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INVOLVEMENT OF ENDOGENOUS OPIOID PEPTIDES IN REGULATION OF PROLACTIN AND LUTEINIZING HORMONE SECRETION

By

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ABSTRACT

INVOLVEMENT OF ENDOGENOUS OPIOID PEPTIDES IN REGULATION OF PROLACTIN AND LUTEINIZING HORMONE SECRETION

by

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1. Serum prolactin (PRL) concentrations in adult male rats were measured after acute administration of morphine (MOR), beta-endorphin (β-END), methionine-enkephalin (MET-ENK), dynorphin, or naloxone (NAL). Intraperitoneal injection of MOR significantly increased serum PRL concentrations. Similarly, serum PRL concentrations were significantly increased by intraventricular injection of β-END, MET-ENK, or dynorphin. In all cases, the stimulatory action of these opiates was blocked by concurrent injection of NAL, a specific opiate antagonist. Moreover, when NAL was administered alone, serum PRL concentrations were significantly reduced. These results suggest that endogenous opioid peptides (EOP) are involved in determining the basal secretion rate of PRL in adult male rats.

2. The effect of the opiate antagonist, NAL, on stress-induced PRL release was determined. Serum PRL levels were increased by ether, heat, or immobilization stress. Pretreatment of stressed rats with NAL significantly inhibited the release of PRL in response to all 3 stresses tested. The ability of NAL to block stress-induced PRL release suggests that EOP are involved in the process by which stress increases PRL secretion.

3. Treatment of male rats with L-DOPA, the precursor of dopamine (DA), piribedil, a DA agonist, or amineptine, a DA reuptake inhibitor, all decreased serum PRL concentrations and blocked the stimulatory action of MOR on PRL release. In a second experiment, the effect of intraventricular injection of β-END on alpha-methyl-para-tyrosine (α-mpt) depletion of median eminence (ME) DA was determined. β-END completely blocked α-mpt depletion of ME DA and significantly increased serum PRL concentration. It is concluded that opiates stimulate PRL release by decreasing tuberoinfundibular DA activity.

4. Injection of NAL increased serum luteinizing hormone (LH) concentration approximately 4-fold. Pretreatment with α-mpt, diethyldithiocarbamate, or phenoxybenzamine, all anti-noradrenergic drugs, inhibited

the stimulatory action of NAL. These results suggest that NAL-induced LH release is produced by activation of hypothalamic noradrenergic neurons.

5. Male rats were castrated and injected once daily with testosterone propionate or twice daily with MOR for 12 days. Blood samples were collected at 2 day intervals, and the hypothalamus was removed after decapitation on the twelfth day. The post-castration rise in serum LH was inhibited by either chronic testosterone or MOR administration. The reduction in hypothalamic LH-releasing hormone (LHRH) concentration in nontreated castrated rats was completely blocked by either testosterone or MOR. It is concluded that both MOR and testosterone inhibited the post-castration rise of LH by inhibiting the release of LHRH from the hypothalamus.

6. Administration of estradiol benzoate (EB) alone or together with progesterone reduced serum LH levels in long-term ovariectomized rats. This inhibitory action of ovarian steroids was reversed by a single injection of NAL. Similarly, NAL blocked the negative feedback action of testosterone propionate on LH release in acutely castrated male rats. These results indicate that EOP may at least partially mediate gonadal steroid inhibition of LH release in male and female rats.

DEDICATION

This thesis is dedicated to my wife, Jann. Her continual support and understanding were of invaluable assistance to the completion of this project. I also am very grateful to my parents, Ernest and Phyllis Van Vugt. Their words of encouragement and expressions of interest in my work were more important to me than perhaps they realized.

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INTRODUCTION

The number of hypothalamic factors which are known presently to influence anterior pituitary (AP) hormone secretion, either by a direct action on the pituitary or via the hypothalamic hypophysiotropic hormones, has grown to a list of over 20. The latest addition to this list of factors is a group of peptides which exhibit morphinomimetic properties. Presently, this group of peptides consists of the 2 enkephalins (methionine-enkephalin and leucine-enkephalin), dynorphin, and the 3 endorphins (alpha-, beta-, and gamma-endorphin). When referring to these brain opioid peptides as a group, it has become acceptable to use the term endogenous opioid peptides (EOP).

The discovery of the EOP was by no means a serendipitous process. Rather, it was a systematic effort by several groups to isolate and characterize an opioid ligand which they were certain existed in the brain. The idea that an endogenous opioid ligand existed in the brain was derived from work showing the presence of specific opiate receptors in brain tissue (Pert and Synder, 1973; Terenius, 1973). This hypothesis was given greater impetus by the finding that

analgesia could be produced by electrical stimulation of the mesencephalic grey area of the brain (Reynolds, 1969).

Hughes et al. (1975) successfully isolated from porcine brain, sequenced, and synthesized 2 opioid peptides in 1975, both pentapeptides with identical structures with the exception of the C terminal amino acid. One of the peptides contains methionine at the C terminus, whereas the other peptide contains leucine. These were named methionine-enkephalin (MET-ENK) and leucine-enkephalin (LEU-ENK), respectively.

A third opioid peptide, beta-endorphin (β-END) was isolated from camel pituitary and its structure identified in 1976 by C.H. Li and co-workers (Li and Chung, 1976). β-END contains 31 amino acids and is identical to $\beta\text{-lipotropin}_{61-91}$ ($\beta\text{-LPH}_{61-91}$). In addition, the first 5 amino acids of β-END are identical to MET-ENK. Guillemin and co-workers (1976) reported the structures of 2 additional endorphins. They were alpha- and gamma-endorphin and are identical in structure to $\beta\text{-LPH}_{61-76}$ and $\beta\text{-LPH}_{61-77}$, respectively. Most recently, a sixth opioid peptide has been partially identified by Goldstein et al. (1979). This peptide was named dynorphin_{1-13} and contains within its sequence the structure of LEU-ENK.

The guinea pig ileum and mouse vas deferens were commonly used for bioassay during the isolation procedure in order to determine opioid activity of the preparations. Like morphine (MOR), the EOP all inhibited smooth muscle contraction induced by electrical stimulation, and this effect was reversed by the specific opiate antagonist, naloxone (NAL). The EOP also mimicked other actions of MOR. These properties include analgesia, miosis, constipation, and catatonia. This demonstration that EOP exhibited MOR-like properties resulted in efforts by several laboratories to discover physiological roles for the EOP. Since MOR previously had been shown to influence anterior pituitary (AP) hormone secretion (Barraclough and Sawyer, 1955; George and Way, 1955; Meites, 1962; Lomax et al., 1970; Martin et al., 1975), the EOP also were tested for their effects on pituitary hormone secretion.

Prior to the discovery of the EOP, it was demonstrated that opiates such as MOR and methadone could stimulate secretion of prolactin (PRL, Meites, 1962; Clemens and Sawyer, 1974), growth hormone (GH, Martin et al., 1975), and adrenocorticotropin (ACTH, George and Way, 1951), and inhibit secretion of luteinizing hormone (LH, Barraclough and Sawyer, 1955), and thyroid stimulating hormone (TSH, Lomax et al.,

1970). Therefore it was logical to determine if the EOP had similar effects on AP hormone secretion. In recent years, it was shown by us and others that acute administration of MET-ENK and β-END could elicit changes in AP hormone secretion that are similar to those produced by MOR. These effects were reversed by NAL. Somewhat surprising was our observation that NAL given alone decreased basal serum PRL and GH levels and increased LH and follicle stimulating hormone (FSH) levels in the blood of mature male rats (Bruni et al., 1977). These results suggested to us that EOP may be involved in regulating the secretion rates of certain AP hormones during basal conditions.

Experiments also were done to determine if EOP were involved in the PRL response during non-basal physiological states. The previous finding that stress could produce analgesia, presumably by stimulating opioid neuronal activity in the brain, was a compelling reason for studying the role of EOP in stress-induced PRL release. Several stresses have been shown to produce a prompt and significant release of PRL (Euker et al., 1973; Krulich et al., 1974). Evidence will be presented in this thesis that EOP are involved in stress-induced PRL release.

The EOP do not alter hormone secretion by a direct

action on the AP. Thus, addition of opiates or NAL to hemipituitaries or pituitary cell cultures does not produce the hormonal response one observes *in vivo*. The logical alternative is that EOP alter AP hormone secretion via a hypothalamic mechanism. Hypothalamic factors important in the regulation of PRL secretion are numerous, and include tuberoinfundibular dopamine (DA), serotonin (5-HT), and possibly other presently unidentified PRL releasing and inhibiting factors. LH secretion is directly controlled by hypothalamic LH-releasing hormone (LHRH) that is released into the portal circulation. Additional factors which influence LH secretion indirectly include the biogenic amines, of which norepinephrine (NE) is stimulatory and appears to be the most important in the control of LH release. DA and 5-HT have been reported to either stimulate or inhibit LH (Meites *et al*., 1977), and hence may be of lesser importance. We have studied the interaction of opiates and hypothalamic catecholamines, and will present evidence in this thesis that opiates stimulate PRL release by inhibiting tuberoinfundibular DA turnover, and inhibit LH release by decreasing hypothalamic NE activity.

It is well known that testosterone tonically inhibits LH secretion in the male by a negative feedback

action on the hypothalamus and pituitary. This is also true of ovarian steroids in the female, except immediately prior to ovulation at which time ovarian steroids exert a positive feedback action on the hypothalamo-pituitary-gonadotropic axis. The stimulatory action of NAL on LH release suggested that it may be tonically inhibited by EOP. It was therefore of interest to determine if the EOP mediate the negative feedback action of gonadal steroids in male and female rats. To test this hypothesis, we determined the effects of NAL on the inhibitory action of gonadal steroids on LH release in castrated male and female rats. Presumably, if gonadal steroids inhibit LH release by activating opiate neurons, NAL should be able to block this action of gonadal steroids. In addition, we compared the effects of MOR and testosterone on the release of LHRH from the hypothalamus of castrated male rats. The results of these experiments suggest that EOP mediate gonadal steroid inhibition of LH release in male and female rats.

LITERATURE REVIEW

I. Hypothalamic Control of Anterior Pituitary Hormone Secretion

A. Classical Observations

It has been known for many years that the pituitary is intimately involved in many important physiological functions and is necessary for the maintenance of normal life. The pituitary was shown to contain hormones that are necessary for body growth (Evans and Long, 1921; 1922), for growth and maturation of the ovaries and testes (Smith, 1926; Zondek and Ascheim, 1926), for normal thyroid (Allen, 1919; Smith and Smith, 1922) and adrenal cortical function (Allen, 1922; Smith, 1926), for milk production (Stricker and Grueter, 1928), and for many other functions. These observations led to the pituitary being called the "master gland" of the body. Subsequent to these studies, it was demonstrated that the central nervous system (CNS), particularly the hypothalamus, exerts a profound control over pituitary function. Although this finding does not diminish the importance of pituitary function, it does make

questionable the appropriateness of referring to the pituitary as the "master gland" since it is itself "mastered" by the hypothalamus.

The importance of the CNS in the control of AP hormone secretion was apparent from experiments in which the hypothalamus was either lesioned or stimulated, or when hypothalamic input to the pituitary was prevented by stalk transection or transplantation of the pituitary to other sites in the body. Appropriate hypothalamic lesion produced atrophy of the gonads (Ascher, 1912) and thyroid (Cahane and Cahane, 1936; Bogdanove and Halmi, 1953), and blocked stress-induced adrenal hypertrophy (Ganong and Hume, 1954). Conversely, electrical stimulation of the hypothalamus resulted in ovulation (Harris, 1937), and increased thyroid (Harris, 1948) and adrenal cortical activity (deGroot and Harris, 1950). These effects of hypothalamic stimulation can not be attributed to nonspecific stimulation of the pituitary, since direct stimulation of the pituitary did not produce these effects (Markee, 1948).

Stalk transection produced endocrine responses similar to those produced by hypothalamic lesions. Thus gonadal (Dott, 1923) and thyroidal (Mahoney and Sheehan, 1936) atrophy, and reduced adrenal activity (Fortier et al., 1957) were observed after stalk transection.

Similar deficiencies in the gonads, thyroid, and adrenals were seen after transplanation of the pituitary to the anterior eye chamber or underneath the kidney capsule (Harris, 1948; 1955).

It would be incorrect to assume from these results that the input from the hypothalamus is only stimulatory. The hypothalamus is primarily inhibitory to PRL secretion. Thus, ectopic pituitary grafts resulted in the maintenance of corpora lutea (Everett, 1954; 1956) and mammary gland function (Meites, 1967), due to increased PRL secretion following removal of hypothalamic inhibitory factors (Meites *et al.*, 1961; Pasteels, 1961). The hypothalamus also contains a GH inhibitory factor (Krulich *et al.*, 1968) which has since been shown to be somatostatin (Brazeau *et al.*, 1973).

There is no evidence for additional hypothalamic inhibiting factors for the AP hormones, but hypothalamic releasing factors have been postulated for all of the AP hormones. Three hypothalamic hypophysiotropic hormones have been purified and sequenced thus far: TRH, LHRH, and somatostatin. Others not yet structurally identified will be discussed in the section on hypophysiotropic hormones.

B. Hypothalamic Anatomy

The hypothalamus is the most ventral portion of the diencephalon and comprises approximately 1% of total brain mass. The anatomical boundaries of the hypothalamus are demarcated rostrally by the optic chiasm and lamina terminalis, caudally by the mammillary bodies, and dorsally by the hypothalamic sulci of the third ventricle. Laterally, the boundary between the hypothalamus and subthalamus is indistinct.

The hypothalamus can be divided into 3 areas. The 3 areas from rostral to caudal are the supraoptic, tuberal, and mammillary regions. The supraoptic hypothalamus lies above the optic chiasm and fuses with the more rostral preoptic area which is usually not considered part of the hypothalamus. The supraoptic area contains 2 paired nuclei. They are the supraoptic and paraventricular nuclei which are dorsal to the optic chiasm with the paraventricular nucleus being dorsomedial to the supraoptic nucleus. The supraoptic and paraventricular nuclei contain the cell bodies which give rise to the supraopticohypophyseal tract which transports antidiuretic hormone and oxytocin to the neurohypophysis.

The tuberal or intermediate hypothalamus lies between the optic chiasm and the mammillary bodies.

Situated at the base of the third ventricle are the arcuate and periventricular nuclei which contain the cell bodies of the tuberoinfundibular DA neurons. Slightly dorsolateral to these nuclei are the ventromedial and dorsomedial nuclei. These nuclei make up the medial border of the lateral hypothalamic nucleus which is bordered laterally by the optic tract and subthalamus. The ventral surface of the tuberal hypothalamus contains the median eminence (ME). The ME can be divided into the following 3 zones: a) inner ependymal zone which contains ependymal cells which line the third ventricle; b) inner palisade layer which contains the hypothalamo-hypophyseal neurons; c) outer palisade layer which contains the junction between the tuberohypophyseal neurons and the capillary plexus (Knigge and Scott, 1970). The mammillary or caudal hypothalamic area contains the caudal hypothalamic nucleus.

The hypothalamus contains both afferent and efferent nerve tracts (Jenkins, 1972). Postcommissural fibers of the fornix terminate in the lateral portion of the mammillary body. A portion of the medial forebrain bundle which originates in the septal area of the olfactory stria innervates several hypothalamic nuclei before continuing on to the midbrain tegmentum. Hypothalamic nuclei receive input from the thalamus via the

periventricular fibers. The stria terminalis originates in the amygdaloid complex and arches caudodorsally prior to terminating mainly in the supraoptic area of the hypothalamus.

Two efferent tracts originate in the mammillary body. The mammillothalamic tract passes rostrodorsally and terminates in the thalamus, whereas the mammillotegmental tract passes caudally and terminates in the brain stem tegmentum. There are lightly myelinated fibers which course from the periventricular nucleus to the dorsomedial thalamic nucleus and a caudal tract which innervates the brain stem via the dorsal longitudinal bundle. The supraoptic and paraventricular nuclei give rise to the supraoptico-hypophyseal tract which is joined in the ME by the tuberohypophyseal tract of the tubercinereum. Upon joining, these 2 tracts make up the hypothalamo-hypophyseal tract which terminates in the posterior lobe of the pituitary. It is this tract which delivers oxytocin and antidiuretic hormone from the paraventricular and supraoptic nuclei respectively to the posterior pituitary for storage.

C. Hypothalamo-Hypophyseal Portal Vessels and Neurosecretion

Unlike the neurohypophysis, the pars distalis has no neural connections with the hypothalamus. Rather, the connection between hypothalamus and AP is vascular. Popa and Fielding (1930) first described a portal system which connected the sinusoids of the AP with a capillary plexus in the ME. Based on morphological evidence alone, they proposed that blood flowed from the AP to the hypothalamus. Houssay *et al*. (1935) were the first to correctly propose that blood flow was from hypothalamus to AP. Definite proof of this proposal was obtained with the use of vital dyes. Systemic injection of vital dyes into the toad resulted in their appearance in the capillary plexus of the ME prior to their appearance in the AP (Wislocki and King, 1936). Green and Harris (1949) subsequently showed that portal blood flow in the mammal was from hypothalamus to AP.

The total blood flow to the AP is by way of the portal vessels. The only exception is in the rabbit (Harris, 1947; Goldman and Saperstein, 1962). The hypophyseal portal system is comprised of long and short portal vessels. The superior hypophyseal artery gives rise to the long portal vessels which travel along the lateral and anterior aspects of the infundibulum to the

sinusoids of the AP (Netter, 1965). The long portal vessels provide 70-90% of the total blood to the AP (Adams et al., 1963; Porter et al., 1967). The remaining blood to the AP is supplied by the short portal vessels which originate in the distal portion of the infundibulum, and travel deep within the infundibulum to the AP (Netter, 1965). Thus, although a neural connection between the hypothalamus and AP does not exist, the hypothalamo-hypophyseal portal vessels provide a means by which CNS activity can infuence AP function.

The concept of neurosecretion was proposed in the late 1930's and early 1940's shortly after the hypophyseal portal vessels and the direction of its blood flow was correctly defined. Haterius (1937) and Hensey (1937) first proposed that CNS activity resulted in the release of factors into the portal vasculature which upon reaching the AP elicited a response. Scharrer and Scharrer (1940) first proposed the existence of neurosecretion into the general circulation. The Scharrers, together with Bargman, demonstrated that antidiuretic hormone and oxytocin were synthesized in the supraoptic and paraventricular nuclei, and were transported by axonal flow to the posterior pituitary where they were stored until released into the general circulation

(Bargman and Scharrer, 1951; Scharrer, 1952; Scharrer and Scharrer, 1954). During this period in which the concept of neurosecretion was beginning to be established, Harris introduced the chemotransmitter hypothesis. Based on the earlier morphological work on the portal circulation and the pioneering work of Bargman and the Scharrers, Harris suggested that hypothalamic activity resulted in the secretion of hormones into the portal circulation, which upon reaching the AP, regulated the secretion of AP hormones (Harris, 1948).

The chemotransmitter hypothesis provided a mechanism to explain how exteroceptive stimuli such as light, temperature, olfaction and sound affect hormone secretion (Marshall, 1942; Harris, 1955). The neurosecretory cells of the hypothalamus are now viewed as transducer cells which convert the electrical signals of the CNS into chemical messages which can be perceived by the AP cells. This function of the neurosecretory cells is necessary for the coordination of the CNS with the endocrine system and for the maintenance of homeostasis (Wurtman, 1973).

D. Hypohysiotropic Hormones

Shortly after the chemotransmitter hypothesis was proposed, several laboratories attempted to show that hypothalamic releasing and inhibiting factors did indeed exist. These studies were done in the 1950's and 1960's. The first releasing factor to be discovered was corticotropin releasing factor (CRF) in 1955 (Saffran and Schally, 1955; Guillemin and Rosenberg, 1955). They showed that the addition of NE to a hypothalamic-pituitary coincubation system resulted in the release of ACTH. This effect of NE was not observed in the absence of hypothalamic tissue, suggesting that NE released an ACTH-releasing factor from the hypothalamus.

Subsequently, releasing factors for thyrotropin (Shibusawa, 1956), PRL (Meites et al., 1960), LH (McCann et al., 1960), FSH (Igarashi and McCann, 1964; Mittler and Meites, 1964), and GH (Deuben and Meites, 1964) were demonstrated. In addition, hypothalamic inhibiting factors were reported for PRL (Pasteels, 1961; Talwalker et al, 1963), and GH (Krulich et al., 1968).

Thus far, only 3 of the 8 hypothalamic releasing and inhibiting factors have been sequenced and synthesized. The sequence and synthesis of thyrotropin releasing hormone (TRH) was performed independently by the laboratories of Guillemin (Burgus et al., 1969) and

Schally (Boler et al., 1969) and shown to be a tripeptide. Luteinizing hormone releasing hormone (LHRH), a decapeptide, was sequenced and synthesized 2 years later by Schally's group (Matsuo et al., 1971). In 1973, GH inhibiting hormone or somatostatin was sequenced (Brazeau et al., 1973) and synthesized (Rivier et al., 1973) by Guillemin and coworkers and shown to be a tetradecapeptide. At present, the structures of the other hypothalamic factors have not been discovered, although partial sequences for CRF and GH releasing factor have been reported.

The structural identification and synthesis of these 3 hypothalamic hormones has made their localization possible through immunohistochemical studies and radioimmunoassay (RIA) of discrete areas of the brain. All 3 peptides are located in the ME in high concentrations (Brownstein et al., 1976b). This would be expected since the ME is the final common pathway all factors must take in their transport to the capillary plexus prior to diffusing into the portal vessels. Lower concentrations of LHRH are found in the preoptic-suprachiasmatic area, and LHRH perikarya have been visualized in this region. Electrical stimulation of the preoptic area was shown to increase LHRH concentration in portal blood (Eskay et al., 1977). Deaffer-

entation of the medial basal hypothalamus (MBH) resulted in decreased LHRH concentration in the MBH (Weiner et al., 1975; Kalra et al., 1977; Brownstein, 1977). Efforts to visualize LHRH cell bodies in additional brain areas of the rat have been unsuccessful. Therefore, it is now believed that LHRH neurons which originate in the preoptic area are the only source of ME LHRH in the rat (Baker et al., 1975; Weiner et al., 1975).

As with LHRH, TRH and somatostatin have high concentrations in the external layer of the ME. TRH and somatostatin also have been localized in brain regions other than the hypothalamus (Hökfelt et al., 1975a; Jackson and Reichlin, 1979). TRH and somatostatin also are distributed outside the brain including gut and pancreas (Hökfelt et al., 1975a; Jackson and Reichlin, 1979) and substantia gelatinosa (Hökfelt et al., 1975b).

Although LHRH, TRH and somatostatin alter the release of their respective AP hormones in a dose dependent manner (Schally, 1973), they are not without effect on other hormones. TRH stimulates PRL secretion in humans in addition to TSH (Jacobs et al., 1971). Furthermore, TRH antiserum was reported to decrease both serum TSH and PRL levels in the rat (Koch et al., 1977). However, it is unlikely that TRH is a major physio-

logical PRL-releasing factor since there is a poor correlation between PRL and TSH secretion during many physiological conditions. Somatostatin, in addition to inhibiting GH release, has been reported to inhibit TRH induced TSH, but not TRH-induced PRL secretion (Vale et al., 1974), whereas somatostatin antiserum increased both GH and TSH levels (Ferland et al., 1976b). LHRH stimulates LH and FSH secretion. However, the secretory patterns of LH and FSH are different, which has led to the speculation of different gonadotropin releasing hormones for LH and FSH. Alternatively, it has been shown that the different secretory profiles of LH and FSH may be due to their different half-lives (Gay et al., 1970), modulation by the steroid environment (Schally et al., 1973; Yen et al., 1975), or a specific inhibitory action of inhibin from the gonads on FSH secretion.

II. Localization of Biogenic Amines and Opiates

A. Dopamine

The presence of DA in the mammalian brain was first demonstrated in 1957 (Montagu, 1957). The observation that DA distribution was distinct from that of NE which

had previously been isolated, suggested that DA may be a neurotransmitter rather than merely the precursor of NE (Bertler and Rosengren, 1959). The distribution of DA in the brain can be divided into 3 distinct systems. The nigrostriatal system contains approximately 80% of the total DA within the brain. Cell bodies within the zona compacta of the substantia nigra (A_9) combine with a cell group (A_8) in the adjacent ventral tegmental area. Together, these give rise to dopaminergic neurons which course rostrally and terminate in the putamen and caudate nucleus (striatum; Andén et al., 1964; 1966c). The nigrostriatal dopaminergic system is involved in the normal control of extrapyramidal upper motor neurons. A deficiency in this system has been shown to be associated with Parkinson's disease (Hornykiewicz, 1963). The mesolimbic dopaminergic system originates in the A_{10} cell group and passes rostrally in close association with the nigrostriatal DA neurons before terminating in the nucleus accubens and olfactory tubercle (Andén et al., 1966; Ungerstedt, 1971; Weiner et al., 1972b).

The third dopaminergic division is the tuberoinfundibular DA system (Fuxé and Hökfelt, 1966). Immunohistofluorescent studies show that the tuberoinfundibular DA system is located completely within the

hypothalamus. Its cell bodies are in the arcuate and periventricular nuclei (Fuxé, 1963). Tuberoinfundibular DA neurons terminate in the external layer of the ME (Fuxé and Hökfelt, 1966; Ungerstedt, 1971), while others continue on and terminate in the neural and intermediate lobes (tuberohypophyseal DA system). Deafferentation of the hypothalamus does not result in a reduction of hypothalamic DA concentration (Weiner et al., 1972b), which further supports the view that tuberoinfundibular DA cell bodies are restricted to the hypothalamus.

B. Norepinephrine

The presence of NE in the brain was demonstrated in 1939 (Holtz, 1939). The cell bodies of NE neurons are located in the pons-medulla (A_1, A_2, A_5, and A_7) and locus coeruleus (A_6). These cell groups give rise to axons which enter the medial forebrain bundle and dorsal bundle which innervate the lower brainstem, limbic system, cerebral cortex, hippocampus and hypothalamus (Fuxé, 1965a, 1965b; Ungerstedt, 1971). In the hypothalamus, NE is concentrated in the retrochiasmatic area of the anterior hypothalamus, the supraoptic, paraventricular, and periventricular nuclei, and in the ME (Fuxé, 1965a, 1965b). NE in the ME is primarily associated with the internal layer in contrast to DA

which is found primarily in the external layer (Jonsson et al., 1972). Lesioning of the locus coeruleus (Loizou, 1969; Korf et al., 1973a; 1973b) or medial forebrain bundle (Kobayashi et al., 1974) reduces hypothalamic NE concentration. Hypothalamic deafferentation causes a complete loss of the enzyme, DA-β-hydroxylase, as well as NE in the hypothalamus (Brownstein et al., 1976a). These results indicate that the source of hypothalamic NE is located completely outside the hypothalamus.

