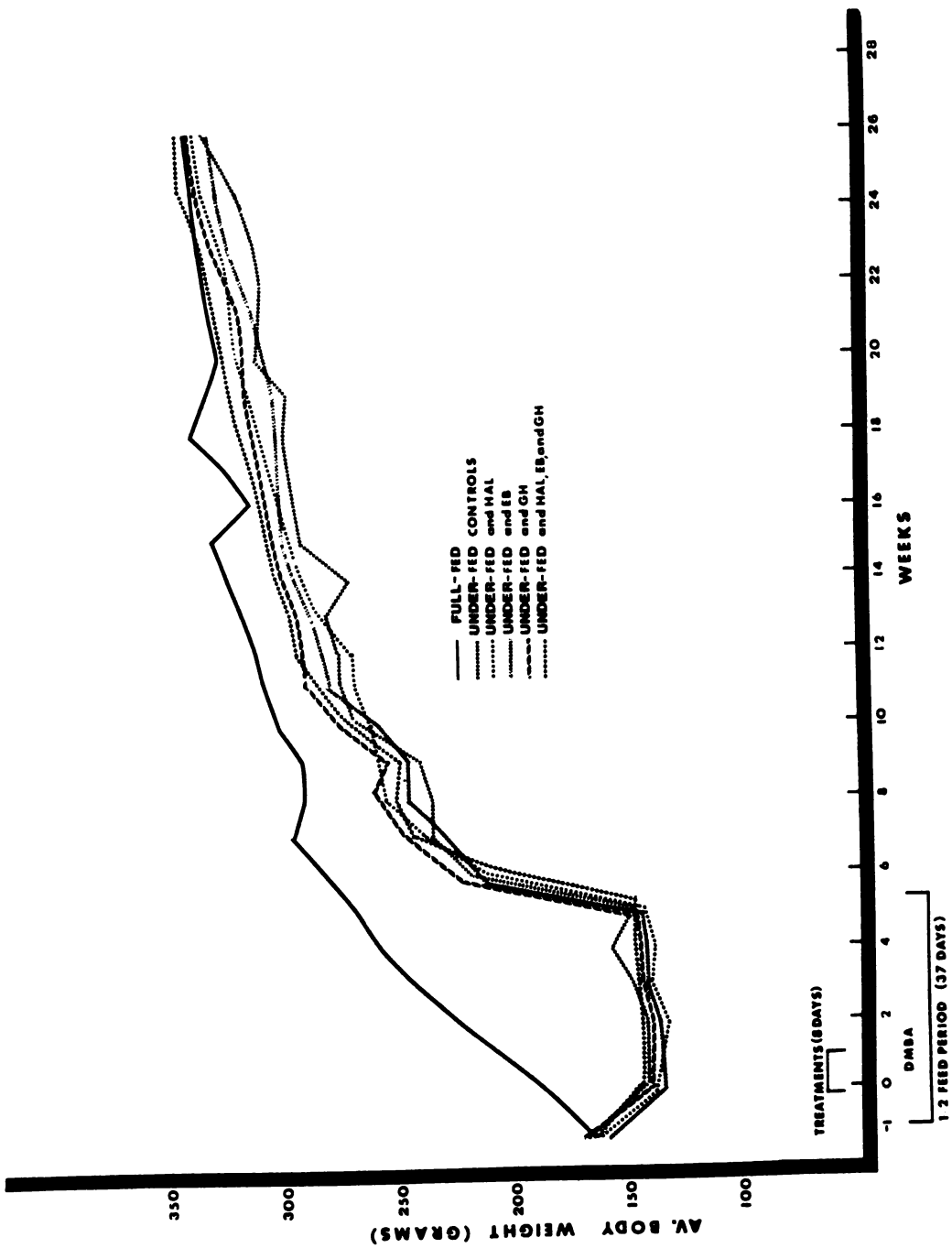
The effects of food restriction on average body weight can be seen in Figure 11. The full-fed controls (Group A) continued to gain weight throughout the entire experiment, when the animals reached approximately 300 g each, at which time further weight gains were minimal. All groups (B to F) on restricted food intake initially showed a reduction in body weight. At the end of 1 week, these food-restricted animals established a lower steady-state body weight which persisted for the duration of the period of restricted food intake. When placed on ad libitum food intake 30 days after DMBA-treatment, these rats gradually reached body weight equal to that of the controls fed ad libitum. Drug and hormone treatment given to the various food-restricted groups had no significant effect on average group body weight.

D. Discussion

This study demonstrates that the inhibitory effect of half-feeding on the formation of DMBA-induced mammary tumors in rats was largely the result of a hormonal deficiency state at the time of tumor initiation and could be counteracted by administering estrogen, HAL, and GH. Animals subjected to food restriction for 7 days before and 30 days after exposure to DMBA showed a significant (and perhaps a permanent) reduction in incidence and growth of mammary tumors during the 26 weeks after DMBA administration. The reduction in body weight of the underfed rats was significant, but body weight increased rapidly after the rats were returned to ad libitum feeding. Treatments that raised serum PRL and estradiol levels in these food-restricted animals for only one day before and 7 days after DMBA injection prevented the decrease in mammary tumor incidence and growth. In fact, underfed groups given the combination of HAL, EB, and GH showed as high an incidence of mammary

FIGURE 11.

Average body weights of under-fed rats given different drug and hormone treatment during the "critical period." The control fed ad libitum initially grew at a faster rate than any of the under-fed rats, whether or not they received hormones or haloperidol (HAL). When underfeeding was terminated 30 days after DMBA-treatment all rats grew quickly and reached ad libitum control values after about 3 weeks.


AV. BODY WEIGHT (GRAMS)
350
300
250
200
150
100
WEEKS
-1 0 2 4 6 8 10 12 14 16 18 20 22 24 26 28
TREATMENTS (8 DAYS)
DMBA
1 2 FEED PERIOD (37 DAYS)
FULL-FED
UNDER-FED CONTROLS
UNDER-FED and HAL
UNDER-FED and EB
UNDER-FED and GH
UNDER-FED and HAL, EB, and GH

tumors as controls fed ad libitum. This indicates that PRL and estrogen are particularly important for mammary tumor induction during the "critical period" following the first 7 days after DMBA-injection. These 2 hormones were shown previously to be essential for carcinogen-induced mammary cancer development and growth in rats (Meites, 1972).

Both HAL and EB injections in food-restricted rats increased serum PRL levels and mammary tumor incidence. Haloperidol, a dopamine receptor blocker, is known to be a potent stimulator of PRL release in rats (Grandison and Meites, 1976). It is also well established that estrogen can increase PRL secretion (Chen and Meites, 1970) and that both estrogen and PRL act directly on the mammary tissue to promote mammary tumor development in rats (Meites, 1972). Estrogen cannot stimulate or maintain mammary tumor growth in the absence of PRL (Meites, 1972), but PRL alone apparently can promote limited development and growth of DMBA-induced mammary cancers in rats after ovariectomy (Meites et al., 1971; Pearson et al., 1969). Therefore, stimulation of mammary tumor development and growth by estrogen administration in food-restricted rats probably resulted from the additive effects of elevated serum PRL and estrogen. In addition, both PRL and estrogen receptors have been shown to be present in mammary tumors (DeSombre et al., 1976), and it is possible that underfeeding reduced these receptors in the mammary tissue. PRL was reported to increase estrogen receptors (Leung et al., 1975), and PRL was shown to increase its own receptors in rat mammary tissue (Kelly et al., 1974).

Treatment of underfed rats for 8 days with the combination of HAL, EB, and GH produced mammary tumor incidence and growth equal to that of full-fed controls. The greater mammary tumor incidence in the underfed

rats given the combined treatment may in part result from enhanced PRL secretion by the estrogenized pituitary in response to HAL stimulation (Grandison and Meites, 1976). This is indicated by the significantly higher serum PRL levels in these animals as compared with rats given HAL or estrogen alone.

