THE EFFECTS OF CHRONIC RESTRAINT STRESS ON REPRODUCTIVE CONTROL MECHANISMS IN THE LABORATORY RAT

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SANDRA M. WOOD
1976

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ABSTRACT

THE EFFECTS OF CHRONIC RESTRAINT STRESS
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IN THE LABORATORY RAT

By

Sandra M. Wood

Effects of chronic restraint stress on serum LH, prolactin and testosterone in male rats, serum LH in female rats, and pituitary responsiveness to LHRH in both male and female rats were determined in young three to five month old Long-Evans rats. Serum samples were taken under light ether anaesthesia via suborbital sinus puncture. Samples were taken before and after two hours of restraint stress on the first day, the seventh day and the thirteenth day of stress. In vivo pituitary responsiveness was measured prior to and after 10 or 12 days of restraint stress. Following a zero time blood sample rats were injected (i.v.) with 100 or 500 ng LHRH and blood samples taken 15, 30 and 60 minutes or 15 and 45 minutes post-injection. Following two weeks of restraint stress the rats were decapitated and their pituitaries challenged with either 0, 5, 25, 50 or 100 ng LHRH. After four hours of incubation the media was decanted and the individual

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pituitary halves homogenized. The incubation media was used to measure pituitary LH and prolactin release, and pituitary LH and prolactin content was measured from the pituitary homogenate. In addition individual hypothalami from control and stressed male and female rats were homogenized and assayed for dopamine and norepinephrine.

Serum LH response was greater to the 500 ng LHRH dose than to the 100 ng LHRH dose in male rats. The restraint stress treatments in the male groups did not alter pituitary release of LH when stimulated with LHRH. Although two weeks of stress decreased LH release in response to 500 ng LHRH injection in one group of female rats, the peak serum LH response to 500 ng LHRH injection did not change between control and stressed females in the second experiment. It was noted that the duration of the response may be different in control and stressed female groups receiving LHRH. The 45 minute postinjection serum LH values from the stress animals were only one half of the control group values. These results suggest that the pituitary's ability to release LH is unaffected by stress in male rats, while the response is altered in female rats as seen as a decrease in peak release or duration of response.

Pituitary content of LH was not affected by stress or LHRH stimulation in either the stress or control male rats. LH release from incubated pituitaries was stimulated

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by LHRH, but the amount of LH released was unaffected by the restraint stress. These data support that of the *in vivo* LHRH data for male rats. Pituitary content was unaffected by LHRH stimulation in female rats, but the pituitary halves from the stress rats contained significantly more LH than the control rats. LHRH stimulated release of LH from the pituitary halves and chronic restraint stress had no effect on pituitary release. These data support that of the *in vivo* LHRH data for female rats that the pituitary LH control mechanisms may be altered by chronic stress.

Although serum LH following the first restraint period was found to be variable, LH was reduced in both the prestress and poststress serum samples taken on day seven of the treatment. Prestress serum LH levels remained lower than control group LH in two of the three male groups after two weeks of restraint treatment and were further reduced following two hours of stress.

Changes in serum LH in response to the first stress period in female rats depended upon the stage of the estrous cycle. Resting serum LH was dramatically reduced in female rats following one week of stress and was unchanged after two hours of stress. Serum LH continued to be low following two weeks of restraint stress. Restraint stress also blocked ovarian cyclicity. Stressed rats had persistent leucocytic vaginal cytology throughout the treatment.

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Pituitary prolactin content and release and serum prolactin concentrations were not altered by chronic stress in male rats.

Serum testosterone increased in response to 500 ng LHRH injection in control animals. Following two weeks of stress resting serum testosterone was significantly reduced and was unchanged by LHRH stimulation. Chronic restraint stress was found to significantly decrease serum testosterone after one and two weeks of restraint stress. These data suggest that the stress-induced decrease in serum LH alters testosterone release at the testes.

Dopamine and norepinephrine content of the hypothalamus were not significantly different in stress and control male rats. Stress also had no effect on hypothalamic content of norepinephrine in female rats.

Both male and female stress groups lost significant amounts of weight during the stress treatment.

These results indicate that chronic restraint stress can interfere with reproduction in male and female rats. Chronic restraint stress decreased serum LH in male and female rats which was manifest as suppressed serum testosterone in male rats and persistent diestrous vaginal cytology in female rats. Chronic restraint stress had no effect on LHRH stimulation of LH release suggesting that the primary effect of stress on reproduction occurs in the nervous system, presumably at the hypothalamus.

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Sandra M. Wood

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INTRODUCTION

Acute stress has been largely used to study the hypothalamo-hypophyseal-adrenal axis as well as the hypothalamo-hypophyseal-gonadal axis and their interactions. Although many investigators have individually considered the effect of stress on serum concentrations of the adenohypophyseal, adrenal and gonadal hormones, our understanding of the effect of stress on central system components remains minimal. The literature in this field is often confusing and conflicting due to the variable stresses studied and inadequate measurement of stress intensity. While acute stress effects have been extensively studied in laboratory rodents, domestic animals and humans, chronic stress has been less well studied.

The objective of these experiments was to determine the effect of a defined stressor of high intensity (physical restraint) on gonadal control system components of the laboratory rat. The rat was chosen for this investigation because of its short reproductive cycle (four to five day estrous cycles in the female). Also normal neuroendocrine control of gonadotropin and prolactin,

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as well as ovulation, has already been fully characterized in this species.

LITERATURE REVIEW

General Considerations

Two different organ systems exist in metazoa for the integration of bodily functions, the nervous system and the endocrine system (Frings and Frings, 1970). These two control systems have developed during the course of evolution, and exhibit some characteristics complementary to each. The nervous system occurs in most multicellular organisms, even coelenterates, and plays an essential role in the control of adaptation to changes in both internal and external environments (Dethier and Stellar, 1970). Neurons have both an excitatory and secretory nature (Mountcastle, 1974). Excitatory neurons can generate action potentials as a result of their excitation and rapidly transmit the signal through the nerve fiber to other cells. Secretory neurons produce various kinds of specific information-carriers called neurohormones (Dethier and Stellar, 1970; Frings and Frings, 1970). These chemical messengers are liberated from fiber endings into the body fluid and exert specific effects on other cells. The neurosecretory system shows characteristics between the nervous and endocrine system. In invertebrates

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neurosecretory neurons and excitatory neurons are sufficient to carry out regulatory function, but as organisms become more complex, the endocrine system develops to regulate certain bodily functions (Inoué and Sekiguchi, 1973; Dethier and Stellar, 1970). These endocrine organs produce and release hormones into the body fluid. Each hormone possesses a specific chemical composition which eliminates the contamination of information as well as specific tissues sensitivity to the specific chemical agents help control selection (Usher, Kasper, and Birmingham, 1967). While neural transmission and control is very rapid (Mountcastle, 1974), endocrine control is followed by a time delay and a longer lasting effect (Nequin, Alerez, and Campbell, 1975). Hormonal information can be transduced into neural information, and neural action potentials into chemical signals. Hormones may also modify the excitability of neurons as well as neural input may alter hormone secretion (Inoué and Sekiguchi, 1973; Allen, Allen, Greer, and Jacobs, 1973).

Neuroendocrinology deals with the interaction of the nervous and endocrine systems in the control of body functions and in the adaptation of an individual in its environment (Donovan, 1970). Intensive study of the relationship between nervous and endocrine systems in mammals has been largely directed toward analysis of the factors involved in the neural control of the pituitary

gland. Meaningful study of brain endocrine relationship began with this century (Meites, Donovan, and McCann, 1975). Although the testicular transplantation experiments by Berthold in 1849 gave information indicative of internal secretion it was not until 1902 that Bayless and Starling proposed the name "hormone" to describe the role of secretion in the control of pancreatic secretion (Donovan, 1970; Mountcastle, 1974). About this time the neurohypophysis was associated with the fast acting and short lived actions of pituitary extract effects on the cardiovascular system, the uterus and mammary glands. It was not until Philip Smith developed a technique for removal of the hypophysis without damaging the hypothalamus that physiological function was described for the adenohypophysis (Brooks, 1975). In 1927 Smith showed that hypophysectomy resulted in retarded growth and atrophy of the gonads, thyroid gland, adrenal cortex, liver, spleen and kidneys. These changes could be reversed by crude pituitary extract injections or by implanting pituitary tissue (Donovan, 1970; Mangili, Motta, and Martini, 1966). In 1936 Marshall concluded that many external influences including emotional stimuli could modify the sexual cycle by acting on the pituitary gland through the central nervous system (Donovan, 1970; Neville and Neathery, 1974; Chatterton, Chien, Ward, and Miller, 1975). Believing the anterior pituitary to be under direct neural control, scientists

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began to search for the connecting link. Effort to locate and identify nerve tracks failed (Jacobsohn, 1975) and examination by the electron microscope ended the dispute over the secretomotor innervation of the anterior pituitary. In 1947 Green and Harris found evidence that the pathway between the hypothalamus and anterior pituitary was the hypophyseal portal vessels (Green and Harris, 1947; Donovan, 1970; Mountcastle, 1974; Jacobsohn, 1975). They found that following transection of the pituitary stalk that anterior pituitary function returned as the portal vessels regenerated. When regeneration was prevented by placing a barrier at the cut stalk, gonadal atrophy and pituitary hypofunction continued. It was then hypothesized that the blood reaching the pituitary contained some special properties. Pituitary tissue transplanted to areas of the body other than the median eminence reestablished its blood supply but not its function (Hinsey, 1975). Evidence of this type supported the neurohumoral theory that the hypothalamus releases chemical agents into the portal vessels and exert their effect on the cells of the anterior pituitary (Donovan, 1970). Confirmation of this hypothesis came from experiments using hypothalamic extracts to alter adenohypophysis function. Appropriate hypothalamic extracts were found to release either follicle-stimulating hormone (FSH-RH) (Johansson et al., 1973), luteinizing hormone (LHRH) (Matsuo et al., 1971), adrenocortocotrophic hormone

(CRH) (Shally et al., 1960; Reniker, Sieber, and Rittel, 1972), or thyrotrophic hormone (TRH) (Bowers et al., 1970; Burgus, 1970), or to inhibit prolactin (PIF) release (Meites, 1972).

Endocrine Control Mechanisms

Endocrine control mechanisms have been examined theoretically and models designed to graphically explain neuroendocrine control systems (Donovan, 1970; Dethier and Stellar, 1970). The model consists of the higher centers of the CNS, the hypothalamus and its releasing or inhibiting factors, the anterior pituitary and its hormones, target tissues and their hormones. The effect of the target tissue hormones at the CNS, hypothalamus and anterior pituitary completes the control system. Both negative and positive feedback have been demonstrated at the anterior pituitary (Price, 1975). When rats were hypophysectomized the gonads atrophied, and administration of fresh pituitary extracts restored the gonads to normal. Implantation of mature rat pituitaries into the median eminence of immature females causes precocious maturity (Price, 1975). These findings established a link on the chain of evidence that provided the background for neuroendocrine control. In the 1920s it was established that the gonads secrete estrogens, progesterone and androgens. It was also demonstrated that the anterior pituitary gland secreted gonadotropins that stimulated the gonads to

secrete sex hormones (Price, 1975; Bogdanove, 1972). Daily injections of estrogen or testosterone were found to have negative effects upon bull testes. Price interpreted this data that estrogen and androgens must act to inhibit pituitary gonadotropins. Negative feedback of adrenal steroids was demonstrated when cortisol implants blocked adrenocortical function such as the stress induced release of adrenal steroids (Sawyer, 1975). While negative feedback is the most common endocrine influence on pituitary hormone release positive feedback can occur with ovarian hormones (Price, 1975; Hohlweg, 1975; Harris, 1964). Estrogen can trigger secretion of LH from the anterior pituitary during follicular development which causes ovulation and stimulates formation of corpora lutea. Single injections of estradiol to immature female rats resulted in corpora lutea formation (Hohlweg, 1975). This positive feedback effect is specific for females, as injection of estrogen into male rats does not cause secretion of LH. Hormonal negative feedback has also been demonstrated at the level of the hypothalamus. Hohlweg (1975) found that pituitaries transplanted to kidney capsules showed no signs of alteration after castration. If pituitaries from castrated animals were transplanted to the kidney capsule it was impossible to observe castration cells in the anterior pituitary after three weeks. These data indicated that the nervous center must monitor the gonadal steroid serum

concentrations and can alter gonadotropin secretion. Experiments of Harris and Jacobsohn (1952) showed a decrease in LH activity and gonadal steroids when rat pituitaries were removed and placed under the kidney capsule. This condition was alleviated when the anterior pituitary was reinstated just below the median eminence. This confirmed the probable existence of a neurohormone which travels to the anterior pituitary by way of the hypophyseal portal vessels. Hypothalamic lesions were found to cause dystrophic changes in both the adrenal cortex and the gonads, especially lesions placed in the medioventral and rostral regions (Ingram, 1975). The first stereotaxically-placed implants of crystalline estradiol and testosterone were placed in the rat brain by Lisk (1960). He was able to differentiate a basal hypothalamic area where steroids induced gonadal atrophy and an area rostral to the preoptic area that influenced sexual behavior. Sawyer (1967) found, in both rabbits and dogs, that gonadal steroids induced gonadal atrophy if placed at the basal hypothalamus but had no effect if placed at the pituitary or other areas of the brain. The effective site of estrogen implantation at the hypothalamus is the same site that electrical stimulation induced ovulation and lesions caused ovarian atrophy (Sawyer, 1963). While intrapituitary estrogen had no effect on the ovary or the uterus, indicating no steroid feedback at the pituitary,

it did activate the mammary glands, indicating prolactin release (Sawyer, Hilliard, Kenematsu, Scaramuzzi, and Blake, 1974). Bioassays of pituitary prolactin and LH showed that intrahypothalamic estrogen interferred with synthesis and release of pituitary LH while intrapituitary estrogen resulted in release and depletion of pituitary prolactin (Kawakami and Sawyer, 1967). Deaffrentation of rostral and preoptic areas of the hypothalamus using the technique of Halaśz were found to eliminate cyclic ACTH and gonadotropin secretion, thus eliminating diurnal changes in levels of adrenal steroids and cyclic ovulation (Sawyer and Gorski, 1971).

Even with the absence of target organ hormone pituitary trophic hormones can still influence hypothalamic activity, indicating a short-loop feedback (Beyer and Sawyer, 1969). Hypothalamic implants of LH decreased multiunit activity when placed in the preoptic area and ventromedial area. ACTH implants were found to increase multiunit activity when placed in the median eminence in adrenalectomized rats, while dexamethasone implants depressed the activity (Sawyer, 1970).

Besides the feedback from the adrenal steroids on ACTH secretion at the pituitary, and gonadal steroid effects on LH and FSH, these systems can interact with each other. In 1939 Selye found that stress induced adrenal corticoid activity was accompanied by gonadal

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atrophy. In 1963 Schreiber found stress increased ACTH secretion and decreased gonadotropin release. Experiments on social behavior with mice and hamsters showed a rapid decrease in animal numbers following a peak population (Scott, 1958; Dethier and Stellar, 1970). Submissive animals became thin, lost hair and often had skin lesions, accompanied by adrenal hyperplasia and gonadal atrophy (Scott, 1958). These endocrine disorders lead to a high rate of mortality, and reduced fertility (Dethier and Stellar, 1970).

Stress Effects on Reproduction

A variety of experiments have shown a relationship between stress or increased adrenocortical function and the reproductive control system. Surgical stress was found to decrease serum LH and FSH in humans (Charters, Odell, and Thompson, 1969) and ACTH could block PMS induced ovulation in immature rats (Hagino, Watanabe, and Goldzeiher, 1969). Injection of hydrocortisone, prednisolone or ACTH during the follicular phase of the estrous cycle in the sow delayed estrous one to three days and shortened the estrus period from three days to one day (Liptrap, 1970). Administration of ACTH in man was found to decrease serum testosterone by inhibiting LH release from the pituitary (Rivarola, Saez, Meyer, Jenkins, and Migeon, 1966). Ajika et al. (1972) found pentobarbitol blocked ether stress-induced LH elevation in rats, but administration of

hypothalamic extracts or LHRH stimulated LH release in these rats. Restraint stress before ovulation and mating in rats had no effect on litter size but did delay ovulation by three days (Euker and Riegle, 1973).

Reproductivity in ewes was affected by excessively high or low ambient temperatures during the breeding season, as exhibited by delayed estrous cycles and increased embryonic mortality (Neville and Neathery, 1974). Restraint stress during the first five days of pregnancy or the fifth through twelfth day of pregnancy of the rat interrupted more than 50 percent of the pregnancies. Animals that remained pregnant had normal litter numbers. This all or none response to stress was further substantiated by the effects of restraint stress on the afternoon of proestrus (McKay et al., 1976) where stress either totally blocked ovulation or had no effect.

