I. LUTEOLYTIC ACTION OF PROLACTIN IN THE MOUSE
II. CHOLINERGIC INHIBITION OF PROLACTIN RELEASE

Thesis for the Degree of M. S.
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ABSTRACT

I. LUTEOLYTIC ACTION OF PROLACTIN IN THE MOUSE

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By

Lindsey Grandison

1. The proestrous surge in serum prolactin was blocked with ergocornine for one or three cycles in Swiss Webster mice. Blockade of prolactin release caused a retention of corpora lutea from earlier cycle(s). Concurrent administration of prolactin and ergocornine left the number of corpora lutea unchanged since no differences were noted between this treatment group and controls. These results indicate that prolactin exerts a luteolytic action on old corpora lutea during the estrous cycle of the mouse.

2. The effects of acetylcholine injected into the lateral ventricle of proestrous female rats were observed on serum prolactin. A 50 μg dose of acetylcholine suppressed prolactin release by 15 and 30 minutes after injection. By 60 minutes after injection the inhibitory response, although still present, was not significant. These observations suggest that cholinergic activity in the hypothalamus suppresses release of prolactin.

3. The effect of a systemic injection of pilocarpine, a cholinomimetic drug, was noted on prolactin secretion in female and male rats. In proestrous female rats, pilocarpine at 9 mg/kg suppressed prolactin release by 15 and 30 minutes after injection. At the end of one hour no depression was observed.

Three doses of pilocarpine were injected into three groups of male rats. The lowest dose, 5 mg/kg, suppressed prolactin secretion whereas the higher doses had no effect. Higher doses of this drug may induce stress, a potent stimulator of prolactin release.

4. The effects of several doses of physostigmine, an acetylcholine esterase inhibitor, were noted on prolactin secretion by 30, 60 and 90 minutes after injection. The 0.5 mg/kg dose suppressed prolactin secretion by 90 minutes, but not by 30 or 60 minutes after injection. Stress probably was responsible for inducing release of prolactin by 30 and 60 minutes with this high dose of physostigmine. These findings indicate that potentiation of cholinergic activity by physostigmine results in decreased release of prolactin, and supports the view that a cholinergic system in the hypothalamus helps regulate prolactin secretion.

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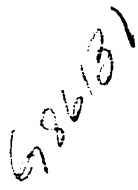

Dedicated

to my

Mother and Father

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INTRODUCTION

Early investigation into the functions of prolactin revealed its involvement in the initiation and maintenance of milk secretion and in the formation and function of the corpora lutea. With the development of a radioimmunoassay (RIA) for prolactin, these basic observations were confirmed. Moreover, the greater sensitivity and specificity of this technique allowed elucidation of the more subtle functions of prolactin. For example, the recent observation of a serum prolactin surge during proestrus in the rat was determined by RIA (Niswender et al., 1969). Thereafter Wuttke et al. (1971) inhibited prolactin secretion with ergocornine and were thus able to show a luteolytic action of prolactin during the estrous cycle of the rat.

Similarly the understanding of hormone regulation has been extended through the use of the RIA. Evidence is accumulating that serotonergic neurons induce the release of prolactin. Thus the tonic inhibition of prolactin release provided by the catecholaminergic neurons is countered by a serotonergic stimulation.

These advances in understanding are refinements of existing concepts. Yet further experimentation is required to substantiate recent advances and to provide details of the mechanisms regulating prolactin secretion. Therefore it was considered appropriate to determine whether prolactin exerted a luteolytic action in a

different species, the mouse. In addition the recent observation of a serotonergic stimulus for prolactin release suggested that other neurotransmitters in the hypothalamus might influence prolactin secretion. Therefore it was of interest to examine the possible effects of the cholinergic system on prolactin secretion.

LITERATURE REVIEW

Luteolysis in Rats and Mice

Regression of Corpora Lutea During the Estrous Cycle

Throughout the reproductive lifespan of laboratory animals such as the rat and the mouse, spontaneous ovulation is an event in anticipation of pregnancy. At each ovulation a set of processes required for pregnancy is begun. However, if mating does not occur, structures such as corpora lutea cease functioning prematurely. During the estrous cycle of a rat, corpora lutea (CL) develop shortly after ovulation. In early diestrus they attain their maximal size (Boling, 1942). Progesterone secretion is maximal on day two of the cycle. Thereafter secretion of progesterone declines and the less active metabolite, 20-α-hydroxypre-4en-3-one (20-OH-P), is secreted (Hashimoto and Weist, 1969). However, the size of the CL is maintained until metestrus of the next cycle (Deane, 1952). Between the second metestrus after formation and the following day, diestrus, a rapid regression occurs. The remaining structure persists for 8 to 9 days since at least three generations of CL are normally observed in rats (Long and Evans, 1922) and mice (Allen, 1922). Although not as well investigated, the phenomena in the mouse are similar. However, maximal CL size is not reached until estrus of the second cycle. As in the rat, CL regress with age. Fat accumulation disappears after the

second metestrus in contrast to the rat where fat accumulation is an indication of aged CL (Greenwald and Rothchild, 1968).

From the above description luteolysis or regression of the CL can be seen to include two phases. Malven (1969) has distinguished between functional luteolysis or cessation of progesterone secretion and structural luteolysis or morphorological regression. This distinction is made since these events occur at different times. During the estrous cycle, pseudopregnancy, pregnancy, and lactation, secretory function ends before structural regression. However, CL can remain intact for a prolonged time even though secretion has ended. Such a condition is encountered in the hypophysectomized rat. The possibility exists that different factors are involved in structural and functional luteolysis.

Regression of Corpora Lutea After Hypophysectomy

The hypophysectomized animal has been used in determining the anterior pituitary hormones responsible for a number of functions. Early research demonstrated that prolactin administration immediately after hypophysectomy was capable of sustaining progesterone secretion from CL in the rat (Astwood, 1941) and the mouse (Robson, 1971). Without exogenous prolactin, the CL of hypophysectomized rats ceased secreting progesterone within 24 hours. However, in the rat the CL structure remains for up to 9 months (Smith, 1930) when hypophysectomy is done during estrus or diestrus. On the other hand, if hypophysectomy is done during proestrus, pseudopregnancy, or if a grafted pituitary is removed from a hypophysectomized rat with a functioning

set of CL, the CL regress immediately (Greenwald and Rothchild, 1968). In the mouse connective tissue invasion occurs 12 days after hypophysectomy.