GH did not appear to stimulate mammary tumor development in the rats in this study. One rat given GH showed early appearance of a single mammary tumor, but this was of doubtful significance in view of lack of tumor development in the remaining animals of this group. A definite role for GH in mammary tumor development and growth in rats has not been demonstrated previously (Evans and Simpson, 1931; Moon et al., 1951), although it was reported to act synergistically with PRL in promoting DMBA-induced mammary tumor development in ovariectomized rats (Talwalker et al., 1964), GH had no effect on growth of existing DMBA-induced mammary tumors in rats (Nagasawa and Yanai, 1970; Iturri and Welsch, 1976).

It is well established that caloric restriction can reduce the incidence of many types of tumors, including non-endocrine-related tumors (Tannenbaum, 1942). The delays in development of tumors by restricted food intake generally have been assumed to be due to the reduced availability of nutrients to the potentially tumorous tissues (Bullough, 1950; Stragard et al., 1979). However, it is clear that reduced food intake also results in a reducted secretion of AP hormones and hormones of their target organs (Campbell et al., 1977). Such a "pseudohypophysectomy" condition can have profound effects on development of endocrine-related tumors, as shown in this study. The reduction in pituitary hormone secretion produced by underfeeding also

may influence development of non-endocrine-related tumors, since a decrease of these hormones results in changes of many metabolic processes.

IV. Influence of Underfeeding During the "Critical Period" or Thereafter on Carcinogen-Induced Mammary Tumors in Rats

A. Objectives

Previously, we demonstrated that animals subjected to a 50% reduction in food intake 7 days prior to and 30 days after DMBA administration showed a significant and apparently permanent reduction in the incidence and growth of mammary tumors, even though the rats were returned to *ad libitum* feeding for the subsequent 26 weeks of the experiment. These observations also provided direct evidence for endocrine involvement in inhibition of mammary tumorigenesis by food restriction. Treatments that increased PRL and estrogen levels, the 2 hormones essential for mammary tumorigenesis (Meites, 1972), for only 1 day before and 7 days after DMBA administration, prevented inhibition of mammary tumorigenesis despite food restriction .

It has been established that the first week after carcinogen administration to Sprague-Dawley rats is critical in terms of hormonal requirements for development of mammary tumors (Dao, 1962). Since food restriction results in decreased secretion of AP hormones (Campbell *et al.*, 1977; Mulinos and Pomerantz, 1940) and inhibition of normal estrous cycles (Piacsek and Meites, 1967), the hormonal deficiencies that develop during the first week after DMBA administrationmay be responsible for the inhibition of mammary tumorigenesis.

The purpose of the present study was to determine if food-restriction begun 1 week before and during the first "critical" week after DMBA administration was as effective for inhibiting mammary tumorigenesis as underfeeding for 1 week before and 30 days after DMBA

administration as shown previously. It also was of interest to determine whether food-restriction imposed for 2 or 4 weeks after the first critical week following DMBA administration had any effect on development of mammary tumors.

B. Materials and Methods

Forty-day old virgin female Sprague-Dawley rats were divided into 5 groups (A to E). Rats were housed in single cages and allowed to drink water ad libitum. All rats were fed laboratory rat chow ad libitum until individual groups were placed on half-feed. Vaginal smears were taken every day and only rats with regular 4-day estrous cycles were used.

At 50 days of age, rats in group A served as full-fed controls, and remained on ad libitum feeding for the entire 21 weeks of the experiment. Rats in Group B were given 10 g of food once daily between 1000 and 1200 hours. This was determined to be approximately 50% of the average daily food consumed by ad libitum rats, as described previously. Rats in group B were placed on this restricted food intake for 1 week before and 1 week after DMBA administration. These rats were begun on half-feeding for 1 week before carcinogen administration to ensure that the effects of underfeeding already were manifested by the first day after DMBA injection. Groups C through E remained on ad libutum food intake, but at progressively lengthened periods of time after carcinogen administration they were placed on half-feed for 2 or 4 weeks, and subsequently were returned to full- feeding.

At 57 days of age, all rats were given a single i.v. injection of 1 ml lipid emulsion, containing 5 mg DMBA. It was noted that in each treatment group, approximately equal numbers of rats were found to be in

each stage of the 4-day estrous cycle at the time of DMBA administration, with the exception of the underfed rats in Group B, which displayed irregular cyclicity. One week after DMBA administration, rats in Group B were returned to full-feed. Rats in Group C were placed on half-feed for 2 weeks, beginning 1 week after DMBA administration, and were then returned to full-feeding. Rats in Group D were placed on half-feed for 2 weeks beginning 3 weeks after DMBA administration, and then returned to ad libitum feeding. Rats in Group E were placed on half feed for 4 weeks, beginning 5 weeks after DMBA administration, and then were returned to full-feed.

Tumor Measurements

Tumor measurements and body weights were recorded at weekly intervals from the beginning until termination of the experiment. Average tumor diameter for each palpable tumor was determined by using the mean of the 2 largest perpendicular diameters measured with vernier calipers. Tumor size was expressed as the summation of average tumor diameter per rat for all tumors found in a treatment group. Average latency period was calculated for all tumors in a group.

Blood Collection and Prolactin Assay

Blood was collected under light ether anesthesia by orbital sinus puncture on the last day of each food- restricted period 1, 3, 5, and 9 weeks after DMBA administration. Blood was collected between 1000 and 1100 hours, when PRL levels in female rats were similar throughout the estrous cycle (Butcher et al., 1974). Serum was separated by

centrifugation and stored at -20°C until assayed for PRL by a standard RIA method.

Statistics

Statistical differences in tumor incidence between treatment groups were determined by X^2 with Yates' correction. Statistical differences in serum PRL levels, average latency period in tumor appearance, number of tumors per rat, differences in average tumor diameter and average body weight between groups were determined by analysis of variance and Student-Newman-Keuls' test for multiple comparisons among groups. The results were considered to be significant if $p<0.05$ when compared to full-fed controls in Group A.

C. Results

The effects of the different periods of underfeeding on mammary tumorigenesis after DMBA administration are shown in Table 8. Mammary tumor incidence in the full-fed controls (Group A) was 80.9%, and the average number of tumors per rat was 3.2. Average tumor latency period was 103 ± 3.6 days. Food restriction for 1 week prior to and 1 week after DMBA administration (Group B) significantly decreased the incidence of tumors to only 27.8%. Average latency period was increased over that of full-fed controls to 125 ± 7.0 days, but this difference was not signficant. Rats underfed for 2 weeks beginning 1 week after DMBA administraton (Group C) showed only a slight reduction in mammary tumor incidence and tumor number, and this was not found to be significantly different from tumor incidence in the full-fed controls (Group A). Similarly, rats underfed for 2 weeks beginning 3 weeks after DMBA administration (Group D), or underfed for 4 weeks starting 5 weeks after DMBA administration (Group E), did not show significant differences in

Table 8.
Effects of Different Periods of Underfeeding After DMBA-Administration on Mammary Tumorigenesis in Rats

Group	Treatment	No. of Rats/ Group Initial	No. of Rats/ Group Survived	No. of Rats With Tumors	% of Rats With Tumors	Total No. of Tumors	No. of Tumors/ Tumor-Bearing Rats	Average Tumor Diameter (cm±S.E.)	Average Latency Period (Days ±S.E.)
A	Full-Fed Controls	21	21	17	80.9	54	4.3	2.00±0.0	103±3.6
B	Half-Fed 1 Wk Prior, 1 Wk After DMBA	20	18	5	27.8*	12	2.4	1.30±0.3	125±7.0
C	Half-Fed 2 Weeks, Starting 1 Week After DMBA	21	21	16	76.2	39	2.4	1.71±0.2	108±3.7
D	Half-Fed 2 Weeks, Starting 3 Weeks After DMBA	21	20	15	75.0	44	2.9	2.09±0.2	107±3.4
E	Half-Fed 4 Weeks Starging 5 Weeks After DMBA	18	16	12	75.0	43	3.6	1.89±0.2	109±3.0

*$p<0.05$ as compared to full-fed controls (group A).

any of the parameters used to evaluate mammary tumor development when compared with full-fed controls (Group A). The number of tumors per tumor bearing rat were not significantly different among these groups.