C. Serotonin

In addition to the catecholamines, 5-HT also is an important hypothalamic neuromodulator of AP hormone secretion. Amin (1954) first showed that 5-HT was present in the CNS of mammals, and that the hypothalamus contained the highest brain concentration. 5-HT cell bodies are localized in the mesencephalic raphé (B_7, B_8, and B_9) and pontine raphé (B_5 and B_6) from which afferents ascend in association with the medial forebrain bundle to innervate forebrain regions and the hypothalamus (Dahlström and Fuxé, 1964; Aghajanian et al., 1969; Ungerstedt, 1971; Kuhar et al., 1972).

The suprachiasmatic, periventricular, and arcuate nuclei, as well as the ME, are areas of the hypothalamus

which contain the highest concentrations of 5-HT. Similar to DA, 5-HT in the ME is localized in the external layer (Saavedra et al., 1974). Stimulation of the raphé produces increased 5-HT turnover in the forebrain (Sheard and Aghajanian, 1963; Andén et al., 1966d), whereas lesioning of the medial forebrain bundle and raphé results in reduced concentrations of 5-HT and tryptophan hydroxylase, as well as 5-HT turnover in the forebrain (Kuhar et al., 1972).

D. Opiates

MET-ENK and LEU-ENK are distributed widely throughout the CNS and periphery. The ratio of MET-ENK to LEU-ENK concentrations in different brain regions varies from 2-9 (Yang et al., 1977; Kobayashi et al., 1978) with MET-ENK concentrations always exceeding LEU-ENK. The distribution of MET-ENK and LEU-ENK are very similar, and they may be synthesized in the same neurons (Akil et al., 1978). Although the distribution of enkephalins in the CNS is diffuse, there are dramatic differences in concentrations from 1 region to another. This was shown to be true of MET-ENK in a study in which concentrations were measured by RIA in 34 different brain regions of the rat (Yang et al., 1978). The highest concentration of MET-ENK was found in the globus

pallidus (76 ng/mg protein). Other areas with high concentrations were the caudate nucleus (10 ng/mg), nucleus accubens (10 ng/mg), medial and lateral preoptic nuclei (6.5 and 4.2 ng/mg, respectively), and hypothalamus. Significant concentrations of MET-ENK were present in all hypothalamic nuclei with the highest concentrations present in the anterior hypothalamic nucleus (6.6 ng/mg), ventral medial nucleus (5.4 ng/mg), and lateral hypothalamic nucleus (5.0 ng/mg). Additional brain regions exhibiting relatively high concentrations included the interpeduncular nucleus, periaqueductal grey, dorsal raphé, and medial raphé. Areas of lowest concentrations were the cerebral cortex (0.98 ng/mg) and cerebellum (0.47 ng/mg).

The distributions of enkephalins as determined by RIA are in good agreement with the immunohistochemical studies (Hökfelt et al., 1977; Johansson et al., 1978). Outside the brain, enkephalins are concentrated in laminae I, II, V, and VII of the spinal cord, and the substantia gelatinosa of the spinal trigeminal nucleus (Hökfelt et al., 1977; Johansson et al., 1978). Peripherally, enkephalins are found in several sympathetic ganglia (Johansson et al., 1978) and gastrointestinal muscle layers. In fact, the concentration of enkephalins in the myenteric plexus of the guinea pig

ileum was shown to be equivalent to the concentration in the striatum (Hughes et al., 1977).

Enkephalin neurons are typically short and are closely associated with opiate receptors (Kuhar et al., 1973; Pert et al., 1974; Watson et al., 1977). It has been suggested that enkephalin neurons may be interneurons which connect local circuitry (Watson et al., 1977). Thus, injection of kainic acid into the striatum reduced MET-ENK and LEU-ENK concentrations approximately 50%, whereas cortical ablation had no effect on enkephalin concentrations in the striatum (Childers et al., 1978). Lesioning of striatal DA neurons with 6-OH-DA dramatically reduced the number of opiate receptors in the striatum (Pollard et al., 1977; Carenzi et al., 1978). These results demonstrate the close association that exists between enkephalins and their receptors in the striatum, and suggest a possible enkephalin-DA circuit in the striatum.

The distribution of endorphins as determined by immunohistochemical and RIA studies is drastically different from the distribution of enkephalins. The highest concentrations of β-END, β-LPH, and α-END are in the pituitary, particularly in the intermediate lobe. Immunohistochemistry revealed the presence of β-END and α-END in every cell of the intermediate lobe, whereas in

the AP, the endorphins were restricted to ACTH producing cells. The posterior pituitary contained insignificant amounts of endorphins which were thought to be due to contamination from the intermediate lobe (Bloom et al., 1978). The relative concentrations of β-END in the pituitary as measured by RIA are in good agreement with the immunohistochemical studies. The concentration of β-END in the intermediate lobe was reported to be in the mg/g wet weight range, as compared to a concentration in the ug/g range for the AP. β-END was undetectable by RIA in the posterior lobe (Rossier et al., 1977). β -END also can be measured in blood with reported values ranging from high picogram/ml to low ng (5.0)/ml. The source of β-END in the blood is thought to be the pituitary (Jeffcoate et al., 1978).

The concentrations of β-LPH and endorphins in the brain were shown to be much lower than in the pituitary (Bloom et al., 1977; Krieger et al., 1977; Rossier et al., 1977; Akil et al., 1978). The concentration of β-LPH in the pituitary was reported to be 50,000 picomoles/g versus 700 and 125 picomoles/g in the hypothalamus and midbrain, respectively (Akil et al., 1978). Of all brain regions, the hypothalamus was shown to have the highest concentrations of α- and β-END (ng/g), which is considerably lower than in the pituitary (Rossier et

al., 1977). β-END cell bodies were demonstrated in the paraventricular, supraoptic, suprachiasmatic, and anterior hypothalamic nuclei, as well as in the ME (Bloom et al., 1978). Nerve tracts which originate in the hypothalamus project throughout the neuraxis and innervate midline structures such as substantia nigra, mesencephalic central grey, raphe and locus coeruleus (Akil et al., 1978). β-END was undetectable in the striatum, brain stem, and spinal cord (Rossier et al., 1977; Akil et al., 1978).

A comparison of enkephalin and endorphin distributions reveals some striking differences. The greatest concentrations of endorphins are in the pituitary, whereas only minimal concentrations of enkephalins are found in the pituitary. The enkephalins and endorphins are distributed fairly evenly throughout the hypothalamus and neuraxis. However, the more lateral brain regions contain primarily enkephalins and not endorphins. Endorphins are not present in the striatum or spinal cord, both of which contain high concentrations of MET- and LEU-ENK.

Shortly after it was recognized that the structure of MET-ENK was identical to the first 5 amino acids of the endorphins, it was suggested that the enkephalins were merely metabolites of endorphins. This proposal

was soon discarded, largely because of the distinctly different distributions of endorphins and enkephalins. The endorphins and enkephalins are now thought to be independent neuronal systems which do not necessarily have identical physiological functions.

III. Biogenic Amine Metabolism

A. Synthesis and Release

The synthesis of DA and NE begins with active uptake of tyrosine into catecholamine neurons (Iverson, 1971). Tyrosine is converted to DA and NE by a chain of enzymatic reactions which occurs at the nerve terminals. The enzymes involved in these conversions are synthesized in catecholamine cell bodies and transported to the nerve terminals (McClure, 1972). Tyrosine is hydroxylated to dihydroxyphenylalanine (DOPA) by the rate limiting enzyme tyrosine hydroxylase (Levitt et al., 1965). Tyrosine hydroxylase is active only when in its reduced form which is accomplished by the co-factor tetrahydropteridine. DOPA is decarboxylated by aromatic-L-amino acid decarboxylase to DA. Unlike tyrosine hydroxylase, aromatic-L-amino acid decarboxylase is a nonspecific enzyme (Carlsson et al., 1972), which is common to all tissues and metabolizes neutral amino acids (Goldstein et al., 1974). DA is hydroxylated to NE by the hydroxylating enzyme, DA-β-hydroxylase. DA-β-hydroxylase is a tetrameric glycoprotein which contains four moles of Cu^{+2} (Goldstein et al., 1965). It is localized on the membrane storage

vesicles of NE neurons only (Potter and Axelrod, 1963; Friedman and Kaufman, 1965), unlike tyrosine hydroxylase which is found in all catecholamine neurons. Epinephrine is the final product of the enzymatic reactions which begin with the hydroxylation of tyrosine. Epinephrine is the product of NE methylation by phenylethanolamine-N-methyltransferase. This enzyme is found in high concentrations in the adrenal medulla, but has a low concentration in the brain (Axelrod, 1962). Therefore, the importance of epinephrine as a brain neurotransmitter is not well established.

Steady state DA and NE concentrations are maintained by product inhibition of tyrosine hydroxylase. Decreased activity of catecholamine neurons results in increased concentrations of intraneuronal DA and NE. DA and NE compete with oxidized tyrosine hydroxylase for binding to tetrahydropteridine and thus decrease the amount of reduced tyrosine hydroxylase (Udenfriend *et al.*, 1965; Costa and Neff, 1966). The end result is a decrease in active tyrosine hydroxylase and reduced synthesis of catecholamines. Conversely, increased release of DA or NE increases tyrosine hydroxylase activity resulting in increased DA or NE synthesis (Sedvall *et al.*, 1968). Thus, acute changes in catecholaminergic activity do not alter catecholamine

stores.

5-HT is synthesized from tryptophan in 2 enzymatic steps. Tryptophan is actively taken up into the nerve terminal and hydroxylated to 5-hydroxytryptophan (5-HTP) by the rate limiting enzyme, tryptophan hydroxylase (Udenfriend, 1959; Ichiyama et al. 1970). 5-HTP is decarboxylated by aromatic-L-amino acid decarboxylase to 5-HT (Udenfriend, 1959). This enzyme also catalyzes the conversion of DOPA to DA. Thus administration of DOPA in order to increase DA synthesis is somewhat non-specific since 5-HT neurons also convert DOPA to DA, and DA inside 5-HT neurons can displace 5-HT (Bartholini et al., 1968).

The synthesis of 5-HT is not as finely controlled as that of DA and NE. The rate limiting enzyme, tryptophan hydroxylase, normally is not saturated (Lovenberg et al., 1968; Fernstrom and Wurtman, 1971). In addition, product inhibition of tryptophan hydroxylase does not exist under normal conditions (Lin et al., 1969; Macon et al., 1971), although product inhibition can be demonstrated when 5-HT levels are elevated by pharmacological methods (Macon et al., 1971).

Monoamines are stored within vesicles at the nerve terminals. Unlike the monoamines which are synthesized at the teminals, the vesicles are synthesized in the

nerve cell body and transported to the terminal by axonal flow (Dahlström et al, 1973). Vesicular storage of the monoamines serves 2 functions: a) prevents enzymatic degradation (Hökfelt, 1968); b) provides a mechanism for quantal release. The existence of 2 pools of monoamines has been suggested (Axelrod, 1974). There is a readily releasable pool characterized by vesicles closely associated with the nerve terminal membrane and believed to be made up of newly synthesized neurotransmitters. There also is thought to be a larger storage pool more distant from the membrane which may serve as a reservoir. Nerve activity results in release of newly synthesized monoamines (Kopin et al., 1968; Glowinski, 1972). The neurotransmitter is released into the extracellular space by the process of exocytosis in which the vesicular membrane fuses with the nerve terminal membrane. This event is calcium dependent (DeRobertes and Vas Ferreira, 1957).

B. Monoamine Inactivation

The action of monoamines is terminated by 3 different means. They are reuptake by the nerve terminal, enzyamtic metabolism, and diffusion from the receptor sites. Reuptake is the most important means of inactivating CNS DA, NE, and 5-HT (Glowinski et al., 1965;

Iverson, 1967; Coyle and Synder, 1969). Reuptake of monoamines, in addition to terminating neurotransmitter action, is also a method for recycling amines and thus is metabolically practical. The reuptake process is saturable, stereospecific and NA^+ dependent (Iverson, 1974). However, the reuptake systems are not totally specific. Reuptake of 5-HT by catecholamine neurons has been demonstrated (Lichtensteiger *et al.* 1967; Bartholini *et al.*, 1968).

Monoamine oxidase (MAO) and catechol-o-methyl transferase (COMT) are the 2 primary enzymes involved in enzymatic degradation of DA, NE, and 5-HT. MAO is intraneuronal and associated with the mitochondria (Nukada *et al.*, 1963). MAO deaminates DA and NE to 3,4-dihydroxyphenylacetaldehyde and 3,4-dihydroxyphenylglycolaldehyde, respectively. Extraneuronally, COMT o-methylates DA to 3-methoxytyramine and NE to normetanephrine (Alberici *et al.* 1955; Axelrod *et al.*, 1959). The primary metabolic product of 5-HT is 5-hydroxyindoleacetic acid and is accomplished by MAO and an aldehyde-dehydrogenase (Sjoerdsma *et al.*, 1955).

Inhibition of catecholamine uptake by cocaine, phenoxybenzamine or imipramine can greatly potentiate adrenergic stimulation (Iversen, 1971), whereas inhibitors of MAO and COMT only slightly potentiate

sympathetic stimulation (Pletscher, 1973). These observations suggest that reuptake of monoamines, and not degradation, is the primary means of monoamine inactivation.

IV. Opioid Peptide Metabolism

A. Synthesis

Although presently incomplete, the area of EOP metabolism has been expanded significantly since their discovery. Synthesis of enkephalins and endorphins appear to be separate processes, and the proposal that enkephalins are synthesized from β-LPH or merely metabolites of the endorphins is no longer accepted. Rejection of this proposal is based on the following observations: The distribution of enkephalins is distinctly different from the distribution of endorphins (Bloom *et al.*, 1976). An endopeptidase capable of processing MET- ENK from either β-LPH or β-END has not been isolated (Smyth, 1980). Furthermore, an opioid peptide of greater molecular weight than the enkephalins has been shown to produce a peptide upon trypsin degradation that is identicle to MET-ENK in terms of immunoreactivity and receptor binding activity (Lewis *et*

al., 1978; Yang et al., 1978). This putative precursor of MET-ENK was shown not to be β-LPH or a fragment of β-LPH.

A potential precursor of LEU-ENK has been identified and was named α-neo-endorphin (Kangawa et al., 1979). The N-terminus contains the structure of LEU-ENK and has a total of 15aa. Dynorphin may also be a precursor of LEU-ENK. Dynorphin has been partially sequenced (first 13aa), and its N-terminus also is identicle to the structure of LEU-ENK (Goldstein et al., 1979). Dynorphin and α-neo-endorphin exhibit significantly greater opioid activity than LEU-ENK, and is thought to be due to increased resistance to enzymatic degradation.

The structure of a MET-ENK precursor has not yet been identified. However, the precursor has been given the provisional name of β-neo-endorphin (Smyth, 1980). It is speculated that α-neo-endorphin and β-neo-endorphin may in turn have a common precursor. This hypothesis is based on the observation that the distributions of LEU- and MET-ENK are identical and may be synthesized in the same cells (Watson et al., 1978; Smyth et al., 1980).

The amino acid sequence of β-END is identical to $\beta\text{-LPH}_{61\text{-}91}$, and β-LPH is believed to be the precursor of

β-END. β-LPH also is a cleavage product of pro-opiocortin, a 31K precursor molecule. Pro-opiocortin, as demonstrated by the pulse-chase experiments of Mains et al. (1977) gives rise to one molecule of β-LPH, which is cleaved to β-END ($\beta\text{-LPH}_{61-91}$) and β-melanocyte stimulating hormone ($\beta\text{-LPH}_{38-58}$). In addition, pro-opiocortin is the precursor molecule of ACTH. That pro-opiocortin is in fact the precursor of both ACTH and β-END is further supported by immunohistochemical data demonstrating the presence of ACTH, β-LPH and β-END in the same cells of the AP (Bloom et al., 1977). Furthermore, ACTH and β-END are released concommitantly from the pituitary. Thus, acute stress, adrenalectomy, and CRF stimulated ACTH and β-END release, whereas dexamethasone inhibited both ACTH and β-END release (Guillemin et al., 1977; Vale et al., 1979).

It has not been firmly established that α-END ($\beta\text{-LPH}_{61-76}$) and γ-END ($^{\beta}\text{-LPH}_{61-77}$) are naturally occurring EOP. There is speculation that they may be artifacts of the procedures used to extract β-END (Rossier et al., 1977). When brain tissues were boiled prior to extraction in order to limit enzymatic breakdown of β-END, α-END was undetectable in the tissue. However, when steps were not taken to limit β-END degradation, α-END was detectable, but at the expense of

reduced β-END concentration (Rossier et al., 1977).

B. Release

Increased nerve electrical activity is believed to release EOP. Thus depolarization of enkephalin neurons by K^+ was reported to increase enkephalin release in vitro. K^+-induced enkephalin release was Ca^{++}-dependent (Smith et al., 1976; Osborne et al., 1980). There are numerous reports containing indirect evidence that opiate release is coupled to neuronal electrical activity. Reynolds (1969) first reported that electrical stimulation of the mesencepalic grey region produced analgesia. Subsequently, Akil (personal communication) showed that β-END concentration in the cerebral spinal fluid was increased by stimulation of the mesencephalic grey region.

Stress also has been shown to produce analgesia. Akil et al. (1976) reported that intermittent footshock evoked analgesia as measured by the tailflick test, and this effect of stress was reversed by NAL. Furthermore, opioid activity in the brain as measured by bioassay was increased by stress. Stress-induced analgesia has been confirmed by others, and is thought to be due to the release of EOP (Madden et al., 1977; Fried and Singer, 1979).

Because of the inherent problems associated with interpreting concentrations of substances in the brain, a number of investigators have studied the effects of different treatments on β-END concentrations in the blood and pituitary in order to evaluate release. The concentration of β-END in the blood was increased by intermittent footshock or immobilization stress (Guillemin et al., 1977; Rossier et al., 1979; Sapun et al., 1981). Stress also was reported to decrease β-END concentration in the pituitary (Baizman et al., 1979), indicating that stress stimulates the release of β-END from the pituitary. Plasma β-END concentration was reported increased after adrenalectomy, in addition to elevated ACTH levels, and this effect of adrenalectomy was reversed by dexamethesone (Guillemin et al., 1977; Akil et al., 1979; Rossier et al., 1979). Adrenalectomy also was reported to increase β-END concentrations in the pituitary, hypothalamus and midbrain (Lee et al., 1980). Blood levels of β-END were reported elevated during pregnancy and parturition (Akil et al., 1979).

There is evidence that hypothalamic factors may be involved in the control of β-END release from the pituitary. Purified CRF and NE were reported to stimulate β-END release from AP cells in vitro, whereas DA and apomorphine inhibited in vitro release of β-END

from the intermediate lobe (Vale et al., 1979). 5-HT has been reported to stimulate β-END release from the pituitary. Administration of 5-HTP or quipazine, a 5-HT agonist, elevated plasma β-END levels, whereas 5,7-dihydroxytryptamine significantly reduced the stress-induced rise of plasma β-END levels (Sapun et al., 1981).

It is not known presently what function circulating β-END serves. Nor is it known whether the factors involved in the regulation of pituitary β-END secretion apply to endorphin and enkephalin neuronal systems in the brain. The answer to this latter question depends on the development of new techniques for measuring opioid activity in the CNS.

C. Inactivation

The enkephalins have extremely short half-lives ($t_{1/2}$). When ^{3}H-MET-ENK was injected iv , 74% of the radioactivity measured 15 sec later migrated with tyrosine, suggesting that the TYR-GLY bond is cleaved very rapidly (Dupont et al., 1977). The tetrapeptide produced by removal of the N-terminal tyrosine was shown to be completely devoid of opioid activity (Hambrook et al., 1976; Terenius et al., 1976). Replacement of GLY^{2} with D-alanine^{2} results in a peptide with increased

activity, and is believed to result from increased resistance to cleavage of the N-terminal tyrosine (Terenius et al., 1976).

There is evidence for a membrane bound aminopeptidase in rat brain that is capable of inactivating MET- and LEU-ENK. Incubation of MET- or LEU-ENK with washed rat brain membranes resulted in rapid inactivation. Substitution of D-alanine for GLY^2 produced a peptide that was resistant to degradation, whereas substitutions at the carboxyl terminal failed to protect the peptide from enzymatic degradation (Pert et al., 1976). These results further substantiate that cleavage of the TYR-GLY^2 bond by an aminopeptidase is the major means of enzymatic inactivation of the enkephalins (Meek et al., 1977).

There is recent evidence that additional membrane bound enzymes are involved in enkephalin inactivation: a dipeptidyl carboxypeptidase (enkephalinase A) which cleaves the GLY-PHE bond and a dipeptidyl aminopeptidase (enkephalinase B) which cleaves the GLY-GLY bond (Gorenstein and Synder, 1980). There also is 1 report that the action of MET-ENK may be terminated by a high affinity uptake system (George and Van Loon, 1981).

The endorphins exhibit prolonged opioid activity compared to the enkephalins. The $t_{1/2}$ of β-END when

injected into the cerebral spinal fluid was calculated to be 60 min (Bloom et al., 1978). A similar $t_{1/2}$ was reported for β-END in vitro, whereas the $t_{1/2}$ of MET- and LEU-ENK under the same conditions was less than 1 min (Hambrook et al., 1976). Exposure of β-END to brain homogenates dramatically reduced the $t_{1/2}$ of β-END. The calculated $t_{1/2}$ was approximately 4 min (Bloom et al., 1976), and believed to be due to enzymatic degradation. Heat inactivation of enzymes by microwave irridiation or boiling prior to homogenization was reported to prevent enzymatic destruction of β-END (Rossier et al., 1977).

At least 3 products of β-END inactivation have been isolated which exhibit immunoreactivity, but are devoid of opioid activity. The C'-Fragment results from enzymatic cleavage of β-END at position 88-89 of β-LPH. The other 2 products of β-END inactivation are the α-N acetyl derivatives of β-END and the C'-Fragment (Zakarian and Smyth, 1980). These investigators observed that the distribution of β-END and its inactive derivatives was not the same in all regions. They reported that the predominant peptide in the hypothalamus was β-END, whereas all 4 peptides were present in the midbrain. In the porcine pituitary, the α-N-acetyl derivatives of β-END and the C'-Fragment were reported to predominate (Smyth et al., 1979). In

contrast, it was shown that the majority of the endorphin immunoreactivity in the neurointermediate lobe of the rat was actually α-N-acetylated derivatives, whereas the anterior lobe was authentic β-END (Akil, personal communication).

V. Hypothalamic Control of Prolactin Secretion

A. Hypothalamic Inhibition of Prolactin Secretion

PRL secretion is inhibited by the hypothalamus during basal conditions. This was clearly demonstrated by the observations that ectopic transplantation of the AP underneath the kidney capsule (Everett, 1954; Chen et al., 1970), sectioning of the pituitary stalk, or lesioning of the ME resulted in enhanced PRL secretion (Meites et al., 1963). Subsequently, the hypothalamus was shown to contain a PRL-release inhibiting factor (PIF) as evidenced by the ability of hypothalamic extracts to inhibit PRL release (Pasteels, 1961; Talwalker et al., 1963). This PIF was believed to be a small polypeptide. However its structure has not been elucidated. Measurement of hypothalamic PIF activity by bioassay methods showed that PIF activity was decreased during states of elevated PRL secreton, such as suckling and estrogen treatment (Ratner and Meites, 1964), and increased after administration of ergot drugs, L-DOPA, or iproniazid, all of which decrease PRL secretion (Meites et al., 1963; Meites and Clemens, 1972).

There is growing evidence that the majority of hypothalamic PIF activity is due to DA. DA was shown to

be a potent inhibitor of PRL release. Intraventricular (ivt) injection of DA decreased PRL release (Kamberi et al., 1971) as did systemic injection of L-DOPA, the precursor of DA (Lu and Meites, 1972). Apomorphine, a potent DA agonist, decreased PRL in rats (MacLeod and Lehmeyer, 1974; Smalstig et al., 1974) and in humans (Martin et al., 1974). Conversely, circulating PRL levels were increased by the DA antagonists, pimozide, sulpiride, and haloperidol (Meites and Clemens, 1972; Meites et al., 1972; Clemens et al., 1974; Mueller et al. 1976b). These findings indicated that hypothalamic DA tonically inhibits PRL secretion during basal conditions.

The role of NE in the regulation of PRL secretion is less clear than DA. NE has been reported to stimulate or inhibit PRL release. Administration of L-DOPS, a precursor of NE (Donoso et al., 1971), or clonidine, an α-agonist (Lawson and Gala, 1975) was reported to stimulate PRL release. Disulfram, a NE synthesis inhibitor, decreased PRL release (Meites and Clemens, 1972), which is in agreement with the above evidence that NE is stimulatory to PRL release.

Conversely, there is evidence that NE inhibits PRL release. High doses of NE were reported to inhibit PRL release in vitro (Koch et al., 1970; Shaar and Clemens,

1974; Labrie et al., 1978), as did clonidine in vivo (Mueller, unpublished). Unlike DA, NE concentration in portal blood is quite low (Ben-Jonathon et al., 1980). Furthermore, NE is approximately 1/10 as effective as DA in reducing PRL release in vitro (Labrie et al., 1979). Therefore the importance of NE in the control of PRL release is presently questionable.

Hypothalamic PIF activity can not be attributed solely to hypothalamic catecholamines. Although Shaar and Clemens (1974) reported that absorption of catecholamines onto alumina removed all PIF activity from hypothalamic extracts, it can not be concluded from this study that other PIFs were not altered by this procedure. Evidence for a non-catecholamine PIF has been reported (Takahara et al., 1974: Schally et al., 1977). In addition, it was reported that PIF activity of hypothalamic extracts could not be blocked by the DA antagonists, pimozide (Vale et al., 1973) or haloperidol (Ojeda et al., 1974). Thus, although DA is a potent inhibitor of PRL release, there are certainly additional hypothalamic factors which exert inhibitory actions on AP PRL release. Acetylcholine is present in the hypothalamus and can inhibit PRL release (Grandison et al., 1974; Kuhn and Lens, 1974), and there is increasing evidence that acetylcholine has a physiological role in PRL

control.

B. Hypothalamic Stimulation of Prolactin Secretion

The hypothalamus, in addition to inhibiting PRL release, also is capable of stimulating PRL release. PRL releasing activity of hypothalamic extracts was reported in 1960 by Meites et al. when such extracts were shown to initiate lactation in estrogen-primed female rats. The factor believed to be responsible for releasing PRL was termed PRL-releasing factor (PRF). Although the structure of PRF is not known, the evidence for a PRF entity has received further validation (Nicoll et al., 1970; Valverde et al., 1972).