The persisting CL of hypophysectomy offer an excellent model for elucidating the pituitary factors involved in structural luteolysis. Malven and Sawyer (1966) postponed prolactin injections for 80 hours and found that prolactin caused regression of CL in such hypophysectomized rats. Also, Piacsek and Meites (1967) found pituitary transplants after hypophysectomy caused luteolysis. LH was not luteolytic under these conditions and LH was unable to synergize with moderate doses of prolactin to induce luteolysis (Malven, 1969). Although a luteolytic activity of prolactin had been clearly demonstrated, its physiological importance remained undetermined. However, the early observations of Long and Evans (1922) implicated a physiological condition during which prolactin was luteolytic. In lactating rats two generations of CL are present, one resulting from pregnancy and another resulting from the parturition-induced ovulation. Normally the CL of pregnancy regress. If the pups are removed and lactation is prevented, the CL of pregnancy regress much more slowly. Presumably the suckling induced prolactin surges cause the regression of the non-functional CL of pregnancy.

After it became well documented that prolactin rose during proestrus (Amenomori et al., 1970; Gay et al., 1970) the physiological significance of this surge was questioned. As a consequence of the luteolytic action of prolactin in the hypophysectomized rat, it was considered possible that prolactin might be involved in luteolysis

during the estrous cycle. Before such an investigation could be undertaken, a means of selectively inhibiting prolactin was needed. With the determination that ergot alkaloids were effective at low doses in preventing the release of prolactin (Nagasawa and Meites, 1970), a pharmacological tool was provided.

Ergocornine: Effects on Prolactin Secretion

Ergocornine and other ergot alkaloids are produced by a fungus which grows on rye and other grains. The ergot alkaloids were the first alpha-adrenergic blockers discovered, but they also have a number of other effects. In the early part of this century, they were used as oxytocics until their untoward side effects were determined. This action on the uterus is an example of the direct stimulation ergots have on smooth muscle, and it is the strongest peripheral side effect. Its activity as a weak agonist (and therefore an occupant of adrenergic receptors, and hence, blocking agent) is suggested to partially explain smooth muscle stimulation. It is also an antagonist of serotonin. The central effects cause medullary depression as evidenced by decreased vasomotor and respiratory activity, and cause inhibition of hypothalamic temperature regulation. However, the central action is not believed to be due to any catecholaminergic effect. Current therapy utilizes ergot alkaloids in treatment of migraine headaches. Its vasoconstrictive activity is responsible for relief of this condition.

In addition a number of endocrine effects have been observed. Ergotoxine, a mixture of ergot alkaloids, inhibited deciduoma formation

(Shelesnyak, 1954) and caused termination of pseudopregnancy and pregnancy when administered early in these stages (Shelesnyak, 1955). Injections of prolactin together with ergotoxine were able to overcome the effects of ergotoxine on pregnancy. During lactation in the rat, ergocornine inhibited deciduoma formation and milk production (Zeilmaker and Carlsen, 1962). These effects were accompanied by morphological changes in the CL of these animals. These observations imply that ergocornine disrupts prolactin secretion since pseudopregnancy, pregnancy, lactation and CL function in the rat are dependent on prolactin. Direct evidence for ergocornine inhibition of prolactin secretion was provided by Nagasawa and Meites (1970). Ergocornine was injected for 15 days into rats bearing DMBA induced mammary tumors. The rats injected with ergocornine had lower serum and pituitary prolactin than rats injected with the control vehicle. A single systemic injection has been shown to inhibit the proestrous rise of prolactin in the serum (Wuttke et al., 1971). The site of action is in part on the median eminence of the hypothalamus since implants there were effective in increasing PIF and reducing serum prolactin levels (Wuttke et al., 1971). A direct inhibitory effect on the pituitary also was observed in vitro (Lu et al., 1971).

Luteolytic Action of Prolactin During the Estrous Cycle of the Rat

The use of ergot alkaloids provided the first evidence that prolactin has a luteolytic role during the estrous cycle of the rat (Wuttke and Meites, 1971; Billeter and Fluckiger, 1971). When the proestrous rise in prolactin is inhibited by ergocornine, the ovaries

retain the old CL and increase in weight as a result. Prolactin injections together with the ergot alkaloids prevent CL accumulation and the gain in ovarian weight. These observations are strong evidence that prolactin during the estrous cycle has the ability to cause regression of the old non-functional CL in the rat.

Luteolysis in the Mouse

Considerably less is known about luteolysis in the mouse than in the rat, and observations in the rat do not always apply to the mouse. For example, the CL of rats are considered to be moderately affected by uterine influences. Hysterectomy, ligation or cutting of the oviduct and utero-ovarian vessels extend pseudopregnancy (Hilliard, 1973). Evidence is accumulating to implicate prostaglandins as the uterine agents responsible for luteolysis of active CL under a number of circumstances in rats and other species (Pharris et al., 1972). So far attempts to show a uterine influence on the CL of mice has failed (Caldwell, 1969; Moor, 1968 a, 1968 b; and Dewar, 1973). These observations on the mouse imply that non-uterine factors are more important in the mouse. However, these agents have not been well defined.

Biogenic Amines and Control of Gonadtropin and Prolactin Secretion

Introduction

The control of anterior pituitary function has been intensively investigated by endocrinologists. Unlike the posterior pituitary, no direct innervation of the anterior pituitary exists. An important advance was made by G. W. Harris (1955) when he showed that vascular

connections between the hypothalamus and anterior pituitary are necessary for normal anterior pituitary function. His observation indicated that factors from the hypothalamus must travel through the portal system to cause the release of anterior pituitary hormones. Further investigation has resulted in the isolation, identification, and synthesis of three hypothalamic factors: luteinizing hormone releasing factor (LRF) (Matsuo et al., 1971), thyrotropic hormone releasing factor (TRF) (Boler et al., 1969), and somatostatin (SRIF) (Brazeau et al., 1973). Studies on hypothalamic extracts indicate that other factors exist in the hypothalamus.

However, these hypothalamic factors are just one system in the control of anterior pituitary secretion. Even before the releasing factors were isolated, Markee et al. (1952) provided evidence of a neutral influence on hormone secretion. These investigators were able to distinguish a critical period on the afternoon of proestrus. During this time injection of drugs such as dibenamine blocked ovulation. Since dibenamine is an adrenergic blocking agent, these results suggested that a catecholaminergic neuron is triggering the release of LH. Using neuroactive compounds, others have been able to implicate neural regulation of the other anterior pituitary hormones as well.

Relation of Catecholamines to Gonadotropin and Prolactin Secretion

Anatomy of Catecholaminergic Innervation of the Hypothalamus

Chemical analysis provided the first evidence that catecholamines existed in the hypothalamus (Vogt, 1954). Subsequent efforts

have shown that both dopamine and norepinephrine are present (Rinne and Sonninen, 1968).