The effects of food-restriction at different periods of time on summation of average mammary tumor diameter after DMBA administration are shown in Figure 12. Tumors first appeared in the full-fed controls (Group A) approximately 8 weeks after DMBA administration, and tumor size continued to increase for the 21 weeks of the experiment. Four tumors in the full-fed group were found to show regression after a period of growth, although regression was not complete. A tumor was considered to display spontaneous regression only when average tumor diameter decreased by more than 0.5 cm. These tumors were included in determining average tumor diameter.

Rats underfed 1 week prior to and 1 week after DMBA administration (Group B) showed a significant reduction in tumor size that remained small for the entire 21 week of the experiment (Figure 12). None of the 12 tumors which appeared in this group showed regression. Rats underfed for 2 weeks starting 1 week after DMBA administration (Group C) displayed a slight but not significant inhibition of average tumor diameter. Four tumors in this group exhibited spontaneous regression. Rats underfed for 2 weeks starting 3 weeks after DMBA administration (Group D), and rats underfed for 4 weeks starting 5 weeks after DMBA administration (Group E), did not show significant differences in average tumor diameter when compared with full-fed controls (Group A). Three tumors in Group D and 2 tumors in Group E displayed spontaneous regression during the course of the experiment.

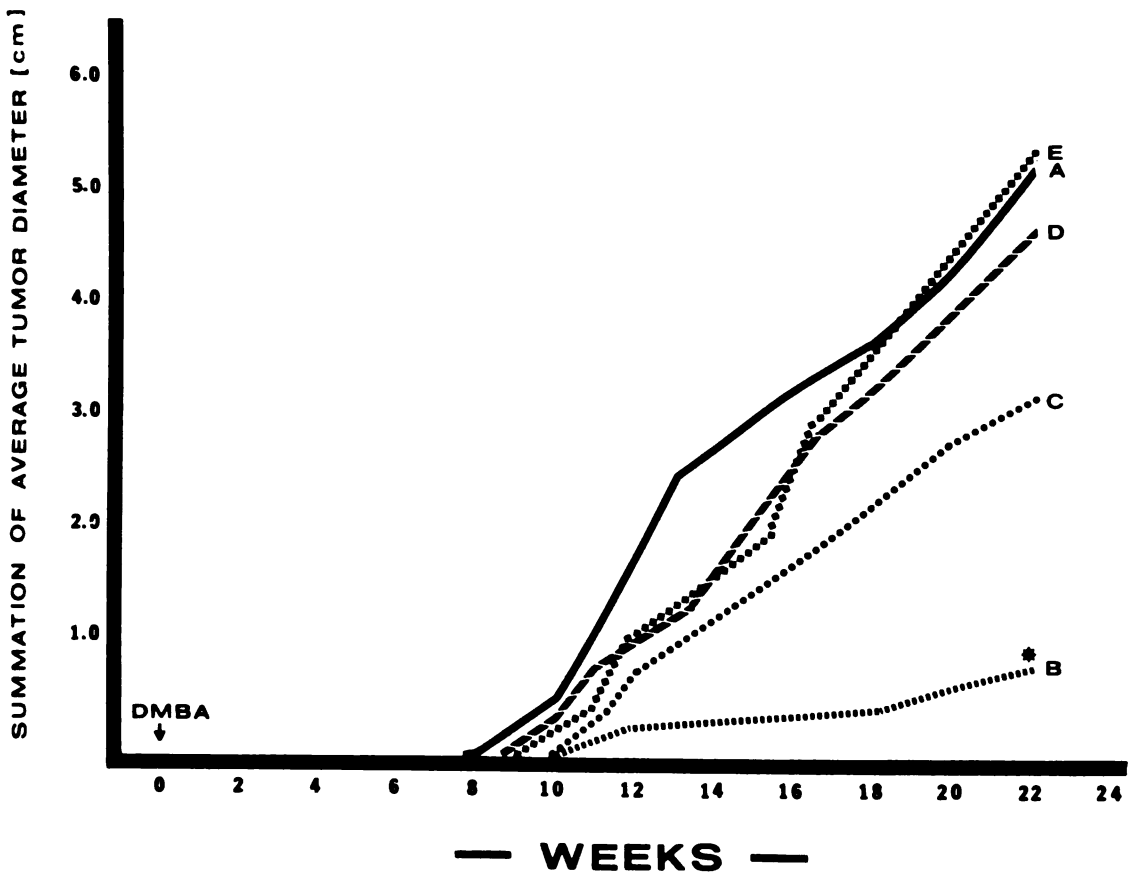


FIGURE 12.

Summation of average tumor diameter per week for all tumors found in the underfed treatment groups, for the weeks following DMBA administration. Group A = full-fed controls. Group B = half-fed 1 week prior to and 1 week after DMBA. Group C = half-fed 2 weeks starting 1 week after DMBA. Group D = half-fed 2 weeks starting 3 weeks after DMBA. Group E = half-fed 4 weeks starting 5 weeks after DMBA. *p 0.05, as compared to under-fed controls (Group A).

Serum PRL levels for each treatment group are shown in Table 9. The first blood samples were collected on the last day of underfeeding in Group B (7 days after DMBA administration), and serum PRL levels were significantly lower than in full-fed controls (Group A) or other treatment groups (C through E). In Group C , when the second blood sample was collected 3 weeks after DMBA administration and on the last day of food-restriction, serum PRL values were significantly lower than in all other groups. Similarly, on the last day of food-restriction for Group D (5 weeks after DMBA admin- istration), and Group E (9 weeks after DMBA administration), serum PRL levels were significantly reduced as compared with full-fed controls. Thus, all treatment groups showed a significant reduction in serum PRL levels at the end of their respective underfeeding period.

Irregularities in cycles occurred both as a result of food-restriction and DMBA administration. Full-fed rats in Groups A,C,D, and E, displayed typical 4-day estrous cycles prior to DMBA administration. After injection of DMBA, most rats (81.3%) showed elongated estrous cycles of 5 to 6 days, characterized by an additional 1 or 2 days of estrus. Food restriction in Groups B through E initially resulted in irregular cycles followed by cessation of cycling. Irregular cycling rats in Group B, upon administration of DMBA, showed continuous diestrus for the remaining 7-day of underfeeding. When Groups B through E were returned to full-feed, prolonged cycles returned in 5 to 7 days. Rats in Group A through D returned to 4-day estrous cycles between 5 to 7 weeks after DMBA administration. Group E rats returned to normal 4-day estrous cycles approximately 2 weeks after being placed on full-feed (11 weeks after DMBA administration).

Table 9.

Serum Prolactin Levels (ng/ml) in Different Treatment Groups Subjected to Different Periods of Underfeeding

Group	Treatment	1 Week	3 Weeks	5 Weeks	9 Weeks
A	Full-Fed Controls	39.18±9.4[b]	37.39± 4.8	49.21± 8.0	42.63±5.5
B	Half-Fed 1 Wk Prior, 1 Wk After DMBA	7.19±2.4[c]	27.60± 9.8	35.44±11.0	40.03±7.2
C	Half-Fed 2 Weeks, Starting 1 Week After DMBA	45.01±6.3	10.35± 1.9[c]	42.11± 6.4	37.33±5.4
D	Half-Fed 2 Weeks, Starting 3 Weeks After DMBA	35.44±9.5	43.82±12.1	9.33± 2.1[c]	41.32±5.5
E	Half-Fed 4 Weeks, Starting 5 Weeks After DMBA	47.63±8.9	32.74± 7.8	37.13± 7.7	9.35±1.3[c]

[a]Blood collected by orbital sinus puncture on last day of underfeeding.
[b]Mean ± S.E. [c]$p<0.05$ as compared to full-fed controls (group A).

The effects of food restriction on average body weight can be seen in Figure 13. The full-fed controls (Group A) continued to gain weight throughout the entire experiment. The animals reached a plateau in average body weight about 300 g. Rats underfed 1 week prior to and 1 week after DMBA (Group B) showed a significant reduction in body weight of about 50 g, but at the end of 1 week, no further weight loss occurred. When these rats were returned to full-feeding, average body weight increased quickly and reached the level of full-fed controls in only 2 weeks. Rats in Groups C through E also lost body weight quickly after being placed on half-feed, but after these rats were returned to ad libitum feeding, average body weights soon returned to those of full-fed controls.