Although Euker et al. (1975) found ether stress, gentle handling and blood sampling increases serum LH in intact male rats, the same treatment resulted in decreased serum LH in castrated male rats. Although rats treated with low doses of dexamethasone could still respond to the handling stress with increased serum corticosterone and LH, dexamethasone treatment reduced the magnitude of the stress-induced response for both hormones.

In another experiment, it was shown that serum LH of intact male rats remained unchanged following two hours

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of restraint stress, as did serum LH in female rats during diestrus or estrus (Euker et al., 1973). Two hours of restraint stress was found to decrease serum LH in proestrus rats (Euker et al., 1973; McKay et al., 1976). In contrast to these findings Neill (1970) found serum LH in female rats unaffected by ether or bleeding stress regardless of the stage of the estrous cycle. These data emphasize differences in stress effects on hormone release between laboratories and the reproductive status of the experimental animals.

Serum prolactin concentration is also affected by stress (Wakabayashi et al., 1971). Acute stress such as handling, ether exposure and serial blood sampling increases serum prolactin within 2 to 4 minutes in male rats (Euker, Meites, and Riegle, 1975; Bellinger and Mendel, 1975). Two hours of restrint stress increased serum prolactin in male rats, in diestrus female rats (Euker, Meites, and Riegle, 1973; Riegle and Meites, 1976), and in ovariectomized rats (Ajika et al., 1972; Riegle and Meites, 1976). Both dexamethasone (Harms, Langlier, and McCann, 1975; Euker, Meites, and Riegle, 1975) and pentobarbitol (Ajika et al., 1972) can block the stress-induced prolactin increase in male and ovariectomized rats. Serial ether stress decreased serum prolactin in proestrous rats, and had no effect on estrous or diestrous rats prolactin concentrations (Euker, Meites, and Riegle, 1975; Riegle and

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Meites, 1976). Serial blood sampling was found to decrease serum prolactin on the afternoon of estrus as well as during proestrus, and had no effect on rats during diestrus or on the morning of estrus (Riegle and Meites, 1976). Two hours of restraint stress decreased serum prolactin in proestrous and estrous rats (Euker et al., 1973; Riegle and Meites, 1976).

Stress is a term used to describe a variety of events that can illicit a multitude of responses. Stress has been defined as a collection of diverse stimuli which damage or potentially damage the organism (Allen et al., 1973). Ether stress, handling stress, novel environment, serial bleeding, surgery, leg break and restraint stress are but a few of the different stressors used (Euker and Riegle, 1973; Baldwin, Colombo, and Sawyer, 1974; Seggie and Brown, 1975; Blake, 1975) in experiments to study stress effects on reproduction. These different stresses have been found to cause conflicting data, as two hours of restraint stress increases serum prolactin in diestrous rats, while serial ether stress had no effect (Riegle and Meites, 1976). In addition restraint stress has been shown to decrease serum LH in proestrous rats, have no effect on serum LH male, diestrous or estrous female rats (Euker, Meites, and Riegle, 1973). Therefore it is important for investigators to carefully define stresses

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so their different effects can be compared between experiments and between various investigators.

Stress Index Identification

The most accepted index of stress is the release of ACTH from the adenohypophysis followed by an increase in adrenal glucocorticoids secretion (Brodish and Redgate, 1973; Donovan, 1970). The central nervous system, peripheral nervous system and the autonomic nervous system transmit and regulate stress-induced impulses to the hypothalamus stimulating the hypothalamic-hypophyseal mechanisms controlling ACTH release (Dethier and Stellar, 1970; Allen et al., 1973). Stressors have been broken down into two main categories, systemic and neural stress (Allen et al., 1973).

I. Systemic stress

A. Internal environmental changes

1. physiological processes

hypotension
hypoglycemia
hypoxia
starvation
exercise

2. pharmacological substances

epinephrine	ether
histamine	parathione
ADH	capsaicin
formalin	
endotoxin	

B. External environmental changes

external temperature

heat
cold

forced immobilization or restraint

II. Neural stress

A. Noxious stimulus

leg break
tourniquet
electric shock
vibration
burning

B. Light

C. Sound

D. Psychological stress--emotional stress

Neurogenic stresses such as leg break or surgery use neural pathways to the hypothalamus by way of the peripheral nervous system to the spinal cord and central nervous system while systemic stress act through the circulation and activates neural signals to the hypothalamus and pituitary (Allen et al., 1973). Maximal stress is found when both neurogenic and systemic pathways are activated as seen with starvation and exercise.

Control of ACTH Secretion

Normally the adrenal glands secrete three groups of steroids, glucocorticoids, mineralocorticoids, and adrenal sex steroids (Mountcastle, 1974). Both stimulated growth of the adrenal cortex at the zona fasciculata and

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zona reticularis, causing synthesis and release of glucocorticoid steroids and adrenal androgens. Secretion of glucocorticoids vary from species to species, as cortisol (hydrocortisone) is the major glucocorticoid secreted in man, dog, and guinea pig and corticosterone the main glucocorticoid in the cat, rat, and mouse (Donovan, 1970).

The chemical structure of ACTH, characterized and synthesized by Schwyzer and Sieber (1963), was found to be a small polypeptide of 39 amino acid residues. Half life studies have shown that 50 percent of ACTH is lost within 5 minutes postinjection in intact rats, 30 seconds in adrenalectomized rats and 10 minutes in man (Evans, Sparks, and Dixon, 1966). This indicates that there must be regular pituitary release of ACTH in order to maintain measurable levels of ACTH in the blood. The action of ACTH at the adrenal cortex is very rapid as the release of steroids into adrenal venous blood can be detected two minutes following intravenous injection of ACTH (Evans et al., 1966; Donovan, 1970).

Hans Selye (1936) initiated the concept of "stress" as he found ACTH release from the anterior pituitary was induced by a variety of stimuli and developed the theory that naturally occurring stresses can cause "diseases of adaptation." It is now clear that ACTH secretion fluctuates in response to a vast number of stimuli which range from psychological conditions such as anxiety, fear and

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anger to cold, heat, and surgical stress or overcrowding (Dethier and Stellar, 1970; Allen et al., 1973; Hearnshaw and Wodzicka-Tomaszewska, 1973; Euker et al., 1973). Increases in serum ACTH and adrenal steroids have been found in people in stressful situations such as students before exams, aircraft pilots, emotionally disturbed patients and relatives accompanying patients at an emergency ward of a hospital (Mason, 1968).

Observations attest to the fact that a balance exists between the blood concentration of adrenal hormones and the secretion of ACTH as chronic administration of adrenal corticoids inhibits pituitary secretion of ACTH and results in atrophy of the adrenal cortex (Donovan, 1970; Tiptaft, 1975). Removal of one adrenal causes compensatory hypertrophy of the remaining adrenal in order to maintain blood corticoid concentration (Mountcastle, 1974). ACTH increases in the blood following bilateral adrenalectomy and injection of corticosteroids can lower ACTH (Tiptaft, 1975). These workers also found that noxious stimulus applied to adrenalectomized rats increased ACTH above the already elevated levels.

Electrical stimulation and lesioning of various parts of the brain has demonstrated that higher centers control secretion of ACTH. Stimulation of the reticular formation in the midbrain can increase release of ACTH (Taylor, 1969). Brown et al. (1974) also found increase

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in ACTH after stimulation of the amygdaloid nuclei, and cingulate gyrus of the frontal lobe in dogs, rabbits and monkeys. Stimulation of the septum, hippocampus, anterior hypothalamus and the midbrain can show a reduction in ACTH and adrenal hormones in most mammals (Mangili, Motta and Martini, 1966).

Lesions in the tuberal and median eminence regions blocks stress-induced ACTH release in all mammalian species tested (Brodish, 1963). In 1967, Usher, Kasper, and Birmingham found lesions in the lateral septal area or anterior cingulate gyrus blocked stress-induced corticosterone release in rats, but Brown, Uhler, Seggie, Schally, and Kastin (1974) found an increase in corticosterone release following stress in rats with septal lesions. Control of ACTH release may be related to the adrenergic system. Stimulation of the adrenergic system was found to block surgically-induced corticosterone release and injection of phenoxbenzamine (α-adrenergic blocker) into the lateral ventricle was found to augment the surgical stress response (Eisenberg, 1975). On the other hand, intraventricular injection of norepinephrine or dopamine had no effect on stress-induced corticosterone release. Administration of 6-hydroxdopamine intracerebrally (depletes brain catecholamines) was found to decrease only norepinephrine in adult rats and norepinephrine and dopamine in immature rats. These depletions

had no effect on the basal diurnal periodicity of ACTH or on responsiveness to stress (Kaplanski, van Delft, Nyakas, Stoof, and Smelik, 1974). Other investigators have found that catecholamines play an important role in ACTH secretion (Ganong, 1972). Abe and Hiroshige (1974) hypothesized that catecholamines may act to stimulate ACTH release but depletion of catecholamines, especially norepinephrine, had no effect on circadian rhythms or stress-induced release of CRF, ACTH or corticosterone (Abe and Hiroshige, 1974; Kaplanski et al., 1974). While it appears that brain catecholamines plays a role in CRF-ACTH secretion, they are not necessary for CRF, ACTH and corticosterone release in response to stress. Perhaps catecholamines are only one of the mechanisms by which CRF and ACTH are regulated and when depleted another mechanism such as the cholinergic system, compensates for it.

Although it is clear that the brain plays a role in ACTH secretion, adrenal corticosteroids can also control ACTH secretion through negative feedback. In rats administration of adrenal corticoids can block the response to stress (Sawyer, 1975). There is a linear relationship between the amount of corticosteroid given and the inhibition observed (Donovan, 1970). Synthetic glucocorticoids such as dexamethasone also block the stress response (Euker, Meites, and Riegle, 1975).

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Adrenal hormone implants have been used to locate areas within the brain where corticosteroids feedback to inhibit ACTH release. Application of cortisone in agar to the tuberal region of the hypothalamus inhibits the secretion of adrenal corticosteroids in rats and cats, while similar implants in the pituitary proved ineffective at blocking ACTH release (Mangili, Motta, and Martini, 1966). Corticosterone implanted in the hypothalamus of rats between the optic chiasm and median eminence was most effective at suppressing ACTH release. The inhibitory action decreased as the implant placement was made closer to the pituitary stalk (Smelik, 1969). Evidence for short-loop feedback for ACTH at the hypothalamus was found by Mess and Martini (1968). These workers showed that ACTH implanted into intact and adrenalectomized rats depressed endogenous release of ACTH, and that CRF secretion following adrenalectomy was blocked by ACTH injection.

Corticotropic-releasing factor (CRF) was found in the median eminence (Shally et al., 1960; Riniker, Sieber, and Rittel, 1972). At one time vassopressin (ADH) was believed to be CRF, as administration of ADH increased the release of ACTH from the pituitary (McCann and Porter, 1969). However rats with diabetes insipidus secrete ACTH and corticosterone following stress. Also it was found that ethanol can block secretion of ADH without blocking ACTH release (Purves and Sirett, 1967). While many agents

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act to release ACTH, such as catecholamines (Abe and Hiroshige, 1974), and ADH (McCann and Porter, 1969) neither of these substances have been shown to be necessary for stress-induced ACTH and plasma corticosterone (Fuxe and Hokfelt, 1969; Abe and Hiroshige, 1974).

Control of LH Secretion

Although there remains some disagreement about the roles of the gonadotropins, it is now generally accepted that FSH stimulates growth of the follicle and LH is responsible for maturation and ovulation of the follicles. Ovulation occurs when LH levels are increased in remales whose ovaries contain mature follicles. LH is also responsible for production and secretion of estrogen from the maturing follicle and it is also necessary for progesterone secretion from corpora lutea in some species. In males FSH acts at the first stages of spermatogenesis and hypertrophies the seminiferous tubules. LH stimulates androgen production and indirectly supports spermatogenesis (Mountcastle, 1974; Ganong, 1971).

The events taking place during the four day estrous cycle of the rat have been summarized by Swartz (1968) as follows:

1. estrus-new follicles begin growing, possibly stimulated by the FSH and LH surge the day before
2. metoestrus and the first day of diestrus-follicle growth continues
3. second day of diestrus-LH begins to stimulate release of estrogen

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4. proestrus-estrogen peak followed by LH and FSH surge between 2:00 and 7:00 p.m. Follicles begin preovulatory swelling
5. ovulation at approximately 2:00 a.m. the morning of estrous.

Kobayashi, Hara, and Miyak (1968) found ovarian venous estrogen increased the morning of proestrus which was followed by increased LH later in the afternoon. Serum progesterone increased in parallel with LH (Swerdloff, 1972). 1972

In women LH is elevated for 24 hours during or just following estrogen elevation. In all cases studied the LH peak never occurred before the estrogen peak (Tamada, Tsukui, Araki, and Matsumoto, 1973). Swerdloff (1972) believes estrogen synergizing with progesterone triggers the preovulatory peak. Plasma levels of LH decreased within 2 to 4 hours following injection with Premarin® (an equine derived hormone complex including conjugated estrogens) in ovariectomized women (Tamada et al., 1973; Swerdloff, 1972). Within 24 hours postinjection LH increased, reflecting the decrease in injected estrogen. This is supported by the data of Odell et al. (1968) who found an FSH-LH peak within 24 hours after a single injection of progesterone. This demonstrates negative feedback mechanism between LH and estrogen. Injection of pentobarbital prior to ovulation can block ovulation in rats (Swartz, Cobb, Talley, and Ely, 1976; Tamada et al., 1973). In experiments using antisera to LH and estrogen,

pentobarbital was found to block progesterone secretion but not estrogen secretion. Even in the presence of anti-LH, LH can be detected in the blood, but not at concentrations that will cause ovulation. Estrogens ability to trigger LH may be a threshold phenomenon or the dynamic rate of change of estrogen may trigger LH release (Tamada et al., 1973).

Gonadotropin secretion is controlled by higher centers. The pituitary secretes little FSH or LH following pituitary stalk lesioning (Donovan, 1970). Ovulation could be induced by stimulation of the medial preoptic area and anterior hypothalamic area, although lesions of the medial preoptic area will block ovarian cycles, severing the posterior connections of the hypothalamus does not alter the estrous cycle in the rat (Everett, 1964). Isolation of the hypothalamus by use of the Halász knife, had no effect on male rat testes histology, but female rats estrous cycles ceased (Butler and Donovan, 1969). If the supra chiasmatic nuclei are included in the Halász knife isolated hypothalamic island, persistent vaginal cornification (estrus) was seen, otherwise persistent corpora lutea (anestrus or diestrus) is observed.

A two level concept of the control of gonadotropins secretion has been theorized. A basal level of secretion is mediated by the median eminence while the preoptic area is necessary for the production of gonadotropin surges in

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female rats (Butler and Donovan, 1969). Using the lesioning technique, FSH-RH has been demonstrated to be synthesized close to the paraventricular nuclei, and LHRH at the suprachiasmatic-preoptic and arcuate ventomedial region (Mess and Martini, 1968).

Estrogen, progesterone and testosterone can inhibit gonadotropin secretion (Yagi and Sawaki, 1973). A number of studies have provided evidence for feedback effects of steroids at the hypothalamus. Implants of estrogen in the arcuate nuclei of the hypothalamus caused atrophy of the gonads in rats (Lisk, 1960). Testicular atrophy follows implantation of testosterone at the median eminence in male rats (Davison, 1967). The anterior hypothalamus is necessary for compensatory hypertrophy following unilateral ovariectomy (Koves and Halász, 1969). Estrogen implantation at the hypothalamus was found to suppress the elevated serum LH following ovariectomy (McCann et al., 1964; Gay and Sheth, 1972). Ovariectomized rats with anterior hypothalamic lesions did not have elevated serum LH (Taleisnuk, 1961).

Estrogen can also stimulate the release of LH (Davison, 1969). A single injection of estrogen can facilitate LH secretion in prepubertal, adult, pregnant, and pseudopregnant rats (Yagi and Sawaki, 1973). Daily injection of estrogen can cause an LH and FSH surge on

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the fifth day of injection in ovariectomized rats (Swerdloff, Jacobs, and Odell, 1972).