With the development of histochemical flourescence, the site of some hypothalamic catecholaminergic tracts was found to be the ventral mesencephalon, the pons and medulla oblongata (Ungerstedt, 1971). These fibers enter the hypothalamus by the medial forebrain bundle and innervate the dorsal medial nucleus, periventricular nucleus, the area ventral to the fornix, the arcuate nucleus and the internal layer of the median eminence, the retrochiasmatic area, the paraventricular nucleus, the supraoptic nucleus and the preoptic area. That these areas are important for hormone regulation has been shown by lesion and stimulation studies. The preoptic area is important in the cyclic release of LH (Halasz, 1969). The whole medial basal area of the hypothalamus is essential for tonic release of anterior pituitary hormones (Halasz, 1969).

In addition there is a dopaminergic pathway extending from the arcuate nucleus and the ventral portion of the periventricular nucleus to the external and internal zones of the median eminence (Fuxe, 1964; Lichtensteiger and Langermann, 1966). The terminals of these fibers end on cells bordering the primary plexus of the hypothalamic-pituitary portal system. Fuxe has proposed that these dopaminergic neurons control the secretion of releasing factors into the portal system connecting the hypothalamus with the pituitary. Other investigators have characterized the remaining tracts in the ventral hypothalamus (Bjorklund, 1968). These tracts include nore-

pinephrine containing neurons extending from above the medio-basal hypothalamus to the external and internal layers of the median eminence. Two other tracts have been observed, one reaching the median eminence, and the other extending only as far as the arcuate nucleus. Although the transmitter in these last two tracts is a catecholamine, its identity has not been established.

Effects of Catecholamines on Gonadotropin and Prolactin Secretion

Direct evidence of catecholaminergic regulation of hormone release has been provided by studies correlating catecholamine administration with hormone secretion. Since neither dopamine nor norepinephrine cross the blood brain barrier, these agents must be given by a central route. When dopamine is injected into the third ventricle of estrogen primed, hypophysectomized female rats, systemic concentrations of LRF and FSHRF increase (Schneider and McCann, 1970a). Likewise collection of blood from the portal system of dopamine-injected animals contained higher levels of LRF than blood from animals injected with the control vehicle (Kamberi et al., 1969). Also administration of dopamine into the third ventricle had been shown to increase serum LH and FSH and decrease prolactin in intact female and male rats (Schneider and McCann, 1970b; Kamberi et al., 1970, 1971). Implantation of catecholamines into the median eminence of the hypothalamus has produced results that do not agree with the above observations, although direct hormone measurements were not taken. Instead vaginal smears and ovarian weight were used to infer long term changes in gonadotropin release in response to a

single implantation (Kobayashi and Matsui, 1969).

Levo-dopa (l-dopa) is the immediate precursor of dopamine and easily penetrates the blood brain barrier. Administration of this compound reduces prolactin secretion from the anterior pituitary and increases the hypothalamic stores of prolactin release inhibiting factor (PIF) (Lu and Meites, 1972). In vitro incubation of dopamine with hypothalamic tissue and anterior pituitary causes an increase in release of LH (Schneider and McCann, 1969).

Effects of Adrenergic Drugs on Gonadotropin and Prolactin Secretion

The use of adrenergic drugs provided the first evidence for catecholaminergic regulation of anterior pituitary secretion. Subsequently it was shown that drugs that interfere with catecholamine synthesis or catabolism, alter gonadotropin and prolactin secretion. Alpha-methyl-para-tyrosine and alpha-methyl-meta-tyrosine are effective in inhibiting catecholamine synthesis through substrate competition. These drugs are also effective in raising serum prolactin levels (Lu et al., 1970). Castration hypersecretion of FSH and LH in parabiotic rats was prevented with alpha-methyl-para-tyrosine (Donoso and Santolaya, 1969). Ovulation in immature rats injected with PMS has been blocked by this inhibitor (Lippman et al., 1967).

The false transmitter, methyl norepinephrine, is synthesized from methyldopa and it has little intrinsic activity at adrenergic receptors. Administration of methyldopa effectively increases serum prolactin levels (Lu et al., 1970). Also LH secretion was lower in PMS treated immature rats given methyldopa (Coppola, 1971).

Reserpine depletes the adrenergic neuron of its catecholamine stores. As with the action of other sympatholytic agents, reserpine inhibits ovulation in PMS-treated immature rats (Hopkins and Pincus, 1963). Reserpine has also been shown to reduce synthesis and release of LH and FSH in intact and castrated rats (Gronroos et al., 1965; Labhsetwar, 1967) and to increase prolactin release in intact rats (Lu et al., 1970).

Adrenergic receptor blockers were very prominent in the investigation of nervous regulation of anterior pituitary secretion. Dibenamine was found to inhibit ovulation presumably because it blocked adrenergic stimulation (Markee et al., 1952). Other alpha-adrenergic blockers have been found to influence hormone secretion. Chlorpromazine blocked the PMS induced rise in LH in immature rats (Zarrow and Brown-Grant, 1964). Since human chlorionic gonadotropin overcame the chlorpromazine effect, the action of this blocker is believed to be central. Prolactin secretion is stimulated by chlorpromazine (Lu et al., 1970) and by the dopaminergic receptor blockers, haloperidol (Dickerman et al., 1972) and pimozide (Meites and Clemens, 1972).

In contrast to the above agents monoamine oxidase inhibitors such as pargyline and iproniazid increase the neuronal concentration of catecholamines by slowing their catabolism. Predictably these drugs decrease prolactin release (Donoso et al., 1971).

The effect of the above drugs which penetrate the blood-brain barrier and of the catecholamines is believed to be central. Many peripheral acting drugs do not have the ability to interfere with

hormone secretion. Several laboratories (Schneider and McCann, 1969; Kamberi et al., 1970) have found dopamine to be ineffective for inducing LH release from the pituitary directly. However, dopamine, norepinephrine, and epinephrine can act directly on the pituitary to inhibit the release of prolactin (Jacobs et al., 1968; MacLeod, 1969; and Birge et al., 1970). As yet no catecholamines have been detected in the hypothalamo-pituitary portal blood, and until they are found in the portal blood catecholamines cannot be considered to have an effect on the pituitary directly under normal psysiological conditions.

Changes in Content and Turnover of Catecholamines Associated With Hormone Secretion

The most physiological approach to hormone control involves correlation of neuronal activity with rates of hormone secretion from the anterior pituitary. In this regard it was noted that after castration the anterior hypothalamic content of norepinephrine is increased (Donoso et al., 1967; Stefano et al., 1965) and tyrosine hydroxlyase activity is increased (Beattie et al., 1972). Synthesis of norepinephrine is also accelerated after castration (Anton-Tay et al., 1970; Wurtman et al., 1969; and Anton-Tay and Wurtman, 1968). Administration of estradiol or estradiol plus progesterone to castrated animals suppresses increased synthesis and content of norepinephrine (Donoso and Stefano, 1967; Bapna et al., 1971; Beattie et al., 1972). During the estrous cycle, norepinephrine content of the anterior hypothalamus is minimal during estrus, increases during diestrus and peaks at proestrus (Stefano and Donoso, 1967). Catecholamine

synthesis from exogenous tritiated tyrosine is highest on proestrus and lowest on diestrus (Zschaeck and Wurtman, 1973). At puberty, when LH and FSH are required for vaginal opening, hypothalamic catecholamine content and synthesis rate are increased (Coppola, 1968, 1969).