D. Discussion

This study provides further evidence that inhibition of mammary tumor development by underfeeding that encompasses the "critical" first week after DMBA administration (Group B) is associated with a reduction in hormone secretion. These rats showed a significant decrease in serum PRL levels and a probable decline in ovarian steroids as indicated by initial irregularity and ultimate loss of estrous cycles. These rats showed a significant and perhaps permanent reduction in mammary tumor incidence, number, and growth rate, even though they were returned to ad libitum feeding beginning 1 week after carcinogen treatment. Thus, a 30-day period of food restriction after DMBA administration not inhibit mammary tumorigenesis.

Animals in treatment groups subjected to similar periods of food-restrictions (Group C through E) for consecutive periods of time following the "critical" first week after DMBA injection, also showed

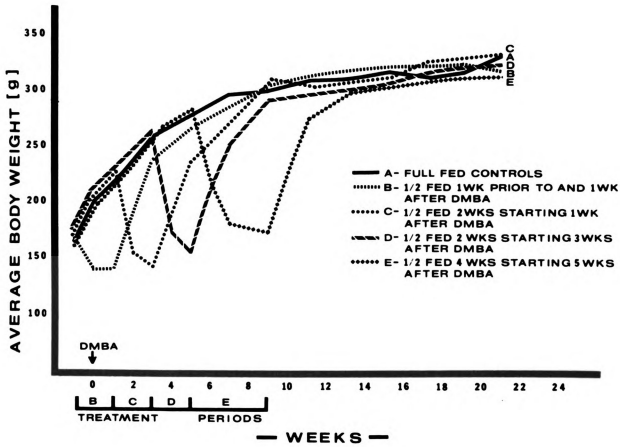


FIGURE 13.

Average body weights of rats subjected to different periods of underfeeding after DMBA administration. Full-fed controls (Group A) continued to gain weight throughout the 21 weeks of the experiment. Animals in treatment groups B - E showed reduction in body weight after being placed on food-restriction, but quickly regained normal weight when returned to ad libitum feeding.

reduced levels of serum PRL and disruption of regular estrous cycles, but this did not result in inhibition of mammary tumorigenesis. This further demonstrates that only the first week after DMBA administration is critical for long-term inhibition of mammary tumor development by underfeeding.

Underfeeding previously was shown to decrease the incidence and growth rate and to increase the average latency period for development of many types of spontaneous, transplanted, and carcinogen-induced mammary cancers in mice and rats (Tannenbaum, 1942; Tannenbaum and Silverstone, 1950; 1953; Tarnowski and Stock, 1956; Welsch and Meites, 1978; White, 1961), whereas food-restriction inhibits the growth of established mammary tumors but once these animals are returned to full-feed, mammary tumor growth resumes (Stragand et al., 1979). The mechanism(s) by which underfeeding inhibits mammary tumorigenesis has not been fully established, but it has been shown that underfeeding depresses secretion of AP hormones (Campbell et al., 1977; Mulinos and Pomerantz, 1940), inhibits normal mammary gland development (Huseby et al., 1945), and results in cessation of normal estrous cycles (Piacsek and Meites, 1967). In the present study, rats underfed for 1 week prior to and 1 week after DMBA administration (Group B) displayed irregular estrous cycles during the first week of food-restriction and showed continuous diestrus during the week after DMBA injection. Thus, during the "critical" week after DMBA administration, cyclic surges of PRL and estrogen did not occur in these animals (Butcher et al., 1974; Nequin et al., 1975; Smith et al., 1975). These hormones have been shown to be essential for the establishment and growth of DMBA induced mammary tumors in rats (Meites, 1972). Abnormalities in estrous cycles

previously were reported to result from DMBA administration (Kerdelhue and El abed, 1979; Stern et al., 1968). The earlier demonstration by us that administration of estrogen and a prolactin release stimulating drug (haloperidol) during the first week after DMBA administration overcame the effects of underfeeding, suggests that the inhibitory effects of underfeeding on mammary tumorigenesis in rats are exerted by decreasing secretion of these hormones. It also is possible that the ACTH-adrenal cortical system is involved, since severe underfeeding has been reported to increase ACTH-adrenal cortical secretion in rats (Tannenbaum and Silverstone, 1957), and glucocorticoid hormones can inhibit growth of mammary tumors in rats (Hilf et al., 1965).

V. Hormone Dependency and Independency During Development and Growth of Carcinogen-Induced Mammary Tumors in Rats

A. Objectives

Development and growth of mammary tumors induced in female rats by administering DMBA are primarily dependent on the presence of hormones, particularly PRL and estrogen (Meites, 1972). A small percentage of DMBA-induced mammary tumors become hormone-independent or autonomous, as indicated by continued growth after ovariectomy. Ovariectomy not only removes the major source of estrogen in the body, but also results in a significant reduction in PRL secretion by the pituitary (Bradley et al., 1976). Estrogen is a potent stimulator of PRL secretion. The mechanism(s) involved in establishment of hormone-independent mammary tumors are not understood. Up to 20% of DMBA-induced mammary tumors in Sprague-Dawley rats show hormone-independency shortly after their appearance, and the incidence of autonomy increases with the age and size of the tumor (Bradley et al., 1976; Griswald and Green, 1970). It has been established that the first week after carcinogen administration to Sprague-Dawley rats is critical for development of mammary tumors (Dao, 1962), and suppression of secretion of estrogen or PRL or both during the "critical period" apparently results in inhibition of mammary tumorigenesis (Experiment III). These observations suggest that the hormonal milieu at the time of tumor induction greatly influences mammary tumor dynamics. The purpose of the present study was to determine whether the hormonal dependency or independency that is observed in DMBA-induced mammary tumors during their growth phase is related to their initial hormonal dependency or independency during the "critical" first week after DMBA administration.

B. Materials and Methods

Tumor Induction and Drug Treatment

Virgin female Sprague-Dawley rats, 55 days old, were given a single i.v. injection of 1 ml lipid emulsion containing 5 mg of DMBA. The rats were housed in plastic cages and fed rat chow and water ad libitum. Rats were divided into 6 groups (A-F) and starting 1 day prior to and continuing for 7 days after DMBA administration, animals were subjected to various drug and hormone treatments.

Group A received a daily 0.1 ml s.c. injection of each vehicle (0.3% ethanol and 0.87% NaCl solution). Group B received a daily 0.1 ml s.c. injection of 0.3% ethanol and a daily s.c. injection of HAL (McNeil Labs, Ft. Washington, PA), at a dose of 0.5 mg/kg, suspended in 0.1 ml 0.87% NaCl solution. HAL, a DA receptor blocker, was administered to increase pituitary PRL release. Group C received a daily s.c. injection of EB (Sigma Chemical Co., St. Louis, MO), at a dose of 1 ug dissolved in 0.1 ml 0.3% ethanol, to raise serum estrogen and PRL levels, together with an injection of 0.1 ml 0.87% NaCl solution. Group D was given a daily s.c. injection of bromocryptine (CB-154) (Sandoz, Ltd., Basal, Switzerland) at a dose of 5.0 mg/kg, suspended in 0.1 ml 0.87% NaCl solution, and an 0.1 ml s.c. injection of 0.3% ethanol. Bromocryptine, an ergot drug and DA agonist, was used to reduce PRL release from the pituitary. Group E received a daily s.c. injection of 20 ug tamoxifen (TAM, ICI, Rotterdam, The Netherlands) suspended in 0.1 ml 0.3% ethanol, together with an 0.1 ml s.c. injection of 0.87% NaCl solution. TAM, an anti-estrogenic drug, was administered to inhibit estrogen action during tumor induction. Group F received a daily 0.1 ml s.c. injection of TAM (20 ug/rat) and CB-154 (5.0 mg/kg) to inhibit both estrogen and PRL

faction. All injections were performed in the morning between 0800 and 1000 hours. After 8 days of treatment injections were terminated.

Tumor Measurement and Classification

Tumor measurements and body weights were recorded at weekly intervals from the beginning until termination of the experiment. Average tumor diameter for each palpable tumor was determined by using the mean of the 2 largest perpendicular diameters as measured with vernier calipers. Average latency period was calculated for all tumors in a group.