Receptor cells for estrogen must be present in hypothalamic-hypophyseal cells for positive and negative feedback mechanisms to exist. These receptors need to be in contact with neurosecretory neurons that secrete gonadotropin releasing factors in the median eminence. Estrogen implants in the arcuate nucleus cuases ovarian atrophy (Lisk, 1960). Estrogen implants at the anterior hypothalamus or medial basal hypothalamus decrease hypothalamic LHRH activity and suppressed LH release following ovariectomy (McCann et al., 1964). Lesions in the suprachiasmatic area and medial basal hypothalamus abolished the compensatory hypertrophy following unilateral ovariectomy Halász et al., 1967). Estrogen responsive neurons have also been found in the medial preoptic and medial basal hypothalamus in castrated female rats (Yagi, 1973). The medial preoptic, anterior and medial basal hypothalamic areas are involved in estrogens negative feedback on gonadotropin release (McCann et al., 1964). Single unit response studies showed decreases in neural firing rates following estrogen implantation at the preoptic and medial basal hypothalamic areas (Yagi, 1973).

There is also evidence that the gonadotropins have negative feedback at neural control centers, independent of gonadol steroids as intermediaries. FSH and LH implanted

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into the hypothalamus depresses the pituitary LH and FSH content (Motta, Franchini, and Martini, 1969). LH also has the ability to decrease neuronal unit activity in the preoptic area and basal medial hypothalamus (Sawyer, 1970).

While stress is known to increase ACTH release its effects on gonadotropin secretion may partially depend on the sex of the animal involved (Euker, Meites, and Riegle, 1975; Neill, 1970; Shaar et al., 1975). In 1965, Christian, Lloyd, and Davis suggested LH secretion was inhibited by ACTH in female mice. Ether stress increased serum LH in intact male rats (Euker et al., 1975). Restraint stress had no effect on serum LH in male rats or estrous or diestrous female rats, but decreased serum LH in proestrus rats (Euker et al., 1973). Baldwin, Colombo, and Sawyer (1974) found moving rats from one room to another had no effect on serum LH of proestrus rats; or female rats primed with PMS and HCG, while ovariectomized rats primed with estrogen exhibited an increase in serum LH. LH is released in a pulsatile manner in ovariectomized rats and surgery, injection of ACTH, or corticosterone treatment does not alter this pulsatile release of LH but restraint stress will block it (Blake, 1975). Further evidence that stress does not need to act through ACTH or corticosteroids to decrease gonadotropins is observed in adrenalectomized female rats (McKay, Wood, and Riegle, 1976). Two hours of restraint stress on the afternoon of

proestrus could equally block ovulation in adrenalectomized and intact rats.

Control of Prolactin Secretion

While both suckling and cervical stimulation illicits prolactin release, the dominant role of the hypothalamus is inhibition of prolactin release. Severing the pituitary stalk increase prolactin release (Donovan, 1970). When pituitaries were transplanted to the kidney capsule gonadotropin secretion ceased and prolactin secretion increased. Return of the pituitary from the kidney capsule to the median eminence reestablished gonadotropin secretion, decreased prolactin release, and returned ovarian cyclicity (Mess and Martini, 1968). Estrogen enhances prolactin release while injections of testosterone, progesterone, and in some cases cortisol and corticosterone do not affect prolactin release (Meites, 1967). Reserpine, and perphenazine which increased prolactin release, induces pseudopregnancy in rats and reduces the amount of norepinephrine at the brain (Eisenberg, 1975; Chatterton, 1975). The CNS dopaminergic system has been implicated as PIF (prolactin inhibiting factor). Antidopaminergic drugs can enhance prolactin secretion (Shaar, 1975). Prolactin is markedly affected by various types of stress (Wakabayashi et al., 1971; Wuttke et al., 1971; Ajika et al., 1972; Linke et al., 1972; Euker et al., 1973). Ether stress and blood sampling increased serum prolactin in male rats,

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decreased serum prolactin in proestrus rats and rats the afternoon of estrus and had no effect on diestrus or rats the morning of estrus (Riegle and Meites, 1976; Euker et al., 1975; Bellinger and Mendel, 1975). Two hours of restraint stress increased serum prolactin in diestrus, and castrated female rats and intact male rats, and decreased serum prolactin in proestrus and estrous rats (Euker et al., 1973; Riegle and Meites, 1976). Harms, Langlier, and McCann (1975) found dexamethasone could block ether stress or disturbance stress induced prolactin release while adrenalectomy augmented the release of prolactin. Serum prolactin stabilized three days following ovariectomy (Lawson and Gala, 1974) and stress by anesthetic or restraint increased prolactin release. Prolactin is considered luteotrophic following ovulation in rats (Chatterton, Chien, Ward, and Miller, 1975) and is capable of promoting progesterone secretion.

Role of Catecholamines in Controlling Hypothalamo-Hypophyseal Secretion

The presence of catecholamines in the central nervous system was demonstrated in 1946 by von Euler, which he attributed to brain vasomotor nerves. In 1950 Sawyer suggested that catecholamines in the brain functioned as neurotransmitters, or humoral transmitters to convey information from the hypothalamus to the anterior pituitary (Hiroshige and Abe, 1973). In 1970 Wurtman described six

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possible mechanisms of action brain catecholamines may use to regulate hypothalamic secretion:

1. As neurotransmitters in a tract that provides an input to hypophysiotropic cells in the hypothalamus.
2. As neurotransmitters that cause the hypothalamic cells to secrete releasing hormones.
3. Act within the hypophysiotropic cell to release releasing hormones.
4. Act as neurohormones directly on the anterior pituitary.
5. Catecholamines delivered to the pituitary through the general circulation and modify secretion of releasing hormones, and
6. Catecholamines alter sensory input to the brain, thus altering secretion of releasing hormones secondarily.

Intrapituitary and intracerebral injections of various catecholamines have supported or disputed Wurtman's hypothesis. In recent years hypothalamic neurotransmitters, particularly the catecholamines have been intimately linked to the control of hypothalamic factors regulating gonadotropin and prolactin secretion. However, the role of the neurotransmitters on ACTH regulation is less clear. Intrapituitary injection of norepinephrine does not affect ACTH release, while L-dopa and epinephrine could increase ACTH release although not significantly (Hiroshige and Abe,

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1973). Serotonin and acetylcholine had no ability to release ACTH, while histamine released ACTH and spermidine proved a potent releasing agent of ACTH (Pearce et al., 1969). In 1972 Ganong hypothesized adrenergic inhibitory, cholinergic stimulatory in CRF regulation. Intraventricular injection of norepinephrine, dopamine, serotonin, and carbachol increase serum corticosterone (Abe and Horoshige, 1974). Treatment with reserpine and 6-hydroxydopamine depletes brain catecholamines but had no effect on circadian rhythms or the stress response (Abe and Hiroshige, 1974; Kaplanski, van Delft, Nyakas, Stoof, and Smelik, 1974).

Palkovits, Kobayashi, Kizer, Jacobowitz, and Kopen (1975) reported that formalin injection, restraint and cold stress decreased norepinephrine and dopamine content of the arcuate nucleus and repeated restraint stress increased tyrosine hydroxylase at the arcuate nucleus which increases catecholamine synthesis. The data presented does not totally support Ganong's (1972) hypothesis that adrenergic neurons in the hypothalamus are inhibitory for ACTH release as depletion of norepinephrine or dopamine did not affect ACTH release. While catecholamines may actually increase plasma corticosterone (Abe and Hiroshige, 1974), depletion of brain catecholamines by reserpine or 6-hydroxydopamine or elevation of brain catecholamines had no effect on circadian rhythms of ACTH or corticosterone,

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MATERIALS AND METHODS

Experimental Animals

All of the Long-Evans rats (Blue-Spruce Farms, Altamont, N.Y.) used in these experiments were bred and raised within the colony at the Endocrine Research Unit at Michigan State University. The rats were housed in a temperature controlled (22°C ± 1°C) environment, in groups of three or four, with a 12:12 light:dark schedule, having lights on at 6 a.m. and lights off at 6 p.m. They were fed a commercial laboratory rat diet (Wayne Lab Blox) and water ad libitum.

The age of the rats used ranged from 3 to 5 months. This age group is considered to be the peak of reproductive performance. Vaginal cytology was monitored daily to determine the reproductive status of the female rats used in this study. Female rats were only used if they showed consistent and regular 4 or 5 day estrous cycles.

Blood Collection

All blood samples were taken under light ether anesthesia via suborbital sinus puncture within 40-60 seconds of initial cage disturbance. This procedure has

previously been shown to minimize the effects of stress on blood hormone concentrations (Euker et al., 1975). Between 1.0 and 1.5 ml of blood was collected from female rats and 1.5 to 2.0 ml from male rats. The blood was allowed to clot at room temperature for 30 to 45 minutes, refrigerated at 4°C overnight and then centrifuged for 10 minutes at 3000 rpm in a refrigerated centrifuge (Sorvall RC 2-B or RC-5). The serum was removed, frozen and stored until assayed for prolactin, LH or testosterone.

Restraint Stress

Restraint stress was achieved by first lightly anesthetizing the rats with ether and then securing them in a supine position on a stainless steel laboratory table by use of masking tape. Animals from the stress groups were restrained in this fashion for two hours each morning and afternoon for two weeks.

Weights

The rats were weighed before any experimentation and at the end of the first and second week of the stress regime.

Radio-Immunoassay for LH and Prolactin

All serum samples and pituitary homogenates were assayed for LH and prolactin. LH and prolactin was measured by double antibody radioimmunoassay as described

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by Monroe et al. (1968) and Niswender et al. (1969) and performed routinely in the laboratory of Dr. J. Meites.

Purified rat prolactin (H-10-10-B-PRL) and LH (LER 1056 LH) were radioiodinated at Dr. J. Meites laboratory with I^{125} on a 1 x 15 cm Bio-Gel P-60 column. The elutant was diluted to a concentration of 30,000 counts per minute per 100 µl with 1% gel PBS as counted on an automatic gamma well counter (Nuclear Chicago, Des Plaines, Ill).

Antiovine LH antiserum was supplied by Dr. G. Niswender (Colorado State University, Fort Collins, Colorado). It was prepared by immunization of rabbits with pituitary ovine LH hormone and was diluted to a 1:28000 concentration. The antirat prolactin antiserum was prepared similarly using purified prolactin and was diluted to a 1:5000 concentration. Antigen-antibody complexes were precipitated using sheep antiserum from specific immunization against rabbit gamma globulin. This ovine anti-rabbit gamma globulin was used at an effective concentration which ranged from 1:35 to 1:60 dilution.

LH and prolactin radioimmunoassays are identical in procedure and differ only in the labeled hormone and specific rabbit antisera. Duplicate aliquots of the pituitary homogenate and incubation media were run at two dilution volumes. Appropriate serum aliquots were estimated from preliminary data. In all cases duplicate samples were assayed if sufficient serum was available.

All unknown samples were placed in 12 x 75 mm disposable culture tubes (diSPo®, Scientific Products, McGraw-Park, Ill.) and made up to a volume of 0.5 ml using 1% gel PBS. Two hundred µl of either LH or prolactin antisera was added, the tubes were vortexed and allowed to equilibrate for 24 hours at 4°C. Then 100 µl of the appropriate radioiodinated hormone was added, mixed and allowed to equilibrate at 4°C for 24 hours. A final incubation of 72 hours at 4°C followed addition of 200 µl of the ovine anti-rat gamma globulin, which precipitates the antigen-antibody complex. All the tubes then received 3 ml of cold 1% gel PBS and were centrifuged for 20 minutes at 22500 x g. The supernatant was poured off and the tubes allowed to drain upside down for 5 to 10 minutes. Care was taken to keep the tubes chilled at all times during this procedure to prevent the precipitate from sliding down the tube wall. The tubes were then counted in the automatic gamma counter.

Included in each assay were the reference standards, total count tubes, total antibody binding tubes and nonspecific binding tubes. Total count tubes received only 100 µl of the labeled hormone and represented the radioactive count recovery. Nonspecific binding was measured by using 200 µl of rabbit serum (NRS) diluted with 1% gel PBS in place of specific hormone antibody. Total antibody binding was determined by using 1% gel PBS instead of

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unknown serum. Reference standards consisted of 5 sets of 15 different quantities of purified NIH rat PRL-RP-1 or NIH-LH-RP-1. Counting time was calculated so that total antibody binding minus nonspecific binding equalled 10,000 counts. Background counts were determined by the non-specific binding tubes and was automatically subtracted from the unknown tube counts by the gamma counter. Standard curves were drawn on 3 cycle semi-log paper plotting counts per minute against the log reference standard concentration. Counts from the unknown samples were then translated into nanogram amounts of hormone and then transformed into ng/ml.

Competitive Binding Assay for Testosterone

Serum testosterone was determined by a single antibody competitive binding assay (Mongkonpunya, 1973, Michigan State University, East Lansing, Michigan). Serum aliquots of 100 µl were extracted with 2 ml Benzene: Hexane (1:1) in 17 x 100 mm DiSPo culture tubes, mixed for 30 seconds and frozen at 0°C for 1 hour. The solvent portion was poured off from the frozen aqueous portion into 12 x 75 mm disposable culture tubes and dryed down under air. A 0.5 aliquot of antitestosterone (diluted 1:3000) was added and mixed. The mixture was allowed to equilibrate at room temperature for 30 minutes, and then 100 µl tritium labeled testosterone (H^3-2,4,7,8,-testosterone, New England Nuclear) was added, mixed, and allowed to

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equilibrate for 18 hours at 4°C. One ml of charcoal with dextran (250 mg charcoal, 25 mg dextran, 100 ml of 1% gel PBS) was added to precipitate the unbound hormone. This was mixed for 10 seconds and chilled on ice for 10 minutes and then centrifuged at 3000 rpm for 10 minutes in a refrigerated centrifuge. Using a repipet diluter (Lab Industries, Berkeley, Calif.) 0.5 ml of the supernatant was removed and added to 10 ml scintillation vials and counted in a automatic beta counter for 4 minutes (Chicago, Nuclear). Reference standards were prepared using a purified bovine testosterone preparation. Three standard curves, consisting of 10 different concentrations were included in each 100 tube run, one at the beginning, the middle and the end.

Blank tubes, total count tubes, recovery tubes and the standard serum tubes were also included so that a correction factor could be determined to compensate for procedural loss at extraction and for background counts. Nonspecific binding was determined by using 100 µl of a pooled rat serum, which is extracted and dried. One hundred microliters (100 µl) of 1% gel PBS buffer was added instead of antibody and the remaining procedure was unchanged. Blank tubes receiving buffer instead of serum or reference standard were used to determine total antibody binding. Total counts were determined by adding 10 µl

of 4 labeled tritium testosterone to scintillation vials, plus 10 ml of scintillation counting cocktail.

Extraction recovery tubes were made with 100 µl pooled rat serum, and 10 µl of 2 labeled tritium testosterone. These were extracted and the solvent phase poured off into scintillation vials, dried down and 10 ml of counting cocktail added and counted.

Log hormone concentration was plotted on three cycle semilog paper against counts per minute. The counts from the serum unknown were then translated to nanograms and multiplied by the correction factor to get nanograms per ml serum.

Catecholamine Assay

Hypothalamic catecholamine was performed at Eli Lily Company (Indianapolis, Indiana) in Dr. James Clemens' laboratory under the direction of Dr. Carl Shaar. A modification of the microfluorescent technique of Laverty and Taylor (1968) was used to measure dopamine and norepinephrine. Seven to nine hypothalamic extracts from control and stress groups from male and female experimental groups were used for norepinephrine quantification, while hypothalamic extracts from control and restraint animals from male experimental groups were used for measurement of dopamine. Individual frozen hypothalamic extracts were thawed, and transferred to a beaker with 2 ml of 1 M sodium acetate buffer and 1 ml 1% disodium EDTA. Heat activated,

neutral grade 1 alumina (240 mg) were added and the mixture swirled for 5 minutes. The supernatant was poured off and discarded. The alumina-catecholamine mixture was applied to a microcolumn containing 140 mg of alumina. The column was washed 3 times with triple distilled water, and then the catecholamines eluted with 5 ml of 0.2 N acetic acid into evaporating tubes. These were evaporated to dryness, and the residue resuspended in 0.4 ml of triple distilled water and catecholamine determination were made by acid fluorescence using a system of reverse blanks and an internal standard. Ten microliters (10 µl) of phosphate buffer at pH 6.52, 10 µl of 0.02 N iodine and 50 µl of alkaline sulfite solution were added to 100 µl of the resuspended extract. After 5 minutes 30 µl of glacial acetic acid were added to develop the fluorescence. Norepinephrine fluorescence was read at excitation wave length of 392 mµ and emission of 490 mµ. Dopamine fluorescence was measured following 40 minutes of heating at 100°C at 320 mµ excitation and 380 mµ emission.

Experimental Design

The experiment was designed so that endocrine parameters could be monitored or challenged while the rats were undergoing a stress regime. The design can be divided into three general areas:

1. response to stress over a two week interval,

2. *in vivo* response to LHRH challenge before and after chronic stress,
3. *in vitro* response to LHRH challenge after chronic stress.