However, not all investigations agree with the above results. Fuxe and Hokfelt (1969), using the semiquantitative histochemical florescence technique, concluded that catecholamines inhibit gonadotropins and promote prolactin secretion. Fuxe and Hokfelt noted no change in norepinephrine content after castration or during pregnancy or lactation. Total catecholamine content was found to increase during diestrus while dopamine was unchanged. Dopamine was increased during pregnancy, pseudopregnancy, and lactation but did not change after castration.

These controversies arise because not all investigators agree on the most appropriate index of neuronal activity. Also recent advances in neurochemistry make interpretation of these results difficult. It has been accepted that the concentration of norepinephrine is under feedback control so that in norepinephrine containing neurons, synthesis rate is an appropriate measure of neuronal activity. On the other hand, Walters and Roth (1973) have recently shown that dopaminergic neurons increase synthesis of dopamine when deprived of presynaptic stimulation. Hopefully further efforts in this area will resolve the existing contradictions and provide direct evidence linking catecholaminergic neuronal activity with anterior pituitary secretion.

Relation of Serotonin to Gonadotropin and Prolactin Secretion

Anatomy of Serotonergic Innervation of the Hypothalamus

Serotonergic innervation of the hypothalamus has not been as thoroughly explored as the catecholaminergic tracts. It has been established that the cell bodies of serotonergic neurons are located on the raphe region of the mesencephalon and project into the hypothalamus via the medial forebrain bundle (Dahlstrom and Fuxe, 1964; Fuxe, 1965). Serotonergic terminals have been observed in the suprachiasmatic nucleus (Fuxe, 1965), in the middle of the retrochiamatic area, and in the anterior median eminence (Fuxe and Hokfelt, 1970). Chemical analysis has also shown the presence of high concentrations of serotonin in the bovine median eminence (Piezzi, 1970). In addition incubation experiments demonstrate the ability of the median eminence to synthesize serotonin from tryptophan (Hamon, et al., 1969).

Effects of Serotonin or Serotonin Precursors on Gonadotropin and Prolactin Secretion

Serotonin like the catecholamines, does not readily cross the blood brain barrier (Douglas, 1971). However, when serotonin is given centrally, it increases prolactin (Kamberi et al., 1970, 1971). Implantation of serotonin directly into the median eminence is followed by a decline in serum LH levels (Fraschini, 1970). Systemic injection of 5 hydroxytryptophan, a precursor of serotonin that crosses the

blood brain barrier, increases serum prolactin (Lu and Meites, 1973) while it blocks ovulation in PMS treated immature rats (Kordon et al., 1968).

Effects of Serotonergic Drugs on Gonadotropin and Prolactin Secretion

Complications arise in interpretation of studies using some drugs. Reserpine and the other rauwolfian alkaloids interfere with the binding of both catecholamines and serontonin in the synaptic vesicle. Since the catecholaminergic and serotonergic system appear to counteract the actions of each other, the observations after reserpine injection might result from an effect on one or both systems. The same problems are encountered with monoamine oxidase inhibitors, since these compounds interfere with the enzyme metabolizing both catecholamines and serotonin. Kordon et al., (1968) have blocked the synthesis of either serotonin or catecholamines prior to injection of a monoamine oxidase inhibitor. They concluded that monoamine oxidase inhibitors block ovulation by increasing extra-neuronal serotonin levels. A more specific drug, p-chlorophenylalanine (p-CPA), which blocks the synthesis of serotonin, increased the number of eggs ovulated by PMS treated immature rats (Kordon and Glowinski, 1972). Furthermore the intensity of provoked ovulation in immature animals is inversely proportional to the concentration of serotonin in the hypothalamus (Kordon, 1970).

Another indication of serotonin involvement in anterior pituitary secretion comes from studies on diurnal variations in

brain serotonin content and prolactin secretion. Brain serotonin concentration is believed to be regulated by the blood levels of tryptophan in the brain (Fernstrom and Wurtman, 1971). It has been found that there is a daily fluctuation in blood levels of tryptophan with a maximum eight hours after lights are turned on and a minimum four hours after lights are turned off (Wurtman et al., 1968; Fernstrom and Wurtman, 1971). A similar change in serum prolactin has been noted with highest values in the afternoon (Koch et al., 1971). Pituitary content of prolactin also varies and reaches its peak late in the afternoon (Clark and Baker, 1964).

Relation of Acetylcholine to Gonadotropin and Prolactin Secretion

Anatomy of Cholinergic Innervation of the Hypothalamus

The cholinergic tracts within the hypothalamus have not been well described. The techniques available for detection of cholinergic neurons are not as sensitive or as specific as histochemical flouresence. However, some information has been gathered using a variety of techniques.

In one approach, areas responsive to cholinergic neurons are revealed by recording from a region while acetylcholine is applied. Such studies have indicated that the dorsal and caudal regions of the hypothalamus are facilitated by acetylcholine whereas the rostral and ventral regions are inhibited (Bloom, et al., 1963). These results show the regions effected by acetylcholine but are not direct

evidence of cholinergic innervation.

A second approach utilizes a histochemical technique for locating acetylcholine esterase (AchE). This enzyme splits acetylcholine and is the mechanism for terminating cholinergic stimulation at a synapse. Both cholinergic and cholinoceptive neurons, those stimulated by acetylcholine, contain this enzyme. These neurons can be distinguished from one another by the intracellular location of AchE. Using this approach Shute and Lewis (1969) demonstrated that the perifornical nucleus and the dorsal-posterior region of the hypothalamus possess many cholinergic fibers. They noted several tracts within the hypothalamus. One extends from the lateral preoptic area to the supraoptic nucleus and then projects to the amygdala. The supraoptic nucleus was also observed to contain cholinoceptive neurons. A second pathway originates in the supramammillary region and leads to the mammillary bodies, specifically the medial and the lateral mammillary nuclei. Additional tracts ascend from the dorsal-caudal hypothalamus to the thalamus. There also is a cholinergic pathway of the reticular system which passes through the hypothalamus. In addition AchE is found in the arcuate nucleus but in low concentration. It was not determined whether fibers in this area are cholinergic or cholinoceptive.