TRH, the first hypophysiotropic hormone to be sequenced and synthesized, was shown to have PRF activity. TRH stimulated PRL release in vitro from a pituitary tumor cell line (Tashjian et al., 1971), and in vivo in humans, rats and cows (Jacobs et al., 1971; Meites et al., 1973; Mueller et al., 1973). It also was reported that injection of TRH antiserum decreased both circulating TSH and PRL levels (Koch et al., 1977), although these results have not been confirmed.

It has been suggested that TRH may be the primary PRF. However, this postulate has been viewed skeptically, primarily because of the poor correlation

between TSH and PRL secretion during many physiological states. TSH secretion is increased during exposure to cold temperatures, whereas PRL is decreased (Mueller et al., 1974). Stresses such as ether, restraint, and heat have been shown to decrease TSH levels, whereas PRL levels are increased (Krulich et al., 1974; Mueller et al., 1974). Thus the different responses of TSH and PRL to the same physiological stimulus argues against TRH as the primary PRF.

There is convincing evidence that hypothalamic 5-HT stimulates PRL release. Kamberi et al., (1970) demonstrated that 5-HT, which does not cross the blood brain barrier, stimulated PRL release when injected ivt. Systemic injection of 5-HT precursors, tryptophan and 5-HTP, which increase brain 5-HT concentrations, produced a similar blood PRL rise (Meites and Clemens, 1972). Clemens et al., (1977) minimized the non-specific effects of high doses of 5-HTP by injecting subeffective doses of 5-HTP, together with fluoxetine, a 5-HT reuptake blocker. These low doses of 5-HTP, which had no effect on PRL levels when given alone, significantly increased PRL levels when injected together with fluoxetine. This suggested that the stimulatory action of 5-HTP on PRL release is due to its conversion to 5-HT rather than to a non-specific effect. Reduction of

hypothalamic 5-HT concentrations decreased blood PRL concentrations. Depletion of 5-HT by injection of PCPA, or lesioning of the raphé nucleus, were shown to reduce PRL levels. This effect was reversed by 5-HTP administration (Caligaris and Taleisnik, 1974; Barophy and Harney, 1975).

There is good evidence that 5-HT is involved in suckling-induced PRL release. Suckling-induced PRL release was blocked by pretreatment with PCPA or methysergide, a 5-HT antagonist (Kordon et al., 1974; Gallo et al., 1975). In addition, the suckling stimulus was shown to increase hypothalamic 5-HT turnover (Mena et al., 1976). Similarly, Mueller et al., (1976a) showed that stress-induced PRL release was associated with an increase in hypothalamic 5-HT turnover. Together, the above results indicate that 5-HT can stimulate PRL release and may very well function physiologically to increase PRL release, probably by enhancing the release of PRF (Clemens et al., 1978).

The EOP are a recent addition to the list of putative hypothalamic PRFs. It was shown prior to the discovery of EOP, that MOR administration stimulated PRL secretion. Meites (1962) reported that MOR initiated lactation in estrogen-primed female rats, presumably by stimulating PRL release. This stimulatory action of an

opiate on PRL release was confirmed by RIA measurement of PRL after MOR (McCann et al., 1974) or methadone administration (Clemens and Sawyer, 1974). The stimulatory action of EOP on PRL release has been demonstrated by our laboratory (Bruni et al., 1977) and others, and is a topic of this thesis.

Opiates do not stimulate PRL release by a direct action on the AP (Grandison and Guidotti, 1977; Rivier et al., 1977; Shaar et al., 1977). Rather, hypothalamic mechanisms are involved. Evidence that opiates stimulate PRL via a decrease in tuberoinfundibular DA turnover will be presented in this thesis.

Estrogens are very important regulators of PRL synthesis and release. They are probably the most important non-hypothalamic agents involved in PRL regulation. Because estrogens regulate PRL secretion via hypothalamic mechanisms, in addition to a direct action on the AP, it is appropriate to discuss the role of estrogens in this section on hypothalamic control of PRL. Reece and Turner (1937) were the first to report that estrogens could stimulate PRL. They reported that estrogens increased pituitary PRL content and induced lactation in rats and guinea pigs. They correctly concluded that estrogens stimulated PRL synthesis and release. These results were confirmed in rabbits by

bioassay (Meites and Turner, 1942), as well as in rats using RIA to measure PRL concentrations in the blood and pituitary (Chen and Meites, 1970).

Blood estrogen and PRL levels are often directly correlated during different endocrine states. Estrogens and PRL levels are low prior to puberty and begin to rise immediately before the onset of puberty (Brown-Grant et al., 1970). There is a distinct surge of PRL on the afternoon of proestrus in rats, mice, hamsters, sheep, goats and cows, but not in primates. This surge is preceeded by increased estrogen titers (Meites and Clemens, 1972). On diestrus, PRL and estrogen levels are both low (Meites et al., 1972). Evidence that the coinciding levels of estrogen evoke, at least in part, the proestrous surge of PRL is that administration of estrogen antiserum blocked the PRL surge (Neil et al., 1971). Furthermore, removal of estrogens by ovariectomy on the morning of proestrus or before, also blocked the PRL surge (Meites et al., 1972).

It has been conclusively demonstrated that estrogens stimulate PRL secretion by a direct action on AP lactotrophs. Addition of estradiol to AP cultures (Nicoll and Meites, 1962) or hemipituitaries (Lu et al., 1971), increased in vitro PRL release. Administration of estrogen to hypophysectomized rats bearing a

pituitary graft underneath the kidney capsule (Chen et al., 1970), or a sc pituitary tumor (Mizuno et al., 1964), significantly increased PRL levels further. Also, uptake of estrogen by AP cells and the presence of estrogen receptors in AP tissue has been demonstrated (Vertes et al., 1973). These observations provide convincing evidence that estrogens stimulate PRL secretion by a direct action on the AP.

The hypothalamic mechanism involved in estrogen stimulation of PRL is less well defined. Implantation of small amounts of estrogen into the ME increased serum PRL concentration 3-fold (Nagasawa et al., 1969), suggesting that estrogens can stimulate PRL secretion via the hypothalamus. However, one can not rule out the possibility that ME estrogen diffused to the AP and acted directly on the AP. Estrogen administration was reported to decrease hypothalamic PIF activity (Ratner and Meites, 1964), which is in agreement with a hypothalamic site of action. Since PIF activity was measured by bioassay, a decrease in PIF activity may also involve an increase in PRF activity, or a combination of both. The effect of estrogens on hypothalamic TRH, 5-HT, and EOP, all of which stimulate PRL release, remains to be thoroughly studied.

A complicating factor in determining the hypo-

thalamic mechanisms by which estrogens increase PRL secretion is the PRL short-loop feedback. Elevated blood PRL levels have been shown to inhibit PRL release (Advis *et al.*, 1977), and probably involves tuberoinfundibular DA. Tuberoinfundibular DA turnover (Eikenberg *et al.*, 1977) and release into the portal vasculature (Gudelsky *et al.*, 1981) were shown to be increased during states of elevated PRL levels. Additional hypothalamic PIFs and PRFs also may be involved in PRL autoregulation. Therefore, it is difficult to determine whether effects of estrogen on PRL secretion are mediated via the hypothalamus, or by a direct action on the AP, or via both actions.

VI. Control of Luteinizing Hormone (LH) Secretion

A. Inhibition of Luteinizing Hormone Secretion by Gonadal Steroids

Feedback inhibition of LH release by gonadal steroids occurs in male and female mammals. Androgen inhibition of LH release in the male is tonic (Bogdanove, 1967; Turner, 1974), whereas ovarian steroids in the female are stimulatory for a short period prior to ovulation, and inhibitory during the luteal phase of the cycle. Removal of steroid negative feedback by ovariectomy or orchidectomy results in a persistent elevation of circulating LH levels (Gay and Midgley, 1969; Yamamoto *et al.*, 1970). Sex steroid replacement returns LH concentration to pre-castration levels (Ramirez *et al.*, 1964; Chowers and McCann, 1967; Ferland *et al.*, 1976a).

Steroid inhibition of LH secretion appears to involve both a hypothalamic and AP site of action. This conclusion is based primarily on studies in which small amounts of steroids were implanted either in the hypothalamus or pituitary. AP implantation of estrogen reduced LH secretion (Rose and Nelson, 1957; Bogdanove, 1963). However, estrogen was shown to be more effective

when implanted in the hypothalamus (Davidson, 1969; Sawyer and Hilliard, 1972). LH levels in the male rat also were reduced by medial basal hypothalamic (MBH) implantation of testosterone (Simpkins et al., 1980). It is doubtful that inhibition of LH release by hypothalamic implants of a steroid is due simply to the steroid being transported to the AP where it exerts a direct action, as has been suggested (Bogdanove, 1964). Although steroids implanted in the hypothalamus do reach the AP (Bogdanove, 1964), steroid inhibition of LH release can be observed prior to delivery of significant amounts of the steroid to the AP (Turner and Simpkins, unpublished). Furthermore, the inhibitory action of estrogen on the AP lasts for only a few hours, and estrogen becomes facilitatory after approximately 8 hours of exposure. However, the inhibitory action of estrogen is maintained in the female. Therefore inhibition is probably maintained by a hypothalamic site of action.

The hypothalamic area thought to be most important in steroid negative feedback is the MBH. Deafferentation of the MBH did not alter ovarian steroid inhibition of LH secretion (Blake, 1977). Furthermore, MBH implants of testosterone were as effective as sc administration of testosterone in reducing post-

castration LH levels (Simpkins et al., 1980). Thus, the present view of gonadal steroid feedback inhibition of LH secretion is that steroids inhibit LH secretion initially by a dual action on the AP and MBH, but the long-term inhibitory action is restricted to a hypothalamic site (Blake et al., 1974; Blake, 1977).

B. Stimulation of Luteinizing Hormone Secretion by Ovarian Steroids

Ovarian steroids can stimulate LH secretion in female mammals (McCann, 1974). The stimulatory action of estrogen on LH secretion was first demonstrated by its ability to cause ovulation, as evidenced by the formation of corpora lutea (Hohlweg, 1934). Subsequently, Everett (1948) and Brown-Grant (1969) showed that estrogen and progesterone could advance ovulation when administered during diestrus. In cycling female rats, basal LH levels are interrupted every 4-5 days by a surge of LH which typically occurs between 1600-1800 hours on proestrus (Monroe et al., 1969; Butcher et al., 1974). Estrogen levels begin to rise on diestrous day 2 and reach a peak level on proestrous morning (Hori et al., 1968). Elevated estrogen levels were shown to be absolutely necessary for the LH surge to occur since administration of antibodies to estrogen blocked the

proestrous LH surge and ovulation in rats (Ferin et al., 1969) and the LH surge induced by estrogen and progesterone in monkeys (Knobil, 1974).

Progesterone by itself has little effect on LH secretion, but potentiates the stimulatory action of estrogen on LH secretion (Döcke and Dörner, 1966; Kalra and Kalra, 1974). There is a small surge of progesterone on the morning of proestrus which is probably adrenal in origin (Barraclough et al., 1971). A second larger progesterone surge occurs which is ovarian in origin, and coincides with the LH surge (Barraclough et al., 1971; Feder et al., 1971; Freeman et al., 1976). Therefore, both estrogen and progesterone levels are elevated during proestrus and probably stimulate LH secretion in a facilitatory manner.

Estrogen and progesterone can produce phasic release of LH in ovariectomized rats which is similar to the proestrous LH surge. In fact, the estrogen-progesterone-primed ovariectomized rat has been used frequently to study the hypothalamic mechanisms involved in ovarian steroid positive feedback. Three different models have been used. One model involves ovariectomy and estrogen administration on the day of diestrus, followed by progesterone on the early afternoon of the next day (expected proestrus). This steroid regimen

produces an LH surge similar to that observed in proestrous rats (Aiyer and Fink, 1974). A second model involves administration of estrogen to long-term ovariectomized rats, followed 72 hours later by progesterone. The LH surge produced is identical in timing to the proestrous surge, but is slightly greater in magnitude (Caligaris et al., 1971). The third model involves long term ovariectomized rats in which estrogen is administered either by daily injections or sc capsule implants. Unlike the other 2 models, this model is characterized by a daily LH surge between 1600-1800 hours (Legan et al., 1975), and demonstrated the need for progesterone to block the daily neural signal produced by estrogen (Freeman et al., 1976).

The site of the positive feedback by estrogen appears to be the hypothalamus and AP. More specifically, the hypothalamic site is probably the preoptic-suprachiasmatic area (Flerko, 1966). Lesioning the afferents to the MBH from the preoptic-suprachiasmatic area blocked ovulation (Hillard, 1949; Halász and Gorski, 1967; Halász, 1972) and the gonadotropin surge (Palka et al., 1969; Blake et al., 1972; Weiner et al., 1972). Similarly, MBH deafferentation blocked the estrogen-progesterone induced LH surge (Taleisnik et al., 1970). Goodman (1978) showed that estradiol

implants in the preoptic area were more effective than MBH implants in producing a LH surge. Furthermore, by measuring pituitary estradiol concentrations, he concluded that exposure of the pituitary to estradiol was not enough to cause LH release, since the pituitary estradiol concentration in the MBH implanted rats was higher than in the preoptic implanted rats. In agreement with a preoptic site of action is the report that estradiol enhanced LHRH release produced by preoptic electrical stimulation, but not ME stimulation. Lastly, the preoptic-anterior hypothalamic area receives substantial innervation from serotonergic and noradrenergic neurons (Ungerstedt, 1971; Brownstein et al., 1976), and there is considerable evidence for a serotonergic and noradrenergic role in phasic release of LH (Kalra and McCann, 1972; Coen and MacKinnon, 1976).

The AP also is most probably a site of estrogen positive feedback. The LH response to LHRH administration was increased by prior exposure of the AP to estrogen. This was shown in vivo (Arimura and Schally, 1971) and in vitro (Labrie et al., 1976; Drouin et al., 1976). These results are in agreement with the finding that the responsiveness of the AP to LHRH was greatest on the afternoon of proestrus, when estrogen levels are high (Aiyer et al., 1974; Zeballos and McCann, 1975).

Thus, estrogen positive feedback at the AP level appears to enhance the action of LHRH, and may do so by increasing LHRH receptor number or affinity (Kyringza et al., 1975; Park et al., 1975).

To summarize the events leading to the preovulatory surge, the blood estradiol level begins to rise on late diestrus/early proestrus. Estradiol increases LHRH release into the portal blood and at the same time increases AP sensitivity to LHRH, resulting in LH release. Increased blood LH stimulates the secretion of progesterone which by a facilitatory action with estradiol, further increases AP responsiveness. The responsiveness of the AP, together with the self-priming action of LHRH, produces a LH surge of high enough magnitude to cause ovulation.

C. Monoaminergic Effects on Luteinizing Hormone Secretion

The biogenic amines have become well established as important regulators of LH secretion. They are localized in close association with LHRH cell bodies in the preoptic-suprachiasmatic area and with LHRH terminals in the ME (Cuello, 1978; Weiner and Ganong, 1978). Drugs which inhibit biogenic amine synthesis or

deplete their stores were shown to produce dramatic changes in LH secretion. Reserpine, which depletes hypothalamic biogenic amine stores (Dahlström et al., 1965), blocked normal ovulation in adult female rats (Brown, 1967) and pregnant mare serum (PMS) induced ovulation in immature female rats (Barraclough and Sawyer, 1957). Alpha-methylparatyrosine (α-mpt), which blocks catecholamine synthesis by competing with the rate limiting enzyme, tyrosine hydroxylase (Spector et al., 1965; Corrodi and Hansen, 1966), was shown to inhibit LH secretion during several different endocrine states. Thus, administration of α-mpt on diestrus blocked the proestrous LH surge (Kalra and McCann, 1973; Kalra and McCann, 1974) and ovulation in rats (Brown, 1967; Lippman et al., 1967). Similarly, α-mpt blocked the LH surge in ovariectomized rats primed with estradiol benzoate and progesterone (EB-P) (Kalra et al., 1972). These results indicate that biogenic amines are important neuromodulators of the hypothalamo-pituitary-LH axis.

The above studies are important since they established the biogenic amines as regulators of LH secretion. However, the action of a particular biogenic amine on LH secretion cannot be determined from these experiments. Subsequent studies were designed to

differentiate the effects of NE, DA, and 5-HT on LH secretion and to determine their physiological significance as it pertained to LH secretion.

It has been firmly established that NE stimulates LH secretion. Intraventricular infusion of NE increased circulating LH levels in the rat and rabbit (Krieg and Sawyer, 1976) and induced ovulation in the rat (Rubinstein and Sawyer, 1970). Results of experiments in which the noradrenergic system was disrupted substantiate a stimulatory action of NE on LH release, and strongly suggest that NE is involved in the phasic release of LH. Thus, deafferentation of the MBH eliminated the proestrous LH surge and produced rats that were anovulatory (Palka et al., 1969; Blake et al., 1972; Weiner et al., 1972a). This surgical procedure depleted hypothalamic NE content by 60% and had no effect on DA content, suggesting that the antigonadotropic effect of MBH deafferentation was due to disruption of the hypothalamic noradrenergic system. Similarly, chemical lesioning of catecholamine axons and terminals by injecting 6-hydroxydopamine (6-OH-DA), a neurotoxin (Thoenen and Tranzer, 1968), into the third ventricle, blocked the proestrous and EB-P induced LH surge (Kalra, 1975). Implantation of 6-OH-DA into the ventral noradrenergic tract, a major source of hypo-

thalamic NE (Fuxé 1965b; Ungerstedt, 1971) or into the anterior hypothalamus, dramatically reduced hypothalamic NE content without affecting DA. This route of administration also blocked the proestrous and EB-P induced LH surge (Martinovic and McCann, 1977; Simpkins et al., 1979a), suggesting that the inhibitory effects of 6-OH-DA on LH secretion are due to its toxic effect on NE axons and terminals.

Kalra and McCann (1972) provided convincing evidence that NE was involved in the phasic release of LH. They showed that inhibition of DA-β-hydroxylase, the enzyme that converts DA to NE, by administration of diethyldithiocarbamate (DDC) or 1-phenyl-3-(2-thiozolyl) thiourea (U-14,624) (Goldstein and Nakajima, 1967), blocked the EB-P induced LH surge in ovariectomized rats. Furthermore, injection of dihydroxyphenylserine (DOPS), which is converted to NE independently of DA-β-hydroxylase, partially reversed the inhibitory effects of DDC on the proestrous LH surge and ovulation in the rat (Terasawa et al., 1975). FLA-63, also a NE synthesis inhibitor, was reported to block the pulsatile release of LH in long-term ovariectomized rats (Drouva and Gallo, 1976), as did phenoxybenzamine, an α -antagonist, in ovariectomized monkeys (Bhattacharya et al., 1972).

Studies in which hypothalamic NE activity was measured during different LH secretory states provided additional evidence that NE plays an important role in LH regulation. Hypothalamic NE turnvoer, as determined by the α-mpt depletion method (Coppola, 1969) or synthesis of ^{3}H-NE after ^{3}H-tyrosine administration (Anton-Tay et al., 1970; Bapna et al., 1971), was increased by castration. This effect of castration was reversed by gonadal steroid replacement. Consistent with these observations are the reports that tyrosine hydroxylase activity was increased in ovariectomized rats (Beattie et al., 1972), and that α-mpt, phenoxybenzamine, and DDC each blocked the post-castration rise of LH (Ojeda and McCann, 1973). An increase in NE turnover in the rostral hypothalamus (Stefano and Donoso, 1967; Coppola, 1969) and ME (Selmanoff et al., 1976; Rance et al., 1981) was reported to occur in association with the proestrous LH surge. Similarly, Simpkins et al. (1979b) observed that anterior hypothalamic NE turnover was increased immediately prior to the EB-P induced LH surge. Hypothalamic NE turnover on the first proestrous day of young female rats also was reported to be increased (Advis et al., 1978). Conversely, hypothalamic NE turnover was reported to be decreased in hyperprolactinemic rats which had reduced

LH levels (Aylsworth and Meites, unpublished). These reported changes in NE activity associated with different LH secretion rates, together with the drug studies, are convincing evidence that hypothalamic noradrenergic neurons are involved in the regulation of LH secretion.

The role of DA in the regulation of LH secretion presently is not well established, mainly due to the contradictory reports on its effects. DA has been reported to stimulate and inhibit LH secretion, while others have reported no effect. It has been shown that the steroid status or steroid environment of the animal is an important factor in determining the response of LH to dopaminergic compounds, although not all discrepancies can be accounted for on this basis alone (Vijayan and McCann, 1978a; 1978b). Intraventricular injection of DA or apomorphine, a DA agonist, was reported to stimulate LH secretion in ovariectomized EB-P primed rats. This stimulatory action of DA or apomorphine was not observed in unprimed ovariectomized rats (Vijayan and McCann, 1978a), suggesting that estrogen and/or progesterone must be present for DA to stimulate LH release.

Similarly, DA stimulated LH secretion when injected on diestrous day 2 or proestrus when circulating

estrogen and progesterone levels are increased, but was ineffective when injected on diestrous day 1 when estrogen and progesterone levels had not yet begun to rise (Schneider and McCann, 1970). Systemic injection of low doses of DA were reported to increase LH levels in EB-P primed ovariectomized rats, whereas high doses actually inhibited LH release in unprimed ovariectomized rats (Vijayan and McCann, 1978b). In agreement with this latter observation is the report that injection of either apomorphine or CB-154, both DA agonists, blocked pulsatile LH release in ovariectomized rats (Drouva and Gallo, 1976), and reduced the post-castration rise of LH in female rats (Beck and Wuttke, 1977). It should be noted that in both of these studies, estrogen was very low due to ovariectomy, and may be the reason why apomorphine and CB-154 inhibited LH release. This tentative conclusion is supported by the observation that apomorphine or piribedil, a DA agonist, were unable to block the proestrous LH surge (Beck *et al.*, 1978) or the EB-P induced LH surge (Simpkins *et al.*, 1979a). Rats in both of these latter 2 models have elevated estrogen and progesterone levels and may be the reason why no inhibitory effect was observed.

Further evidence that DA is stimulatory to LH secretion is that HAL, a DA antagonist, decreased basal

LH levels and decreased hypothalamic LRF activity (Dickerman et al., 1974). It also was reported that HAL blocked ovulation in the rat (Boris et al., 1970) and that pimozide, also a DA antagonist, reduced the preovulatory LH surge (Beattie et al., 1976).

DA does not influence LH secretion by a direct action on the AP. Addition of DA to a hypothalamo-pituitary coincubation system was reported to stimulate LH release in vitro, presumably by stimulting LHRH release into the medium (Schneider and McCann, 1969). This effect of DA on LHRH was confirmed in vivo by Kamberi et al. (1969) who reported that ivt injection of DA increased LRF activity in portal blood. More recently, Rotsztejn et al. (1976) reported that DA stimulated LHRH release from incubated MBH of ovariectomized EB-P primed rats, but not from MBH of unprimed ovariectomized rats. Still others have reported that DA either inhibited (Miyachi et al., 1973) or had no effect (Quijada et al., 1974) on LHRH release in vitro.

The inverse correlation between LH secretion and hypothalamic DA turnover strongly suggests that DA is inhibitory to LH secretion. DA turnover is reduced after castration (Fuxé, 1965b) and on proestrous afternoon (Fuxé et al., 1967; Rance et al., 1981), whereas circulating LH levels are increased. Conversely, LH

secretion is reduced and DA turnover is increased during lactation (Ben-Jonathan and Porter, 1976).

It has been suggested that the LH stimulatory effect of DA administration may be due to the conversion of DA to NE upon its uptake into NE neurons (Fuxé and Hökfelt, 1970). This hypothesis is suported by the finding that α-antagonists can block the stimulatory action of DA (Schneider and McCann, 1969). However, this hypothesis can not account for the stimulatory effect of apomorphine. Furthermore, DDC, which should block the conversion of DA to NE, only partially reduced the stimulatory action of DA (Vijayan and McCann, 1978a). Lastly, one should consider the possibility that DA is both stimulatory and inhibitory to LH secretion. A possible explanation may be that 2 functionally different dopaminergic neuronal systems are involved in LH control. There is some evidence that the incerto- hypothalamic DA neurons in the preoptic area are stimulatory (Kawakami et al., 1975), whereas the tuberoinfundibular DA system is inhibitory (Ojeda et al., 1974).

Until recently, the effect of 5-HT on LH secretion was believed to be strictly inhibitory. Systemic injection of 5-HTP, the precursor of 5-HT, blocked cyclic ovulation, whereas parachlorophenylalanine

(PCPA), a 5-HT synthesis inhibitor, facilitated ovulation in PMS treated immature rats (Kordon et al., 1968). Furthermore, electrical stimulation of the raphé, which increases 5-HT turnover, blocked ovulation (Carrer and Taleisnik, 1970). Different methods of increasing hypothalamic 5-HT content, such as systemic injection of 5-HTP or ivt injection of 5-HT decreased circulating LH levels, blocked the proestrous surge of LH, and inhibited ovulation (Kamberi et al., 1970; Kamberi, 1973). 5-HT concentration in the ME was reported to be decreased prior to the LH surge in sheep, and may reflect decreased 5-HT synthesis which may serve to facilitate LH secretion (Wheaton et al., 1972). 5-HT turnover as determined by the ratio of the 5-HT metabolite, 5-hydroxyindoleacteic acid (5-HIAA), to 5-HT was reported to be increased during suckling and may account for the reduced LH levels observed during suckling (Mena et al., 1976).

More recently, there is increased evidence that under certain conditions 5-HT may be stimulatory to LH secretion. Kordon showed, in contrast to his earlier report (Kordon et al., 1968), that injection of PCPA 20 hours before the critical period was able to block PMS-induced ovulation (Kordon and Glowinski, 1972). Injection of either parachloroamphetamine (PCA) or

5,6-dihydroxytryptamine, both 5-HT neurotoxins, or PCPA, blocked the daily LH surge in estrogen-primed, ovariectomized rats (Coen and MacKinnon, 1976). Similarly, Héry et al. (1976) reported that inhibition of 5-HT by injection of either PCPA or methiothepin, a 5-HT antagonist, also inhibited the estrogen-induced LH surge in ovariectomized rats, and that the inhibitory effect of PCPA was reversed by 5-HTP. Chen et al. (1981) published similar results using PCPA and PCA, and reported that 5-HTP not only reversed the effects of PCPA and PCA, but greatly potentiated the LH surge. Lastly, Fajor et al. (1970) reported that PCPA administration delayed the onset of puberty.

These results indicate that 5-HT may play a stimulatory, as well as an inhibitory role. The stimulatory role of 5-HT may be limited to situations where LH is released in a phasic manner, and may mediate the stimulatory feedback action of estrogen (Kalra and McCann, 1972). Estrogen was reported to increase 5-HT turnover in ovariectomized rats (Fuxé et al., 1974).