The design was basically the same for both male and female groups. Animals were selected by age, weight, and for the females, regularity of estrous cycles. This was to ensure that regular cyclicity did exist prior to experimentation and that change in cyclicity could be attributed to the stress regime. Minimum weight for the males was 225 grams and for the females was 195 grams. Minimum weights were selected to ensure a greater homogeneity of size in the groups and to ensure that no animal was too small to undergo the repetitive blood sampling. Mean weights taken before and during the stress regime helped monitor the effect of stress on the rats.

Each experimental group was divided into two sections, one acting as control animals and the other as the stress group. Both groups were subjected to *in vivo* LHRH challenge before and after the stress treatments, weekly blood samples and the *in vitro* LHRH challenge. Stress was achieved by restraining the rats as previously described.

Model of the experimental design is as follows:

1-2 weeks before stress	Selection of rats by age, weight and vaginal cytology. First *in vivo* LHRH challenge.
Day 1	First day of stress, blood sample from controls and following 2 hours of stress.
2	
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6	Weigh rats
7	Blood samples before and following stress, and from controls.
8	
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12	Weigh rats
13	Second *in vivo* LHRH challenge.
14	Blood samples following 2 hours of stress; *in vitro* LHRH challenge.

Definition of Terms

C.R.	Chronic Restraint Group
♂	male
♀	female
I	first experimental group
II	second experimental group
III	third experimental group

In vivo LHRH Challenge

Before beginning the stress regime, all animals from both control and stress group were challenged with an intravenous (i.v.) injection of synthetic LHRH (Eli Lily Inc., Indianapolis, Ind.). LHRH challenge occurred in the afternoon for both male and female groups. Female rats were subjected to the first LHRH challenge only if they were in the diestrus stage of the estrous cycle.

Following a control blood sample either 100 or 500 ng LHRH was injected via an exposed jugular vein. Serial blood samples were taken at 15, 30 and 60 minutes or 15 and 45 minutes postinjection. Following injection and bleedings rats were returned to their cages. The same process was repeated on the afternoon of day 12 or 13 of stress.

In vitro LHRH Challenge

Following the final morning of stress animals from both the stress and control groups were decapitated, and their hypothalami and anterior pituitaries were removed. Control rats were decapitated within 30 seconds of initial cage disturbance, while stress rats were decapitated immediately following removal from the stress table. This was done in a room adjoining the colony to minimize stress effects on basal levels of pituitary hormone in the control animals, since this type of disturbance has been shown to alter serum concentrations of prolactin and LH in male rats.

Hypothalamic Extract Preparation

The top of the skull was removed and the brain was teased from the cranial cavity. The "hypothalamic island" was located via the optic nerve tract on the ventral surface. Iris scissors were used to remove this island at a depth of 2 mm, and weighed to the nearest half milligram. The hypothalami were hand homogenized in a total of 0.4 ml cold 0.4 N perchloric acid and then centrifuged for 20 minutes at 20,000 rpm in a refrigerated ultra-centrifuge (Sorvall RC2-B or RC-5). The supernatant was removed and placed in 12 x 75 mm disposable culture tubes with 0.1 ml EDTA, and then frozen and stored for catecholamine assay.

Anterior Pituitary Collection and Incubation

The anterior pituitary (AP) was teased from the base of the cranium and placed in a petri dish on a piece of filter paper soaked with medium 199 (1.1% Medium 199, Difco Laboratories, Detroit, Mich.). The bilobar AP was bisected at the isthmus with a scalpel blade and each half was placed in a 12 x 75 mm diSPo culture tube containing 2 ml of medium 199 that had been previously incubated in a Lab-line shaker water bath at 37°C with 5% CO_2 in O_2 at a rate to maintain a pH of 7.25-7.35. The tubes were returned to the water bath at the same condition for one hour. At the end of one hour the incubation-media was poured off and the last remaining drops wiped from the

tube. The odd number tubes, containing one half of each AP from the stress and control rats, 2 ml of medium 199 was added, and acted as control or untreated AP half. The even number tubes containing the remaining AP half were treated with 5, 25, 50 or 100 μg LHRH and medium 199 to 2 ml. The tubes were returned to the water bath and incubated for 4 hours. The media was removed and diluted 1:10 with phosphate buffered saline (PBS) with 1% gelatin. This solution was assayed for prolactin and LH released. Pituitary halves were weighed and homogenized in a total of 1.0 ml 1% PBS and then further diluted to 0.5 ml PBS/ mg pituitary. This solution was prepared for LH and prolactin assay by dilution 1:40 with 1% PBS.

Plasma LH, Prolactin and Testosterone Response to Stress

Blood samples were taken at intervals to monitor plasma LH, prolactin, and testosterone concentration as affected by chronic stress. Blood samples were taken before and after stress on days 1, 7 and 13 of the treatment regime. Control animals were bled only once on the same days. Pre-injection samples from the *in vivo* LHRH challenges were used as the control samples for days 1 and 13; this was done to minimize stress to the control animals.

Statistical Analysis

Pituitary content, pituitary release and serum hormone concentration were analyzed for drug and stress treatment by analysis of variance. A critical alpha probability value of 0.05 was selected. In studies where experimental factors were determined to be significant, the differences among the means were tested by the least significant difference test (LSD) (Sokal and Rohlf, 1969). In some cases the student t test alone was used.

RESULTS

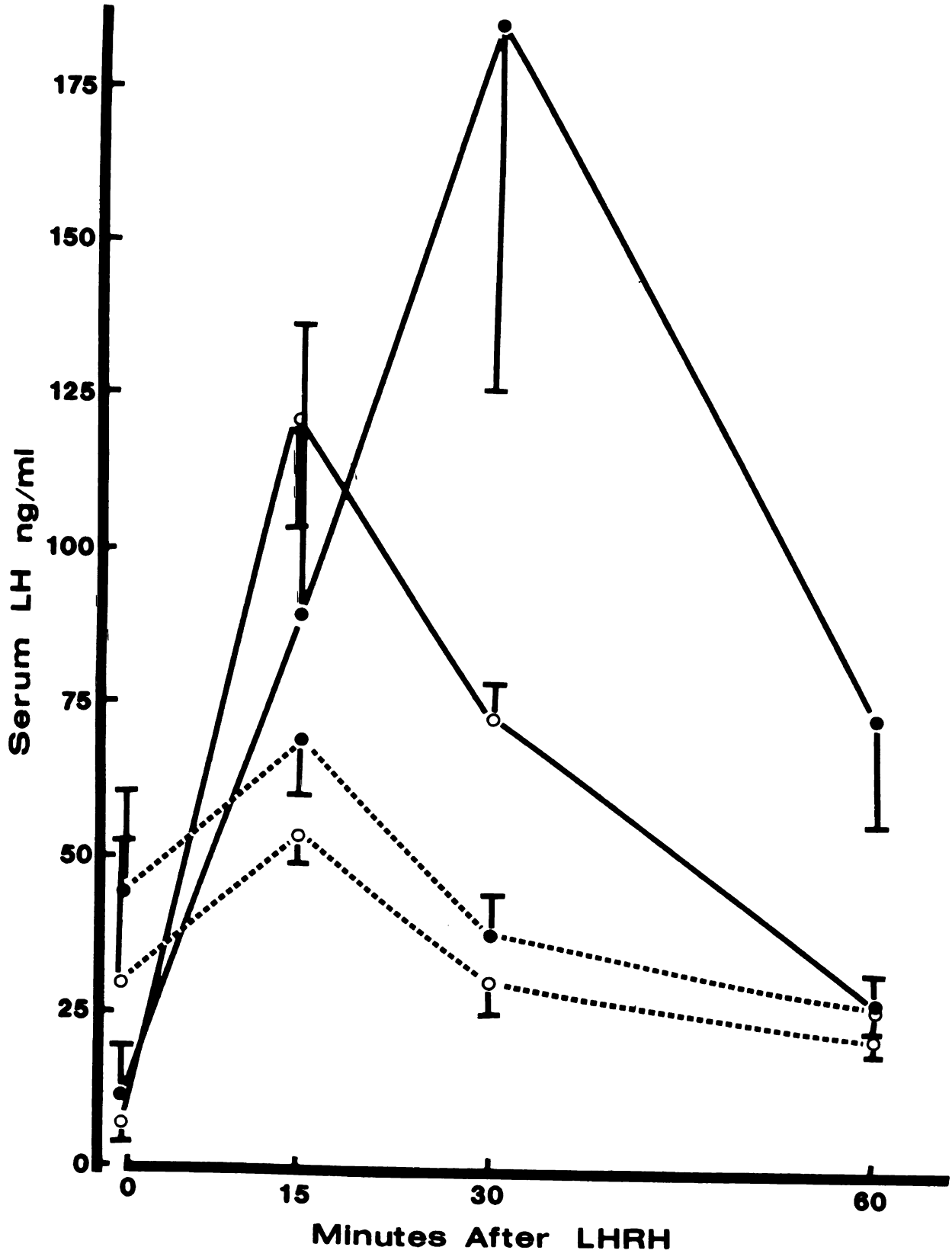
LH Data

In Vivo Pituitary LHRH Challenge

Two doses of LHRH, 100 ng and 500 ng, were given to the first experimental male group (C.R.♂ -I). Blood samples were taken before and 15, 30 and 60 minutes post-injection. This experimental group was subjected to the in vivo LHRH challenge only on day 10 of the stress regime. A dose response was observed as both stress and control groups had a greater response to the 500 ng dose of LHRH than to the 100 ng dose (Figure 1). Preinjection value for the 100 ng control group was 43.51 ± 17.86 ng LH/ml serum and 11.96 ± 7.09 ng LH/ml serum for the 500 ng LHRH control group. Resting values for the restraint groups were 29.75 ± 23.41 and 7.09 ± 2.10 ng LH/ml serum for the 100 ng and 500 ng LHRH doses respectively. Although the restraint groups arithmetic means are less than those of the control group, the differences were not significant. The response to the 100 ng LHRH dose did not differ significantly from the control to the restraint group. Peak response occurred at 15 minutes postinjection at

Fig. 1.--Effects of 100 ng and 500 ng LHRH on serum LH in young male rats--C.R.♂-I.

Mean serum LH concentrations expressed as ng/ml and their standard errors appear on the ordinate as a function of a time relative to injection of 100 or 500 ng LHRH. Blood samples were taken before injection (0 time) and 15, 30, and 60 minutes after intravenous LHRH injection. Solid circles represent nonstress control animals, while open circles signify chronically restrained rats. The broken line represents the 100 ng LHRH dose and the solid line the 500 ng LHRH dose.

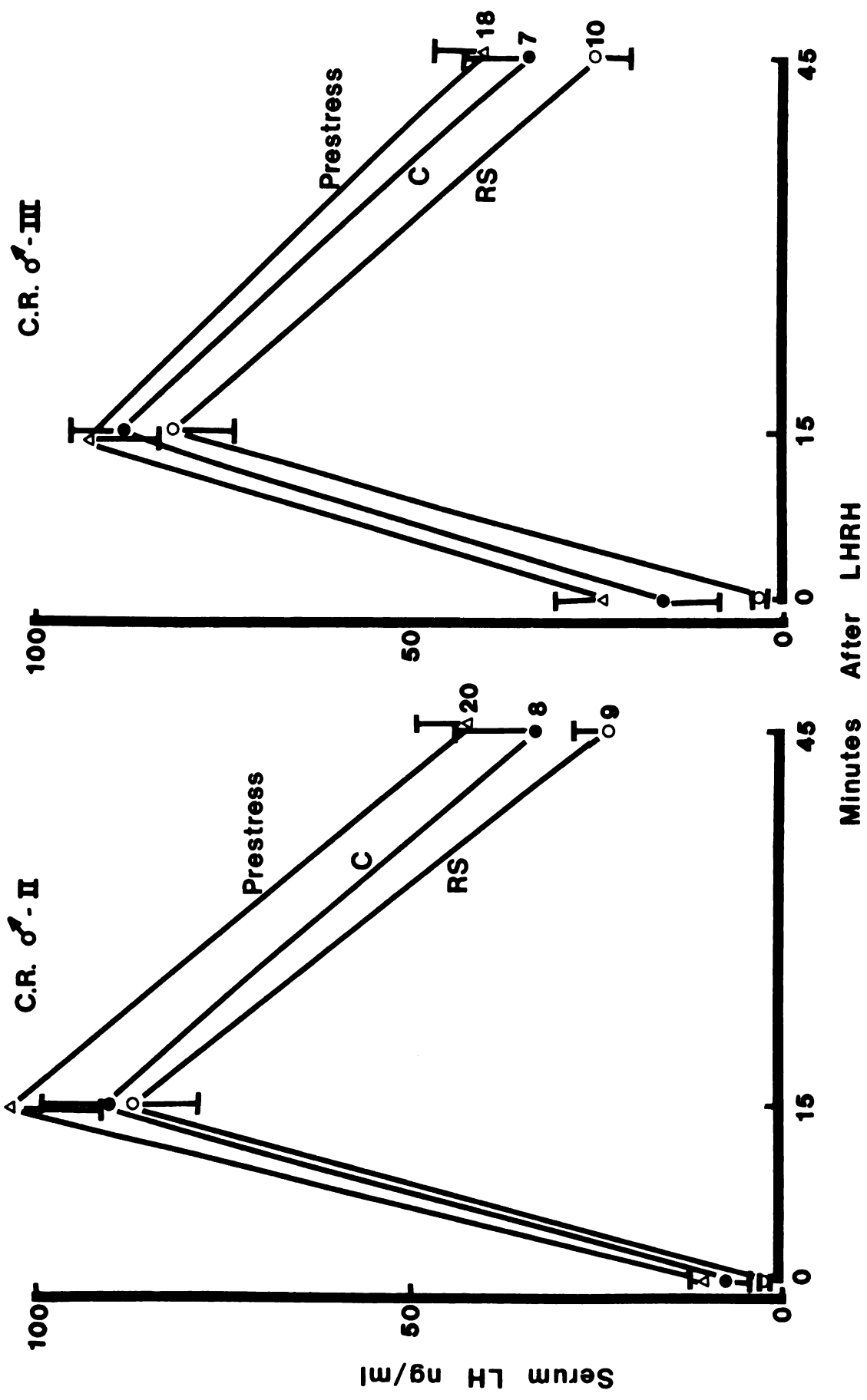

175
150
125
100
75
50
25
0
Serum LH ng/ml
0
15
30
60
Minutes After LHRH

68.50 ± 8.19 and 54.0 ± 5.08 ng LH/ml serum for the 100 ng LHRH control and stress groups. By 60 minutes both control and stress rats serum LH had returned to resting levels. The response to the 500 ng LHRH dose did not differ significantly at 15 minutes from control to stress group, the control group having 92.88 ± 43.69 ng LH/ml serum and the restraint group 122.17 ± 17.34 ng LH/ml serum. The stress groups peak response was measured at the 15 minute bleeding while the control group peak response was measured at 30 minutes at 185.44 ± 58.77 ng LH/ml serum. The value from the stress group at 30 minutes was 73.63 ± 4.50 ng LH/ml serum which was significantly less than control value at the same time. By 60 minutes the restraint group had returned to the equivalent of resting levels while the control group still had elevated levels, 73.69 ± 17.53 ng LH/ml serum.

Since the preliminary data suggested that there were differences in stressed male rats response to 500 ng LHRH the experiment was repeated in duplicate (C.R. ♂ -II, C.R. ♂ -III, Figure 2). Blood samples were taken before and 15 and 45 minutes postinjection. In both C.R. ♂ -II and C.R. ♂ -III experimental groups stress had no effect on the pituitaries ability to respond to a bolus injection of LHRH. The prestress LHRH challenge response did not differ significantly from control or stress response at any of the time sampled. While the control rats resting

Fig. 2.--Effects of 500 ng LHRH on serum LH in young male rats--C.R.♂-II and C.R.♂-III.

Mean serum LH concentrations (ng/ml) and their standard errors appear on the ordinate as a function of time after injection. Blood samples were taken before injection (0 time) and 15 and 45 minutes postinjection. Solid circles represent control animals, open triangles represent pre-stress values and open circles represent chronically stressed rats following injection with 500 ng LHRH. The numbers indicate the number of animals per group.