Although lesion and diafferentation studies emphasize the importance of the anterior and basomedial hypothalamus, lesions in the thalamo-hypothalamic border and dorsocranial mesencephalon, the sites of cholinergic neurons, produce increased prolactin release (Chen et al., 1970; Flerko, 1966; Flerko and Bardos, 1966).

Others have found acetylcholine in the median eminence (Kobayashi et al., 1966). Kobayashi and Matsui (1969) noted axonal endings in the median eminence with small vesicles. These vesicles resemble synaptic vesicles containing acetylcholine in the periphery and are distributed similarly to AchE. It was suggested by these investigators that such axons are cholinergic.

Changes in Enzyme Activity Associated With Different Reproductive States

Several investigators have monitored acetylcholine esterase or choline acetyltransferase activity during various reproductive states in order to determine whether any relationship exists. Kobayashi et al. (1966) measured the activity of choline acetyltransferase, the enzyme synthesizing acetylcholine, in the anterior and posterior hypothalamus. During the estrous cycle, choline acetyltransferase activity was lowest in proestrus and estrus in the anterior hypothalamus while the posterior hypothalamus showed no change in the activity of this enzyme. After castration choline acetyltransferase activity increased in the posterior hypothalamus. When estrogen was given to these castrated animals, the choline acetyltransferase activity decreased. In prepuberal females, estrogen caused ovulation and increased choline acetyltransferase activity at the same time.

Recently Libertun et al. (1973) measured choline acetyltransferase and acetylcholine esterase activity in the anterior and the posterior hypothalamus. Both enzymes showed higher activity

during diestrus in the preoptic-suprachiasmatic area while no variation was seen in the arcuate-mammillary region. Lower enzyme activity was seen in males than in females. This difference in enzyme activity between sexes might be a reflection of hypothalamic maturation. After birth, testosterone from the male testes causes the hypothalamus to induce secretion of gonadotropins and prolactin at a constant baseline level. In the female the hypothalamus induces a cyclic pattern of hormone release. The rise in enzyme activity by 25 days of age in males castrated at birth suggests that hypothalamic maturation might determine cholinergic enzyme activity. However, a similar rise occurs in the females when castrated early. Consequently the relationship between enzyme activity and hypothalamic maturation is not clear.

Effects of Cholinergic Drugs on Gonadotropin and Prolactin Secretion

In the early studies on anterior pituitary regulation, atropine, a cholinergic blocking agent was observed to inhibit ovulation in the rat (Everett et al., 1949) and in the rabbit (Sawyer et al., 1949). Synthetic antimuscarinic agents such as methantheline and pathelon, which act like atropine, also block ovulation (Sawyer, 1963; Gitsch and Everett, 1958). Recently injections of atropine into the third ventricle or beneath the skin were observed to inhibit the proestrous surge of the gonadotropins and prolactin (Kamberi and Bacleon, 1973; Libertun and McCann, 1973). These effects of atropine are believed to be central since LRF can overcome the atropine

blockade of ovulation (Libertun and McCann, 1973). Under in vitro conditions, atropine inhibited LH secretion from pituitaries co-incubated with ventral hypothalamic tissue (Kamberi and Bacleon, 1973). In contrast acetylcholine co-incubated with pituitary tissue and hypothalamic tissue stimulated LH secretion.

Prolactin secretion is influenced by atropine but the type of effect is dose-dependent. High doses injected systemically induced lactation (Meites et al., 1960) and pseudopregnancy (Gitsch and Everett, 1958), while low doses inhibited lactation (Jacobson, et al., 1950) and pseudopregnancy (Grosvenor and Turner, 1958). Cholinomimetic agents such as pilocarpine, a direct agonist, or physostigmine, a acetylcholine esterase inhibitor, induced lactation in rats (Meites et al., 1960). Lactation is dependent on other hormones, principally adrenal cortical steroids, besides prolactin and is induced by stress also. Thus lactation is not a specific indicator of prolactin secretion.

When these cholinomimetic agents are given to ovariectomized, estrogen primed rats, LH and prolactin decrease and then increase (Libertun, 1973). Such results are inconsistent with the implications of atropine administration. However, the use of atropine has some objections since it has a number of side effects, especially at the high doses used. Consequently more research is required to establish the role of cholinergic neurons on pituitary function.

MATERIALS AND METHODS

Animals

Mature 2 to 3 month old virgin female Swiss-Webster mice and mature virgin female and mature male Sprague-Dawley rats were obtained from Spartan Research Animals, Inc., Haslett, Michigan. All animals were housed in a temperature controlled (75±1°F.) artificially illuminated room with 14 hours of light (5:00 a.m. to 7:00 p.m.) and 10 hours of darkness. Wayne Lab Blox pellets (Allied Mills, Chicago, Illinois) and water were provided *ad lib*.

Estrous cycles were determined by taking daily vaginal smears and only those females showing at least two regular 4 or 5 day cycles were used for experimentation

Histological Examination of Ovaries

For histological examination of the ovaries from mice, they were removed, cleaned, rolled dry on a piece of kemi-wipe and weighed on a torsion balance. The tissues were then fixed in Bouin's fluid and stained with hematoxylin and eosin. Sectioning was done parallel to the longest axis at the center of each ovary.

Radioimmunoassay of Prolactin

Serum prolactin concentration was determined by radioimmunoassay as described by Niswender *et al*. (1969). Serum samples were diluted with 0.1% gelatin in phospho-buffered saline. At least two

dilutions of each sample were made and the mean of the dilutions was used as the sample value. The sample values were expressed in terms of a standard: National Institute of Arthritis and Metabolic Diseases-Rat Preparation-1 (NIAMD-RP-1) obtained from the National Institutes of Health, Bethesda, Maryland. The prolactin used for iodination was provided by Dr. S. Ellis of NASA Ames Research Center, Moffet Field, California. Radioactive iodine, I^{125}, was supplied by Amersham/Searle, Chicago, Illinois. The first antibody was collected from a rabbit and characterized by Niswender et al.(1969). The second antibody was collected from a sheep kept locally and diluted as titration indicated.

Cannulation of the Lateral Ventricle of Rats

Lateral ventricle cannulation followed the description of Verster et al. (1971). Female rats weighing 200 to 225 grams were anesthetized with sodium pentobarbital (45 mg/kg). After placement in a Stoelting or Neuman stereotaxic instrument the skin was retracted and two holes were drilled through the skull with a small hand drill. The hole receiving the cannula was drilled 1 millimeter behind and 2 millimeters lateral to bregma. The cannula was made from polyethylene tubing (PE 10) by inserting a wire in the tubing, heating over a soldering gun and compressing the heated region so that a bulb formed. The tubing was cut 4 millimeters from the bulb with a 1 millimeter bevel. Five centimeters of tubing were left on the other side of the bulb and this end was heat sealed. When placed in the hole, the bulb of the cannula rested on the surface of the skull, and the

short end protruded into the lateral ventricle. The second hole was fitted with an anchor screw. Both were cemented in place with dental cement. The skin was sewn and the animals were injected with 0.2 milliliter of Longicillin, (Fort Dodge Laboratories, Inc., Fort Dodge, Iowa) an antibiotic. Vaginal smears were taken after cannulation to ascertain that cycling continued.