5-HT does not alter LH secretion by a direct effect on the AP. Infusion of 5-HT into the portal vessels had no effect on LH secretion, whereas ivt injection of 5-HT reduced circulating LH levels (Kamberi et al., 1971). Thus, 5-HT is believed to influence LH secretion via

hypothalamic mechanisms, presumably by altering LHRH release into the portal blood. 5-HT neuron terminals are closely associated with LHRH cell bodies in the preoptic-suprachiasmatic area and LHRH terminals in the arcuate-ME region (Dahlström and Fuxé, 1964; Aghajanian, 1969; Ungerstedt, 1971; Kuhar et al., 1972; Saavedra et al., 1974), which gives anatomical support for a hypothalamic mechanism.

The inhibitory 5-HT input to the hypothalamus appears to be limited to the arcuate-ME region. This conclusion is based on the observation that this region was the only hypothalamic area in which 5-HT implantation inhibited LH secretion and blocked ovulation (Kordon, 1969). The hypothalamic region involved in 5-HT stimulation of LH release is believed to be the suprachiasmatic nucleus. The integrity of this nucleus is essential for phasic release of LH (Clemens et al., 1976; Coen and MacKinnon, 1971). Furthermore lesioning the raphé eliminated 5-HT input into this area (Bjorklund et al., 1973), blocked PMS-induced ovulation (Meyer, 1978), and eliminated the estrogen-induced LH surge in ovariectomized rats (Coen and MacKinnon, 1976).

D. Opiate Effects on Luteinizing Hormone Secretion

Barraclough et al. (1955) reported that MOR administration during the critical period of proestrus

prevented ovulation in rats. More recently, Pang et al. (1977) demonstrated this action of MOR was due to inhibition of the proestrous LH surge, and that NAL counteracted the inhibitory effects of MOR on ovulation and LH release. MOR or methadone administration was shown to reduce basal LH and testosterone levels in the male rat, and reduce seminal vesicle and ventral prostate weight (Cicero et al., 1976). We also have observed that MOR reduced basal LH levels in male rats. In addition, MET-ENK similarly reduced serum LH concentration (Bruni et al., 1977). Stubbs et al. (1978) reported similar observation in the human after administration of DAMME, a MET-ENK analog. Concurrent injection of NAL was shown to reverse the inhibitory effects of opiates on LH release (Bruni et al., 1977; Pang et al., 1977; Stubbs et al., 1978), and when given alone, NAL significantly increased serum LH levels (Bruni et al., 1977; Cicero et al., 1979). This effect of NAL suggested that basal LH secretion may be tonically inhibited by EOP.

Experiments subsequent to the studies described above have further established the EOP as important regulators of LH secretion. Included in this thesis are results of experiments in which hypothalamic mechanisms

involved in opiate inhibition and NAL stimulation of LH secretion were studied. Results indicating that EOP may mediate the negative feedback of gonadal steroids on LH secretion also are included. Pertinent findings from other laboratories will be discussed in each of these sections.

MATERIALS AND METHODS

I. Animals, Treatment, and Blood Collection

Mature male and female Sprague-Dawley rats were purchased from either Spartan Research Animals (Haslett, MI) or Harlan Industries (Indianapolis, IN), and housed in our facility for at least 1 week prior to experimentation. Rats were kept in temperature (25°C) and light (14 h light/10 h dark) controlled rooms. Ralston Purina Rat Chow (Ralston Purina Co., St. Louis, MO) and water were provided ad libitum throughout the periods of acclimatization and experimentation.

Drugs and steroids were administered by several different routes. The diluent and route of administration are stated in the Materials and Methods section of each experiment. β-END and dynorphin were injected ivt via the right lateral ventricle by a slightly modified procedure of deBalbian Verster et al. (1971).

Lateral ventricular cannulae were made from PE-20 tubing (inside dia. = .015", outside dia. =.043"). A wire was inserted into the tubing in order to keep the

tubing patent while it was heated and compressed to form a bulb approximately .08" in diameter. A beveled tip was cut 4 mm from the bulb. The total length of the cannula was 20 mm and had a dead space volume of 3 µl. Rats were anesthetized with chloral hydrate (8% solution, 1 ml/200 grams BW), and the cranium was exposed by making a 2 cm longitudinal cut. A hole was drilled 2 mm lateral to the sagital suture and 1 mm caudal to the coronal suture. The beveled cannula was inserted to a depth of 4 mm and secured in place with dental cement and one anchoring screw. The incision was closed with wound clips. Rats were allowed to recover for at least 1 week. Injections were performed without anesthesia. Rats were gently held while a 20 ul Glenco micro syringe was attached to the cannula. A volume of 13 µl was injected and the cannula was immediately heat sealed.

Blood was collected by decapitation, orbital sinus puncture under light ether anesthesia, or from a chronic right atrial cannula. Cardiac cannulae were made from silastic tubing (Dow Corning, Midland, MI) having an inside dia. of .025" and an outside dia. of .047". The saline filled cannula was inserted into the right atrium via a small incision in the right external jugular vein approximately 32 mm above the right atrium. The cannula

was secured in place by sutures above and below the incision. The free end was passed underneath the skin and exited approximately 2 cm posterior to the base of the skull. Rats were housed in individual cages and allowed to recover for at least 2 days. Cannulae were flushed daily with sterile saline. On the day of the experiment, a 1 ml syringe with a 30 cm extension of silastic tubing was attached to the cannula and exited outside the cage. Rats were able to move freely about their cage while injections and blood samples were accomplished without disturbing the animals. Blood was stored overnight at $4^{\circ}C$ and serum was separated and frozen at $-20^{\circ}C$ until assayed.

II. Radioimmunoassay of Hormones

Serum PRL and LH were measured by a double antibody technique described in the NIAMDD RIA kits. These assays were nonequilibrium assays which used specific antibodies to rat-PRL and rat-LH. Rat PRL and LH were iodinated using chloramine-t, followed by separation on a P-60 bio-gel column (Bio-Rad Labs, Richmond, CA). Antibody-antigen complexes were precipitated by addition of rabbit gamma globulin antiserum produced in sheep.

Serum samples were run in either triplicate or quadruplicate. Only those volumes which gave hormone values which corresponded to the linear portion of the standard curve were used. Hormone concentrations were expressed as the mean ± standard error of the mean (SEM). Differences between group means were determined by one-way analysis of variance and Student-Newman-Keuls' test when multiple comparisons were made. Students "t" test was used only when comparing the means of 2 groups alone. The level of significance chosen in all experiments was $p < 0.05$.

III. Assay of Dopamine, Norepinephrine and LHRH in Brain

A. Isolation and Preparation of Brain Tissue

Brains were removed from the cranium after decapitation and laid dorsal side down. The hypothalamus was removed by cutting at the following landmarks: a) anterior, 2 mm rostral to the optic chiasm; b) posterior, immediately rostral to the mammillary bodies; c) lateral, the lateral hypothalamic sulci; d) dorsal, at the level of the anterior commissure (3-4 mm). The hypothalamus was divided into the anterior

hypothalamic-preoptic area (AH) and medial basal hypothalamic (MBH) area by cutting immediately caudal to the optic chiasm (Experiments IV and V).

The stalk-ME (Experiment III) was dissected using fine iris scissors with the aid of a dissecting microscope. Cuts were made at a 20° angle from the ventral surface along the lateral aspects of the tuber-cinereum beginning at the posterior border of the infundibulum. The stalk-ME contained approximately 20 ug protein as determined by a micro-protein assay (Lowry et al., 1951).

The ME was homogenized in 30 µl perchloric acid containing 10 mg% EDTA. The AH and MBH regions were homogenized in 10 µl perchloric acid plus 10 mg % EDTA /mg wet tissue weight. 1 N acetic acid was used to extract hypothalamic tissue for LHRH measurements. All tissues were sonified in a Cell Disruptor (Branson Sonic Power Co., Plainview, NY) and centrifuged at 2000 x g for 15 min at 4°C.

B. Radioenzymatic Assay of Dopamine and Norepinephrine

Tissue DA and NE were assayed by a modification of the method of Coyle and Henry (1976) (see appendix A). Ten ul aliquots of supernatant or standard DA and NE (Sigma Chemical Co., St. Louis, MO) were incubated in the

presence of buffered COMT and ^{3}H-S-adenosyl- methionine (New England Nuclear, Boston, MA), a methyl donor. COMT was partially purified from rat liver by the method of Nikodejevic et al. (1970).

The DA and NE metabolites, methoxytyramine and normetanephrine respectively, were separated by solvent extraction and thin layer chromatography. Amine content was determined by counting the chromatographic spots containing ^{3}H-labeled metabolites in glass scintillation vials containing 10 ml aqueous counting scintillant (Amersham Corp., Arlington Heights, IL). Samples were counted in a Beckman LS-100 liquid scintillation counter (Beckman Instruments, Palo Alto, CA).

C. Radioimmunoassay of Luteinizing Hormone Releasing Hormone

Tissue supernatant was diluted 1 to 20 in 0.02 M borate buffer containing 0.1% gel (pH 8.4) and assayed in quadruplicate. LHRH antiserum (R-42 pool) was generously supplied by Dr. G.D. Niswender (Colorado State Univ., Ft. Collins, CO). Synthetic LHRH (Beckman) was iodinated using the lactoperoxidase-glucose method (Tower et al., 1977) and purified on a 0.5 x 30-CM (CM-22, Whatman Inc., Clifton, NJ) column. The specific activity of the tracer was 1800 uCi/ug (Marshall and

Odell, 1975). Tracer-antibody binding was inhibited in a dose-related manner by synthetic LHRH. The minimum detectable dose was 1.0 pg/tube, and 50% inhibition of tracer binding was achieved with 16 pg/tube. Unknowns were parallel to the standard curve and expressed as pg/mg wet tissue weight.

EXPERIMENTAL

I. Initial Studies on Opiate Stimulation and Naloxone Inhibition of Prolactin Release

A. Objectives

The discovery of the EOP has generated great interest in their possible physiological functions. Although much research has centered on the effects of these compounds on pain perception, behavior, and psychiatric disease (Frederickson, 1977), their relatively high concentrations in the hypothalamus prompted us to investigate their endocrine effects. MOR and methadone were shown previously to stimulate PRL secretion (Meites, 1962; Clemens and Sawyer, 1974). Therefore, it was of interest to determine the effects of several different EOP on PRL secretion.

B. Materials and Methods

Male Sprague-Dawley rats (200-225 g, Spartan Research Animals, Haslett, MI) were injected with MOR (Mallinkrodt Labs., St. Louis, MO), MET-ENK (Bachem, Marina Del Ray, CA), or NAL (Endo Labs., Garden City, NY) alone or together with MOR or MET-ENK. The drugs

were given ip in 0.1 ml saline/100 g BW. The rats were injected in a randomized block design and decapitated 20 min after injection. Trunk blood was collected, and serum was separated and frozen at -20°C until assayed for PRL.

Because MET-ENK is metabolized very rapidly, MET-ENK was injected ivt or the MET-ENK analog, [D-Ala^2, MePhe^4, Met(O)-ol] enekphaline (DAMME, Sandoz Inc., East Hanover, NJ) was injected ip in a second experiment in order to determine the effect of MET-ENK on PRL release. The lateral ventricle of male Sprague Dawley rats was cannulated as described in Materials and Methods, and rats were allowed to recover for 10 days. Rats were injected with 150 ug MET-ENK dissolved in saline or an equivalent volume (13 ul) of saline. Blood was collected upon decapitation 10 min after injection. Alternatively, rats were injected ip with the enkephalin analog, DAMME. Doses of DAMME ranged from 1 ug to 1 mg/kg BW. Rats injected ip with saline served as controls. Rats were bled by orbital sinus puncture under light ether anesthesia 30, 60 and 120 min after injection.

In a third experiment, the effects of β-END and dynorphin on PRL release were tested. The right lateral ventricle was cannulated. Ten days later, a silastic

cannula for withdrawing blood was placed into the right atrium, and rats were allowed to recover for 2 days. Rats were divided into 6 groups with 8 rats in each group and injected ivt with either 1 or 10 ug dynorphin (US Biochemical Corp., Cleveland, OH), 1 or 10 ug β-END (provided by Dr. C.H. Li, Univ. of Calif., San Francisco, CA), 10 ug dynorphin plus 20 ug NAL, or 13 ul saline. One ml of blood was withdrawn via the atrial cannula 10, 30, 60, and 120 min after injection. One ml of saline was replaced immediately after each bleeding in order to minimize extracellular fluid changes. Blood was allowed to clot overnight at $4^{\circ}C$, and serum was separated and frozen at $-20^{\circ}C$ until assayed for PRL.

C. Results

The results of the first experiment are shown in Table 1. The 2 higher doses of MOR significantly increased serum PRL concentrations 20 min after injection, whereas MET-ENK had no effect. NAL completely blocked the stimulatory action of MOR when injected together with MOR. Injection of NAL alone significantly reduced serum PRL concentrations at all doses tested.

MET-ENK, when injected ivt, significantly increased serum PRL concentration. The mean serum PRL concentration 10 min after injection of MET-ENK was 44.0 ± 2.5

ng/ml, and was significantly greater than the saline injected controls (10.6±1.2 ng/ml).

Table 1. Dose-Response Effects of Morphine, Methionine-Enkephalin, and Naloxone on Serum Prolactin

Group	Prolactin
Controls	9.0 ± 0.4[a]
NAL (0.2 mg/kg)	4.6 ± 0.4[b]
NAL (2.0 mg/kg)	4.2 ± 0.3[b]
NAL (5.0 mg/kg)	4.0 ± 0.3[b]
MOR (2.0 mg/kg)	10.1 ± 0.8
MOR (10.0 mg/kg)	20.2 ± 2.3[b]
MOR (15.0 mg/kg)	18.5 ± 1.2[b]
MET - ENK (5.0 mg/kg)	11.5 ± 0.6
NAL and MOR (0.2+2.0 mg/kg)	3.6 ± 0.4[b]
NAL and MOR (0.2+10.0 mg/kg)	9.0 ± 1.1
NAL & MET-ENK (0.2+5.0 mg/kg)	5.4 ± 0.8[b]

[a] x ± SEM; all data are expressed in ng/ml serum.
[b] $p<0.05$ compared with controls. MOR = morphine; NAL = naloxone; MET-ENK = methionine-enkephalin. n = 10 animals per group.

The effects of the MET-ENK analog, DAMME, on serum PRL levels is shown in Table 2. The 2 lower doses of DAMME did not significantly increase serum PRL levels. However, injection of DAMME at doses of either 0.5 or

1.0 mg/kg BW significantly increased serum PRL concentrations 30 and 60 min after injection. The stimulatory effect of DAMME was no longer evident 120 min after injection.

Table 2. Effect of DAMME on Serum Prolactin Concentrations (ng/ml)

Group	Time in Min After Injection		
	30	60	120
Control	11.6± 6.4[a]	23.7± 8.5	7.0±3.2
DAMME:			
0.001 mg/kg	22.8±10.4	12.7± 3.0	11.4±4.2
0.1 mg/kg	27.2± 6.2	10.1± 4.3	6.0±3.0
0.5 mg/kg	76.3± 6.1[b]	71.5±15.0[b]	6.8±3.7
1.0 mg/kg	92.5±18.2[b]	84.9±28.3[b]	12.0±9.4

[a] $\bar{x}$ ± SEM; [b] $p<0.05$ compared with controls.
n = 8 animals per group.

The effects of dynorphin and β-END on PRL release are shown in Table 3. Both 1 and 10 ug doses of dynorphin significantly elevated serum PRL concentrations 10 min post-injection as compared to control values. Serum PRL concentrations in the rats given 1 ug dynorphin returned to control values by 30 min, whereas the mean serum PRL level of the group given 10 ug dynorphin was approximately 3 times greater than con-

trols at 30 min. However, this difference was not statistically significant. Serum PRL levels in both dynorphin treated groups at 60 and 120 min post-injection were not different from the controls. Both doses of β-END significantly increased serum PRL concentrations at 10 min, although the higher dose appeared to be less effective than the lower dose. PRL levels in the β-END treated rats remained significantly elevated 30 and 60 min after injection in contrast to the loss of effect of dynorphin at these 2 periods. Injection of 20 ug NAL together with 10 ug dynorphin, completely blocked the stimulatory action of dynorphin, and resulted in a reduction in serum PRL values.

D. Discussion

These results show that the EOP have in common with MOR the ability to stimulate PRL release in the rat. MET-ENK is inactivated very rapidly when administered systemically, and this could account for its inability to increase serum PRL levels when injected ip. However, when MET-ENK was injected ivt or the MET-ENK analog was injected ip, serum PRL levels were significantly elevated. These results are in agreement with those of Ferland *et al*. (1977) and Stubbs *et al*. (1978) who reported that MET-ENK and DAMME significantly increased

PRL levels in the blood.

Table 3. Acute Effects of Dynorphin and β-Endorphin on Prolactin Release

Treatment	10 min	30 min	60 min	120 min
Saline (13 µl)	16.1± 1.9[a]	12.5± 1.8	10.9± 2.2	7.1±0.9
DYNOR (1 µg)	52.4± 8.4[b]	16.5± 3.6	5.9± 1.8	4.8±1.3
DYNOR (10 µg)	82.6±15.2[b]	34.3± 6.3	10.8± 1.8	5.1±0.7
β-END (1 µg)	76.1±12.6[b]	63.3±15.4[b]	39.8± 7.2[b]	6.1±0.8
β-END (10 µg)	42.2± 4.4[b]	56.6±12.2[b]	63.4±15.9[b]	8.7±1.2
DYNOR+NAL (10µg+20µg)	6.2± 1.8	2.0± 1.1	1.7± 0.5	4.3±0.6

[a] x ± SEM; [b] $p<0.05$ compared with controls.
DYNOR = dynorphin; β-END = β-endorphin. n = 8 animals per group.

Serum PRL concentrations also were significantly increased by β-END and dynorphin. The stimulatory effect of β-END on PRL secretion has been reported previously (Rivier et al., 1977; Dupont et al., 1979; Van Vugt et al., 1979), whereas this is the first report that dynorphin stimulates PRL secretion. Furthermore, this action was specific since NAL completely blocked dynorphin-induced PRL release.

Dynorphin was reported to be 30 times more potent than β-END in the guinea pig ileum bioassay (Goldstein et al., 1979). It is interesting that this rank order of potency was not observed by us in vivo. A possible explanation is that β-END may be more resistant to peptidase degradation by brain tissue than dynorphin.

The reduction of serum PRL concentrations produced by NAL at 3 different doses in the first experiment is of great interest. This effect of NAL has been observed by others (Grandison and Guidotti, 1977; Shaar et al., 1977; Guidotti and Grandison, 1978; Meltzer et al., 1978; Gold et al., 1979; Blank et al., 1980), and suggests that basal PRL levels are under the tonic stimulatory action of EOP. There also are reports that NAL had no effect on basal PRL levels and thus disagree with this conclusion (Martin et al., 1979; Blankstein et al., 1979). A possible explanation may involve stress due to bleeding since different methods of blood collection were used.

II. Role of Endogenous Opioid Peptides in Stress-Induced Prolactin Release

A. Objectives

EOP have been shown to be potent stimulators of PRL secretion (Cusan et al., 1977; Rivier et al., 1977; Shaar et al., 1977; Dupont et al., 1979). The inhibitory effect of NAL on PRL secretion suggests that EOP may regulate PRL secretion during basal conditions (Bruni et al., 1977; Grandison and Guidotti, 1977; Shaar et al., 1977; Guidotti and Grandison, 1978; Meltzer et al., 1978; Blank et al., 1980). It also was of interest to determine if EOP are involved in PRL secretion during non-basal states.

Several different stresses have been shown to stimulate PRL secretion (Euker et al., 1973; Krulich et al., 1974; Mueller et al., 1974). More recently, stress was shown to produce analgesia and increase opioid concentrations in the brain (Akil et al., 1976; Madden et al., 1977; Fried and Singer, 1979; Wesche and Frederickson, 1979). Thus, activation of opioid neurons in the brain by stress may be part of the mechanism by which stress increases PRL secretion. In order to test this hypothesis, we determined if pretreatment with NAL

could block stress-induced PRL secretion.

B. Materials and Methods

The effect of NAL on stress-induced PRL release was studied in the first experiment in which the stress used was exposure to ether. Male Sprague Dawley rats weighing 250-300 g were divided into 2 groups of 20 each. One group was injected with 0.2 mg NAL/kg BW in 0.2 ml saline/100 g BW. The other group was injected with an equivalent volume of saline. Thirty min after injections, half of the rats in the 2 groups were bled by decapitation, and the remaining rats were bled by orbital sinus puncture under light ether anesthesia.

In a second experiment, we investigated the effect of NAL on PRL release in male Sprague-Dawley rats stressed by immobilization. Thirty rats were randomly divided into 3 groups of 10 each. One group was injected ip with 0.2 mg NAL/kg BW and immediately subjected to 30 min restraint stress. The 2 remaining groups were injected with the saline vehicle, and 1 of these 2 groups was stressed for 30 min. Trunk blood was collected by decapitation 30 min after the time of injection.

The effect of NAL on PRL release in heat-stressed rats was tested in a third experiment. Two groups of 10

rats were injected with either 0.2 mg NAL/kg BW or an equivalent volume of saline and placed in an oven at $40^{\circ}\pm2^{\circ}C$ for 20 min. A third group was injected with saline and returned to their cage and served as ambient temperature controls. Blood was collected by decapitation 20 min after the saline or NAL injection.

In a fourth experiment, we determined the effect of NAL on the time response of PRL release in male rats stressed by immobilization. Male Sprague Dawley rats, 250-300 g each, were fitted with an intra-atrial cannula and allowed to recover from surgery for 3 days. Rats were randomly divided into 4 uniform groups of 4 rats each. Two groups were given 0.2 mg NAL/kg BW by ip injection. The other 2 groups were injected with an equivalent volume of the saline vehicle. At the time of the injections, a 0.3 ml blood sample was collected via the cannula from each rat. The rats were then bled a second time, 5 min after the initial blood sample was collected. At this time, restraint stress was imposed on 1 group injected with NAL and on another group given saline. Blood samples were collected from all 4 groups at 5 min intervals during the next 30 min. The 2 restrained groups remained immobilized during the 30 min sampling period while the unrestrained animals were allowed to move freely in their cages.

In all 4 experiments, blood was allowed to clot at 4°C, and the serum was separated and frozen at -20°C until assayed for PRL. The data were expressed as ng/ml in terms of NIAMDD-PRL-RP-1. Analysis of variance and Student-Newman-Keuls' test for multiple comparisons between groups were used to analyze the data. Differences were considered significant at $p<0.05$.

C. Results

The effects of ether exposure and NAL on serum PRL concentrations are shown in Table 4. Ether exposure elevated PRL levels approximately 2-fold when compared to decapitated controls. NAL administration significantly reduced basal PRL levels and partially blocked the stress-induced increase in serum PRL concentration.

Table 4. Effects of Naloxone on Basal Serum Prolactin Concentrations

	Serum Prolactin (ng/ml)	
Method of Bleeding	Saline	Naloxone
Decapitation	16.3 ± 4.0[a]	3.7 ± 0.6[b]
Orbital Sinus	35.1 ± 4.3[b]	23.9 ± 3.2[c]

[a] x ± SEM. [b] $p<0.05$ compared to decapitated controls.
[c] $p<0.05$ compared to orbital sinus bled controls.

Table 5. Effects of Naloxone on Stress-Induced Prolactin Release

	Serum Prolactin (ng/ml)		
Treatment	Non-Stressed	Stressed	Stressed + NAL
Restraint Stress	9.8±1.6[a]	55.0±14.0[b]	11.6±2.4[c]
Heat Stress	18.8±2.1	91.0± 8.8[b]	38.6±1.4[c]

[a] x ± SEM. [b] $p<0.05$ compared to non-stressed controls. [c] $p<0.05$ compared to stressed controls. NAL = naloxone; n = 10 animals per groups.

Table 5 shows that both restraint and heat stress produced about a 5-fold elevation in serum PRL levels. Injection of NAL prior to each of these stresses completely prevented the rise in serum PRL induced by restraint stress, and significantly reduced the increase in serum PRL evoked by heat stress.

The results of the experiment in which chronic cannulas were used to take blood samples (Figure 1), indicate that restraint stress increased serum PRL concentrations above control values. These PRL levels remained elevated throughout the experiment. NAL given prior to restraint stress depressed the stimulatory effect of restraint on PRL release. When administered to unstressed rats, NAL consistently reduced serum PRL concentrations below control levels.

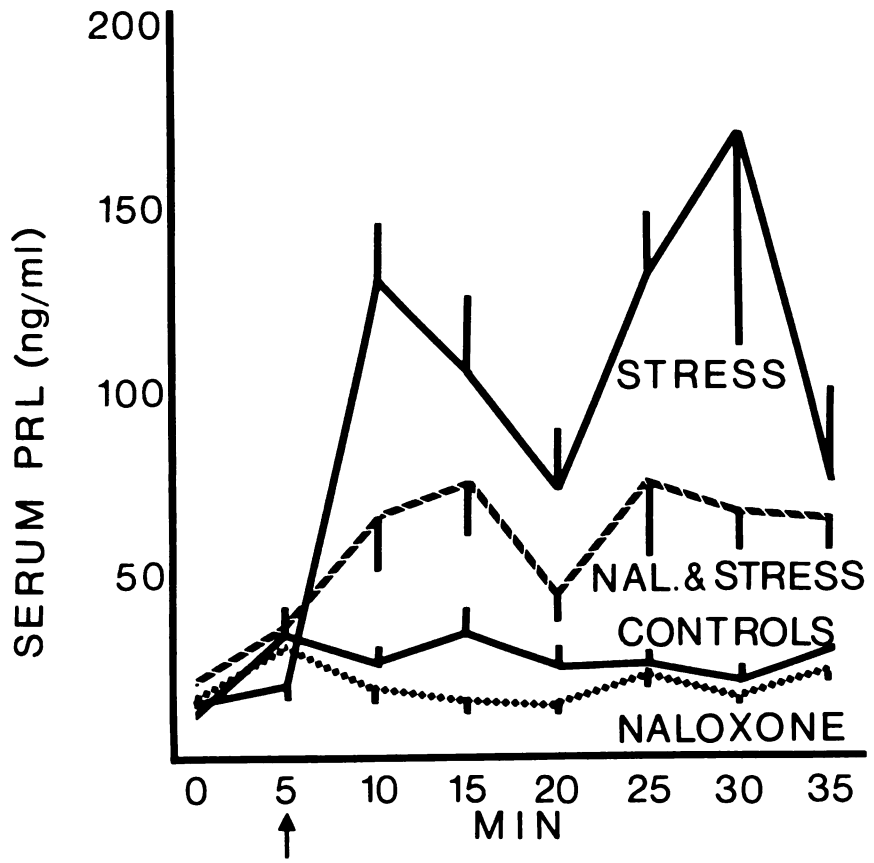

FIGURE 1.

Effect of naloxone (NAL) on restraint stress-induced prolactin (PRL) release (n = 4). Arrow indicates time of immobilization, and vertical bars indicate SEM.