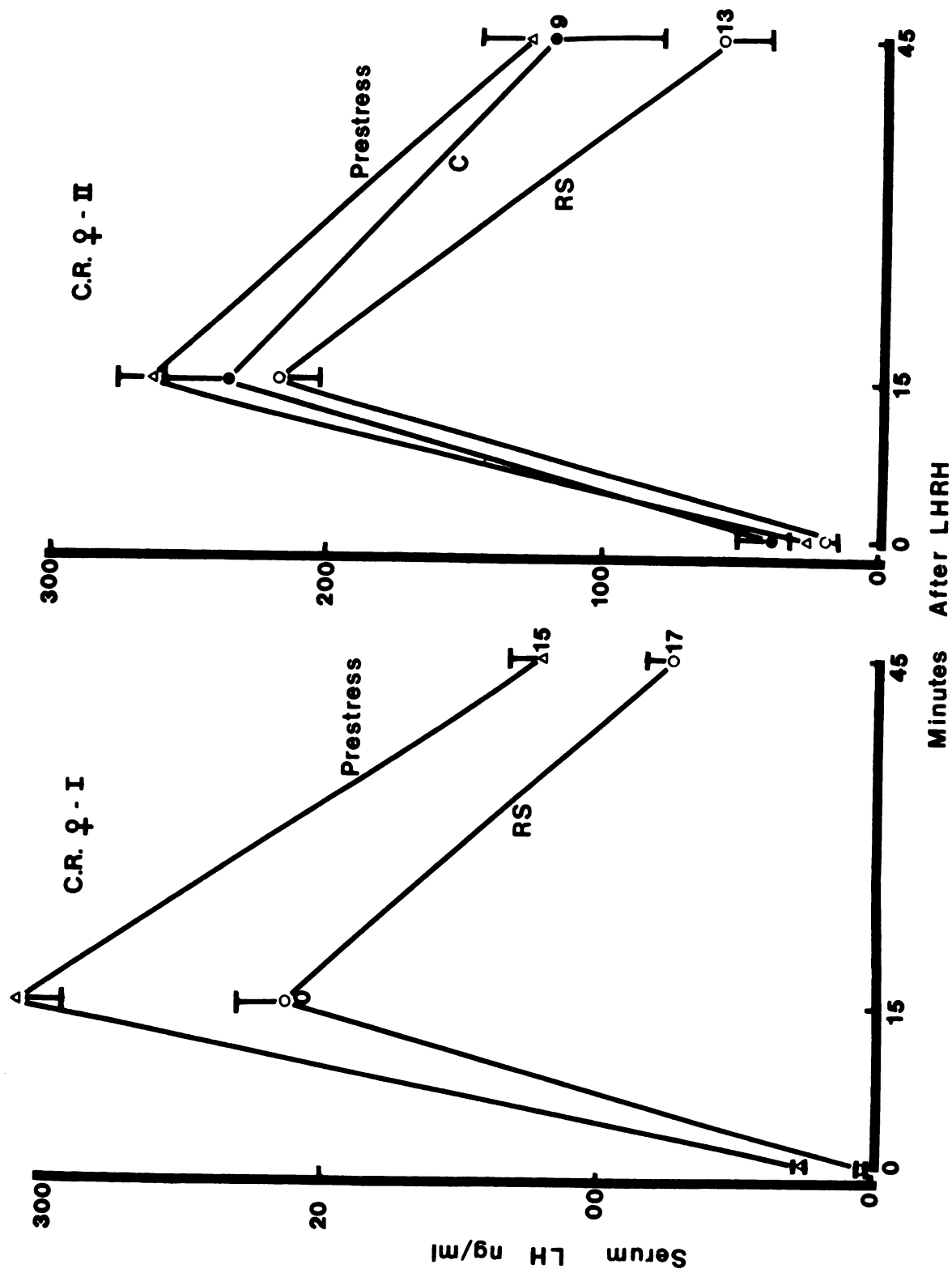

C.R. ♂- II
C.R. ♂- III
Prestress
C
RS
Serum LH ng/ml
Minutes After LHRH
100
50
0
0
15
45
20
8
9
18
7
10

levels of LH were 8.37 ± 2.95 and 16.74 ± 7.28 ng LH/ml serum for C.R. ♂ -II and C.R. ♂ -III respectively, the stress groups resting levels were 3.33 ± 0.0 and 4.09 ± 0.39 ng LH/ml serum for the two groups. Although the resting levels of the stress group were less than the controls there was no significant difference. There was also no significant difference in peak values measured at 15 minutes as controls peak values were 87.81 ± 9.09 and 89.14 ± 6.44 ng LH/ml serum for C.R. ♂ -II and C.R. ♂ -III and 90.17 ± 8.84 and 82.40 ± 8.27 ng LH/ml serum for the two stress groups. The 45 minute postinjection serum concentration also showed no alteration in the pituitaries ability to respond to LHRH. In both control and stress animals from both experimental groups serum LH was returning towards resting levels. Although the resting serum LH concentrations were suppressed by the chronic stress, the pituitaries ability to respond to LHRH is unaltered.

Less consistent data came from the 2 female experimental groups (Figure 3). Only the restraint animals from the first group (C.R. ♀ -I) were challenged with LHRH, once before the stress regime and then on day 13 of stress. For the first LHRH challenge all of the rats were diestrus day one of their estrous cycle, and were in constant diestrus following chronic stress for the second LHRH challenge. Resting serum LH concentrations were 26.10 ± 3.47 ng LH/ml serum before the stress regime and had

Fig. 3.--The effects of LHRH on serum LH concentrations in young female rats.

Mean serum LH concentrations (ng/ml) and their standard errors are plotted on the ordinate as a function of time in control and chronically stressed female rats. Blood samples were taken before injection (0 time) and at 15 and 45 minutes postinjection. Open triangles represent prestress from LHRH challenge before the stress regime. The solid circles indicate control animals and open circles the stress rats following stress treatment. The number of rats in each group are indicated.


C.R. ♀ - I
C.R. ♀ - II
Prestress
RS
C
Serum LH ng/ml
Minutes After LHRH
300
200
100
0
15
45
13
9
15
17

dropped significantly to 3.78 ± 0.29 ng LH/ml serum after 13 days of stress. Peak values were measured with the 15 minute blood sample. The prestress peak value of LH was 312.60 ± 18.40 ng LH/ml serum. Following 13 days of stress the peak value was only 212.90 ± 19.23 ng LH/ml serum, which is significantly less than the prestress peak value. Forty-five minutes postinjection the prestress values decreased to 73.40 ± 10.0 ng LH/ml serum.

In the second group, C.R. ♀ -II, the LHRH challenge gave somewhat differing results. The control, prestress and stress groups resting serum LH concentrations did not differ significantly. Resting LH levels for the control group were 38.06 ± 12.23 ng LH/ml serum and 21.01 ± 6.66 ng LH/ml serum for the stress groups. Peak values of LH following LHRH injection were measured at 15 minutes post-injection in both control and stress groups. There was no significant difference between peak values following 2 weeks of stress as controls had 237.78 ± 23.26 ng LH/ml serum and the stress group 219.68 ± 16.43 ng LH/ml serum. At 45 minutes postinjection the stress group had significantly less LH than the control group. Control rats serum LH values at 45 minutes were 120.34 ± 39.61 ng LH/ml serum while the stress groups peak value was 60.22 ± 18.54 ng LH/ml serum. In both chronic restraint female groups, stress decreased the pituitaries ability to release LH by LHRH

stimulation by either decreasing the maximal response or duration of response.

In Vitro Pituitary LHRH Challenge

The anterior pituitary halves from C.R.♂ -I were incubated with either 0, 25 or 100 ng LHRH. Both of the doses of LHRH stimulated release of LH maximally, so in C.R.♂ -II and C.R.♂ -III 5 and 50 ng of LHRH were used to incubate the anterior pituitaries.

Pituitary content of LH was not affected by LHRH or stress treatment. Control animals from C.R.♂ -I without LHRH treatment had 84.15 ± 7.70 µg LH/mg pituitary tissue, pituitaries treated with 25 ng LHRH contained 90.20 ± 4.54 µg LH/mg pituitary and pituitaries treated with 100 ng LHRH had 81.55 ± 4.23 µg LH/mg pituitary tissue (Figure 4). Stress animals had 85.67 ± 2.82, 79.77 ± 4.55 and 80.56 ± 5.60 µg LH/mg pituitary after treatment with 0, 25, and 100 ng LHRH respectively. While pituitaries from C.R.♂ -II and C.R.♂ -III contained approximately one half as much LH as rats from C.R.♂ -I, they also showed the consistent LH content regardless of LHRH dose or stress.

LH released from the anterior pituitary halves incubated with LHRH showed a dose response relationship (Figure 5). Pituitary halves treated with 5 ng LHRH released significantly more LH than those receiving none.

Fig. 4.--The effect of LHRH on pituitary content on LH in young male rats.

The height of each bar represents mean LH concentration in the pituitary (μg LH/mg pituitary tissue) with brackets corresponding to the standard error of the mean. Each bar indicates pituitary content of LH after treatment with LHRH. Open bars represent pituitary halves receiving no LHRH treatment, lined bars represent either 5 or 50 ng LHRH treatment, and shaded bars indicate 50 or 100 ng LHRH treatment. Numbers at the base of the bars indicate the numbers of animals per group.

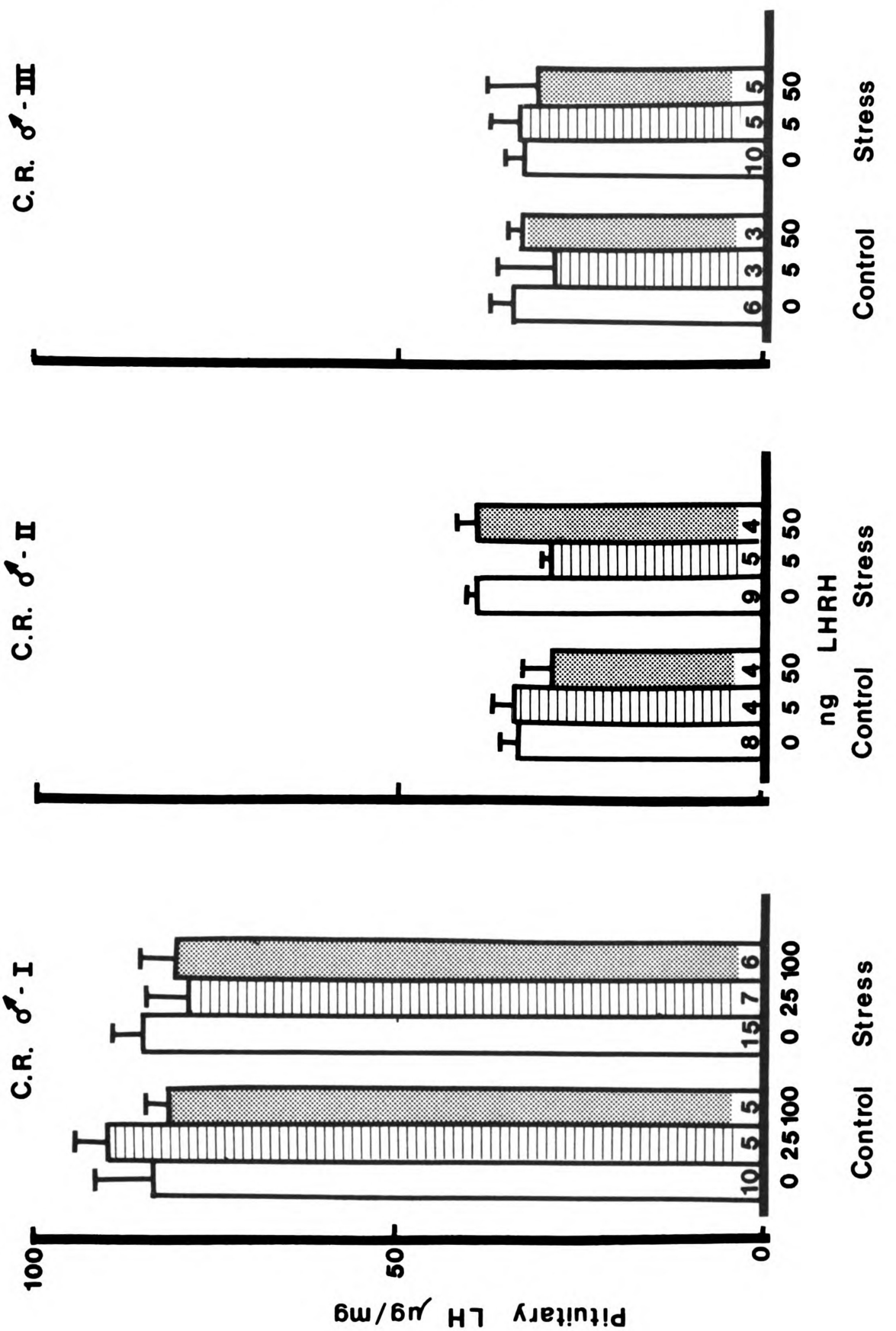
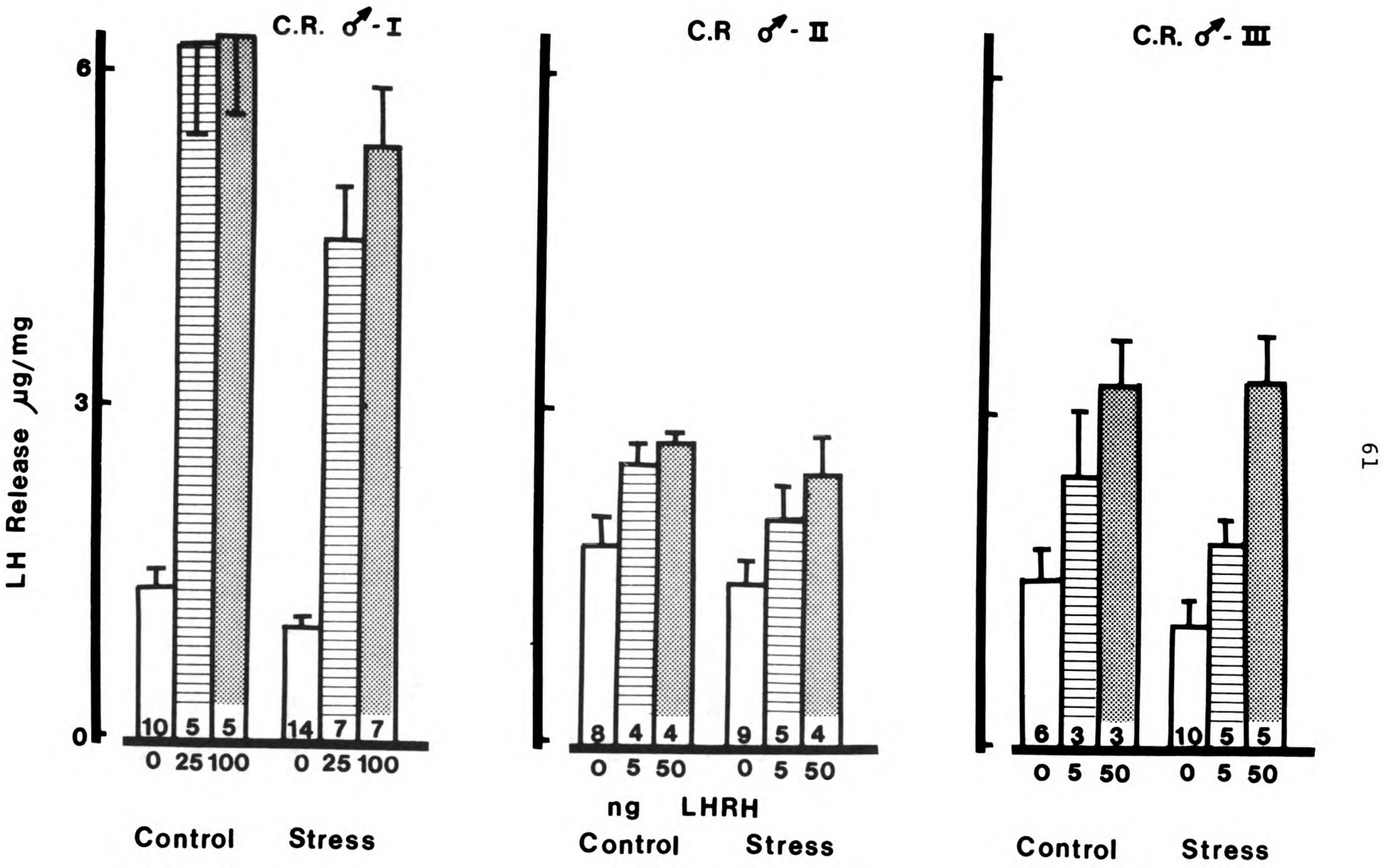

C.R. ♂- I
C.R. ♂- II
C.R. ♂- III
Pituitary LH µg/mg
100
50
0
0 25 100
0 5 50
Control
Stress
ng LHRH

Fig. 5.--The effect of LHRH on pituitary release of LH in young male rats.