A tumor which had decreased by 5 mm or more in average diameter was classified as regressing. A tumor that had increased by more than 5 mm in average diameter was classified as growing, and a tumor that had changed less than 5 mm in average diameter was considered stable. Upon termination of the experiment, tumors were removed for routine histological examination.

Evaluation of Hormone-Dependency of Mammary Tumors

Sixteen weeks after DMBA administration, all animals were bilaterally ovariectomized to determine hormonal-dependency of the mammary tumors. This period of time after DMBA administration was chosen because at least 94% of the tumors have been classified as adenocarcinomas at this time (Griswald and Green, 1970). The percentage of mammary adenocarcinomas decreases progressively 16 weeks after carcinogen administration. Tumor growth was followed for 4 weeks after ovariectomy.

Blood Collection and Hormone Assay

Blood was collected under light ether anesthesia by orbital sinus puncture on the last day of drug and hormone treatment (7 days after

DMBA administration), prior to ovariectomy (16 weeks after DMBA administration) and upon termination of the experiment (4 weeks after ovariectomy).

In all 3 sampling periods blood was collected between 1000 and 1100 hours, when serum PRL levels in female rats are approximately equal throughout the estrous cycle. Serum was separated by centrifugation and stored at -20°C until assayed for PRL by a standard RIA method.

Statistical differences in tumor incidence between treatment groups were determined by X^2 with Yates' correction. Statistical differences between treatment groups were determined by analysis of variance and Student-Newman-Keuls' test used for multiple comparisons among groups. The differences were considered to be significant if $p < 0.05$ when compared to vehicle treated controls.

C. Results

The effects of the various hormone and drug treatments given to rats during the "critical" first week after DMBA administration on mammary tumorigenesis are shown in Table 10. Tumor incidence in vehicle treated controls (Group A) 16 weeks after DMBA administration was 72.2% and the average number of tumors per rat was 3.8. Spontaneous regression was found in 4 tumors in the control animals (Group A). Rats which received daily injections 1 day prior to and 7 days after DMBA administration of either HAL (Group B), EB (Group C), or CB-154 (Group D), showed slight alterations in mammary tumor development. However, these differences were not significant when compared to controls (Group A).

Animals injected during the "critical" period after DMBA administration with TAM (Group E) showed significant reductions in

Table 10.
Effects of Different Drug and Hormone Treatments During the "Critical Period" on Development of DMBA-Induced Mammary Tumors

Group	Treatment During the "Critical" Period[a]	No. of Rats per Group	No. of Rats With Tumors	% of Rats With Tumors	Total No. of Tumors	No. of Tumors Tumor-Bearing Rat	Average Tumor Diameter (cm)	Average Latency Period (days)	No. of Regressing Tumors
A	Control (0.87% NaCl)	18	13	72.2	49	3.8	1.12	77.8	4
B	Haloperidol (0.5 mg/kg)	21	17	77.3	78	4.6	1.37	80.3	8
C	Estradiol Benzoate (1 ug/rat)	22	16	72.7	57	3.7	1.59	82.4	3
D	CB-154 (5.0 mg/kg)	21	12	57.1	31	2.6	1.25	89.0	5
E	Tamoxifen (20 ug/rat)	45	10	22.2[a]	25	2.5	1.69	77.4	0
F	Tamoxifen (20 ug/rat) +CB-154 (5.0 mg/kg)	47	11	23.4[a]	22	2.0[a]	1.38	90.6	0

[a] $p<0.05$ as compared to controls (group A). "Critical Period" = first week after DMBA injection.

incidence (22.2%), as compared with controls (Group A). Similarly, rats given the combination of TAM and CB-154, 1 day prior to and 7 days after DMBA administration (Group F), showed significant reductions in tumor incidence (23.4%) and number of tumors per rat (2.0), as compared with controls (Group A). This combined treatment was no more effective for inhibiting mammary tumor development than TAM treatment alone (Group E). Animals in Groups E and F had no tumors that displayed spontaneous regression.

Sixteen weeks after DMBA administration, tumor-bearing rats in all treatment groups were ovariectomized to determine mammary tumor hormone-dependency. During the 4 week period after ovariectomy, one rat in Group A, 3 rats in Group B, 1 rat in Group C, 2 rats in Group D, 1 rat in Group E, and no rats in Group F died. Rats which died during this time were not included in calculations of mammary tumor hormone-dependency in their respective groups.

The effects of ovariectomy on mammary tumor growth in rats of the various treatment groups are shown in Table 11. Ovariectomy resulted in regression of 75% of the mammary tumors in control rats (Group A), whereas 13.9% were stable and 11.1% showed continued growth. Over 80% of the tumors found in rats treated with HAL (Group B) and EB (Group C) during the first "critical" week after DMBA administration showed regression 4 weeks after ovariectomy, while less than 10% of the tumors were stable or showed autonomous growth. Rats treated with CB-154 during the "critical" period (Group D) showed little differences in tumor response to ovariectomy as compared with controls (Group A). Rats injected with TAM during the "critical" period (Group E) showed a 1/3 reduction in the incidence of mammary tumors that regressed after ovari-

Table 11.
Effect of Ovariectomy on Mammary Tumor Growth 16 Weeks After DMBA Administration in Rats Given Different Drug and Hormone Treatments During the "Critical Period" After Carcinogen Administration

Group	Treatment During the "Critical" Period[a]	No. of Rats	Total No. of Tumors	Regressed	Stabilized	Grew
A	Control (0.87% NaCl)	12	36	27 (75.0)[b]	5 (13.9)	4 (11.1)
B	Haloperidol (0.5 mg/kg)	14	67	56 (83.6)	5 (7.5)	6 (9.0)
C	Estradiol Benzoate (1 ug/rat)	14	36	29 (80.6)	3 (8.3)	3 (8.3)
D	CB-154 (5.0 mg/rat)	10	24	17 (70.8)	3 (12.5)	4 (16.7)
E	Tamoxifen (20 ug/rat)	9	21	11 (52.4)	3 (14.3)	7 (33.3)
F	Tamoxifen (20 ug/rat) +CB-154 (5.0 mg/kg)	11	22	6 (27.3)	4 (18.2)	12 (54.5)

[a]16 weeks after DMBA administration, rats in each group were ovariectomized and mammary tumor responses were followed for 4 weeks. "Critical Period" = first week after DMBA. [b]Percentage.

ectomy (52.4%) and a 3-fold increase in the number of autonomous tumors (33.3%) as compared to controls (Group A). Rats treated with the combination of TAM and CB-154 during the "critical" period (Group F) showed regression of only 27.3% of the mammary tumors after ovariectomy. This is nearly a 2/3 reduction in the incidence of hormone-dependent tumors as compared to controls (Group A). Interestingly, the incidence of autonomous tumors found in these rats (Group F) was 54.5% or a 5-fold increase over controls (Group A).

The percentage change in average tumor diameter in the 4 week period after ovariectomy in rats of the various treatment groups is shown in Figure 14. Ovariectomy significantly decreased average tumor diameter by 50% in control rats (Group A) as compared to initial preovariectomy values. A significant reduction of average mammary tumor diameter was also found in rats treated during the "critical period" with HAL (Group B), EB (Group C), and CB-154 (Group D). Ovariectomized rats treated with TAM during the "critical period" (Group E) demonstrated a reduction in average tumor diameter from that of pre-ovariectomy values, but this was not found to be significant. In contrast to all other groups, rats treated with the combination of TAM and CB-154 (Group F) during the "critical" period demonstrated a significant increase in average tumor diameter over that of pre-ovariectomy values and ovariectomized control rats (Group A).