D. Discussion

These results demonstrate that ether, restraint and heat stress each increased PRL release, and are in agreement with previous reports (Euker et al., 1973; Krulich et al., 1974; Mueller et al., 1974). They also

show that NAL significantly inhibited the increase in PRL release induced by stress, in addition to reducing PRL levels in non-stressed rats. These results indicate that stress may increase opioid neuronal activity in the brain. Increased opioid activity could explain stress-induced PRL release, in addition to stress-induced analgesia.

The ability of NAL to block stress-induced PRL release has been confirmed (Grandison and Guidotti, 1977; Dupont et al., 1979; Ragavan, 1981). In addition, ivt injection of β-END antiserum was reported to partially block the response of PRL to stress, suggesting that stress-induced PRL release is due in part to activation of β-END neurons (Ragavan, 1981). Similar experiments using antiserum to the other EOP have not been done, although stress has been reported to increase MET-ENK concentrations in the brain of rats (Wesche and Frederickson, 1979).

Results of NAL administration during other states of elevated PRL secretion further suggest that EOP participate in the regulation of PRL secretion during non-basal conditions. NAL was reported to reduce the suckling-induced release of PRL in post-partum lactating rats (Dupont et al., 1979; Miki and Meites, unpublished observation). NAL also was reported to block the

nocturnal PRL surge in humans (Dupont et al., 1979). Multiple injections of NAL were shown to completely block the PRL surge on the afternoon of proestrus (Ieiri et al., 1980). In this regard, hypothalamic MET-ENK concentrations were reported to be increased on proestrous morning and may be involved in producing the proestrous afternoon PRL surge (Kumar et al., 1979).

III. Effects of Opiates on Hypothalamic Dopamine Activity; Evidence That Opiates Stimulate Prolactin Release Via a Dopaminergic Mechanism

A. Objectives

Opioid stimulation of PRL release is not due to a direct action on the AP. Thus, incubation of opiates or opiate antagonists with hemi-pituitaries or pituitary cell cultures does not elicit the response observed in vivo (Grandison and Guidotti, 1977; Rivier et al., 1977; Shaar et al., 1977). If a direct action on the AP is not the mechanism by which opiates stimulate PRL release, the logical alternative is that opiates stimulate PRL release via hypothalamic mechanisms. In agreement with this conclusion is the report by Grandison and Guidotti (1977) that β-END and MOR stimulated PRL release in a NAL reversible manner in

rats with a deafferentated hypothalamus.

Basal secretion appears to be under the tonic inhibitory influence of tuberoinfundibular DA. Administration of DA antagonists increased serum PRL levels above basal PRL levels (Meites and Clemens, 1972; Mueller et al., 1976a), whereas DA agonists significantly decreased PRL levels (Lu and Meites, 1971; Mueller et al., 1976b). Since DA can inhibit PRL release by a direct action on the AP lactotrophs (Koch and Meites, 1970; Smalstig et al., 1974), a possible mechanism by which opiates stimulate PRL release could be by decreasing tuberoinfundibular DA activity. We investigated this problem by utilizing central acting drugs, as well as measuring tuberoinfundibular DA turnover.

B. Materials and Methods

In the first experiment, male Sprague-Dawley rats weighing 200-250 g, were given a single sc injection of 50 mg L-DOPA/kg (Hoffman-LaRoche, Nutley, NJ), 3 mg piribedil/kg (Les Laboratories Servier, Neuilly-sur-Seine, France), 20 mg amineptine/kg (Les Labs. Servier), 1 mg haloperidol (HAL)/kg (McNeil Labs., Ft. Washington, PA), or 10 mg MOR/kg (Mallinkrodt Labs., St. Louis, MO) alone or in combination with the above drugs. One ml of

blood was collected by orbital sinus puncture under light ether anesthesia at time 0, 1, and 2 hrs after drug administration.

In a second experiment, male Sprague-Dawley rats, 225-250 g, were injected sc with 0.025 mg HAL/kg, 0.05 mg HAL/kg or 5 mg MOR/kg alone or in combination with the 2 doses of HAL. A sixth group was injected sc with saline and served as controls. Rats were decapitated 1 hr after injection and trunk blood was collected.

The effect of β-END on ME DA turnover was investigated in a third experiment. A cannula was placed in the right lateral ventricle of male Sprague-Dawley rats by the method of deBalbian-Verster et al. (1971). They were allowed to recover for 10 days. During the recovery period, rats were handled daily to minimize stress from injection without anesthesia. Rats were randomly divided into 4 groups. Two groups were injected ip with 250 mg α-mpt/kg (Sigma Chemical Co., St. Louis, MO). At the same time, these rats were injected ivt with either 20 ug β-END (provided by Dr. A.A. Manian, Dept. of Psychopharmacology, NIH) in 13 ul of saline or with the vehicle. The ivt injection was repeated 30 min later. The 2 remaining groups were treated similarly except that saline was injected ip instead of α-mpt. Rats were decapitated 1 hr after the

initial injections and blood was collected from the trunk. The ME were removed with fine iris scissors and immediately homogenized in 30 ul of 0.4 N perchloric acid containing 10 mg EDTA/100 ml.

Opiate stimulation of 5-HT activity could be an alternative mechanism by which opiates stimulate PRL release. To test this hypothesis, the following 2 experiments were done. A cannula was placed in the right lateral ventricle of male Sprague Dawley rats, and rats were allowed to recover for 10 days. In the first experiment, 1/2 of the rats were injected ip with 300 mg PCPA (Sigma Chemical Co., St. Louis, MO)/kg BW. The remaining rats were injected with an equivalent volume of saline at this time. Rats were divided into 3 groups (n = 14) consisting of 7 PCPA pretreated and 7 saline pretreated rats and injected ivt with either 10 ug MOR, 150 ug MET-ENK, or 13 ul saline. Rats were decapitated 20 min after ivt injection and trunk blood was collected for PRL determinations.

In a second experiment, the same protocol as above was followed except rats were injected ivt with either 20 ug MOR, 20 ug β-END, 20 ug NAL, or 13 ul saline. Blood was collected by decapitated 20 min after ivt injection, approximately 48 hours after PCPA injection.

Serum PRL was assayed by a standard RIA pro-

cedure using the NIAMDD kit provided by A.F. Parlow (Harbor General Hospital, Torrance, CA). DA was assayed by the radioenzymatic method of Coyle and Henry (1973), using COMT isolated from the rat liver by the method of Nikodijevic et al. (1970). Methoxytyramine was separated utilizing the solvent extraction and thin layer chromatography method of Ben-Jonathan and Porter (1976; Appendix B). The protein content of the ME was determined by the method of Lowry et al. (1951). Results were expressed as ng DA per mg protein. Analysis of variance and Student-Newman-Keuls' test for multiple comparisons between groups, or Students "t" test when appropriate, were used to analyze the data. The results were considered to be significant at $p<0.05$.

C. Results

L-DOPA, piribedil, and amineptine each significantly decreased serum PRL concentrations 1 and 2 hrs after injection (Table 6). HAL and MOR each significantly increased serum PRL levels at the 1 hr sampling period. PRL remained elevated 2 hrs in the HAL treated, but not in the MOR treated rats. When MOR was injected in combination with each of the above drugs, the inhibitory effects of L-DOPA and piribedil on serum PRL levels were not altered. MOR prevented amineptine

from lowering serum PRL levels at 1 hr, but not at the 2 hr sampling period. It should be noted that MOR given alone had no effect on serum PRL at 2 hrs. MOR in combination with HAL did not increase PRL levels above that produced by HAL alone.

Table 6. Effects of Dopaminergic Drugs on Morphine Induced Increase in Serum Prolactin

	0 hr	1 hr	2 hr
Controls saline	11 ± 1[a]	17 ± 3	13 ± 3
L-DOPA 50 mg/kg	15 ± 4	3 ± 1[b]	6 ± 2[b]
PIR 3 mg/kg	15 ± 3	3 ± 1[b]	8 ± 1[b]
AMIN 20 mg/kg	16 ± 4	6 ± 1[b]	5 ± 1[b]
HAL 1 mg/kg	14 ± 3	37 ± 3[b]	33 ± 3[b]
MOR 10 mg/kg	18 ± 3	36 ± 5[b]	17 ± 7
L-DOPA+MOR	13 ± 3	6 ± 2[b]	7 ± 2[b]
PIR + MOR	16 ± 2	4 ± 1[b]	4 ± 2[b]
AMIN + MOR	16 ± 4	15 ± 2	5 ± 1[b]
HAL + MOR	15 ± 2	36 ± 2[b]	34 ± 2[b]

[a] $\bar{x} \pm$ SEM; [b] $p<0.05$ compared to saline controls;
PIR = pipiribedil; AMIN = amineptine; HAL = haloperidol;
MOR = morphine; n = 6 animals per group.

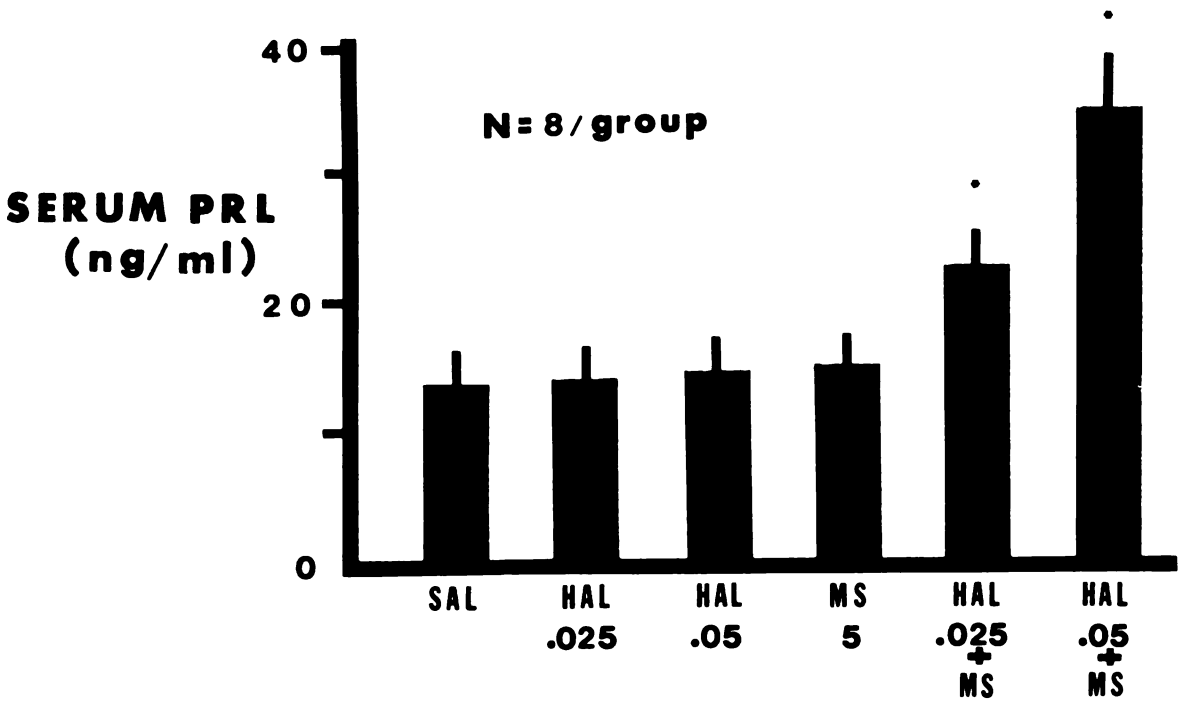

FIGURE 2.

Effects of haloperidol (HAL) and morphine (MS) on serum prolactin (PRL) concentrations. vertical bars represent SEM. *represents $p < 0.05$ as compared to controls. Number below each treatment indicates dose of drug (mg/kg).

When subeffective doses (0.025 or 0.05 mg) of HAL/ kg were injected, serum PRL levels were not altered 1 hr later (Figure 2). A subeffective dose (5 mg/kg) of MOR

also did not increase serum PRL concentrations. However, when doses of these two drugs were combined, serum PRL levels were significantly elevated.

In the third experiment α-mpt, β-END, and β-END together with α-mpt each significantly increased serum PRL concentrations over control values (controls = 9.7±0.5 ng/ml, α-mpt = 72.1±10.3 ng/ml; β-END = 109.7±10.2 ng/ml; β-END + α-mpt = 126.9±4.8 ng/ml). DA content in the ME of rats treated with β-END was less than 2% depleted 1 hr after α-mpt, but DA was depleted approximately 31% 1 hr after administration of α-mpt alone (Figure 3).

The results of PCPA pretreatment on MOR and MET-ENK stimulation of PRL release are shown in Table 7. Intraventricular injection of either 10 ug MOR or 150 ug MET-ENK significantly increased serum PRL levels 20 min after injection. Blockade of 5-HT synthesis by pretreatment with PCPA had no effect on the stimulatory action of these 2 opiates, nor did it effect basal PRL levels.

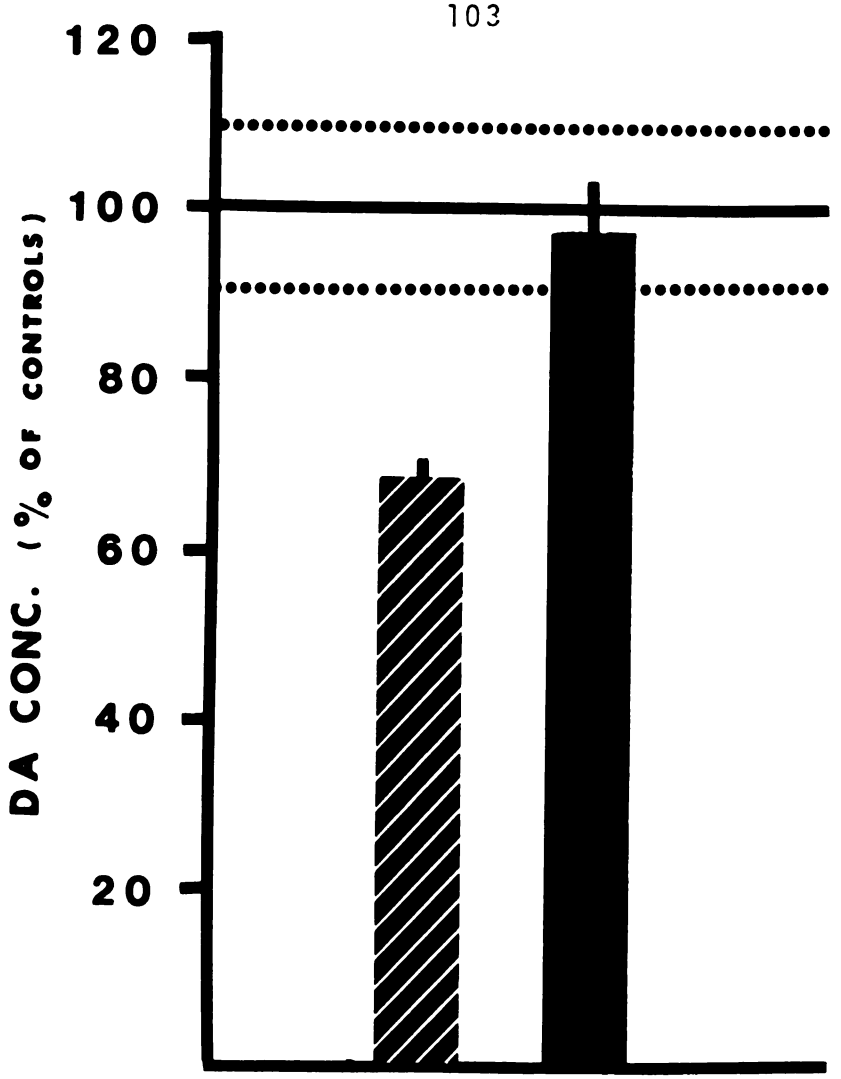

FIGURE 3.

Effects of β-END on ME DA turnover: Since there was no significant difference in the DA concentration of rats treated with saline and rats treated with β-END (131.4 ±12.7 vs 118.3±18.1 ng/mg protein), these values were combined. The mean was set at 100% and is represented by the solid horizontal line. The dotted horizontal lines represent ± 1 SEM. The solid verticle bar represents ME DA concentration (122.2±7.4 ng/mg protein) 1 hr after α-mpt and β-END administration. The hatched bar represents DA concentration (85.8±2.7 ng/mg protein) in the ME 1 hr after α-mpt injection. Both values are presented as % of controls. The vertical lines represent ± 1 SEM based on six determinations. DA concentrations in the ME of rats treated with α-mpt and rats treated with α-mpt together with β-END were significantly different ($p<0.01$) from each other, as determined by Students "t" test for unpaired data.

Table 7. Effects of PCPA on Morphine and Methionine-Enkephalin Stimulation of Prolactin Release

Treatment	Serum Prolactin (ng/ml)
Saline + Saline	10.4 ± 1.0[a]
Saline + PCPA	7.8 ± 0.9
MOR + Saline	34.4 ± 8.9[b]
MOR + PCPA	36.0 ± 7.9[b]
MET-ENK + SALINE	39.9 ± 2.7[b]
MET-ENK + PCPA	36.4 ± 3.6[b]

Doses were PCPA = 300 mg/kg; MOR=10 ug; MET-ENK=150 ug.
[a] = x ± SEM; [b] $p<0.05$ vs control.

The effects of PCPA pretreatment on serum PRL concentrations 20 min after injection of either saline, MOR, β-END, or NAL are shown in Figure 4. Intraventricular injection of 20 ug MOR or 20 ug β-END resulted in a 4-5 fold increase in serum PRL concentrations 20 min after injection. Conversely, ivt injection of 20 ug NAL significantly reduced serum PRL concentration. Pretreatment with PCPA had no effect on the stimulatory action of these 2 opiates, nor on the inhibitory action of the opiate antagonist, NAL. Once again, PCPA pretreatment did not alter basal PRL levels.

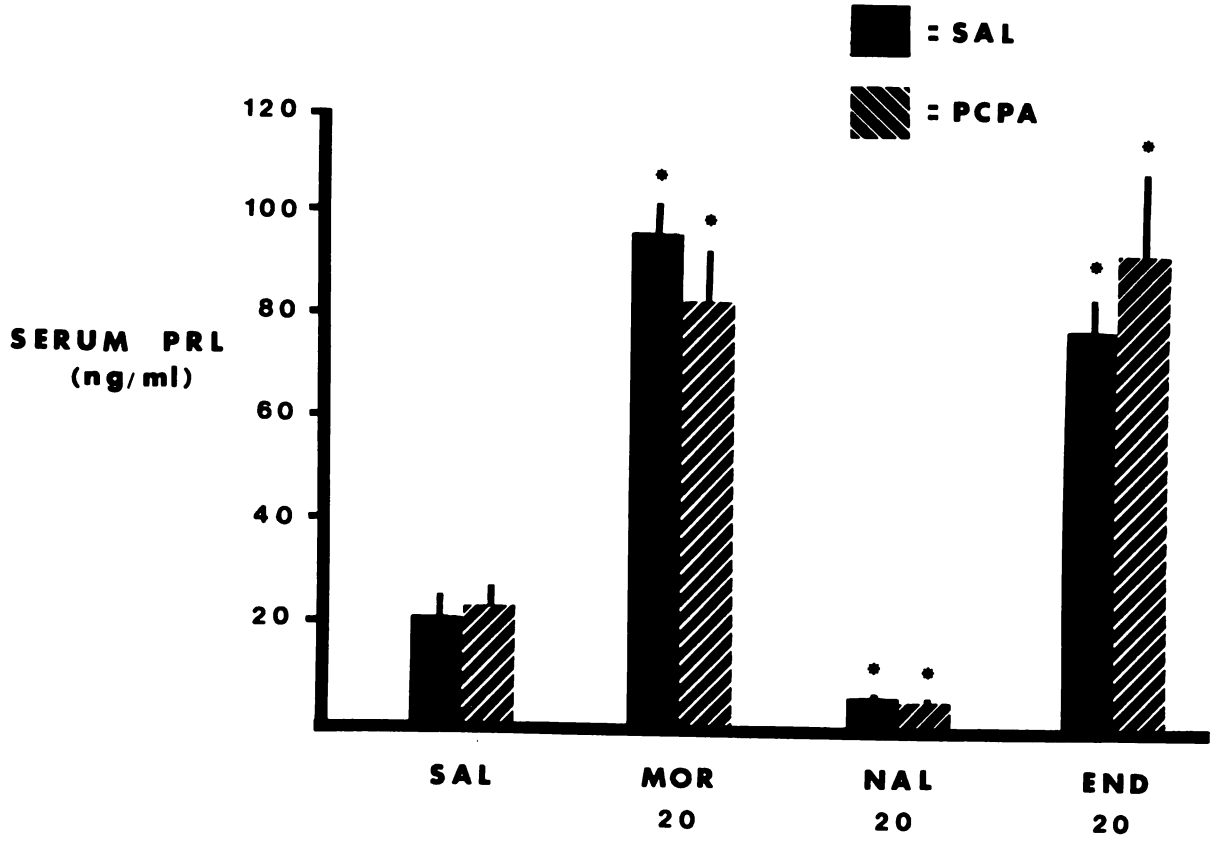

FIGURE 4.

Effects of PCPA on MOR and β-END stimulation and NAL inhibition of PRL release. Rats were pretreated with either PCPA (hatched bars) or saline (solid bars), 48 hrs prior to ivt injection of MOR, NAL, and β-END.
* $p<0.05$ vs controls; n = 7.

D. Discussion

In general, these results indicate that opiates increase PRL release by reducing tuberoinfundibular DA activity. MOR stimulation of PRL release was blocked by L-DOPA and piribedil, both dopaminergic agents. MOR prevented amineptine, a DA reuptake inhibitor, from reducing serum PRL levels, presumably by decreasing the amount of DA available at the amineptine site of action. MOR had no effect on PRL levels when given together with a large dose of HAL. HAL alone may have inhibited maximally DA receptors, and thus further inhibition of tuberoinfundibular DA by MOR had no effect. When subeffective doses of MOR or HAL were injected, no increases in serum PRL were observed, but when the 2 drugs were administered concurrently, serum PRL levels were increased significantly. This effect is believed to indicate that the combination of the 2 drugs was able to reduce DA activity sufficiently to elevate serum PRL values.

A direct demonstration of the ability of opiates to reduce hypothalamic DA activity was provided in the experiment with β-END which decreased DA turnover in the ME and increased serum PRL levels approximately 10-fold. These results are in agreement with those recently reported by Ferland *et al.* (1977), who showed that MET-ENK

infusion reduced DA activity in the ME as measured fluorimetrically, and also increased serum PRL in rats. The effect of opiates on tuberoinfundibular DA activity also has been confirmed by several other laboratories (Deyo et al., 1979; Van Loon and Kim, 1980). Furthermore, Gudelsky and Porter (1979) reported that DA in pituitary stalk plasma was significantly reduced by MOR, β-END or an enkephalin analog. Together, these results strongly suggest that the stimulatory action of opiates on PRL secretion is mediated via a reduction of tuberoinfundibular DA activity.

Additional hypothalamic neurotransmitters, most notably 5-HT, also may mediate the stimulatory action of opiates on PRL secretion. β-END has been reported to stimulate hypothalamic 5-HT turnover (Van Loon and de Souza, 1978). However, we failed to observe any effect of 5-HT synthesis blockade by PCPA on opiate stimulation of PRL release. Cusan et al. (1977) also observed no effect of PCPA on opiate-induced PRL release, whereas Spampinato et al. (1979) reported that PCPA potentiated the response of PRL to MET-ENK. In contrast to these results which suggest 5-HT does not mediate the PRL stimulatory action of opiates, it was reported that metergoline and methysergide, both 5-HT antagonists, or 5,6-dihydroxytryptamine, a 5-HT neurotoxin, blocked the

stimulatory action of MET-ENK on PRL release (Spampinato et al., 1979; Koenig et al., 1979). Although inferential, data suggesting that EOP are involved in stress-induced (Grandison and Guidotti, 1978; Van Vugt et al., 1979) and suckling-induced PRL release (Dupont et al., 1979) are consonant with the proposal that 5-HT mediates the stimulatory action of opiates on PRL release, since 5-HT activity appears to be increased during both of these states (Mena et al., 1976; Mueller et al., 1976a). Thus, in addition to reducing tuberoinfundibular DA activity, opiates may increase PRL release by stimulating hypothalamic 5-HT activity.

An interesting observation by Demarest and Moore (1981) suggests that these opposite effects of opiates on DA and 5-HT activity may in fact be related. They reported that disruption of 5-HT neuronal activity by injection of either metergoline or 5,7-dihydroxytryptamine blocked the inhibitory effect of MOR on DA turnover in the ME. Since 5-HT can not stimulate PRL secretion by a direct action on the AP, this proposed interaction could explain how increased hypothalamic 5-HT activity produced by opiates stimulates PRL secretion.

IV. Evidence That Stimulation of Luteinizing Hormone Release by Naloxone is Mediated by Norepinephrine

A. Objectives

Our laboratory reported that NAL, a specific opiate antagonist, not only blocked the inhibitory action of MOR on LH release, but when administered alone, stimulated LH release (Bruni et al., 1977). This effect of NAL on LH release has been confirmed in intact male and female rats (Blank et al., 1979; Cicero et al., 1979; Ieiri et al., 1979), in castrated male rats (Van Vugt et al., 1978), and in human subjects (Mirin et al., 1976). We have suggested that LH release is under tonic inhibition by hypothalamic EOP (Meites et al., 1979).

MOR, the brain opioid peptides, and NAL do not alter AP hormone secretion via a direct action on the pituitary (Grandison and Guidotti, 1977; Rivier et al., 1977, Shaar et al., 1977), but are believed to act via hypothalamic mechanisms. Several recent reports have shown that opiates depress hypothalamic DA turnover (Ferland et al., 1977; Gudelsky and Porter, 1979; Van Vugt et al., 1979) and elevate 5-HT turnover (Van Loon and DeSouza, 1978; Spampinato et al., 1979). The effects of DA on LH release are controversial (McCann and Moss, 1975; Meites et al., 1977), but there are many

indications that 5-HT can inhibit LH release under a variety of physiological conditions (Kamberi et al., 1970; Schneider and McCann, 1970; Müller et al., 1977; Weiner and Ganong, 1978).

The possible effects of opiates on hypothalamic NE activity are not clear at present. Since there is considerable evidence that hypothalamic NE stimulates LH release (Schneider and McCann, 1970; Krieg and Sawyer, 1976; Müller et al., 1977; Weiner and Ganong, 1978), it was of interest to determine whether drugs which depress the hypothalamic noradrenergic system could prevent NAL from promoting LH release.

B. Materials and Methods

Male Sprague-Dawley rats (Harlan Industries, Cumberland, IN), weighing 225-250 g each, were housed 1 week prior to the experiments in a temperature (25°C) and light (14 hr daily) controlled room. Food and water were provided ad libitum both before and during experiments. During the 1 week period of acclimatization, all rats were injected daily with saline in order to minimize any stress of handling or injection. In all experiments, drugs were given by ip injection in saline (0.2 ml/100 g BW).