The height of each bar represents mean LH released from the pituitary expressed as μg/mg pituitary tissue, with brackets corresponding to the standard error of the mean. Each bar indicates pituitary release of LH after treatment with LHRH. Open bars represent pituitary halves receiving no LHRH treatment, lined bars represent either 5 or 50 ng LHRH treatment and shaded bars indicate 50 or 100 ng LHRH treatment. Numbers at the base of the bars indicate the numbers of animals per group.


C.R. ♂- I
C.R ♂- II
C.R. ♂- III
LH Release µg/mg
6
3
0
10 5 5
14 7 7
8 4 4
9 5 4
6 3 3
10 5 5
0 25 100
0 25 100
0 5 50
0 5 50
0 5 50
0 5 50
ng LHRH
Control
Stress
Control
Stress
Control
Stress

LHRH doses of 25, 50 and 100 ng stimulated maximal amounts of LH release, which was greater, but not significantly than the amount released by 5 ng LHRH. Two weeks of restraint stress had no apparent effect on pituitary responsiveness to LHRH stimulation in male rats.

Chronic stress affected the pituitary content of LH in female rats differently. LH content of pituitary halves from control rats was unaffected by LHRH dose, as was the content in pituitary halves from stressed rats; but pituitary halves from stress rats contained significantly more LH than pituitary halves from control rats (Figure 6). Control rats pituitary content ranged from 5-9 μg LH/mg pituitary while pituitary content of stressed rats ranged from 12-18 μg LH/mg pituitary tissue.

LH release from pituitary halves also showed the stepwise increase release of LH with increasing LHRH dose stimulation (Figure 7). Stress had no effect on the amount of LH released in response to LHRH as control values and stress values are similar for each dose of LHRH.

Stress Effects on Serum LH

Blood samples were taken once a week from both the control and restraint stress animals from all experimental groups to monitor stress effects on resting serum LH concentrations. The effect of stress on serum LH on day one of treatment was variable (Figure 8). In both C.R. ♂-I and C.R. ♂-III serum LH dropped significantly following

Fig. 6.--The effect of LHRH on pituitary content of LH in young female rats.

The height of each bar represents mean LH concentration in the pituitary (μg LH/mg pituitary tissue) with brackets corresponding to the standard error of the mean following treatment with LHRH. Open bars represent pituitaries receiving no LHRH treatments, while lined bars and shaded bars indicate LHRH doses of 5 and 50 ng respectively. Numbers at the base of the bars indicate the number of animals in each group.

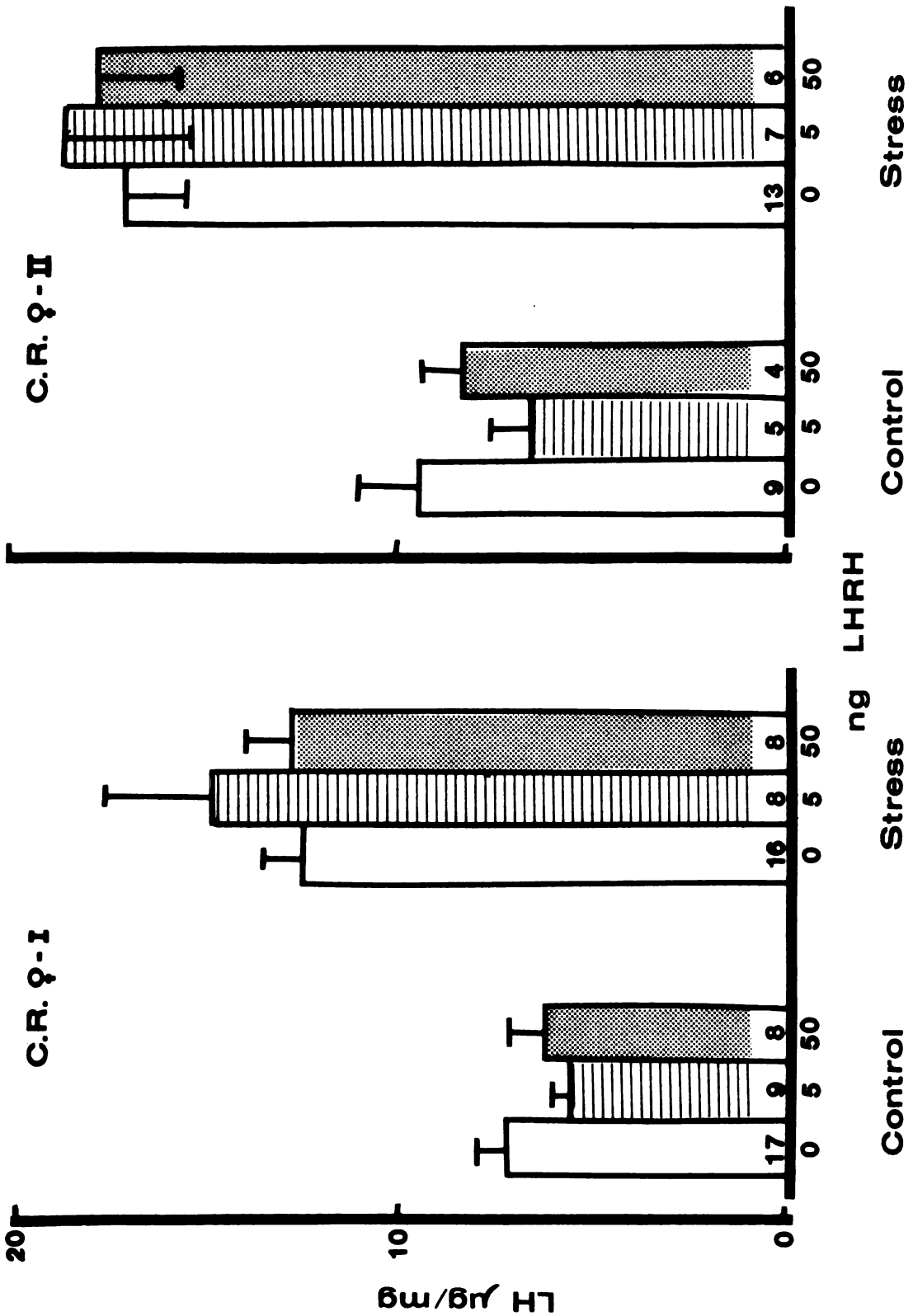

C.R. ♀-I
C.R. ♀-II
LH µg/mg
20
10
0
Control
Stress
ng LHRH
0
5
50
17
9
8
16
8
8
13
7
6
9
5
4

Fig. 7.--The effect of LHRH on pituitary release of LH in young female rats.

The height of each bar represents mean LH concentration released from pituitary halves (μg LH/mg pituitary tissue) with brackets corresponding to the standard error of the mean. Each bar represents pituitary release of LH after treatment with LHRH. Open bars represent pituitary halves receiving no LHRH treatment, while lined bars and shaded bars indicate LH release from pituitary halves treated with 5 and 50 ng LHRH respectively.

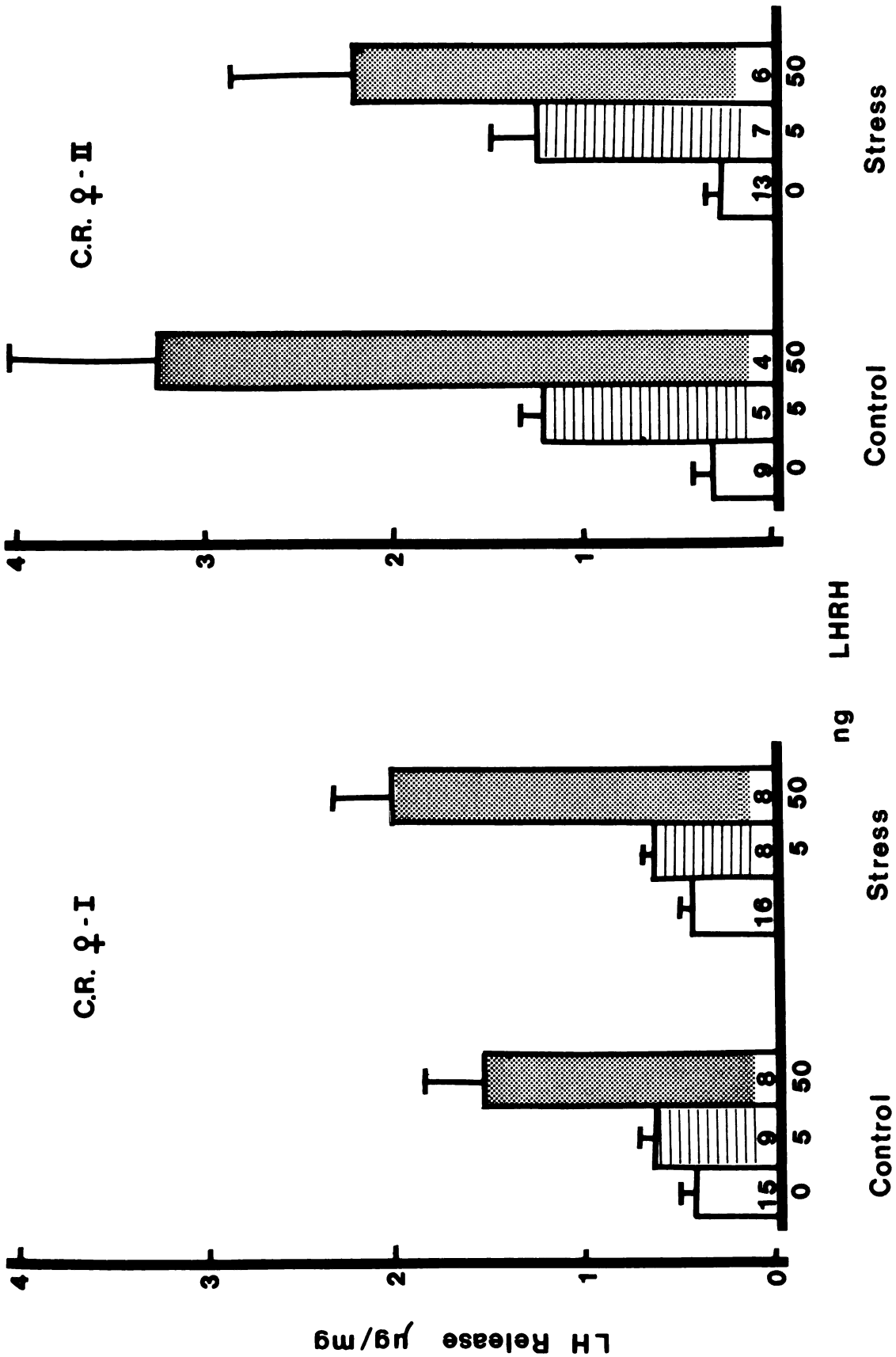
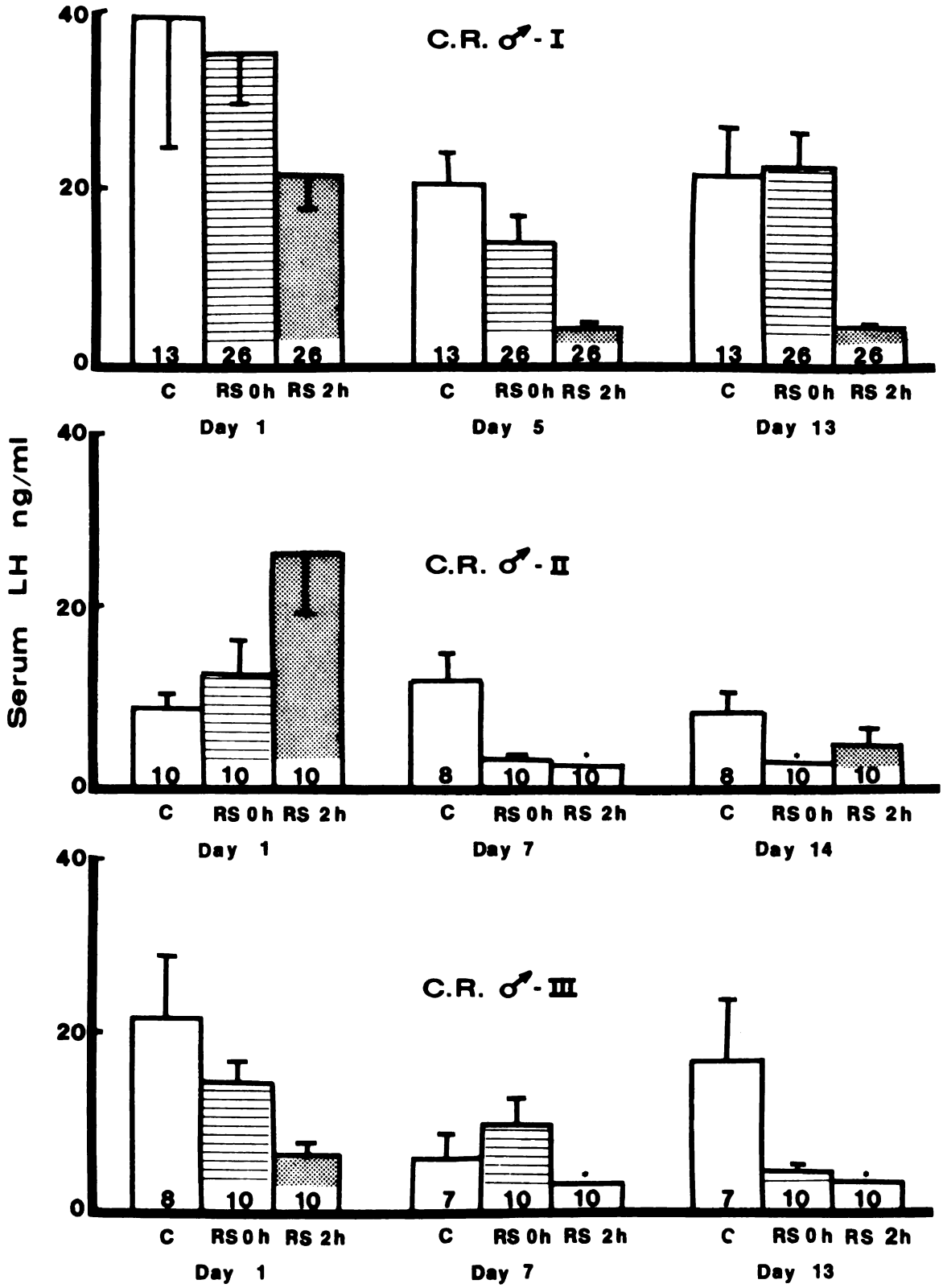

C.R. ♀-I
C.R. ♀-II
Control
Stress
ng LHRH
LH Release µg/mg
0
1
2
3
4
0
5
50
15
9
8
16
8
8
13
7
6

Fig. 8.--The effect on chronic restraint stress on serum LH in young male rats.

The height of each bar represents mean serum LH concentration (ng LH/ml) with brackets corresponding to the standard error of the mean. Each bar represents serum LH on different days of stress treatment. Open bars represent control animals resting serum LH levels, lined bars represent restraint rats resting serum LH concentrations, and shaded bars represent restraint rats serum LH concentrations following 2 hours of restraint stress. Asterisk marks serum samples having too little LH concentration to be measured by the assay so there is no standard error of the mean.