Serum PRL levels for each treatment groups are shown in Figure 15. The first blood sample collected was on the last day of drug and hormone treatment (7 days after DMBA administration). Serum PRL levels were significantly elevated by daily injections of either HAL (Group B) or EB (Group C), as compared to controls (Group A). Daily injections of

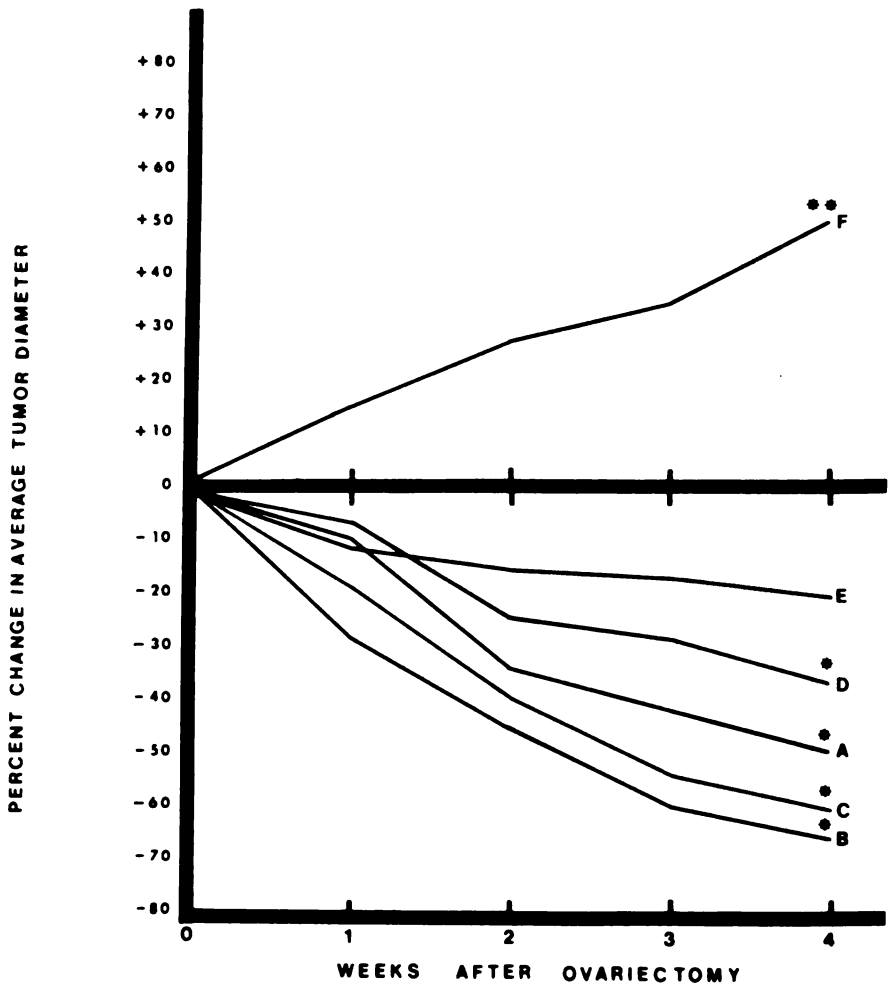


FIGURE 14.

The percentage change in average tumor diameter in the 4 week period after ovariectomy in rats of the various treatment groups. Group treatments during the "critical period" after DMBA administration. A = vehicle treated controls; B = haloperidol (HAL, 0.5 mg/kg); C = estradiol benzoate (EB, 1 ug/rat); D = bromocryptine (CB-154, 5.0 mg/kg); E = tamoxifen (TAM, 20 ug/rat). F = TAM, 20 ug/rat plus CB-154 (5.0 mg/kg). *$p<0.05$ as compared to initial pre-ovariectomized values. **$p<0.05$ as compared to initial pre-ovariectomized values and with controls (Group A).

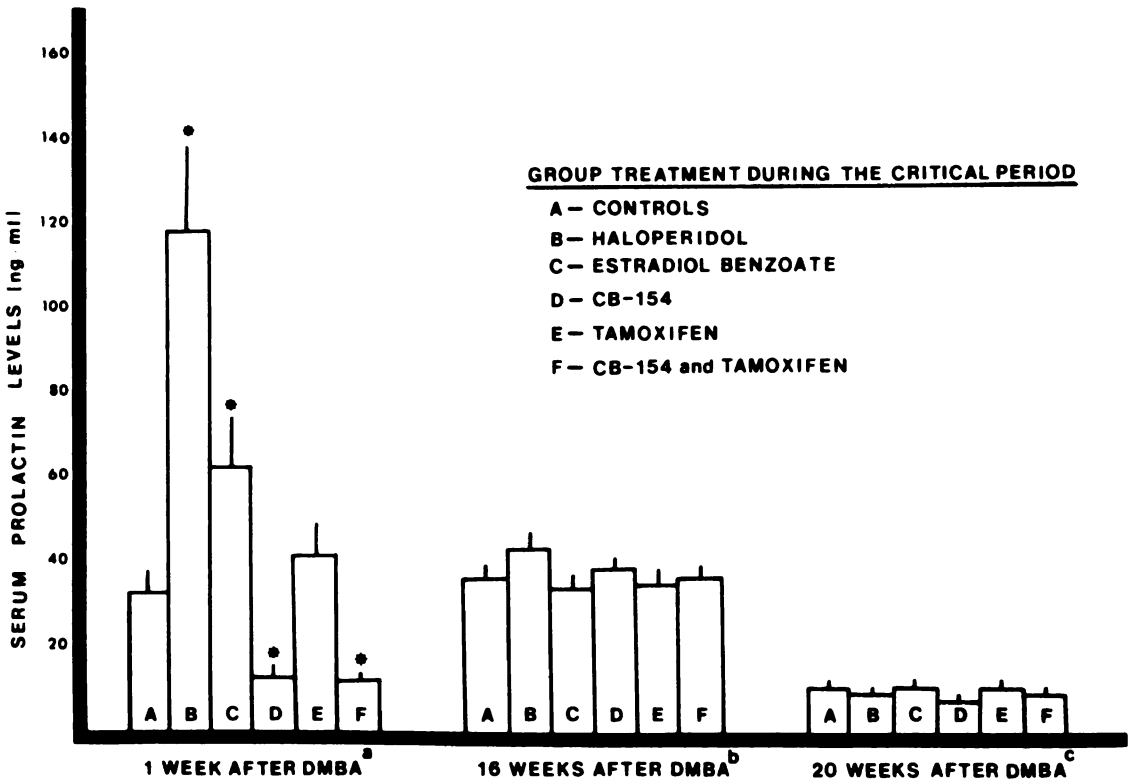


FIGURE 15.

Serum prolactin (PRL) levels for each treatment group at various time periods. [a]Blood collected on the last day of drug and hormone treatment (7 days after DMBA administration). [b]Blood collected just prior to ovariectomy (16 weeks after DMBA administration). [c]Blood collected upon termination of the experiment (20 weeks after DMBA administration and 4 weeks after ovariectomy). *$p < 0.05$ as compared to controls (Group A).

CB-154 resulted in significant reductions in serum PRL when given alone (Group D) or in combination with TAM (Group F). Rats treated with TAM alone (Group E) showed no significant changes in blood serum PRL levels from that of controls (Group A).

The second blood sample was taken 16 weeks after DMBA administration, just prior to ovariectomy. At this time, when all animals had long since been removed from drug and hormone treatment, no differences in serum PRL levels were found among treatment groups (Figure 15). The last blood sample was taken upon termination of the experiment, 20 weeks after DMBA administration and 4 weeks after ovariectomy. All rats showed suppressed PRL levels in response to ovariectomy and no differences appeared among treatment groups (Figure 15).

Histological examination of tumor samples taken from all rats at the end of the experiment showed that 98% of the tumors were adenocarcinomas. These tumors contained characteristic columns of epithelial cells many cell layers thick. Little fibrosis was present and only 1 carcinosarcoma was found in Group E and 1 sebaceous cell carcinoma in Group B.

D. Discussion

This study demonstrates that suppression of estrogen and PRL at the time of tumor initiation in rats not only reduces the incidence and number of mammary tumors, but tumors that developed in these animals were less dependent on estrogen and PRL for subsequent growth. Control animals which received injections of vehicle 1 day prior to and 7 days after DMBA administration (Group A) had a 75% incidence of mammary tumors and only 11% of these tumors showed hormone-independent growth

after ovariectomy. In contrast, rats which received daily injections of the combination of CB-154 and TAM during the "critical" first week after DMBA administration (Group F), while showing significantly lower incidence of mammary tumors(23%), exhibited about a 5-fold greater number of autonomous tumors (54%) after ovariectomy than control rats (Group A). In general, these results indicate that autonomy of carcinogen-induced mammary tumors is determined by the hormonal environment during the first week after DMBA treatment. It has been shown that at 3 months after DMBA administration, 94% of the mammary tumors found in rats are adenocarcinomas (Griswald and Green, 1970). At 5 months this percentage drops to 80% and by 9 months only 40% of the tumors are adenocarcinomas. We chose to examine tumor response to ovariectomy 16 weeks after DMBA administration because tumors at this early stage of development are nearly all frank adenocarcinomas and highly hormone-dependent (Bradley et al., 1976).