Statistical Analysis

Means and standard errors of the means were calculated. The significance of difference between a control group and a treated group was determined by Student's t test. Where numerous treatment means were compared to a single control mean, Dunnett's multiple range test was used after an analysis of variance indicated treatment differences.

The following formulae were used in determining statistically significant differences:

1. Student's t test:

t value for the Student's t test

$$= \frac{\overline{Y} - \mu 0}{\sigma \overline{Y}}$$

where $\overline{Y}$ represents the treatment mean

$\mu 0$ represents the control mean

$\sigma \overline{Y}$ represents the standard derivation.

Sokal and Rohlf, 1969.

2. Dunnett's multiple range test:

d' the difference which must be found between a treatment mean and the control mean before significance is achieved according to the Dunnett test.

$$= t\ D\alpha/2;\ k,v\sqrt{2\left(\frac{MS\ error}{n}\right)}$$

t Dα/2; k,v - a value obtained from a Dunnett's table

n = number of animals per group

MS error - the error mean square

Dunnett, 1970.

EXPERIMENTAL

Luteolytic Action of Prolactin During The Estrous Cycle of the Mouse

Effects of Ergocornine Treatment

Objective

Recent work has shown that the proestrous surge of prolactin during the estrous cycle of the rat is responsible for luteolysis of old corpora lutea (Wuttke and Meites, 1971; Billeter and Fluckiger, 1971). Prolactin also rises on proestrus in the mouse (Kwa and Verhofstad, 1967) and regression of old corpora lutea occurs. It was of interest, therefore, to determine in the mouse whether inhibition of prolactin secretion by the drug ergocornine during proestrus and estrus could prevent luteolysis of the old corpora lutea, and whether concurrent administration of prolactin could induce luteolysis. Previously ergocornine was reported to decrease pituitary prolactin levels in the mouse (Yanai and Nagasawa, 1970).

Materials and Methods

Twenty mature Swiss-Webster mice were injected intraperitoneally with 200 mgs. of ergocornine methanesulfonate base (Sandoz, Ltd., Basel, Switzerland) daily, dissolved in 70% ethanol first and then in 0.9% saline to give a final concentration of 4% ethanol. Treatment

was begun on the afternoon of the diestrous day prior to proestrus and continued for three days. Ten of these mice received 1 mg. of prolactin (NIH-P-S-8 ovine prolactin, 28 IU/mg) dissolved in 0.9% saline on the afternoon of proestrus and on the morning of estrus. In a group of ten control mice, each was injected intraperitoneally with the saline-ethanol solution during the same period. At the end of treatment, on the first day of diestrus, the mice were killed and the ovaries were removed, weighed and prepared for histological examination.

In addition, 16 mice were injected intraperitoneally daily with 200 µg of ergoncornine methanesulfonate per mouse beginning on the afternoon prior to the expected day of proestrus and continuing for three estrous cycles. During each cycle, 9 of the 16 mice were injected with 1 mg. of prolactin on the afternoon of proestrus and on the morning of estrus during each cycle. A control group of 8 mice was injected daily for three cycles with the saline-ethanol solution. The mice were killed on the first day of diestrus after the third cycle, and the ovaries were removed, weighed and prepared as above.

Results

Ergocornine treatment during a single cycle (Table 1) led to a significant increase in the amount of corpora lutea (9.7 ± 0.7) compared to the number of corpora lutea (6.0 ± 0.4) in control mice. Injections of prolactin on the days of proestrus and estrus in ergocornine-treated mice resulted in a return of the number of corpora

Table 1. Effects of Ergocornine (EC) and Prolactin During the Estrous Cycle on Number of Corpora Lutea

Treatment and No. of Mice	Average		
	Body wt. (g)	Ovarian wt. (mg)	No. of Corpora lutea[a]
	One Estrous Cycle		
Controls (10)	22.4 ± 1.5*	24.4 ± 3.2	6.0 ± 0.4
EC (10)	22.6 ± 1.6	23.2 ± 2.3	9.7 ± 0.7[b]
EC + prolactin (10)	22.7 ± 1.5	17.0 ± 1.5	5.3 ± 0.5[c]
	Three Estrous Cycles		
Controls (8)	36.9 ± 0.6	24.8 ± 1.0	6.9 ± 0.7
EC (9)	34.6 ± 0.5	23.9 ± 1.0	9.6 ± 0.8[b]
EC + prolactin (7)	34.3 ± 0.5	22.6 ± 1.0	6.2 ± 0.7[c]

*mean ± standard error of the mean.

[a]Represents single cross section count.

[b]EC vs controls = $p < .05$

[c]EC vs EC + prolactin = $p < .05$

lutea to about the same number as in control mice. No differences were apparent in the appearance of the corpora lutea as a result of ergocornine treatment. Ovarian weight was slightly reduced in the group given ergocornine and prolactin compared to the other two groups. None of the groups differed significantly in body weight.

Ergocornine treatment during three cycles had the same effect as treatment during one cycle, resulting in a significant increase in number of corpora lutea compared to those in control mice. Prolactin administration to ergocornine-treated mice on the days of proestrus and estrus during each of the three cycles resulted in a return of corpora lutea to about the same number as in normal mice. No significant differences were observed in ovarian or body weights in any of the three groups of mice.

Conclusion

These results demonstrate that a reduction in prolactin secretion in cycling mice induced by ergocornine results in an increase in numbers of corpora lutea and that injections of prolactin on the days of proestrus and estrus returns the number of corpora lutea to normal. It is apparent, therefore, that prolactin promotes luteolysis of corpora lutea during the estrous cycle of the mouse as it does in the rat. Unlike the rat, the accumulation of corpora lutea in the ovaries in the mouse as a result of ergocornine treatment was not accompanied by increased ovarian weight. Others also found no increase in ovarian weight in ergocornine-treated mice (Yanai and Nagasawa, 1970). No obvious differences were observed in histological appearance of the corpora lutea of the mice treated with ergocornine as compared

with the corpora lutea of control mice.