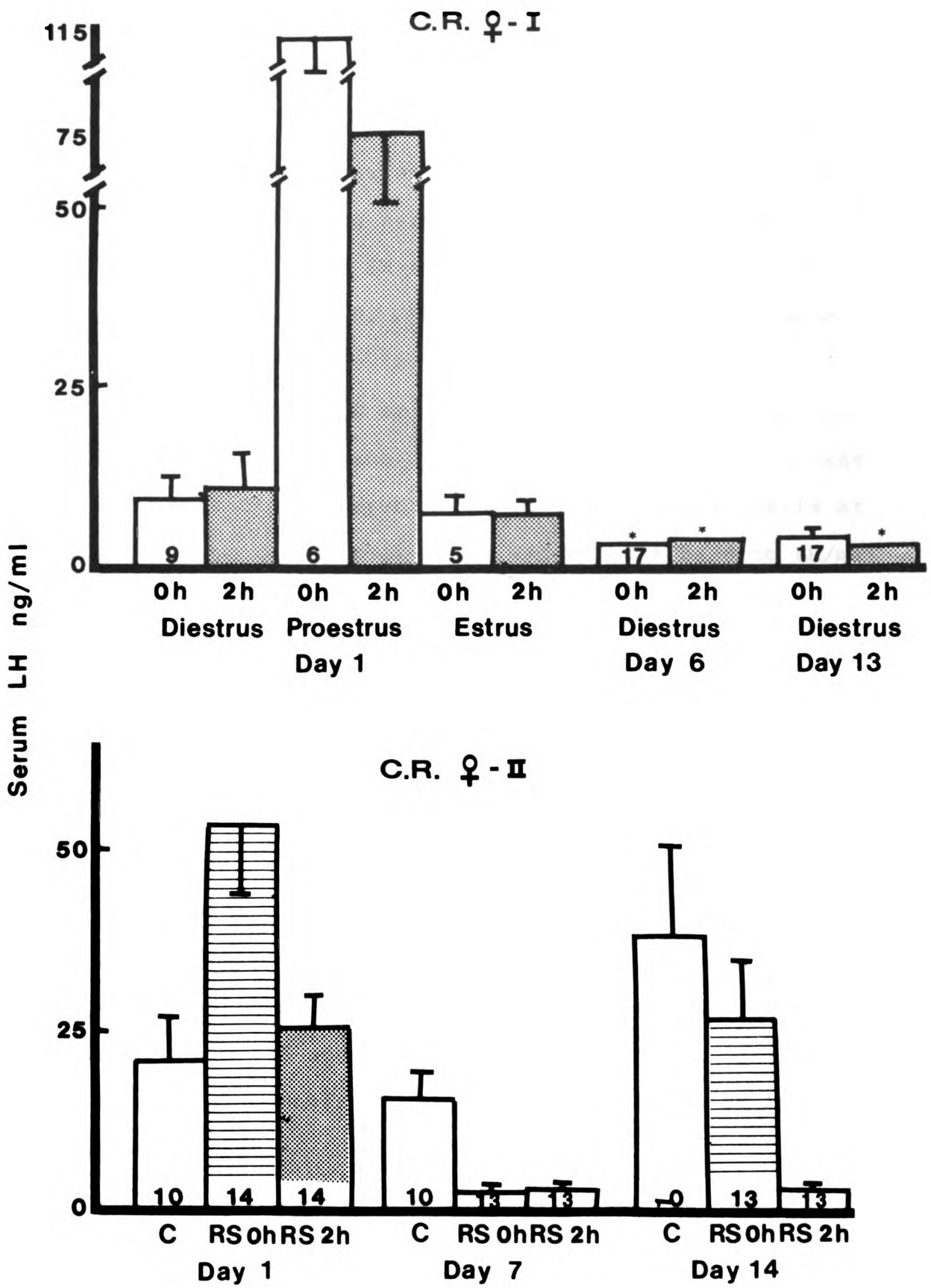

C.R. ♂-I
C.R. ♂-II
C.R. ♂-III
Serum LH ng/ml
40
20
0
C
RS 0h
RS 2h
Day 1
Day 5
Day 13
Day 7
Day 14
13
26
26
10
8
7

two hours of restraint stress from 35.89 ± 5.95 to 21.40 ± 2.79 ng LH/ml serum and 22.07 ± 7.08 to 6.61 ± 1.08 ng LH/ml serum respectively, while C.R. ♂ -II demonstrated a significant increase in serum LH following two hours of stress. After one week of stress resting levels of LH had decreased from 35.89 ± 5.95 to 14.19 ± 2.72 ng LH/ml serum for C.R. ♂ -I, from 21.77 ± 3.40 to 3.63 ± 2.10 ng LH/mg serum for C.R. ♂ -II and 13.35 ± 3.7 to 9.52 ± 3.29 ng LH/mg serum for C.R. ♂ -III. Following two hours of restraint serum LH decreased from the already lowered resting levels to the minimum sensitivity of the LH assay. Results from the end of the second week were similar for C.R. ♂ -II and C.R. ♂ -III where resting serum LH levels from the restraint groups were less than those after controls and following two hours of restraint serum LH concentrations remained lowered. Although C.R. ♂ -I resting LH concentration of the restraint group is not different from the control, two hours of stress decreased serum LH.

Changes in serum LH in response to stress in female rats were found to be dependent upon the stage of the estrous cycle at the time of stress (Figure 9). Restraint stress on diestrus either decreased serum LH as in C.R. ♀ -II where serum LH decreased from 54.17 ± 9.63 to 25.13 ± 4.64 or had no effect as with C.R. ♀ -I where serum LH was 9.48 ± 3.06 before stress and 11.62 ± 3.73 ng LH/ml following stress. Restraint stress on the afternoon of

Fig. 9.--The effects of stress on serum LH in young female rats.

The height of the bar represents mean serum LH concentration expressed as ng LH per ml serum with brackets corresponding to the standard error of the mean. Each bar represents serum LH on different days of stress treatment. Open bars represent control animals resting serum LH levels, lined bars represent restraint rats (RS) resting LH levels, and shaded bars represent restraint rats serum LH levels following 2 hours of restraint stress. Asterisks mark groups with no standard error of the mean due to serum samples containing too little LH to be measured.


C.R. ♀ - I
115
75
50
25
0
Serum LH ng/ml
9
6
5
17
17
0h 2h 0h 2h 0h 2h 0h 2h 0h 2h
Diestrus Proestrus Estrus Diestrus Diestrus
Day 1 Day 6 Day 13
C.R. ♀ - II
50
25
0
10
14
14
10
13
13
10
13
13
C RS 0h RS 2h C RS 0h RS 2h C RS 0h RS 2h
Day 1 Day 7 Day 14

proestrus decreased serum LH from a proestrus peak of 114.47 ± 18.83 ng LH/ml serum to 76.89 ± 26.35 ng LH/ml serum. Restraint stress during estrus appeared to have no significant effect on serum LH concentration. After one week of stress, resting serum LH was decreased to the minimum sensitivity of LH assay and two hours of stress did not change serum LH. Resting LH concentrations were also at a minimum following two weeks of stress for C.R.♀ -I and two hours of stress did not alter serum LH. Resting serum LH from the stress animals of C.R.♀ -II was not significantly different from control resting levels at the end of the second week as it was 26.57 ± 8.28 ng LH/ml serum while the control value was 38.06 ± 12.25 ng LH/ml serum. Two hours of restraint stress did decrease serum LH to the lowest sensitivity of the LH assay.

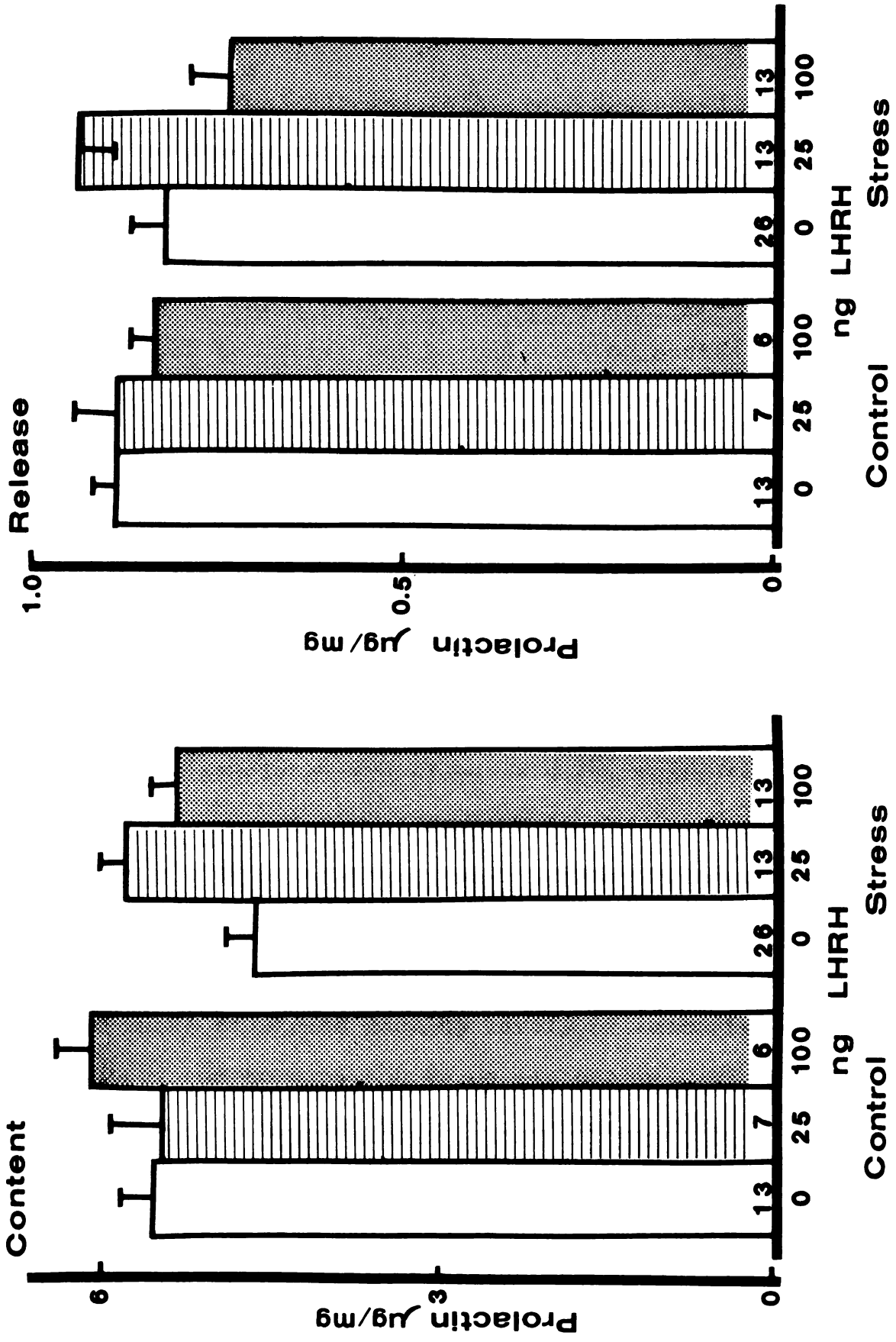
PRL Data

In Vitro Pituitary LHRH Challenge

Pituitary prolactin content of male rats was not affected by LHRH stimulation and was not altered by two weeks of chronic stress (Figure 10). The pituitaries contained between 4.6 and 6.0 ng PRL/mg pituitary. Prolactin release was also unaffected by LHRH stimulation or by restraint stress.

Fig. 10.--The effects of stress on pituitary content of prolactin and ability to release prolactin in young male rats.

The height of the bar represents mean pituitary content and mean pituitary release as μg prolactin per mg pituitary tissue, with brackets corresponding to the standard error of the mean. Open bars represent pituitaries without LHRH treatment, lined bars represent pituitary halves treated with 25 ng LHRH and shaded bars indicate pituitaries treated with 100 ng LHRH. Numbers at the base of the bars indicate numbers of animals per group.


Release
Content
Prolactin µg/mg
1.0
0.5
0
6
3
0
13
7
6
26
13
13
0
25
100
0
25
100
ng LHRH
Control
Stress

Stress Effects on Serum Prolactin

Serum prolactin was relatively unchanged throughout the two week stress in male rats (Figure 11). Resting levels of prolactin in stress and control rats were similar and two hours of restraint stress had no significant effect on day 1 of stress in C.R.♂ -I or C.R.♂ -III. At the end of the first and second week of stress, serum prolactin concentrations was not significantly different from control to stress rats. Two hours of restraint stress did not alter serum prolactin from the resting values.

Testosterone Data

Serum Testosterone Response to LHRH Challenge

LHRH significantly elevated serum testosterone over the 45 minute interval sampled in the control animals from 1.55 ± 0.34 ng testosterone/ml serum preinjection to 3.0 ± 0.44 ng testosterone/ml serum at 15 minutes and 4.10 ± 0.56 ng testosterone/ml serum (Figure 12). Following two weeks of stress resting levels of testosterone were suppressed to 0.56 ± 0.05 ng testosterone/ml serum. Injection of 500 ng LHRH tended to increase serum testosterone but not significantly to 0.65 ± 0.08 ng testosterone/ml serum at 15 minutes and 0.70 ± 0.10 ng testosterone/ml serum at 45 minutes postinjection.

Fig. 11.--The effect of stress on serum prolactin in young male rats.

The height of the bar indicates mean serum prolactin concentration expressed as ng prolactin per ml serum, with brackets corresponding to the standard error of the mean. Each bar represents serum prolactin on different days of stress treatment. Open bars represent control animals resting serum prolactin, lined bars represent restraint rats resting levels, and shaded bars represent rats serum prolactin levels following 2 hours of restraint stress. Numbers at the base of the bars represents the number of animals in each group.

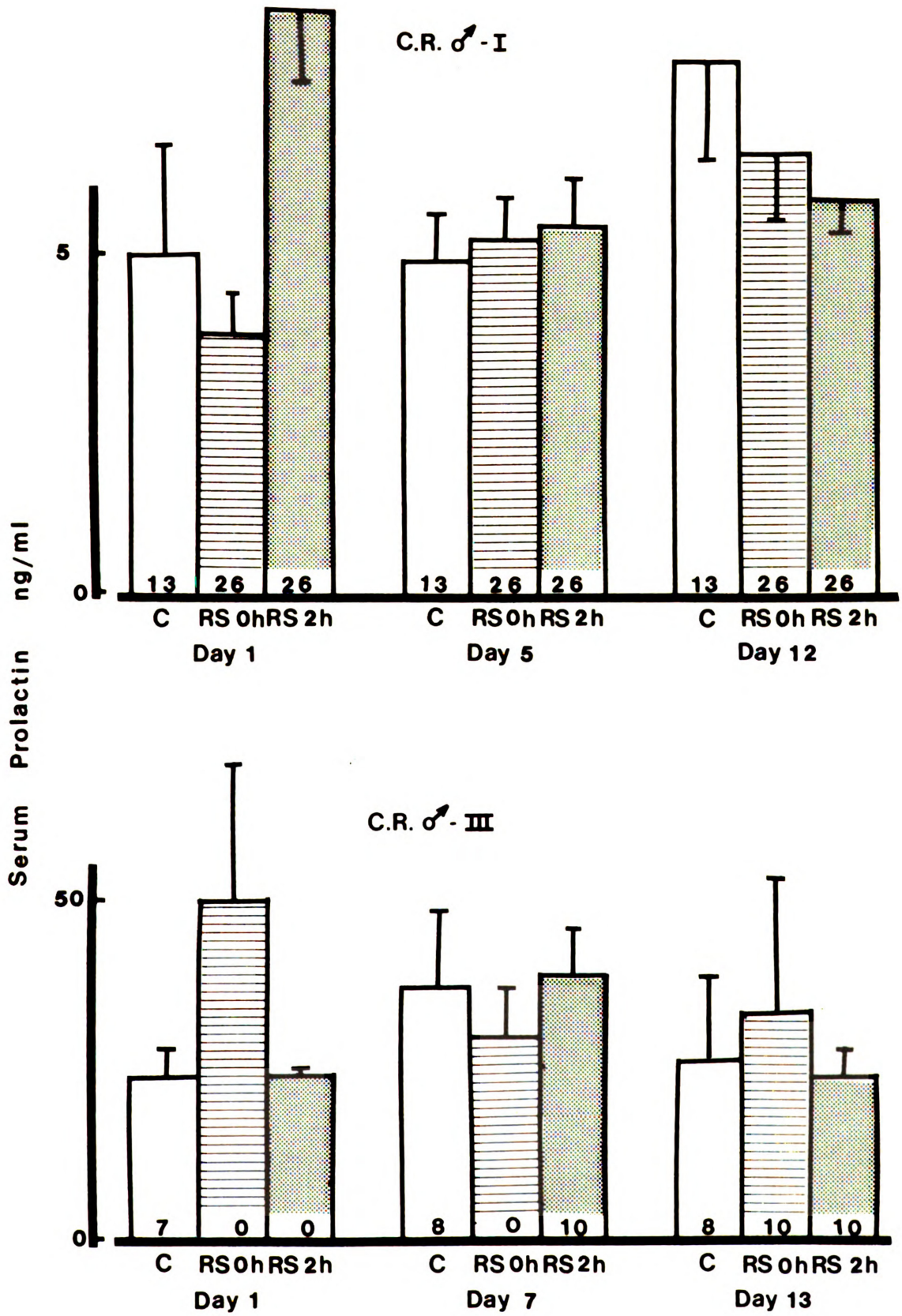
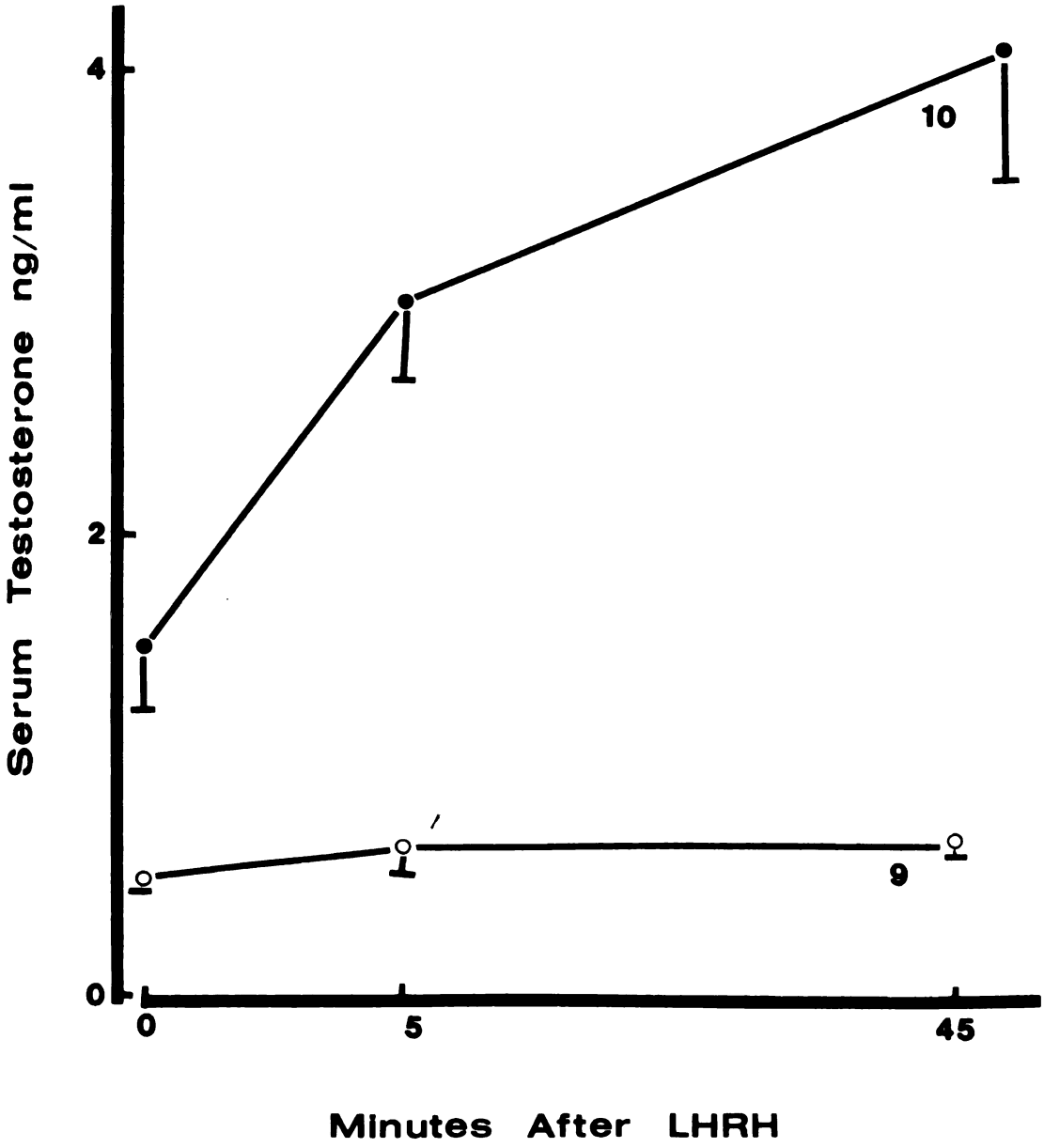