Our results are in agreement with previous reports showing that removal of estrogen influence by anti-estrogenic drugs (Jordan, 1976) or ovariectomy (Dao, 1962) shortly after carcinogen administration in rats results in significant inhibition of mammary tumorigenesis. Suppression of serum PRL for several weeks prior to and after carcinogen administration, also was reported to inhibit mammary tumorigenesis (Clemens and Shaar, 1972; Kledzik et al., 1974). These investigators, however, did not determine the subsequent response of these tumors to ovariectomy. The reason I was unable to suppress mammary tumor development in rats given daily injections of CB-154 during the "critical" period after DMBA administration may have been due to the experimental model used. Previous investigators induced

hypoprolactinemia for 1 week prior to, as well as after DMBA administration (Clemens and Shaar, 1972; Kledzik et al., 1974), and this probably delayed maturation of the mammary glands, rendering them less succeptible to the carcinogen (Cohen, 1981). My results show that increased circulating levels of estrogen and PRL produced by estrogen and HAL administration did not greatly alter mammary tumor development or hormone-dependence.

Early removal of the estrogen influence by TAM during the first "critical" week after DMBA-administration was more effective than similar early removal of the PRL influence by CB-154 in determining subsequent autonomy of the mammary tumors. Daily injections of CB-154 during the "critical period" after carcinogen administration (Group D) caused significant reductions in serum PRL levels, but mammary tumor incidence and hormonal-dependency did not significantly differ from controls (Group A). Treatments with TAM 1 day prior to and 7 days after DMBA administration (Group E) significantly decreased mammary tumor incidence without altering basal PRL values. These tumors, however, failed to show significant regression in average tumor diameter in response to ovariectomy. The combined treatment of TAM and CB-154 (Group F) was no more effective in inhibiting mammary tumor development than TAM treatment alone (Group E), but these tumors displayed the greatest autonomy. These results suggest that during the early events of tumor initiation, estrogen rather than PRL is the more importanat influence in development of hormone-dependent tumors. This is supported by the observation that inhibition of mammary tumor development by underfeeding is reversed by treatment with estrogen, but not by PRL treatment during the "critical period" after DMBA administration.

The majority of established DMBA induced mammary tumors regressed after ovariectomy. Both hormone-dependent and independent tumors were found in the same animals, regardless of the treatment given. It is know that mammary gland susceptability to carcinogen induction of tumors is highest when the mammary gland contains the largest number of undifferentiated mitotically active terminal end-buds (Russo and Russo, 1978). This occurs in the female rat at approximately 55 days of age (Huggins et al., 1961; Janss and Hadaway, 1977). Estrogen and PRL stimulate mitotic activity in normal and neoplastic mammary tissue (Lee et al., 1975; Welsch et al., 1977). DMBA-induced tumors contain a heterogeneous cell population and it has been suggested that within a single tumor, growth in response to stimulatory hormones depends on the rate of cell division of the hormone-dependent cells within that tumor (Leung et al., 1975; Minasian-Batmanian and Jabara, 1981). It has been found that at the time of DMBA administration, the greater the rate of mitotic activity in the terminal end-buds, the greater the rate of DNA synthesis. This has been correlated positively with carcinogen binding and tumor incidence (Janss and Ben, 1978).

Suppression of mitotic activity by combined TAM and CB-154 treatment at the time of mammary tumor initiation could decrease the number of hormone-dependent cells affected by DMBA action and may reflect the variability in hormone-dependency of DMBA induced mammary tumors. This could be responsible for the differences in concentrations of hormone-dependent versus hormone-independent cell populations in the tumors. A cell's response to a hormone is mediated by interactions of that hormone with a specific receptor found on or within the cell. Estrogen and PRL binding has been found to be generally lower in

hormone-independent than hormone-dependent mammary tumors (McGuire et al., 1971; Turkington, 1974). Identification of PRL receptor sites in DMBA-induced mammary tumors by autoradiography showed that in some tumors, all cells contained PRL receptors, while in other tumors up to 50% of the cells remained unlabelled (Costlow and McGuire, 1977). Thus, within a given mammary tumor, individual cells display wide variability in hormone binding and dependence. These heterogeneous cell populations appear to be in a dynamic state, since mammary tumor responsiveness to ovariectomy declines with increased age and size of the tumor (Bradley et al., 1976; Griswald and Green, 1970).

In conclusion, I have demonstrated that the hormonal milieu in rats at the time of initiation of mammary tumorigenesis determines not only tumor incidence, but also hormone-dependency in these animals. Animals deficient in estrogen and PRL at the time of DMBA administration develop fewer tumors, but these tumors are less dependent on these hormones for subsequent growth. In other words, the mammary tumors that develop in response to DMBA initially may contain a large percentage of hormone-independent cells and hence apparently remained hormone-independent during their subsequent growth phase.

GENERAL DISCUSSION

I. Role of Endogenous Opioid Peptides in Regulation of Phasic and Pulsatile Release of Gonadotropins

The data presented in the first part of the thesis indicate that the EOP are involved in the regulation of both phasic and pulsatile release of gonadotropins. Previously it was demonstrated that administration of MOR or the EOP inhibits, whereas NAL or naltrexone stimulates basal secretion of LH and FSH in normal male rats (Bruni et al., 1977). Naloxone and naltrexone are specific opiate receptor antagonists. Thus, it can be concluded that the EOP acts to tonically inhibit basal release of LH and FSH. Our results demonstrate that the EOP also are involved in modulating the secretion of gonadotropins during dynamic physiological states.

Previously it was shown that MOR or EOP block ovulation an the preovulatory gonadotropin surge on the afternoon of proestrus (Barraclough and Sawyer, 1955; Pang et al., 1977). Our laboratory recently demonstrated that MOR when, administered once during the "critical period" for LH release, at 1400 hours on the afternoon of proestrus, delayed the surge of serum LH by approximately 2 hours and lowered the peak LH values (Ieiri et al., 1980). This effect of MOR was reversed by NAL. Administration of NAL alone did not alter the peak of the surge on proestrus, but maintained serum LH at significantly higher levels than that seen in control rats (Ieiri et al., 1980).

In experiment I, the effects of MOR and NAL on the estrogen-induced gonadotropin surge in long-term ovariectomized rats was examined. In ovariectomized rats injected with EB followed 3 days later by a second injection of EB or P, MOR completely blocked the LH and FSH surges on the day of drug treatment. However, on the following afternoon, a large rebound surge of these hormones occurred. In contrast, NAL treatment resulted in a potentiated gonadotropin surge on the day of drug treatment, but no subsequent surge of LH or FSH occurred on the following day. These effects of MOR and NAL were found not to be the result of a build-up or depletion of pituitary stores of gonadotropins, since administration of GnRH released similar amounts of LH and FSH from drug or saline-treated rats. Thus, it can be concluded that the EOP are involved in modulating the neural surge signal for the release of gonadotropins during ovarian steroid induced positive feedback.

The effects of the opiates and their antagonists does not appear to result from a direct action on the pituitary. Incubations of MOR, EOP or NAL with hemi-pituitaries or pituitary cell cultures does not alter the release of LH and FSH into the surrounding medium (Shaar et al., 1977; Grandison and Guidotti, 1977). In addition, analogs of opiates or opiate antagonists which do not cross the blood brain barrier produce characteristic changes in hormone release when administered intraventricularly, but not systemically (Panerai et al., 1981). Thus, the action of opiates and their antagonist appear to be mediated via hypothalamic mechanisms.