Effects of Cholinergic Drugs on Serum Prolactin

Effects of Intraventricular Injection of Acetylcholine on Serum Prolactin

Objective

Presence of biogenic amines within the hypothalamus suggests they may regulate anterior pituitary function. Infusion into the brain of catecholamines and serotonin has demonstrated that they alter anterior pituitary secretion (Kamberi et al., 1970, 1971). Therefore, it was of interest to determine whether acetylcholine, which is also present in the hypothalamus, could influence prolactin release from the anterior pituitary when given intraventricularly.

Materials and Methods

Mature female rats (200-225 grams) were implanted with polyethylene tubing for lateral intraventricular injection. At 11:00 a.m. on proestrous day the rats were slowly infused with 50 µgs. of acetylcholine bromide (K and K Laboratories, Inc., Cainscrew, New York) in a volume of 8 µl of 0.85% NaCl. Controls were infused with an equal volume of 0.85% NaCl. Blood was collected under light ether anesthesia by cardiac puncture 15, 30 and 60 minutes after infusion.

Results

Injection of acetylcholine directly into the lateral ventricle of the brain produced a 47% decrease in serum prolactin

by the end of 15 minutes (Table 2). This reduction in serum prolactin was also observed at 30 and 60 minutes after injection, although the 60 minute values were not different statistically from the control values.

Conclusion

Acetylcholine does not cross the blood brain barrier when given systemically (Koelle, 1970). However, the present results show that acetylcholine given intraventricularly does cause a rapid depression in serum prolactin. The short duration of this effect is probably due to its rapid metabolism by the choline esterases. Previous studies showed no direct effect of acetylcholine on in vitro pituitary release of prolactin (Talwalker et al., 1963) or gonadotropins (Kamberi and Bacleon, 1973). The effect on prolactin release may be the result of acetylcholine acting directly on the hypothalamus to increase PIF release or to decrease release of PRF.

Table 2. Serum Prolactin in ng/ml of NIAMD-RP-1 After Intraventricular Injection of Acetylcholine Bromide (50 μg) Into Proestrous Female Rats

Treatment and No. of Rats	15 min.	30 min.	60 min.
Controls (15)	30 ± 3*	43 ± 7	40 ± 7
Acetylcholine Bromide (12)	16 ± 2[a]	22 ± 3[b]	22 ± 5

*mean ± standard error of the mean

[a] $p < 0.001$ controls vs drug, Student's t test

[b] $p < 0.02$ controls vs drug, Student's t test

Effects of Pilocarpine on Serum Prolactin Levels of Male and Female Rats

Objective

Pilocarpine is an agonist of acetylcholine which can pass through the blood brain barrier. Systemic administration of this drug would be expected to reproduce cholinergic activity within the central nervous system. Therefore, this drug was used to test its effect on prolactin release by the pituitary.

Materials and Methods

Mature male rats were injected with pilocarpine nitrate (Nutritional Biochemical Corporation, Cleveland, Ohio) intraperitoneally at doses of 5, 10 and 18 mg/kg. Female rats were injected with 9 mg/kg of the drug intraperitoneally at noon on the day of proestrus. Blood was collected by cardiac puncture under light ether anesthesia 15, 30 and 60 minutes after injection.

Results

In male rats pilocarpine at the 5 mg/kg dose significantly reduced serum prolactin (Table 3). A dose of 10 mg/kg also reduced serum prolactin values although these differences were not significant. The highest dose, 18 mg/kg, apparently had no effect on serum prolactin. This may be due to the stressful effects of this dose since diarrhea and piloerection were observed in these rats. When pilocarpine was administered systemically to female rats at a dose

Table 3. Serum Prolactin in ng/ml of NIAMD-RP-1 After Intraperitoneal Injection of Pilocarpine Nitrate in Male Rats

Treatment and No. of Rats	Body wt. (g)	15 min.	30 min.	60 min.
Controls (6)	448 ± 12*	26.9 ± 4.5	25.9 ± 4.3	11.1 ± 3.0
5 mg/kg (7)	443 ± 10	9.9 ± 2.5(1)[a,b]	8.4 ± 3(3)[a,c]	11.0 ± 4.8(3)[a]
10 mg/kg (5)	449 ± 12	17.1 ± 3.3	18.6 ± 1.9(1)[a]	--------
18 mg/kg (6)	453 ± 8	30.8 ± 5.2	18.2 ± 4.1	15.0 ± 3.6

*mean ± standard error of the mean

[a]Rats with serum levels of prolactin too low to be detected. No. of rats indicated in ( )

[b]$p < 0.01$ controls vs drug, Dunnett's multiple range test

[c]$p < 0.02$ controls vs drug, Student's t test

of 9 mg/kg, significant reductions in serum prolactin were observed after 15 and 30 minutes but not by 60 minutes after injection (Table 4).

Conclusion

These results show that pilocarpine can cause a reduction in serum prolactin in both male and female rats. A low dose (5 mg/kg) was most effective in males, whereas 9 mg/kg was effective in females. A dose of 10 mg/kg did not produce a statistically significant reduction in serum prolactin in males. The difference in the number of animals used in the two experiments may be the explanation for this inconsistency. The highest dose of pilocarpine appeared to stress the rats. Since stress is a potent stimulus for prolactin release, the depressive effect of this dose might have been overcome by the stress. The results suggest that cholinergic stimulation produced by pilocarpine reduces release of prolactin from the anterior pituitary.

Effects of Physostigmine on Serum Prolactin Levels in Male Rats

Objective

An injection of cholinomimetic drugs can stimulate cholinoceptive neurons, but they might also have a nonspecific effect. For this reason another approach was sought to test the effect of cholinergic stimulation on prolactin secretion. Physostigmine is a reversible inhibitor of choline esterase, the enzyme degrading

Table 4. Serum Prolactin in ng/ml of NIAMD-RP-1 After Intraperitoneal Injection of Pilocarpine Nitrate (9 mg/kg) Into Proestrous Female Rats.

Treatment and No. of Rats	Body wt. (g)	15 min.	30 min.	60 min.
Controls (11)	276.4 ± 7*	24.7 ± 2.7	25.6 ± 4.1	15.8 ± 2.6
Pilocarpine Nitrate (14)	272.0 ± 4	11.7 ± 1.4[a]	11.7 ± 1.8[b]	10.1 ± 2.0

*mean ± standard error of the mean

[a] $p < 0.005$ controls vs drug, Student's t test

[b] $p < 9.001$ controls vs drug, Student's t test

acetylcholine in the synaptic cleft and terminating its action. Injection of physostigmine can prolong the effect of acetylcholine released at a synapse, thus potentiating the effects of cholinergic activity. Therefore, physostigmine was used to determine the effect on prolactin secretion of normal cholinergic activity.

Materials and Methods

Male rats were injected intraperitoneally with physostigmine sulfate (Merck and Co., Rahweg, New Jersey) in doses of 0.3, 0.4 and 0.5 mg/kg. Blood samples were taken under light ether anesthesia by cardiac puncture 30, 60 and 90 minutes after injection.