Three anti-noradrenergic drugs were used to assess

whether the hypothalamic noradrenergic neuronal system mediates the stimulatory effect of NAL on LH release. In a preliminary experiment, rats were divided into 4 groups of 8 rats. One group was injected with the tyrosine hydroxylase inhibitor, α-mpt (Sigma Chemical Co., St. Louis, MO), using a dose (250 mg/kg) which has been shown to reduce hypothalamic DA and NE concentrations (Brodie et al., 1966; Fuxé and Hökfelt, 1969; Donoso et al., 1971). A second group was injected with 5 mg NAL/kg. A third group received a combination of the 2 drugs while a fourth group received saline. NAL injections were repeated 30 min later. All rats were decapitated 60 min after the initial injections and trunk blood was collected for LH RIA.

It was previously reported that α-mpt and phenoxybenzamine, an alpha receptor blocker, effectively reduced the post-castration rise of LH for at least 6 hrs, presumably by inhibiting the stimulatory action of NE (Ojeda and McCann, 1973). Using a similar time course and identical doses, we determined if these 2 drugs could block the stimulatory effect of NAL on LH release. Rats were divided into 4 groups of 8 rats. One group was injected with 250 mg α-mpt/kg BW. A second group was injected with 20 mg phenoxybenzamine hydrochloride (PBH, Smith, Kline and French Labs,

Philadelphia, PA)/kg BW in order to block alpha receptors. A third and fourth group were injected with saline at this time. Five hrs later, all groups, except 1 of the 2 groups pretreated with saline, were injected with 2 mg NAL/kg BW. Controls were injected with an equivalent volume of saline. All rats were decapitated 20 min later and trunk blood was collected.

Since α-mpt depletes DA in addition to NE, a third experiment was done in an effort to deplete only NE. Diethyldithiocarbamate (DDC, Fisher Scientific Co., Fair Lawn, NJ) was used in this experiment since it does not interfere with DA synthesis, but inhibits synthesis of NE. Male Sprague-Dawley rats were divided into 2 groups of 16 rats each. One group was injected with 500 mg DDC/kg BW. The second group received an equivalent volume of saline. Two hrs later, half of the rats in each group were injected with 2 mg NAL/kg BW. The remaining rats were injected with saline. Rats were decapitated 20 min later and trunk blood was collected for serum LH RIA.

In order to determine the efficacy of DDC in depleting hypothalamic NE, brains were removed immediately after decapitation. The hypothalamus was removed by cutting at the following landmarks: a) anterior, 2 mm rostral to the optic chiasm; b)

posterior, immediately rostral to the mammillary bodies; c) lateral, the lateral hypothalamic sulci; d) dorsal, at the level of the anterior commissure (3-4 mm). The hypothalamus was divided into an anterior hypothalamic portion (AH) and medial basal hypothalamic portion (MBH) by cutting immediately caudal to the optic chiasm. The tissues were weighed and frozen within 2 min of decapitation, and were assayed for DA and NE on the same day.

DA and NE were assayed by the radioenzymatic method of Coyle and Henry (1973). Tissues were extracted in 0.4 N perchloric acid containing 10 mg% EDTA. All tissues were diluted to a concentration of 1 mg/10 ul perchloric acid to prevent possible false readings due to tissue competition. DA and NE concentrations were expressed as ng/mg wet weight.

Blood from the trunk of decapitated rats was allowed to clot at $4^{o}C$. Serum was separated 24 hrs later and stored at $-20^{o}C$ until the LH RIA could be performed. Serum LH was assayed by a standard double antibody RIA technique. LH values were expressed in terms of NIAMDD-LH-RP-1. Statistical differences between groups were determined by analysis of variance and Student-Newman-Keuls' test. The level of significance chosen was $p<0.05$.

C. Results

The results of the first experiment are illustrated in Fig. 5. NAL caused a 4-5 fold increase in serum LH concentration as compared to controls (60.5±9.2 vs 13.1±1.9 ng/ml). Injection of α-mpt had no effect on basal serum LH levels (16.7±2.2 vs 13.19 ng/ml). However, α-mpt completely blocked the stimulatory action of NAL on LH release (60.5±9.2 vs 20.4±1.1 ng/ml).

The effects of α-mpt or PBH pretreatment on NAL-induced LH release are shown in Figure 6. A single injection of NAL increased serum LH concentrations approximately 4-fold (18.1±1.8 vs 76.1±11.1 ng/ml) 20 min after injection. Pretreatment with either α-mpt or PBH 5 hrs prior to NAL injection significantly inhibited the stimulatory action of NAL on LH release (76.1±11.1 vs 30.1±10.3 and 30.3±8.9 ng/ml, respectively). Statistical analysis indicated that serum LH levels of rats treated with NAL alone were significantly greater than LH levels in the other 3 groups.

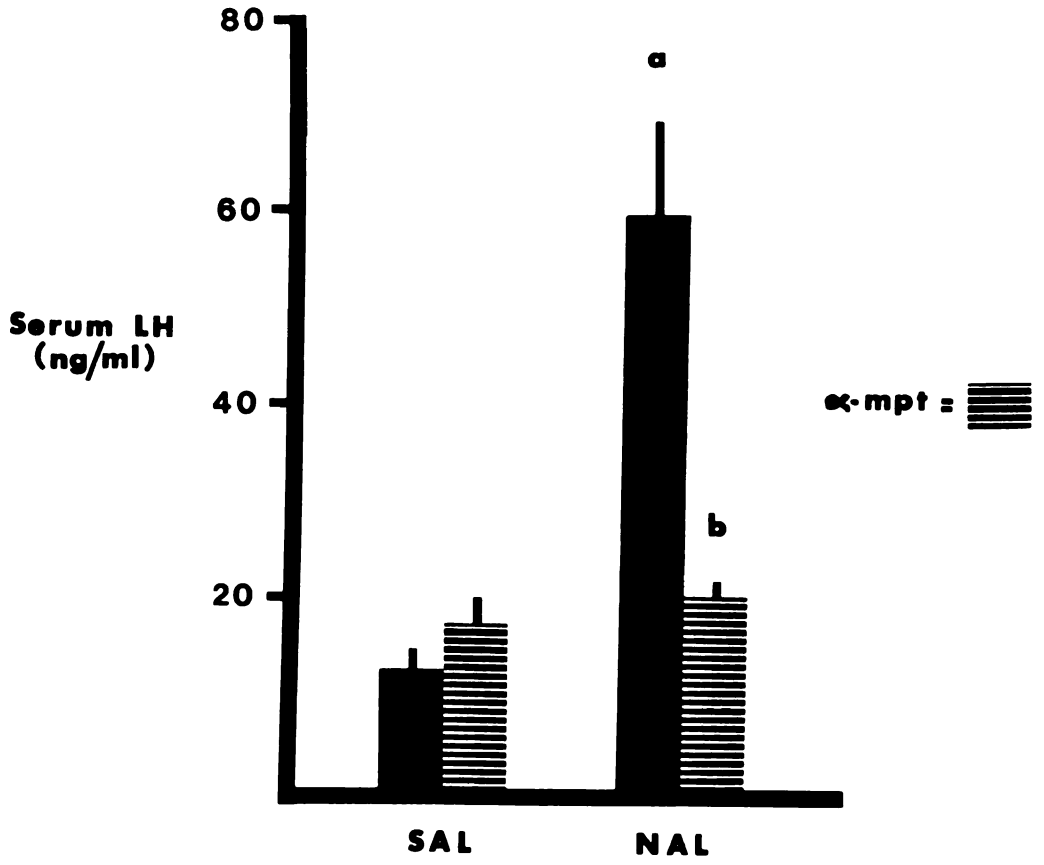

FIGURE 5.

Effect of α-methyl-para-tyrosine (α-mpt) on NAL-induced release of LH. Vertical bars represent SEM.
[a] Represents $p<0.05$ as compared to controls.
[b] Represents $p<0.05$ as compared to NAL-treated rats.
Doses used were: NAL=5 mg/kg BW 2x; α-mpt=250 mg/kg BW.
n = 8 animals / group.

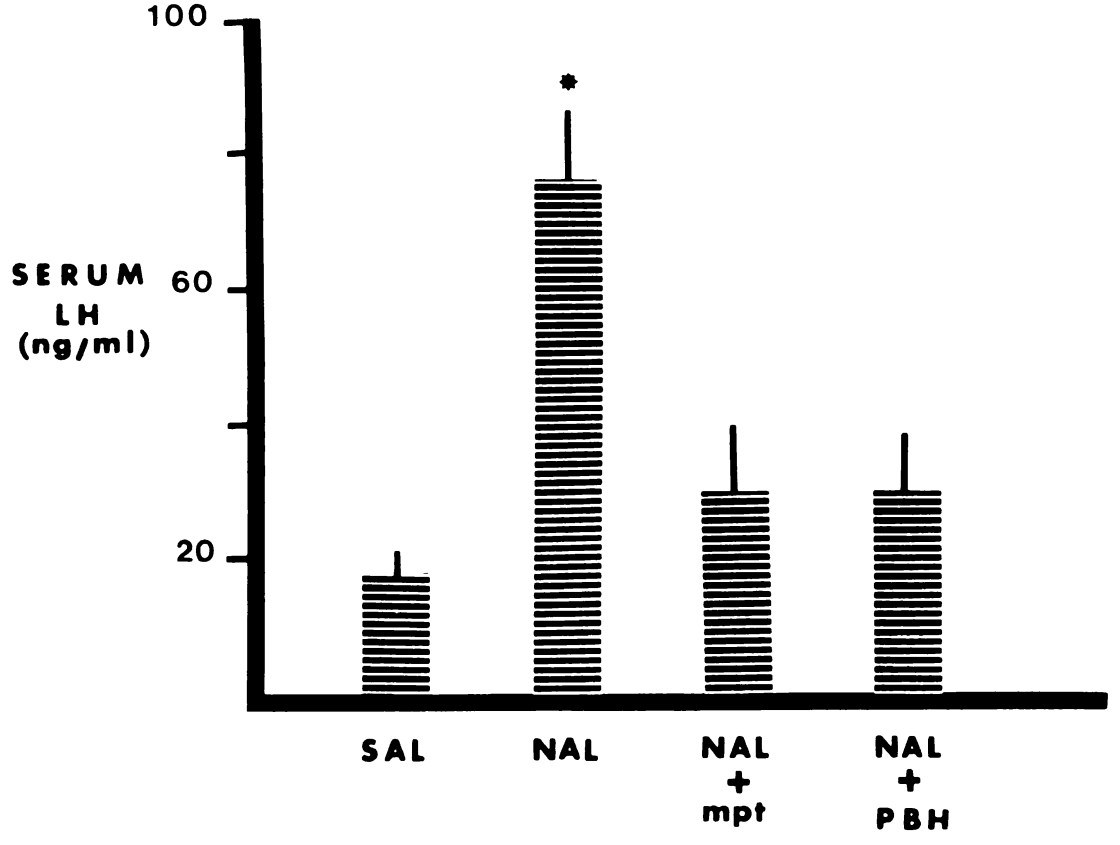

FIGURE 6.

Effect of α-methyl-para-tyrosine (α -mpt) and phenoxybenzamine hydrochloride (PBH) on NAL-induced release of LH. * Represents $p < 0.05$ as compared to controls. Doses used were NAL = 2 mg/kg BW; α-mpt = 250 mg/kg BW; and PBH = 20 mg/kg BW. n = 8 animals per group.

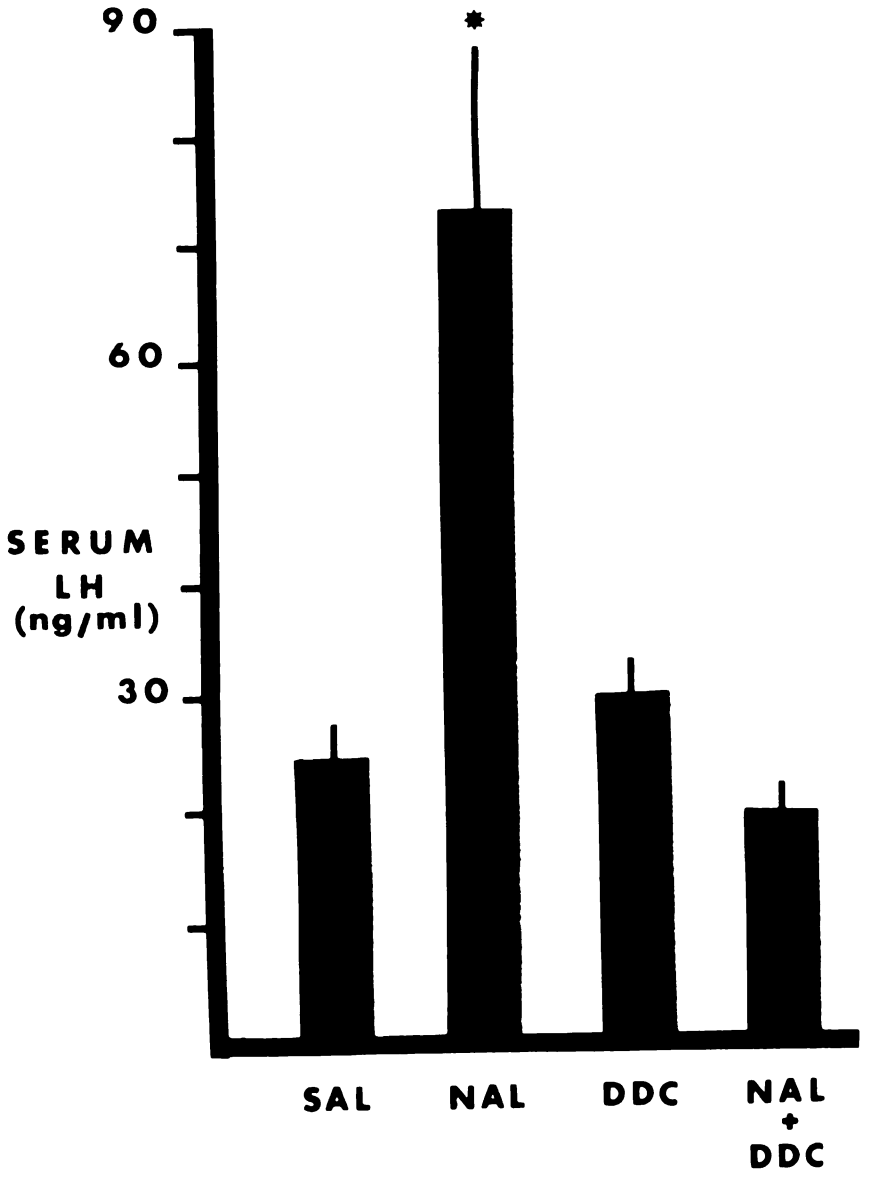

FIGURE 7.

Effect of diethyldithiocarbamate (DDC) on NAL-induced release of LH. Dose of DDC=500 mg/kg BW; NAL=2 mg/kg BW. n = 8 animals per group. *Represents $p < 0.05$ as compared to controls.

The effect of DDC administration on NAL-stimulated LH release is shown in Figure 7. NAL alone significantly increased serum LH concentrations 20 min after injection, as compared to control values (73.9±13.5 vs 25.2±3.4 ng/ml). Injection of DDC alone had no effect on basal serum LH levels (30.3±3.0 vs 25.2±3.4 ng/ml). However, pretreatment with DDC 2 hrs prior to injection of NAL completely blocked the stimulatory action of NAL on LH release (73.9±13.5 vs 19.8±2.4 ng/ml).

The acute effects of NAL and DDC on hypothalamic NE and DA concentrations are shown in Figures 8 and 9. NAL significantly reduced AH NE concentration, but significantly increased MBH NE concentration as compared to control values. DDC alone or together with NAL significantly reduced NE concentrations in both areas of the hypothalamus. NAL had no effect on AH or MBH DA concentrations. However, DDC alone or together with NAL significantly increased DA concentrations in both hypothalamic areas.

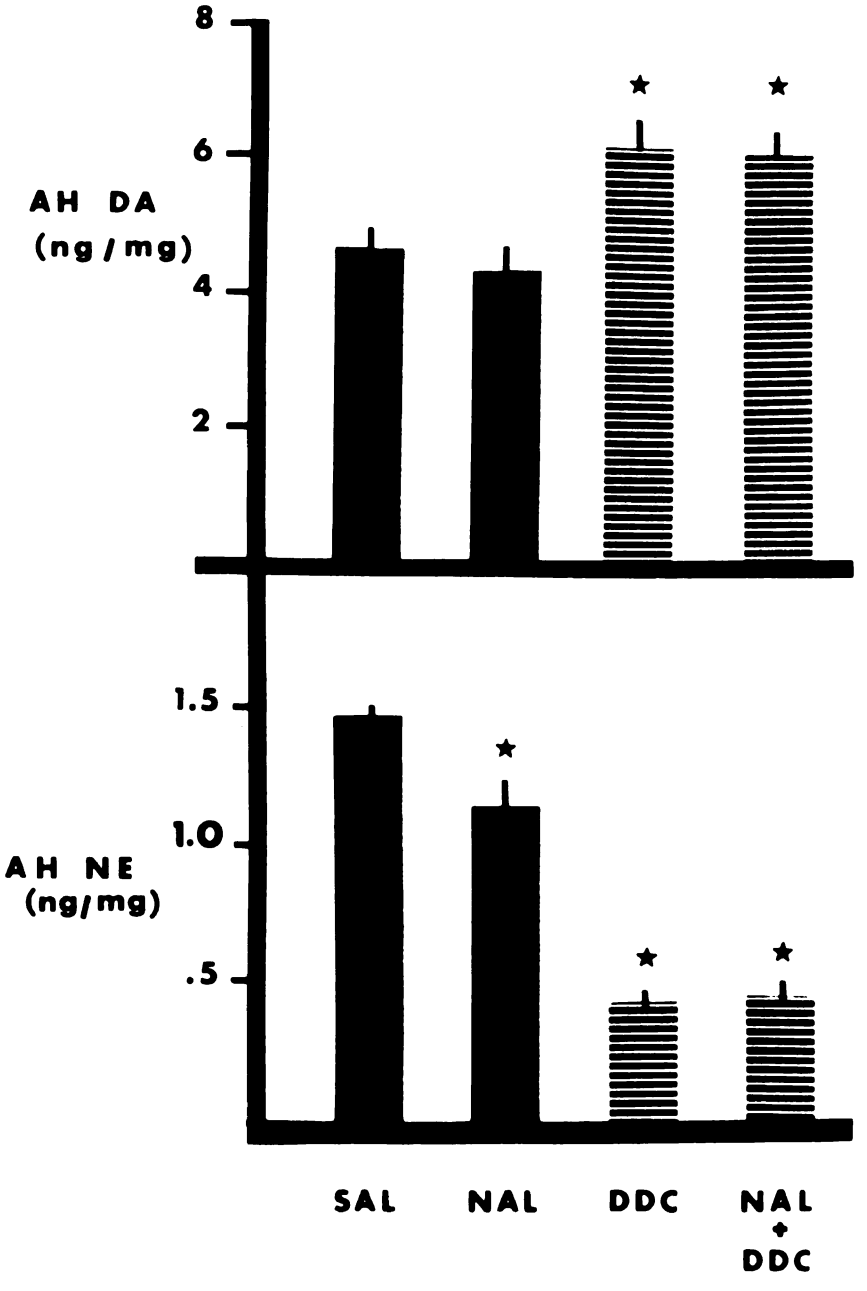

FIGURE 8.

Effects of NAL, DDC, or NAL plus DDC on anterior hypothalamic (AH) NE and DA concentrations. Concentrations are expressed as ng/mg wet weight. *Represents $p<0.05$ as compared to controls. n = 8 animals per group.

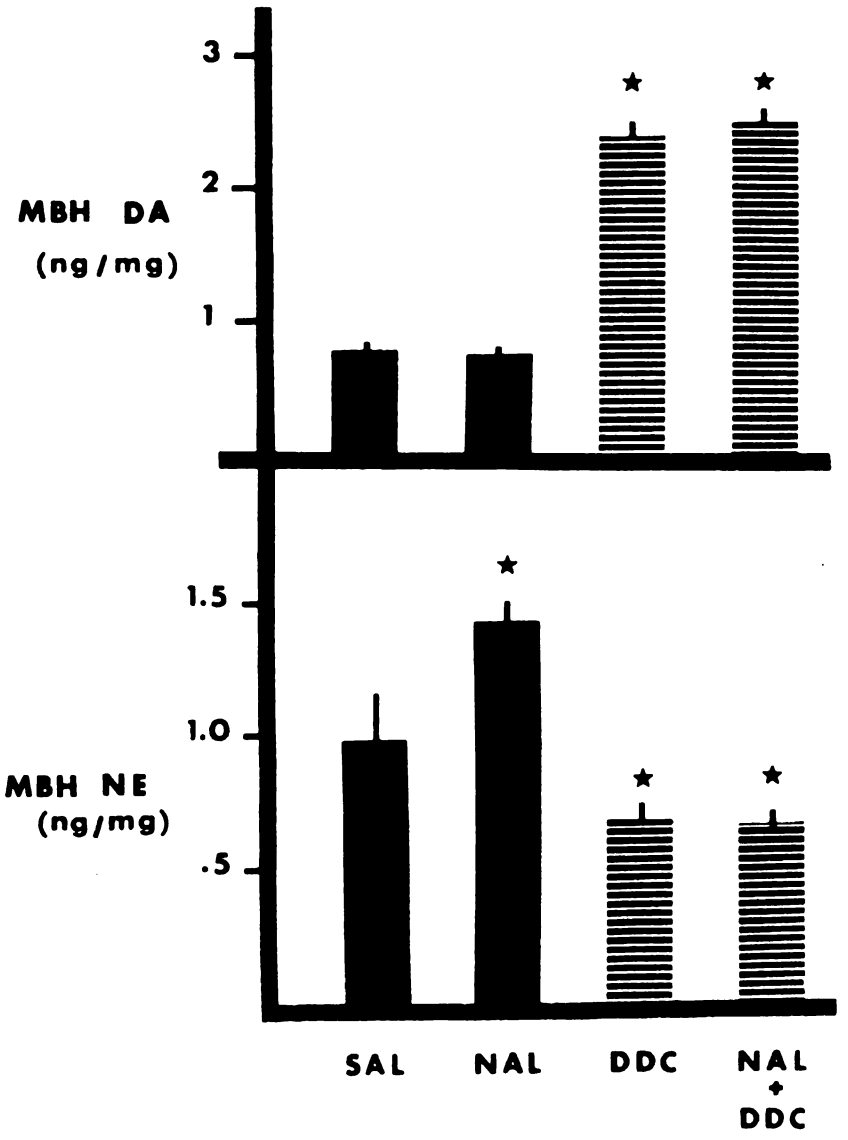

FIGURE 9.

Effects of NAL, DDC, or DDC plus NAL on medial basal hypothalamic (MBH) NE and DA concentrations. Concentrations are expressed as ng/mg wet weight. * Represents $p<0.05$ vs controls. n = 8 animals per group.

D. Discussion

These results show that NAL-induced LH release is blocked by drugs which disrupt noradrenergic neuronal activity. It is well established that α-mpt inhibits catecholamine synthesis (Brodie et al., 1966; Fuxé and Hökfelt, 1969; Donoso et al., 1971), whereas DDC specifically inhibits NE synthesis (Donoso et al., 1971; Negro-Vilar et al., 1979). However, we believe that the inhibitory action of α-mpt and DDC is due to their effect on NE synthesis. This conclusion is supported by our finding that PBH, which blocks alpha receptors, also inhibited NAL-induced LH release. Therefore, the ability of these 3 different anti-noradrenergic drugs to prevent the increase in serum LH produced by NAL is believed to indicate that hypothalamic NE is involved in mediating the stimulatory effect of NAL on LH release. Although it would be incorrect to assume that the 3 anti-noradrenergic drugs used in these experiments are specific in their actions and influenced only the noradrenergic system, their similar ability to block NAL-induced LH release suggests that NE is involved in NAL stimulation of LH release.

Neither α-mpt nor DDC altered basal serum LH levels, suggesting that NE may not be an important factor in basal LH release. However, there are many

indications that when LH release is increased, as after castration (Anton-Tay and Wurtman, 1968; Donoso et al., 1969), or during the proestrous surge of LH (Donoso and deGutierrez-Moyano, 1970; Blake et al., 1972; Kalra and McCann, 1974), NE is an important mediator of LH release. Our results suggest that this is also true of the stimulation of LH release by NAL. This conclusion is in agreement with the findings of Simpkins and Kalra (1980). They reported that stimulation of LH release by NAL in the estrogen-progesterone-primed ovariectomized rat was blocked by DDC. Furthermore, the LH inhibitory action of MOR in their model was reversed by clonidine, a NE receptor stimulator. It also was reported that MOR reduced the firing rate and release of NE from the locus coeruleus (Korf et al., 1974), which is believed to be a major source of hypothalamic NE.

Hypothalamic NE concentrations were significantly reduced by DDC, whereas DA concentrations were increased. An increase in DA concentration after DDC administration has been reported (Negro-Vilar et al., 1979), and is believed to be due to accumulation of DA in NE neurons since DDC blocks the conversion of DA to NE by inhibiting DA-β-hydroxylase. NAL alone had no effect on hypothalamic DA concentrations. However, NAL significantly decreased AH NE concentration and

increased MBH NE concentrations. Although this dual effect of NAL on NE concentration is interesting, a valid conclusion concerning the effect of NAL on hypothalamic NE activity can not be made based on concentration data alone.

5-HT also may be involved in NAL stimulation of LH release. Several reports have indicated that opiates can increase hypothalamic 5-HT activity (Van Loon and de Souza, 1978; Spampinato et al., 1979; Ieiri et al., 1980), and 5-HT has been shown to inhibit LH release under a variety of experimental conditions (Kamberi et al., 1970; Schneider and McCann, 1970; Müller et al., 1977; Weiner and Ganong, 1978). Ieiri et al. (1980) demonstrated that 5-HTP, the precursor of 5-HT, inhibited the action of NAL on LH release in prepubertal female rats, whereas PCPA, which depletes 5-HT, potentiated the release of LH in response to NAL. It is possible, therefore, that NAL promotes LH release not only by stimulating hypothalamic NE neurons, but also by reducing 5-HT metabolism in the hypothalamus.

V. Morphine Exerts a Testosterone-Like Effect on Luteinizing Hormone Release; Involvement of Luteinizing Hormone Releasing Hormone

A. Objectives

It was shown previously that MOR and MET-ENK reduced serum LH levels in noromal adult male rats (Cicero *et al.*, 1976; Bruni *et al.*, 1977). Furthermore, MOR was reported to block the preovulatory LH surge and ovulation in a NAL reversible manner in adult female rats (Barraclough and Sawyer, 1955; Pang *et al.*, 1977). The effects of opiates on the post-castration rise of serum LH and hypothalamic LHRH were studied in castrated male rats to further access the inhibitory action of opiates on LH secretion.