Ovariectomized rats release LH in a pulsatile manner and administration of ovarian steroids abolish this episodic release of LH (Gay and Sheth, 1976). In experiment II, the effects of MOR and NAL on

pulsatile LH release and the interaction of these drugs with ovarian steroids was examined. Morphine treatment significantly decreased the frequency of LH pulses and decreased mean serum LH levels in non-primed ovariectomized rats. Naloxone treatment in non-primed ovariectomized rats significantly increased the magnitude of LH pulses and mean serum LH levels, but did not alter pulse frequency when compared to saline treated controls. The combination of MOR and NAL showed LH pulses similar in frequency and amplitude as saline treated controls.

Administration of EB in long-term ovariectomized rats abolished pulsatile LH release and significantly decreased mean serum LH concentrations. Naloxone treatment reversed this inhibitory effect of EB on episodic release of LH. Similarly, suppression of pulsatile LH release in EB-P treated ovariectomized rats was reversed by NAL administration. These results demonstrate that MOR, like the ovarian steroids, can inhibit the pulsatile release of LH. Administration of NAL blocks this inhibitory effect of MOR, EB or EB-P on pulsatile LH secretion. This suggests that the EOP can modulate the inhibitory effects of ovarian steroids on pulsatile LH release. This suggestion is supported by the finding that NAL increases the frequency and amplitude of LH and FSH pulses during the luteal and late folliculary phase of the human menstrual cycle (Quigley et al., 1980; Ropert et al., 1981). In addition, NAL has been shown not only to block the inhibitory effects of testosterone on the post-castration rise of LH in male rats (Cicero et al., 1980), but also can block the negative feedback inhibition of estrogen or the combination of estrogen plus progesterone in castrated female rats (Van Vugt et al., 1982).

The EOP are highly concentrated in hypothalamic and preoptic areas of the brain, and their neurons are in close association with steroid concentrating, aminergic and GnRH containing neurons (Sar et al., 1977). Previously it was demonstrated that opiates decrease hypothalamic turnover of catecholamines (Van Vugt, 1977) and increase the turnover of serotonin (Ieiri et al., 1980), resulting in the inhibition of serum LH release. It is possible that during positive feedback, ovarian steroid reduced brain opioid activity to stimulate LH release, whereas during negative feedback ovarian steroids stimulate brain opioid activity and inhibit LH release.

In conclusion, the observations in experiment I and II demonstrate that the EOP are intimately involved in the regulation of both phasic and pulsatile gonadotropic hormone secretion. Furthermore, the hypothesis that gonadal steroids and the EOP inhibit GnRH release in the hypothalamus by a common mechanism is supported by the finding that opioids mimic, whereas NAL antagonizes the effects of gonadal steroids on gonadotropin release. It is possible that both EOP and gonadal steroid receptors are present in GnRH containing neurons and that the EOP tonically regulate the activity of these cells. Thus changes in activity of GnRH neurons in the brain could represent interactions among EOP, neurotransmitters, and gonadal steroids. Whether the action of the EOP is exerted directly on GnRH secreting neurons, or on brain neurotransmitters, or on a combination of these, remains to be determined.

II. Relation of Hormones and Food Intake to Development and Hormone-Dependency of Carcinogen-Induced Mammary Tumors

The role of diet in mammary tumorigenesis has been investigated for many years, and it is well established that caloric-restriction inhibits the formation of spontaneous and carcinogen-induced mammary tumors in laboratory rodents (Tannenbaum, 1940; Dunning et al., 1949). The exact mechanism by which underfeeding inhibits mammary tumor development, however, has not been firmly established. The subject of the research reviewed in the second part of this thesis, focused upon the involvement of the endocrine system and nutrition at the time of tumor induction on the subsequent development of mammary tumors and on their hormone dependency.

It had been suggested that the effects of caloric-restriction on mammary tumor development result from the reduced intake of some essential nutrients for the growth of potentially tumorous mammary tissue (Bullough, 1950). However, underfed rats and mice may live longer than full-fed animals and remain in good health (McCoy and Crowell, 1934). Most of the previous work dealing with the effects of caloric-restriction on mammary tumor development utilized hormone-dependent tumors. Therefore, it was important to determine the effects of food-restriction on hormone secretion in rats with carcinogen-induced mammary tumors.

Animals on restricted food intake have shown changes in ovaries, uterus, and mammary tissue analogous to that seen in hypophysectomized animals (Huseby et al., 1945). Food-restriction has also been shown to decrease secretion of AP and ovarian hormones (Campbell et al., 1976;

Piacsek and Meites, 1967). In addition, food-restricted animals display adrenal hyperfunction which could contribute to inhibition of mammary tumor development (Boutwell, 1948).

It has been established that the first week after carcinogen administration to Sprague-Dawley rats is critical in relation of hormonal requirements for development of mammary tumors (Dao, 1962). In general, physiological or pharmacological treatments that increase estrogen and PRL levels at this time promote, whereas treatments that inhibit the circulating levels of these hormones reduce mammary tumorigenesis (Meites, 1972). Thus hormonal deficiency in food-restricted rats at the time of tumor induction may be responsible for the inhibition of tumor development.

In experiment III, we investigated the effects of hormone replacement given during the critical first week after carcinogen administration in food-restricted rats on development of mammary tumors. We showed that food-restriction for 7 days prior to and 30 days after DMBA exposure significantly reduced mammary tumorigenesis. Treatment for 8 days after DMBA with EB produced a significant increase in tumor incidence in the half-fed rats, while the combination of HAL, EB and GH returned tumor incidence to that of full-fed controls. These results suggest that the underfeeding induced suppression of AP function during the critical first week after DMBA administration was responsible for inhibition of mammary tumorigenesis.

In experiment IV, we examined whether food restriction begun 1 week before and 1 week after DMBA administration was as effective for inhibiting mammary tumor development as underfeeding for 1 week before and 30 days after DMBA, as shown in Experiment III. Rats in different

treatment groups subjected to underfeeding for 2 or 4 weeks, at consecutive periods of time before and /or after DMBA administration, all showed reduced serum PRL levels and ovarian function (as indicated by cessation of estrous cycles) at the end of their respective underfeeding periods. However, only rats underfed during the week before and the critical first week after DMBA treatment showed significant reduction in mammary tumor development. The suppression of mammary tumors that resulted from food restriction during the early period after DMBA administration apparently resulted in permanent suppression of mammary tumorigenesis both in experiments III and IV. This further emphasizes the importance of hormones and nutrition during the critical early period after DMBA tumor induction.

It must be stressed however, that in these experiments, a 50% reduction in calories during the time of tumor induction was necessary for the inhibition of mammary tumorigenesis. The severity of such a restricted diet produces drastic alterations in the basic physiology of these animals. While it is possible that the inadequate calories or undernutrition in many developing countries of Asia and Africa may explain their low incidence in breast cancer, it is obvious that severe caloric restriction is not a practical method for the prevention of human breast cancer. Our studies however, do provide a mechanism by which underfeeding inhibits breast cancer and firmly establishes and clarifies the involvement of the endocrine system.

The majority of DMBA-induced rat mammary tumors are dependent on estrogen and PRL for development and growth, but a small percentage of tumors that develop are hormone independent. We were interested in

determining whether hormonal dependency during a tumor's growth phase was related to hormonal-dependency during the first critical week after carcinogen administration. Our results indicate that suppression of estrogen and PRL at the time of tumor induction not only significantly reduced tumor incidence and number, but the tumors which developed were less dependent on these hormones for their growth, as tumors develop and grow in size, more of them become independent of hormones. This could be due in part to loss of hormone receptors. It is possible that the early hormone-independent tumors dealt with in the present study were autonomous because they had few hormone receptors at the initiation of tumor development of DMBA.

In contrast to the rat, approximately 30-50% of human breast tumors respond to endocrine therapy and predictability for a particular breast cancer hormone-dependency is low (Costlow and McGuire, 1978). Whereas PRL and estrogen both have been shown to be essential for mammary tumor development and growth in rats, PRL has not been shown to be important in human breast cancer, even though a small number of PRL receptors have been shown to be present in human breast cancer tissue (Holdaway and Friesen, 1977). On the other hand, estrogen is of definite importance in human breast cancer. The observation in Experiment V that estrogen is more important than PRL in determining hormone dependency in early development of rat mammary tumors, suggests that in both rat and human breast cancer, estrogen may be more important than PRL in determining autonomy.

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