Results

The two lower doses of physostigmine, 0.3 and 0.4 mg/kg, were effective in suppressing prolactin release during the three time intervals observed (Table 5). The highest dose, 0.5 mg/kg, was ineffective at 30 minutes. At 60 minutes a non-significant depression was noted, but by 90 minutes this dose also caused a depression of prolactin release.

Conclusion

These results show that physostigmine causes a depression in serum prolactin. The highest dose may have a stress effect which stimulates prolactin, thus causing a competition of effects. The suppression of serum prolactin by physostigmine is probably related to its potentiation of cholinergic activity. Thus these results suggest that cholinergic activity can produce a depression in serum prolactin.

Table 5. Serum Prolactin in ng/ml of NIAMD-RP-1 After Intraperitoneal Injection of Physostigmine Sulfate in Male Rats

Treatment and No. of Rats	Body wt. (g)	30 min.	60 min.	90 min.
Controls (8)	447 ± 10*	39 ± 8.6	21.8 ± 2.0	31.3 ± 6.1
0.3 mg/kg (8)	439 ± 11	16.8 ± 2.6[b]	8.2 ± 1.7(2)[a,b]	11.1 ± 3.0[c]
0.4 mg/kg (7)	447 ± 8	17.9 ± 2.9[b]	12.8 ± 1.9[c]	10.2 ± 1.7(1)[a,b]
0.5 mg/kg (8)	438 ± 11	36.8 ± 6.4	13.3 ± 3.3	11.8 ± 4.0(3)[a,c]

*mean ± standard error of the mean

[a]Rats with serum levels of prolactin too low to be detected. No. of rats indicated in ( )

[b]$p < 0.01$ controls vs drug, Dunnett's multiple range test

[c]$p < 0.05$ controls vs drug, Dunnett's multiple range test

DISCUSSION

A luteolytic role for prolactin has previously been demonstrated in the rat but not in the mouse. The results reported here indicate that prolactin also causes regression of old, non-functional corpora lutes in the mouse. This supports the view that the pro-estrous surge of prolactin is the physiological mechanism for accomplishing luteolysis during the estrous cycle of the mouse. A possible complication is the involvement of LH in luteolysis. Rothchild (1965) has proposed that LH is the luteolytic agent of the pituitary in the rat. A recent study by Hausler and Malven (1971) clearly demonstrates a synergism between prolactin and LH in luteolysis of functioning corpora lutea in this species. Still undetermined, however, is the question of the action of LH on non-functioning corpora lutea. If one can consider structural regression as a phenomenon separate from cessation of progesterone secretion, then much of the accumulated data on the luteolytic action of LH relates to functional luteolysis but not to non-functional corpora lutea. Malven (1969) found that LH was unable to induce regression of non-functional corpora lutea and that it was even unable to synergize with sub-minimal doses of prolactin in inducing regression. Before one can propose that luteolysis during the estrous cycle is due entirely to prolactin, the involvement of LH must be excluded.

Also unresolved is the apparent opposite actions of prolactin on luteal tissue. It is luteotrophic on newly developed corpora lutea, while in older corpora lutea it is luteolytic. The length of the time interval before prolactin is present may explain the difference in reactivity of the corpora lutea. Thus within 56 hours after hypophysectomy an injection of prolactin is luteotrophic, but after 80 hours a prolactin injection is luteolytic. Another possibility is that the corpora lutea may need to be primed by other hormone(s) in order to grow and secrete progesterone under the influence of prolactin. Hilliard (1973) suggested that estrogen is important in determining the reactivity of luteal tissue to prolactin. As evidence, she cited a study by Hixon and Armstrong (1971) in which prolactin was found to be luteolytic when administered 4 days after destruction of antral follicles by irradiation. If, however, estradiol was given daily after the radiation treatment, prolactin was luteotropic. Armstrong (1968) also found that prolactin does not increase progesterone release in hypophysectomized rats unless LH is administered 28 hours earlier. The LH in this case would cause an increase in estrogen synthesis.

During the estrous cycle the old corpora lutea are subjected to estrogen action since estrogen rises late on diestrus day two and is the stimulus for the proestrous release of LH and prolactin. Either the corpora lutea are incapable of being primed by estrogen because of age or the corpora lutea do not react soon enough to the estrogen before prolactin induces regression.

Obviously additional research is required to determine the factors regulating the reactivity of the corpora lutea to prolactin. This study clearly demonstrates luteolytic action of prolactin during the estrous cycle of the mouse.

The present work provides evidence for a cholinergic control of prolactin secretion. Direct infusion of acetylcholine decreased serum prolactin. Similar results were obtained by systemic injection of other cholinomimetic agents. However, high doses of physostigmine or pilocarpine did not change serum prolactin, since stressful agents generally increase serum prolactin levels. Although these results agree with the very recent observations of Libertun (1973), they contradict the implications of studies using the cholinolytic drug, atropine. This drug blocked ovulation and decreased prolactin in estrogen-primed ovariectomized animals, thus implying that cholinergic neurons stimulate prolactin secretion. Resolution of this controversy necessitates additional work. However, some comments are appropriate in respect to atropine administration. The dose of atropine given subcutaneously to inhibit ovulation was 90% of the LD 50 (Cahen and Tvede, 1952). Stress alone is a potent inhibitor of ovulation and it cannot be overlooked in this case. Also noteworthy is the antiserotonergic (Jacques, 1956) and anticatecholaminergic properties of atropine. These latter properties of atropine could explain its action on ovulation.

The site of cholinergic action induced by the cholinomimetic agents is probably the central nervous system as indicated by intraventricularly administered acetylcholine. Although there

are cholinergic neurons in the hypothalamus, these may not be the ones causing the response to pilocarpine and physostigmine. Cholinoceptive neurons outside the hypothalamus but with connections to it, might be the initial site of action. The effects on LH secretion of pituitaries in vitro co-incubated with hypothalamic tissue and acetylcholine argue against an extra-hypothalamic site of action (Kamberi and Bacleon, 1973). It is unlikely that these agents are directly effecting the pituitary since acetylcholine does not induce release of LH when incubated with pituitary tissue (Kamberi and Bacleon, 1973), and early work showed no change in prolactin secretion when acetylcholine was added to incubated pituitaries (Talwalker et al., 1963).

There remains the more difficult task of relating these observations to physiological phenomena. It is interesting that cholinomimetic agents depress prolactin. However, no physiological role for cholinergic activity on prolactin secretion has yet been demonstrated. There also is the need to learn the possible relationships between acetylcholine and the important catecholaminergic and serotonergic systems in the control of prolactin secretion.

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