Opiates presumably inhibit LH release by inhibiting the release of LHRH from the hypothalamus. Thus electrical stimulation of the ME blocked the anti-ovulatory action of MOR, suggesting that MOR inhibits the release, but not necessarily the synthesis of LHRH (Sawyer, 1963). In addition, the inhibitory effect of MOR is not at the level of LHRH receptors, since Cicero *et al.* (1979) showed that MOR had no effect on LHRH stimulation of LH release *in vitro*.

Castration results in elevated circulating LH levels (Gay and Midgley, 1969; Yamamato et al., 1970), whereas hypothalamic LHRH concentration is reduced (Araki et al., 1975; Chen et al., 1977). The reduced hypothalamic LHRH concentration is believed to be due to increased release. This interpretation is supported by the recent observation that castration increases LHRH concentrations in portal blood (Sarkar and Fink, 1980). Therefore, we reasoned that the castrated male rat would be a good model to study the inhibitory actions of opiates on LH and LHRH release.

B. Materials and Methods

Male Sprague-Dawley rats, weighing 250-275 g each, were divided into 4 groups of 10 each. Three of the groups were castrated and given either saline, 10 mg MOR/kg B.W. (Mallinckrodt Labs., St. Louis, MO) or 0.2 mg NAL/kg B.W. ip once every 6 hours. The fourth group remained intact and received saline at 6 hour intervals. Blood was collected by orbital sinus puncture under light ether anesthesia at the times indicated in Figure 10. In all cases, blood samples were taken 6 hours after the previous injection.

In a second experiment, castrated male rats were divided into 4 groups and injected ivt with either

saline, 20 ug NAL, 20 ug MOR, or 20 ug β-END 24 hours after castration. A fifth intact group was injected with saline. Trunk blood was collected upon decapitation 10 min after injection.

In a third experiment, male Sprague Dawley rats were castrated and divided into 3 groups (n = 8). One group was injected with 1 mg testosterone propionate (TP, Sigma Chemical Co., St. Louis, MO)/day. A second group received 10 mg MOR/kg BW twice daily. A third castrated group and a fourth intact group were injected once daily with oil (TP vehicle) and twice daily with saline (MOR vehicle). In order to control the various injections, the TP treated group was injected twice per day with saline, and the MOR treated group was injected once per day with oil. TP and oil were injected at 0900 hours each day and MOR and saline were injected at 0900 and 1700 hours each day. All injections were given sc. Injections were begun immediately after castration and continued for 12 days. One ml blood samples were collected by orbital sinus puncture 1 hour after the 1700 hour injection, beginning 2 days after castration, and at 2 day intervals thereafter.

Rats were decapitated on the morning of the twelfth day 1 hour after the 0900 hour injection and trunk blood was collected. The hypothalamus was quickly removed by

making cuts immediately rostral to the mammillary body, 2 mm rostral to the optic chiasm, laterally at the hypothalamic sulci, and dorsally at the level of the anterior commissure. The hypothalamus was divided into an anterior hypothalamic-preoptic area, and a mid-hypothalamic portion by cutting immediately caudal to the optic chiasm. Tissues were weighed and frozen on dry ice within 2 min after decapitation.

Blood was allowed to clot overnight at 4°C, and serum was separated and frozen at -20°C until assayed for LH. Hypothalamic tissues were homogenized in 1 N cold acetic acid and centrifuged at 2000 x g for 15 min at 4°C. The supernatant was diluted 1 to 20 in 0.02 M borate buffer containing 0.1% gel (pH 8.4) and assayed in quadruplicate. LHRH antiserum (R-42 pool) was generously supplied by Dr. G.D. Niswender (Colorado State Univ., Ft. Collins, CO). Synthetic LHRH (Beckman Instruments, Palo Alto, CA) was iodinated, using the lactoperoxidase-glucose method (Tower et al., 1977) and purified on a 0.5 x 30-CM (CM-22, Whatman Inc., Clifton, NJ) column. The specific activity of the tracer was 1800 uCi/ug. Tracer-antibody binding was inhibited in a dose-related manner with synthetic LHRH (Beckman). The minimum detectable dose was 1.0 pg/tube, and 50% inhibition of tracer binding was achieved with 16 pg/tube.

Unknowns were parallel to the standard curve. Analysis of variance and Student-Newman-Keuls' test for multiple comparisons between groups were used to analyze the data. The results were considered significant if $p<0.05$.

C. Results

The results of the first experiment are shown in Figure 10. Castration resulted in a rapid rise in serum LH. Multiple injections of MOR attenuated the increase, whereas NAL administration increased serum LH concentrations as compared to castrated rats injected with saline.

The effects of NAL, MOR, and β-END on the post-castration rise of LH are shown in Table 8. Serum LH concentration was significantly elevated 24 hrs after castration. Acute administration of NAL further elevated LH levels, but this difference was not statistically significant. Intraventricular injection of MOR and β-END each significantly reduced serum LH concentrations as compared to saline injected castrate controls.

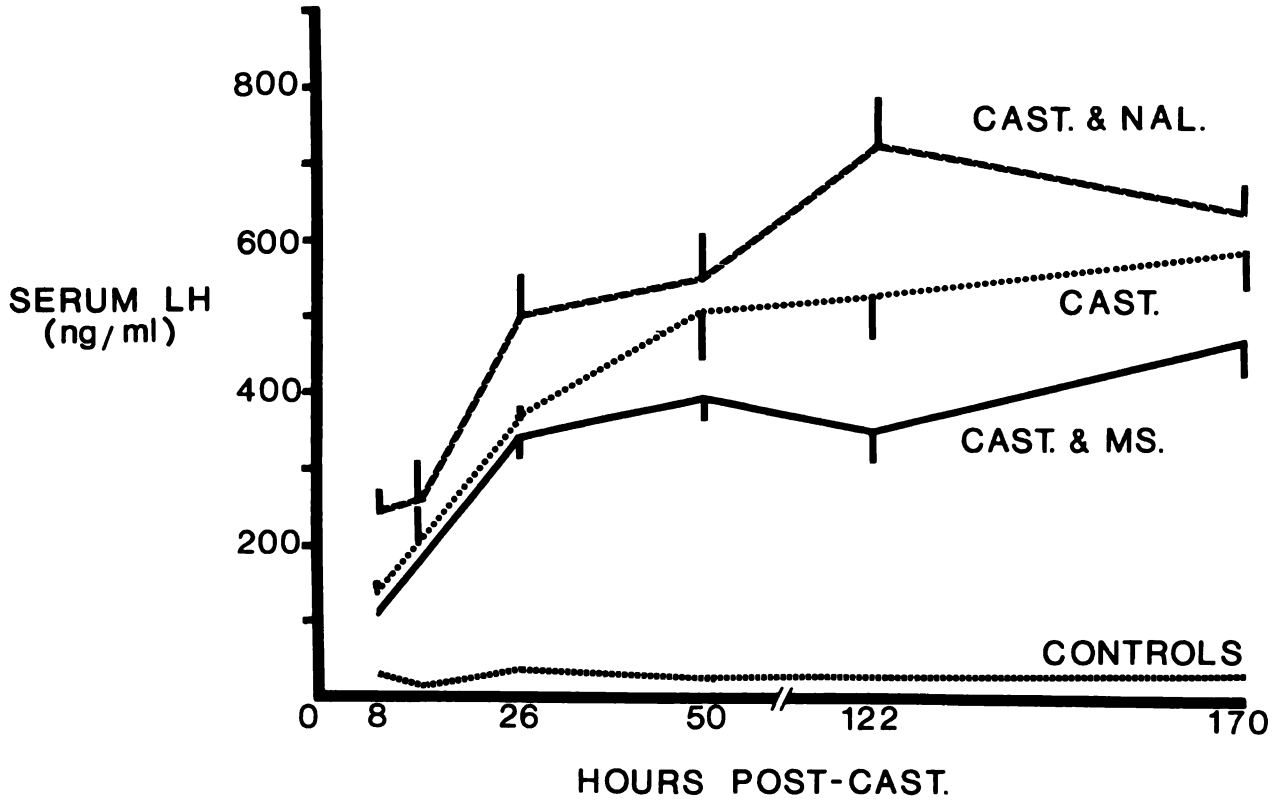

FIGURE 10.

The Effects of morphine sulfate (MS) and NAL on the post-castration rise of serum LH. Drugs and saline were injected at 6 hour intervals. Blood samples were collected by orbital sinus puncture at the indicated times prior to the next injection.

The effects of chronic MOR or TP administration on serum LH concentrations of castrated male rats are shown in Figure 11. Serum LH levels were elevated in castrated rats by day 2, and continued to rise through the tenth day. TP completely blocked the post-

castration rise of serum LH, and serum LH concentrations were actually lower than in intact controls. Similarly, MOR significantly reduced the post-castration rise of serum LH, although inhibition of LH release was not as complete as in the TP treated group.

Table 8. Effects of Naloxone, Morphine, and β-Endorphin on the Post-Castration Rise of Serum LH

Treatment (n = 6)	Serum LH (ng/mg)
Intact	26 ± 2[a]
Castrated + saline	669 ± 66
Castrated + NAL	836 ± 127
Castrated + MOR	421 ± 30[b]
Castrated + β-END	381 ± 44[b]

[a] = x ± SEM; [b] $p<0.05$ vs castrated controls.
NAL = naloxone; MOR = morphine; β-END = β-endorphin.
Blood was collected by decapitation 10 min after ivt injection. Injections were given 24 hours after castration.

Figure 12 shows the effects of castration and chronic MOR or TP administration on anterior hypothalamic-preoptic area and mid-hypothalamic LHRH concentrations. Neither castration, nor chronic MOR, or TP altered anterior hypothalamic-preoptic LHRH concentration. However, mid-hypothalamic LHRH concentration

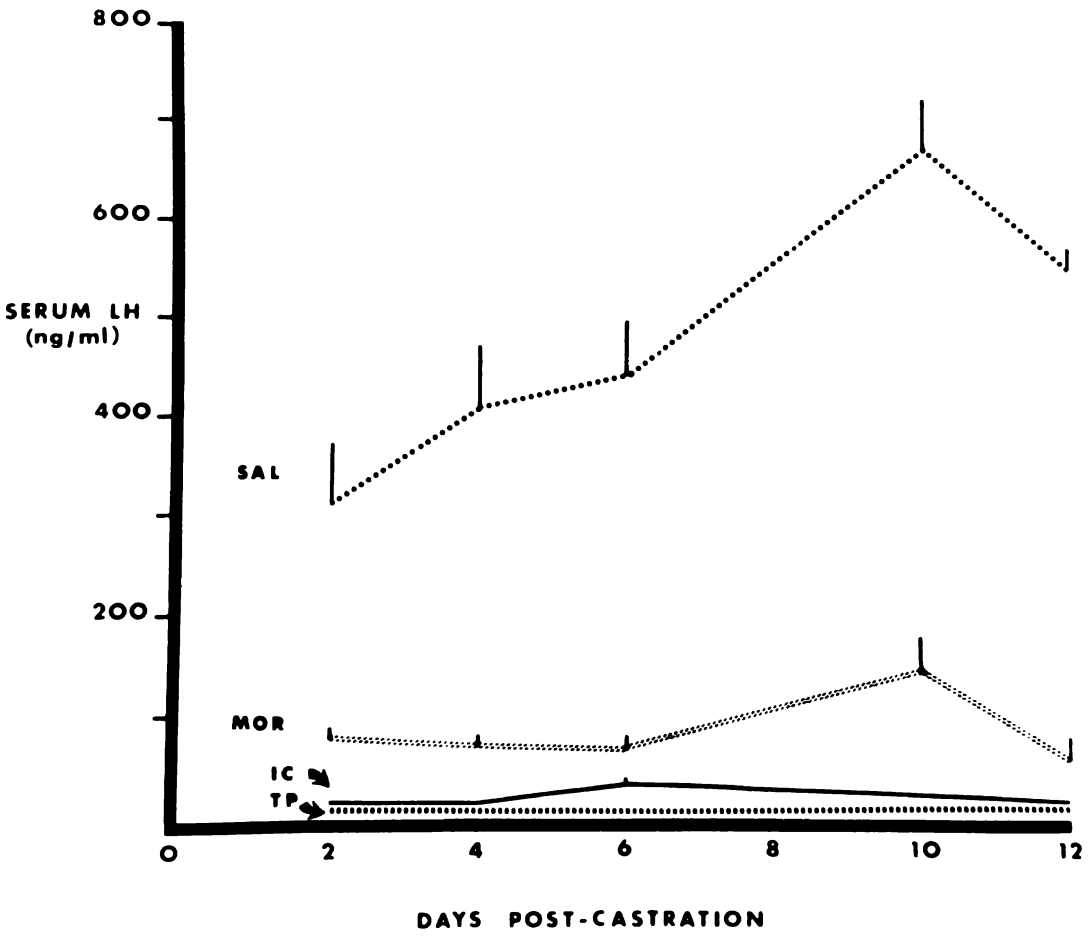

FIGURE 11.

Inhibition of the post-castration rise of serum LH by chronic administration of testosterone or MOR. Male rats were castrated and injected once daily with either 1 mg testosterone propionate (TP), or twice daily with 10 mg morphine sulfate (MOR)/kg BW. A third castrated group (SAL) and a fourth intact control (IC) were injected with appropriate volumes of vehicle. Blood was collected at 2 day intervals 1 hour after the 1700 hour injection (see Materials and Methods for more details).

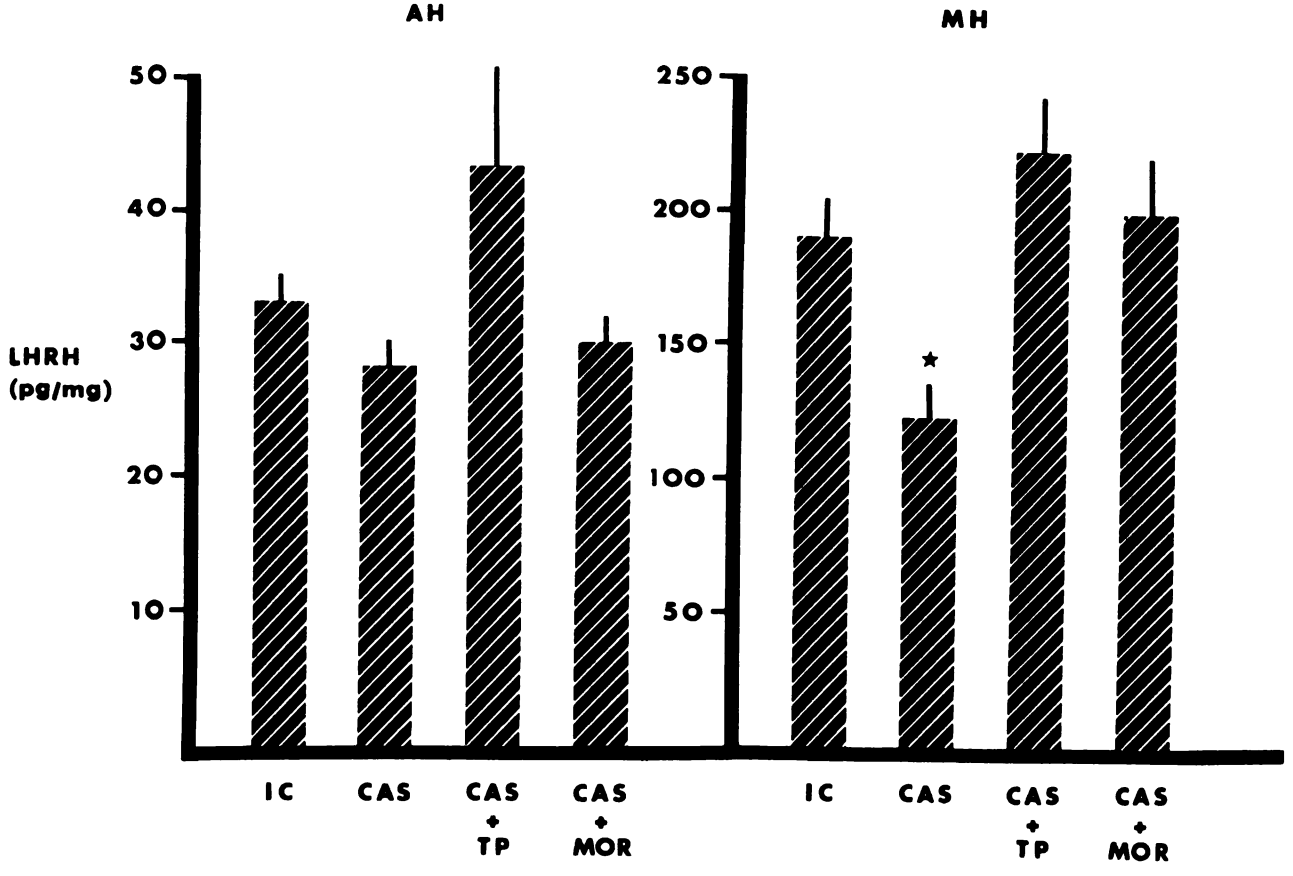

FIGURE 12.

Inhibition of the castration-induced reduction of hypothalamic LHRH concentration by testosterone propionate (TP) or morphine sulphate (MOR) administration (see Materials and Methods and Legend to Figure 11 for more details). Rats were decapitated on day 12 and LHRH concentrations in the anterior hypothalamic-preoptic area (AH) and mid-hypothalamic portion (MH) were measured. * $p<0.05$ vs all other groups.

was reduced by approximately 35% 12 days after castration, as compared to intact controls. Chronic administration of either TP or MOR completely blocked this castration-induced reduction of mid-hypothalamic LHRH.

D. Discussion

Acute administration of either MOR or β-END significantly reduced serum LH levels in castrated male rats, whereas NAL further increased serum LH levels. MOR most effectively reduced the post-castration rise of LH when administered chronically. Although MOR did not reduce serum LH levels to the same extent as TP, this may be due to the inability of multiple MOR injections to produce sustained MOR levels in the blood. Thus, in Experiment 3 in which blood samples were taken 1 hour after TP or MOR injection, inhibition of the post-castration rise of LH was more complete than in Experiment 1 in which blood samples were collected 6 hours after drug injection.

Cicero et al. (1980b) reported that pellet implantation of MOR effectively blocked the initial post-castration rise of serum LH (days 1-5), but saw increased serum LH levels above castrate control levels during the period that followed. We did not observe any stimulatory action of MOR on LH release, since twice

daily MOR injections effectively reduced the post-castration rise of serum LH throughout the 12 days of administration. The explanation for this difference is presently unknown.

In Experiment 3, mid-hypothalamic LHRH concentration was significantly reduced by castration, whereas chronic MOR or TP administration completely prevented this decrease in LHRH (Figure 12). Castration was reported to increase LHRH levels in the portal blood (Sarkar and Fink). Therefore, the castration induced reduction of mid-hypothalamic LHRH concentration is probably a result of increased release, and is prevented by either MOR or TP.

Unlike mid-hypothalamic LHRH concentration, LHRH concentrations in the anterior preoptic hypothalamic area were not altered after castration, in agreement with another report (Araki et al., 1975). In the present experiment, neither MOR nor TP administration produced any significant change in this area. Thus, the major effects of the opiates and testosterone on LHRH release appear to be exerted on the mid-hypothalamic area.

There are several reports which substantiate our conclusion that opiates inhibit LHRH release. Cicero et al. (1980) also reported that chronic MOR administration

blocked the castration induced reduction of hypothalamic LHRH. Rotsztejn *et al*. (1978) reported that MET-ENK blocked DA stimulated release of LHRH from the MBH *in vitro*. Similarly, several opiates were reported to block K^+ induced LHRH release from MBH *in vitro* (Drouva *et al*., 1981). Wilkes and Yen (1981) reported that addition of NAL to superfused MBH increased the efflux of LHRH into the medium. Addition of β-END had no effect on basal release of LHRH, but did block NAL-induced release of LHRH in a dose related manner. The ability of NAL to stimulte LHRH release from the MBH *in vitro* is consistent with its stimulatory action on LH release *in vivo*.

Using the male castrated rat as a model, we have shown that opiates, like testosterone, are able to inhibit the post-castration rise of serum LH. Moreover, chronic MOR administration was as effective as TP in blocking the release of LHRH in castrated male rats. As will be discussed in the following section, the similar inhibitory actions of MOR and testosterone on LH and LHRH release may in fact be due to an interaction between gonadal steroids and hypothalamic opioid peptides.

VI. Evidence That Brain Opiates Mediate Gonadal Steroid Inhibition of Luteinizing Hormone Release

A. Objectives

The stimulatory action of NAL on LH release in male and female mammals suggested to us and others that LH secretion is tonically inhibited by EOP. It is well known that androgens tonically inhibit LH secretion in the male, and ovarian steroids tonically inhibit LH secretion in the female, except immediately prior to ovulation at which time ovarian steroids exert a positive feedback action on the hypothalmo-pituitary-LH axis. Confronted by the observation that both EOP and gonadal steroids tonically inhibit LH secretion, it occurred to us that hypothalamic opioid peptides may mediate the negative feedback action of gonadal steroids.

To test this hypothesis, we determined the effects of NAL on the inhibitory action of gonadal steroids on LH release in castrated male and female rats. Presumably, if gonadal steroids inhibit LH release by activating opiate neurons, NAL should block this action of gonadal steroids.

B. Materials and Methods

In Experiment I, Sprague-Dawley female rats (Harlan Ind. Cumberland, IN), weighing 300-325 g each, were ovariectomized and 4 weeks later were divided into 3 groups. One group (n = 20) was injected sc with 20 μg estradiol benzoate (EB, Sigma Chemical Co., St. Louis, MO). A second group (n = 20) was injected sc with 2 mg TP (Sigma Chemical Co.). The third group (n = 10) was injected sc with an equivalent volume (0.2 ml) of corn oil, the vehicle for EB and TP. These injections were repeated 24 hours later. Three hours after the second injection, half of the rats in the EB or TP treated groups were injected sc with 2 mg NAL (Endo Labs, Garden City, NY)/kg BW. The remaining rats, including the oil treated ovariectomized controls, were injected sc with saline, the vehicle for NAL. One ml blood samples were collected via orbital sinus puncture under light ether anesthesia 20 and 40 min after the injection of NAL or saline.

In Experiment II, Long-term ovariectomized rats were injected sc with 2.5 mg progesterone (P, Sigma Chemical Co.), together with 1 or 10 μg EB. A third ovariectomized group (n = 10) was injected sc with 0.2 ml oil. These injections were repeated 24 hours later. Half of the rats (n = 10) in the 2 EB+P treated groups

were injected sc with 2 mg NAL/kg BW 3 hours after the second EB+P injection. The remaining rats were injected sc with saline. One ml blood samples were collected via orbital sinus puncture immediately prior to the NAL or saline injections and 20 min later.

In Experiment III, Male Sprague-Dawley rats weighing 225-250 g were castrated and a silastic cannula was inserted into the right atrium via the jugular vein. Rats were housed in individual cages during the 2 day recovery period and during the experiment. Rats were divided into 3 groups (n = 10), and a 1 ml blood sample was withdrawn via the atrial cannula immediately prior to treatment. One group was injected iv with 2 mg TP/kg BW. A second group received an iv injection of 2 mg TP/kg BW together with 2 mg NAL/kg BW. The third group was injected iv with an equivalent volume of vehicle. TP and NAL were initially dissolved in alcohol and further diluted in saline to a final alcohol-saline ratio of 1:50. The NAL and vehicle injections were repeated 1 and 2 hours after the initial injection. TP was administered only once. One ml blood samples were taken 60 min after the initial injection and at 3 consecutive 30 min intervals thereafter. When sampling and injection times coincided with each other, blood samples were collected immediately prior to

injection. One ml of saline was replaced immediately after each bleeding so as to minimize extracellular fluid changes.

Blood was allowed to clot overnight at 4°C, and serum was separated after centrifugation. Serum LH concentrations were assayed in triplicate by a double antibody RIA technique (Niswender et al., 1968) using reagents provided by NIAMDD. Serum LH values were expressed as ng/ml in terms of NIAMDD-RP-1. Statistical significance between group means was determined by one-way analysis of variance and Student-Newman-Keuls' test for multiple comparison. The level of significance chosen was $p<0.05$.

C. Results

The effects of NAL on inhibition of LH release by either EB or TP in ovariectomized rats are shown in Figure 13. Injection of either EB or TP reduced serum LH concentrations to levels approximately 50 and 30%, respectively, of LH concentrations of ovariectomized controls. A single injection of NAL to EB or TP treated rats returned serum LH values to approximately those in non-steroid treated ovariectomized controls. The effect of NAL was apparent 20 min after injection, and was still seen by 40 min after treatment.

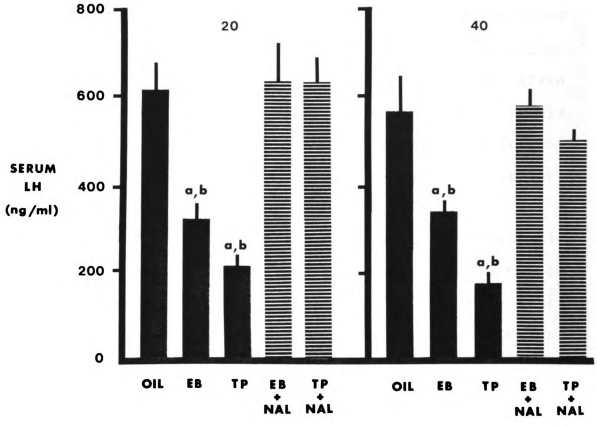

FIGURE 13.

Effects of NAL on EB or TP inhibition of LH release in ovariectomized rats. EB or TP were injected twice at 24 hour intervals. NAL was injected 3 hours after the second steroid injection. Blood samples were collected 20 and 40 min after injection of NAL. Doses were EB = 20 μg; TP = 2 mg; NAL = 2 mg/kg. [a] $p<0.05$ vs ovariectomized controls; [b] $p<0.05$ vs EB + NAL or TP + NAL.

The effects of EB + P alone, or EB + P and NAL on serum LH concentrations in ovariectomized rats are shown in Figure 14. Serum LH levels were decreased by injection of EB + P. The higher dose of EB (10 ug) given with P was more effective in decreasing serum LH levels than the lower dose of EB (1 ug). A single injection of NAL partially, but significantly reversed the inhibitory effect of EB + P administration.

The effect of NAL on TP inhibition of LH release in castrated male rats is shown in Figure 15. Serum LH levels in castrated controls were elevated by 48 hours after castration and remained at this level during the 150 min sampling period. (The range of LH levels of intact male rats measured in our laboratory is 15-30 ng/ml). A single injection of TP reduced serum LH levels. This reduction was first evident by 90 min after injection. Multiple injections of NAL completely blocked the inhibitory action of TP, and actually increased serum LH above concentrations exhibited by castrated controls.

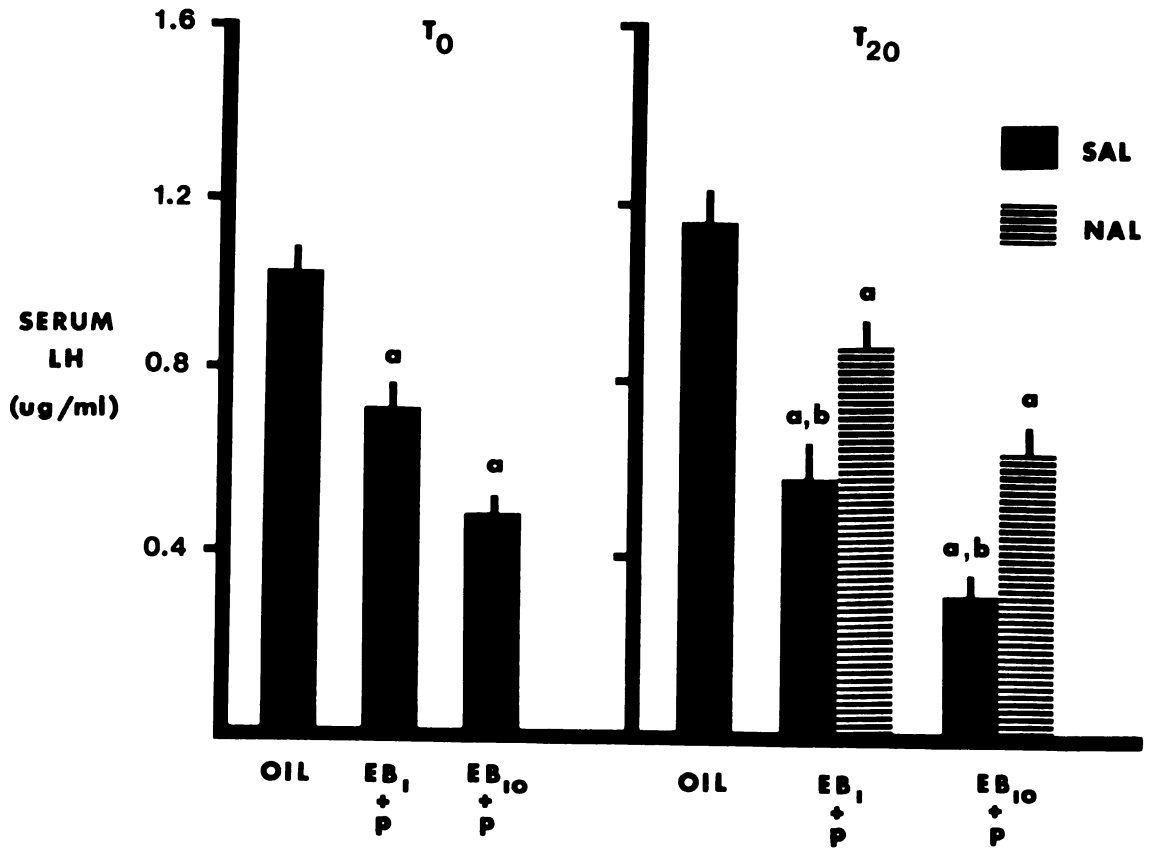

FIGURE 14.

Effect of NAL on the combined inhibitory action of estradiol benzoate (EB) and progesterone (P) on LH release in ovariectomized rats. EB and P were injected twice at 24 hour intervals. NAL was injected 3 hours after the second steroid injection. Blood samples were collected immediately prior to and 20 min after NAL injection. Doses were EB = 1 or 10 ug; P = 2.5 mg; NAL = 2 mg/kg. [a] $p<0.05$ vs ovariectomized controls; [b] $p<0.05$ vs EB + P + NAL.

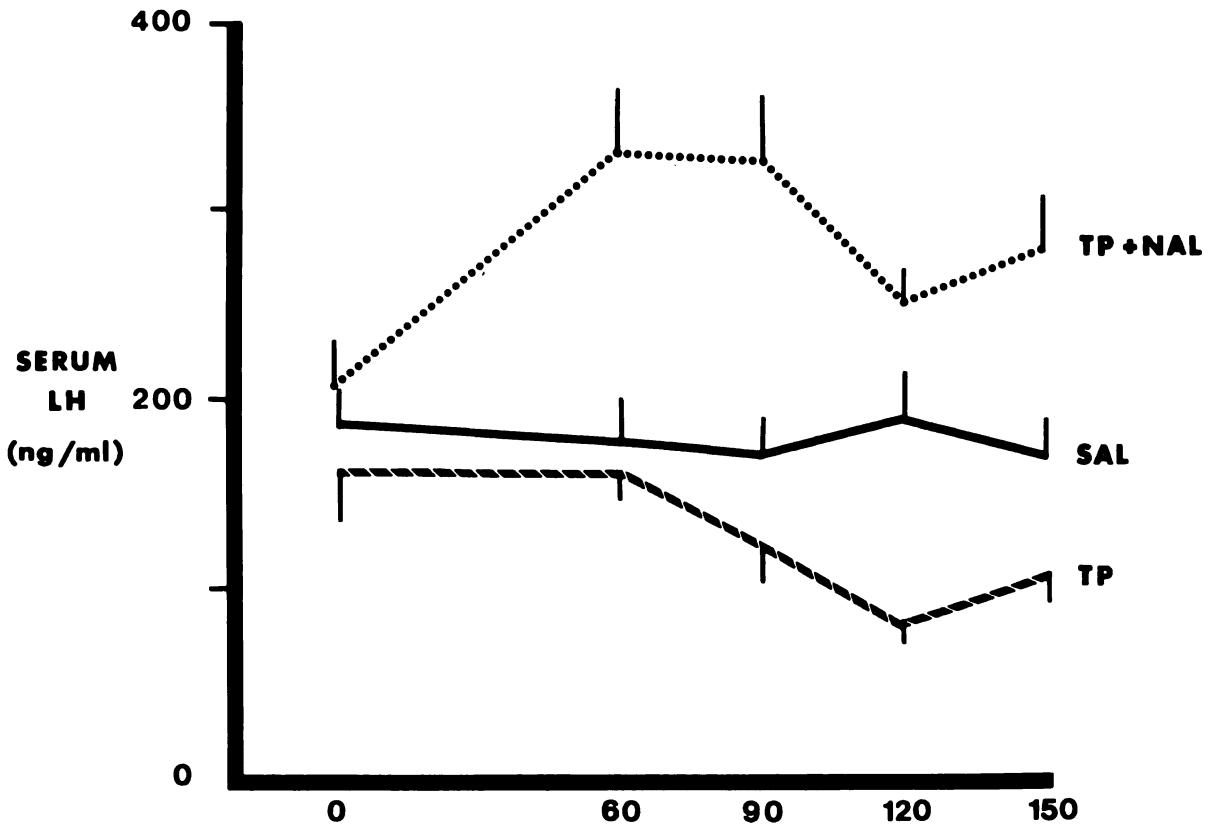

FIGURE 15.

Effect of NAL on testosterone propionate (TP) inhibition of LH release in acutely castrated male rats. TP was injected alone or together with NAL at time 0. NAL was injected 60 and 120 min later. Doses were TP = 2 mg/kg; NAL = 2 mg/kg. n = 10.

D. Discussion

The present results show that NAL can block the inhibitory effect of TP on LH release in castrate male rats and completely or partially reverse inhibition of LH release by estrogen or estrogen in combination with progesterone in castrate female rats. These results indicate that EOP mediate, at least in part, gonadal

steroid inhibition of LH secretion in male and female rats.

NAL-reversal of testosterone inhibition of LH secretion in male castrate rats was first reported by Cicero *et al*. (1979). Moreover, they reported that NAL was unable to reverse the inhibitory effect of dihydrotestosterone on LH release from pituitary cells *in vitro*, suggesting that NAL antagonizes the action of testosterone at the hypothalamic level.

The ability of NAL to block the inhibitory action of gonadal steroid on LH release may be explained by 2 different hypothalamic mechanisms. 1) The inhibitory action of gonadal steroids on LHRH release is mediated by hypothalamic opioid peptides. 2) Alternatively, EOP and gonadal steroids may each inhibit LH release by 2 separate inhibitory inputs to the same hypothalamic site. Either explanation could account for the counteraction by NAL of gonadal steroid inhibition of LH release.

Additional evidence for the first hypothesis above, namely that the endogenous opiates help mediate the negative feedback of gonadal steroids, is that ^{3}H-naltrexone binding to opiate receptors in the brain of male rats was signifiantly increased after castration, and this effect was reversed by TP replacement (Hahn and

Fishman, 1979). It was suggested that this effect of castration was due to an increase in availability of unoccupied opiate receptors, resulting from a decrease in concentration of an endogenous opioid ligand. In agreement is the observation that chronic administration of 17β-estradiol increased MET-ENK concentrations in the lateral and medial preoptic nuclei, areas which contain LHRH cell bodies (Savard et al., 1980). Furthermore, it was reported in a recent abstract that ovariectomy reduced the concentration of β-END in portal blood, whereas estrogen and P replacement increased β-END concentration (Wehrenberg et al., 1981). It was suggested from these results that ovarian steroids stimulate β-END neuronal activity in the hypothalamus, and that increased concentrations of β-END in the portal blood are a reflection of increased activity of hypothalamic β-END neurons.

We have not determined precisely the mechanism by which EOP mediate the negative feedback of gonadal steroids. As suggested in the previous chapter, opiates inhibit LH release by inhibiting LHRH release into the portal blood. However, we cannot say for sure that opiates act directly on LHRH neurons. There is circumstantial evidence which suggests otherwise. Castration has been reported to increase NE turnover in the

hypothalamus (Anton-Tay and Wurtman, 1968; Donoso et al., 1969), whereas estrogen or androgen replacement decreased hypothalamic NE turnover (Bapna et al., 1971). Opiates also may decrease hypothalamic NE activity (de Wied et al., 1974; Korf et al., 1974; Simpkins and Kalra, 1980; Van Vugt et al., 1981). Since NE is stimulatory to LH release (Weiner et al., 1972; Krieg and Sawyer, 1976; Müller et al., 1977; Weiner and Ganong, 1978), it is tempting to speculate that opiate mediation of gonadal steroid inhibition of LH release is in turn mediated by a decrease in hypothalamic NE activity. This decrease in hypothalamic NE activity may be responsible for reduced LHRH release.

General Discussion

The data presented in this thesis indicate that EOP are important neuromodulators of PRL and LH secretion. Serum PRL concentrations were significantly increased by acute administration of MOR, β-END, dynorphin, MET-ENK, and the enkephalin analog, DAMME. Concurrent administration of the opiate antagonist, NAL, blocked the stimulatory action of these opiates on PRL release, indicating that the observed effect is opiate specific. Moreover, administration of NAL alone significantly decreased serum PRL concentration. This effect of NAL suggests that there is present a tonic stimulatory input from opioid neurons to the hypothalamic components controlling PRL release, and thus, EOP appear to be involved in determining the secretion rate of PRL during basal conditions.

There also is evidence that EOP are involved in the regulating of PRL release during non-basal conditions. Stress-induced PRL release was completely blocked by NAL (Experiment II; Grandison and Guidotti, 1977; Dupont *et al.*, 1979). In addition, stress has been shown to produce analgesia and elevated opiate activity in the brain (Akil *et al.*, 1976; Madden *et al.*, 1977; Fried and

Singer, 1979; Wesche and Frederickson, 1979). Brain opioid peptides also have been implicated in the release of PRL during suckling (Dupont et al., 1979; Miki and Meites, unpublished observations), in the nocturnal rise of PRL (Dupont et al., 1979), and in the surge of PRL that occurs on the afternoon of proestrus (Ieiri et al., 1980). NAL was shown to block the release of PRL during each of these different states. Changes in hypothalamic and pituitary MET-ENK concentrations were reported to occur on the morning of proestrus and may be involved in producing the proestrous afternoon PRL surge (Kumar et al., 1979).

Opioid stimulation of PRL release is not accomplished by a direct action on the AP. Incubation of opiates or opiate antagonists with hemipituitaries or pituitary cell cultures does not elicit the response observed in vivo (Grandison and Guidotti, 1977; Rivier et al., 1977; Shaar et al., 1977). Furthermore, MOR-methyliodide, which does not cross the blood brain barrier, increased serum PRL levels when given ivt, but did not when injected systemically. NAL-bromide, which also does not cross the blood brain barrier, was unable to block the stimultaory action of ivt MOR-methyliodide when given systemically but did when injected ivt (Panerai et al., 1981). Lastly, β-END, which stimulates

PRL release in the intact monkey, failed to do so in the stalk sectioned monkey (Wardlaw et al., 1980).

The logical alternative to a direct action on the AP is that opiates stimulate PRL release via hypothalamic mechanisms. In agreement with this conclusion is the report that β-END and MOR stimulated PRL release in a NAL reversible manner in rats with a deafferentated hypothalamus (Grandison and Guidotti, 1977).

The results of Experiment III of this thesis indicate that EOP stimulate PRL release by reducing tuberoinfundibular DA turnover. Dopaminergic agonists were shown to block MOR stimulation of PRL release, whereas HAL, a DA antagonist, produced a synergistic effect on PRL release when given together with MOR. Tuberoinfundibular DA turnover was dramatically decreased by ivt injection of β-END and serum PRL concentration was significantly increased. Other laboratories have shown that opiates reduced ME DA turnover (Ferland et al., 1977; Deyo et al., 1979; Van Loon and Kim, 1980), and Gudelsky and Porter (1979) reported that administration of MOR, β-END, or an enkephalin analog each significantly reduced the concentration of DA in pituitary stalk plasma. Together, these results are convincing evidence that opiate stimulation of PRL release is mediated via a reduction of tuberoinfundibular DA

activity.

Opiate stimulation of PRL release also may be mediated by an increase in hypothalamic 5-HT activity. β-END was reported to increase hypothalamic 5-HT turnover (Van Loon and DeSouza, 1978), and an increase in 5-HT activity could account for the increased serum PRL concentration produced by β-END . However, neither we (Experiment III) nor Cusan et al. (1977) observed any effect of 5-HT synthesis blockade by PCPA on opiate stimulation of PRL release. The reason for this lack of effect is not apparent, but PCPA inhibition of 5-HT synthesis may be only partial and supersensitivity of the remaining 5-HT neurons may have occurred.

Spampinato et al. (1979) and Koenig et al. (1979) reported that metergoline and methysergide, both 5-HT antagonists, or 5,6-dihydroxytryptamine, a 5-HT neurotoxin, blocked the stimulatory effect of MET-ENK on PRL release. These results are in agreement with the hypothesis that opiates stimulate PRL release by increasing 5-HT activity. Therefore, in addition to decreasing DA turnover, opiates may increase 5-HT turnover. Both mechanisms offer plausible explainations of how opiates increase PRL release.

As suggested by Demarest and Moore (1981), the opposite effects of opiates on hypothalamic DA and 5-HT

activity may in fact be related. They reported that disruption of 5-HT neuronal activity by injection of either metergoline or 5,7-dihydroxytryptamine blocked the inhibitory effect of MOR on DA turnover in the ME. Since 5-HT does not stimulate PRL secretion by a direct action on the AP, this proposed interaction could explain how increased hypothalamic 5-HT activity produced by opiates stimulates PRL release.

LH secretion also may be under the tonic influence of EOP. But unlike PRL secretion, LH secretion appears to be tonically inhibited by EOP. Administration of opiates decreases LH levels in a NAL reversible manner, whereas NAL alone stimulates LH release (Bruni et al., 1977; Blank et al., 1979; Cicero et al., 1979; Ieiri et al., 1979).

The mechanism involved in NAL stimulation of LH release may involve hypothalamic NE. As shown in Experiment IV, the stimulatory action of NAL was blocked by pretreatment with α-mpt, DDC, or phenoxybenzamine, all of which disrupt noradrenergic activity. These results suggest that hypothalamic noradrenergic neurons are tonically inhibited by EOP. This inhibition of noradrenergic neurons may explain why further inhibition by α-mpt or DDC did not decrease basal serum LH levels. However, these antinoradrenergic drugs did inhibit the

stimulatory action of NAL on LH release presumably because NE activity was increased by NAL.

Since NE does not act directly on the AP to influence LH release, an additional hypothalamic factor presumably LHRH, must be evoked to explain opiate inhibition and NAL stimulation of LH release. We used the castrated male rat as a model to study LHRH release from the hypothalamus. Since LHRH concentration is inversely correlated to the LHRH release during states of increased LH release, this model allows us to make a valid interpretation about LHRH release from LHRH concentration data.

Mid-hpyothalamic LHRH concentration was significantly reduced 12 days after castration in vehicle treated rats. Chronic administration of MOR or testosterone blocked the castration-induced reduction of LHRH, suggesting that MOR, as well as testosterone, blocked the release of LHRH into the portal blood. This interpretation of LHRH concentration is suported by the observation that MOR and testosterone both effectively blocked the post-castration rise of serum LH.

Evidence that EOP mediate the negative feedback of gonadal steroids also is included in this thesis (Experiment VI). The similar ability of MOR and testosterone to block the post-castration rise of serum

LH and the castration-induced release of LHRH, and the fact that EOP and gonadal steroids tonically inhibit LH secretion, suggested to us that the inhibitory feedback of gonadal steroids at the hypothalamic level may be mediated by an increase in hypothalamic opioid activity. To test this hypothesis, we determined the effect of NAL on LH levels in ovariectomized rats treated with estrogen and progesterone or orchidectomized rats treated with testosterone. The inhibitory effect of these gonadal steroids on serum LH was blocked by NAL, suggesting that gonadal steroids inhibit LH secretion by activating hypothalamic opioid neurons.

Based on the results of Experiments IV-VI the following working hypothesis is proposed. Gonadal steroids feed back on hypothalamic opioid neurons and increase their activity. Increased hypothalamic opioid activity results in a decrease in hypothalamic noradrenergic activity. This reduction of noradrenergic activity reduces the release of LHRH into the portal blood which results in a decrease in LH release from the anterior pituitary.

Since the discovery of the EOP in 1975 and 1976, many different physiological roles for the EOP have been proposed. Much of the evidence for the proposed functions of EOP, including their endocrine functions

published in this thesis, is based on the criterion that the opiate antagonist NAL is a specific antagonist with relatively few nonspecific actions.

We believe that the use of NAL in the experiments described in this thesis, both our own and those from other laboratories, is a valid first approach to determining if EOP are involved in a particular neuroendocrine process. Our reasons for this belief are several. There is no doubt that NAL competes with opioid ligands for opiate receptors. This has been conclusively shown from binding studies in the CNS and periphery. There is little doubt that antagonism by NAL of opiate stimulation of PRL release or inhibition of LH release also is due to NAL competing with opiates for specific opiate receptors. The effects of opiates and NAL on PRL and LH release are opposite. This also strongly indicates that these effects are specific. Intraventricular injection of ug quantities of opiates or opiate antagonists produces the same effect on PRL and LH release as systemic injection of mg quantities. This certainly eliminates the possibility that altered hormone levels are the result of a nonspecific peripheral action, perhaps at the lung, liver, or kidney.

Antagonism of EOP by NAL does not discriminate between the different opioid ligands, rather NAL

inhibits the binding of all opioid ligands to their receptors. This property of NAL can be viewed as both desirable and undesirable. It is desirable because it allows one to determine if an EOP is involved in a particular process. However, after making that determination, one is unable to conclude which particular opioid peptide is involved. Therefore, while NAL is useful as a first step approach to a problem, more refined experimental approaches must be incorported in order to extend our understanding of EOP function.

One such improvement is the use of passive immunization. Injection of antiserum to a particular opioid peptide, and observing the response makes it possible to conclude which EOP is involved. Passive immunization of rats with β-END antiserum was reported to reduce basal PRL levels and block stress-induced PRL release (Ragavan, 1981). These results suggest that β-END is involved in the control of basal PRL secretion and stress-induced PRL release. They also indicate that the similar effects produced by NAL (Experiments I and II) were indeed due to NAL blockade of opiate receptors. Further use of passive immunization as a probe should clarify the role of EOP.

In addition to using opiate antagonists and passive immunization, several laboratories have used RIA

measurements of EOP in tissues and biological fluids to access opiate neuronal activity. While this procedure may be suitable for determining the release of β-END from the pituitary, its adequacy for measuring opiate activity in the brain is questionable. As with the biogenic amines, a better understanding of opiate synthesis, release, and inactivation should result in the development of new and improved methods for accessing opiate activity.

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APPENDICES

APPENDIX A

COYLE AND HENRY CATECHOLAMINE ASSAY PROCEDURE

1. Homogenize pieces of brain tissue in desired volume of 0.4 N perchloric acid (plus 10 mg % EDTA) using matched glass microhomogenizers (Micrometric Instruments, Cleveland, OH).

2. Transfer homogenate to microcentrifuge tubes (Kew Scientific, Columbus, OH) and centrifuge for 45 sec in a microcentrifuge (Coleman Inst., Oak Brook, IL).

3. Transferr 10 ul of supernatant (or of working NE and DA standard solution) to glass culture tubes and add 25 ul of the following mixture:

Reagent	Proportion
20 mM EGTA-Na salt (0.760 g / 100 ml H_2O and pH to 7.2)	1
Pargyline Solution (to 4mg pargyline add 25 ul β-mercaptoethanol and 225 ul H_2O)	1
1 M Tris base (with 3 mM $MgCl_2$) (to 6.05 g Tris add 50 ml H_2) plus 30.5 mg $MgCl_2$)	6.5
S-adenosyl-l-methionine-(Methyl-^3H) 11.6 Ci/m mole in Sulfuric acid: ethanol solution (90:10, v:v), pH 1-3	3.0
Catecholamine-o-methyl transferase (COMT, partially purified by the method of Nikodijevic *et al.*, 1970)	2.5
1 mM sodium phosphate buffer	2.5

4. Incubate for 40 min at 37°C.

5. Add 30 ul of mixture of 5 volumes 0.45 M borate buffer (pH 10.0) and 1.0 volumes of carrier meth-

oxyamine mix prepared as follows:

add 5.0 ml H_2O to the following salts:
50 mg 3-methoxytyramine, 50 mg DL-metanephrine, 50 mg DL-normetanephrine and 5 mg Na-bisulfite

6. Add 500 ul to toluane: isoamyl alcohol solution (3:2, v:v), vortex for 30 sec and centrifuge for 5 min at 3,000 RPM (RC2-B, Sorvall, Dupont Inst., Newtown, CT).

7. Transfer 400 ul of organic phase to conical centrifuge tubes containing 400 ul borate buffer (pH 10.0), vortex for 30 sec and Centrifuge at 5/7 speed in IEC clinical centrifuge (International Equipment Co., Needham Hts., MS).

8. Transfer 300 ul of organic phase to conical centrifuge tubes containing 500 ul of 0.1 N HCl, vortex for 30 sec and centrifuge as in step 7.

9. Aspirate organic phase.

10. To remaining aqueous phase add 7 ml toluene: isoamyl alcohol (3:2, v:v), vortex, centrifuge as in step 7 and discard organic phase.

11. To remaining aqueous phase neutralize with 500 ul of 0.5 M sodium phosphate buffer (pH 7.5), add 50 ul of 3% sodium metaperiodate; wait 2 min and add 50 ul of 10% glycerol.

12. Add 10 ml toluene, vortex for 30 sec, centrifuge as in step 7.

13. Transfer 9 ml of organic phase to conical centrifuge tubes containing 1 ml of 1 N NaOH for final extraction of NE metabolites. Vortex for 30 sec, centrifuge and discard organic phase. Add 100 ul glacial acetic acid, 10 ml scintiverse (Fisher Scientific, Livonia, MI), and transfer to 20 ml glass scintillation vials for counting.

14. From the remaining aqueous phase of step 12, the residue toluene is aspirated, 500 ul of 1 M borate buffer is added and tubes are vortexed. Add 8 ml of toluene isoamyl alcohol (3:2 v:v), vortex and centrifuge. 0.6 ml of the organic phase is added to 10 ml of scintiverse in 20 ml glass scintillation vials and counted for dopamine.

APPENDIX B

BEN-JONATHEN AND PORTER CATECHOLAMINE ASSAY PROCEDURES

1. Same as step 1 in Appendix A.

2. Same as step 2 in Appendix A.

3. Transfer 10 ul of supernatant (or of working NE and DA standard solutions) to conical centrifuge tubes and add 25 ul of the following mixture:

Reagent	Proportion
20 mM EGTA-Na salt (0.760 g/100ml H_2O and pH to 7.2)	1
Pargyline solution (to 4 mg pargyline add 25 ul β-mercaptoethanol and 225 ul H_2O)	1
1 M Tris base (with 3 mM $MgCl_2$) (to 6.05 g Tris add 50 ml H_2O plus 30.5 mg $MgCl_2$)	6.6
S-adenosyl methionine (Methyl-^3H) (11.6 ci/m mole diluted 1:3:5 with H_2O)	3.0
Catecholamine-o-methyl transferase (COMT; partially purified by the method of Nikodijevic et al., 1970)	5.1

4. Incubate for 60 min at 37°C.

5. Add 30 ul of 0.45 M borate buffer (pH 10.0) and 5 ul of carrier methoxyamine mix (50 mg 3-methoxytyramine, 50 mg DL-metanephrine and 50 mg DL-normetanephrine; 10 mg of each amine/ml of 0.1 N HCl). Add 500 ul of toluene: isoamyl alcohol (3:2, v:v), vortex for 30 sec, and centrifuge at 5/7 speed on IEC clinical centrifuge (International Equipment Co., Needham Hts., MA).

6. Transfer 400 ul of organic phase to conical centrifuge tubes containing 40 ul of 0.1 N HCl. Vortex for 30 sec and centrifuge as in step 5. Carefully aspirate organic phase.

7. Apply 25 ul of acid phase to LQ-60 silica gel plates previously spotted with 5 ul of carrier methoxyamine mix. Allow plates to dry.

8. Place plates in thin-layer chromatography tanks containing chloroform, ethanol and methylamine (40:18:5 by volume). Allow plates to run 1½ to 2 hrs and remove from tank to allow for drying.

9. Visualize and outline spots under ultraviolet light.

10. Scrape plates and place scrapings into scintillation vials containing 1.0 ml of ethlacetate acetic acid and H_2O (3:3:1 by volume) and shake for 30 min. Add ~10 ml of scintiverse and count.

APPENDIX C

Publications

Bruni, J.F., D.A. Van Vugt, S. Marshall, and J. Meites: The effects of naloxone, morphine and methionine enkephalin on serum prolactin, luteinizing hormone, follicle stimulating hormone. Life Sciences 21: 461-466, 1977.

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