C.R. ♂ - I
5
0
13
26
26
13
26
26
13
26
26
C
RS 0h
RS 2h
Day 1
Day 5
Day 12
Serum Prolactin ng/ml
C.R. ♂ - III
50
0
7
0
0
8
0
10
8
10
10
Day 1
Day 7
Day 13

Fig. 12.--The effects of 500 ng LHRH on serum testosterone in young male rats.

Mean serum testosterone values (ng/ml) and their standard errors of the mean appear on the ordinate as a function of time. Blood samples were taken before injection (0 time) and 15 and 45 minutes postinjection with 500 ng LHRH. Solid circles represent control animal values, while open circles represent serum testosterone values from chronically restrained rats. Numbers indicate number of animals per group.


Serum Testosterone ng/ml
4
2
0
10
9
0
5
45
Minutes After LHRH

Effect of Stress on Serum Testosterone

Serum testosterone from control animals taken at one week intervals remained unchanged, ranging between 1.00 to 2.00 ng testosterone/ml serum (Figure 13). The first two hour restraint period had no significant effect on serum testosterone. After one week of stress resting testosterone concentrations in the stress group was significantly decreased to 0.36 ± 0.03 ng testosterone/ml serum. Further stress did not change serum testosterone. Serum testosterone was similarly suppressed at the end of two weeks of stress.

Catecholamine Data

Hypothalamic extracts from control and stress male and female rats were assayed for dopamine and norepinephrine content (Table 1). Chronic stress had no effect on hypothalamic content of norepinephrine in male and female rats. Dopamine content was also unaffected by restraint stress in male rats.

Vaginal Cytology Data

Regular estrous cyclicity was part of the criteria in the selection of female rats. Restraint stress interrupted the estrous cycles in all of the female rats that underwent the two week stress treatment. By the morning of day 4 of stress vaginal cytology of the restraint female groups were of the leukocytic cell type, which is

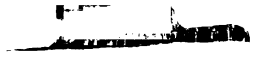


Fig. 13.--The effect of stress on serum testosterone in young male rats.

The height of the bars represents mean serum testosterone as ng testosterone/ml serum with brackets corresponding to the standard error of the mean. Each bar represents serum testosterone on different days of stress treatment. Open bars represent control animals resting serum testosterone values, lined bars represent restraint rats resting levels, and shaded bars represent restraint rats serum testosterone concentrations following 2 hours of restraint stress. Numbers at the base of the bars indicate the number of animals in each group.

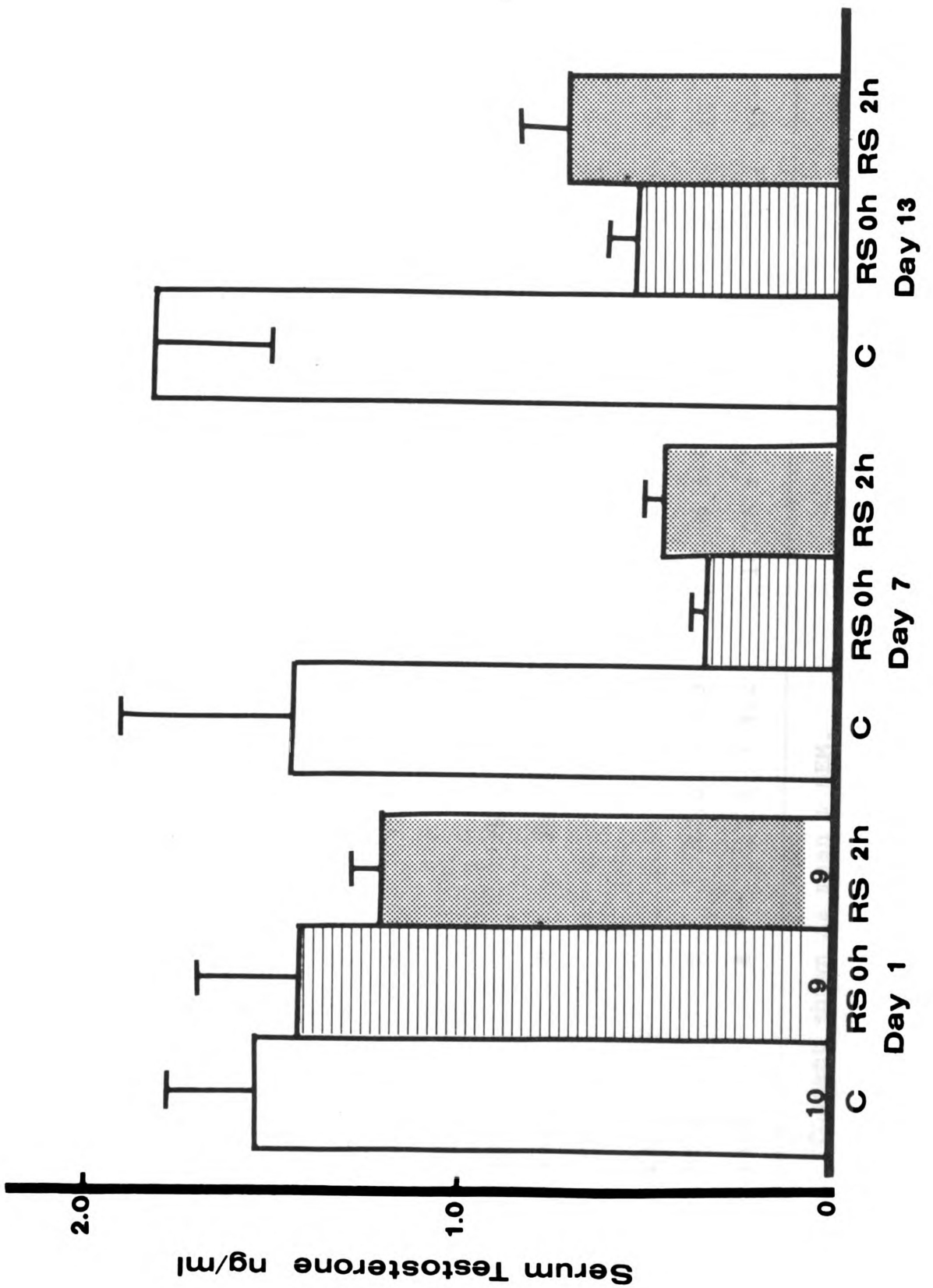

Serum Testosterone ng/ml
2.0
1.0
0
C
RS 0h
RS 2h
10
9
9
Day 1
C
RS 0h
RS 2h
Day 7
C
RS 0h
RS 2h
Day 13

Table 1.--The Effects of Chronic Restraint Stress on Hypothalamic Catecholamines in Young Female and Male Rats.

	Number	ng/hypothalamus	Mean hypothalamic weight (mg)	ng/mg hypothalamus
Young				
Dopamine				
Controls	7	33.14 ± 4.80	18.77 ± 0.55	1.80 ± .29
Stress	7	32.37 ± 5.78	19.46 ± 1.28	1.69 ± .29
Norepinephrine				
Control	7	29.8 ± 2.52	19.49 ± 1.25	1.53 ± .07
Stress	8	29.80 ± 2.34	18.54 ± 1.11	1.61 ± .09
Young				
Norepinephrine				
Control	9	47.0 ± 4.3	22.06 ± 0.73	2.12 ± .15
Stress	8	37.8 ± 4.2	18.06 ± 1.14	2.08 ± .16

Content shown as mean ± SEM.

representative of the diestrus state. All rats remained diestrus throughout the two week interval except for 2 of 16 rats from C.R.♀ -I that had proestrus smears on day 8 of stress, and 5 of the 13 rats from C.R.♀ -II were proestrus or estrus on day 8. On day 8 of stress the restraint rats were bled twice and this may have caused the change from diestrus cytology. The control groups for each stress group continued to cycle.

Chronic Stress Effects on Body Weights

Two weeks of restraint stress caused a significant decrease in body weight in female rats from 230.21 ± 6.74 g to 214.08 ± 5.96 g (Table 2). Over the same two week span the control females had a significant increase in body weight from 244.40 ± 8.82 g to 268.0 ± 7.69 g.

The control male groups also showed no loss of body weight (Table 2). C.R.♂-I controls weighed 487.07 ± 18.7 g at the beginning of the experiment and 465.77 ± 15.14 g at the end. C.R.♂-II and C.R.♂-III showed similar results. The chronically restrained male rats had a significant decrease in body weight as C.R.♂-I dropped from 453.38 ± 11.31 g to 399.0 ± 10.02 g, while C.R.♂-II had a decrease from 478.0 ± 20.54 g to 413 ± 16.99 g and C.R.♂-III 496.5 ± 21.20 g to 423.0 ± 20.07 g.

Table 2.--The Effects of Chronic Restraint Stress on Body Weight of Male and Female Rats.

	No.	Body Weight (grams)		
C.R. ♂-I		Day 1	Day 5	Day 12
Control	13	487.07 ± 18.70	481.15 ± 15.97	465.77 ± 15.41
Restraint	26	453.3 ± 11.31	429.62 ± 10.83*	399.0 ± 10.02*
C.R. ♂-II		Day 1	Day 6	Day 14
Control	10	472.03 ± 9.75	461.0 ± 13.47	456.25 ± 21.03
Restraint	10	478.0 ± 20.54	427.7 ± 19.65*	413.33 ± 6.99*
C.R. ♂-III		Day 1	Day 8	Day 14
Control	7	444.29 ± 17.30	445.0 ± 14.23	465.0 ± 12.04
Restraint	10	496.5 ± 21.20	445 ± 20.62*	423 ± 20.09*
C.R. ♀-II		Day 1		Day 13
Control	10	244.4 ± 8.82		268 ± 7.69**
Restraint	14	230.21 ± 6.74		214.08 ± 5.96*

Weights are shown as mean ± SEM in grams.

*Denotes a significant decrease in body weight from day 1 values.

**Denotes a significant increase in body weight from day 1 values.

DISCUSSION

The results indicate that chronic restraint stress can effectively interfere with reproduction in both male and female rats. Stressed female rats did not have regular estrous cycles, stressed male rats had lowered serum testosterone, and serum LH was suppressed in both female and male rats. Stress has been shown to interfere with reproduction in rats. Restraint stress during pregnancy has been found to increase embryonic mortality in the rat (Euker and Riegle, 1973). Plasma corticosterone increases during pregnancy in the hamster, but 24 hours of restraint stress, or isolation without food, or injection of corticosterone on day 14-15 of gestation increases corticoster-ne levels even more and significantly increases the incidence of cleft palate in the offspring (Barlow, McElhalton, Morrison and Sullivan, 1974). Heat stress has been found to impair reproduction in ewes (Neville and Neathery, 1974) and increase infant mortality and decrease weaning weight in rats and hamsters (Hearnshaw, Wodzicka-Tomaszewska, 1973; Barlow, Morrison and Sullivan, 1975). Stress in the form of handling, ether or serial blood samples increases serum LH in male rats (Euker, Meites and

Riegle, 1975). Two hours of restraint stress had no effect on serum LH in male, diestrus, or estrus rats and decreased serum LH in proestrus rats (Euker, Meites and Riegle, 1973).

Resting levels of LH were affected by chronic stress. The response of male rats to the initial stress was variable as both elevation and suppression of serum LH occurred, which correlates with the ether stress data and restraint stress data of Euker et al. (1973, 1975). These data suggest that the intensity of the stress can affect serum LH levels in the male rat. Restraint stress may be considered more stressful than either ether stress or handling, as these are carried out over a very short time, generally not exceeding 15 minutes, while restraint stress was carried out for 2 hours. It is possible that restraint stress initially elevated serum LH, but after 2 hours the serum LH was returning to resting values or had returned to resting values, or was suppressed by the sustained stress. Following 1 or 2 weeks of stress most male rats did experience a decrease in resting serum LH and in many instances two hours of stress could further decrease serum LH. In some rats serum LH appeared unaffected by the chronic stress as resting LH levels following a week of stress were not significantly different from control animal resting levels. This suggests that serum LH secretion could recover from stress-induced suppression in

male rats and that stress may have only a temporary effect on LH secretion.

In comparison to the male rat the female rat appears to be more sensitive to stress-induced inhibition of LH secretion. Restraint stress caused complete suppression of resting levels of serum LH far below serum LH concentrations found in estrous or diestrous female rats or chronically stressed male rats. Where two hours of restraint stress could further decrease serum LH in male rats it could not decrease serum LH in female rats. Resting serum LH concentrations in female rats were found to be at the lower limits of the LH assay sensitivity, which suggests that chronic stress can completely suppress LH release in female rats where it partially or temporarily decreased serum LH in male rats. Resting serum LH blood samples were taken four hours following the morning restraint period. Although the suppressed serum LH following two hours of restraint in male rats returned toward normal resting levels four hours post stress, female rats serum LH remained maximally suppressed four hours following completion of the stress regime.

Pituitary responsiveness was tested by measuring the increase in serum LH stimulated by an intravenous injection of 100 or 500 ng LHRH. If stress was exerting an effect at the pituitary, we would expect pituitary responsiveness to LHRH to be affected. No significant

difference was observed between the control or stress rats receiving 100 ng LHRH over the sampling times used in this study. Stress and control animals receiving 500 ng LHRH released more LH at peak response than the rats receiving the 100 ng LHRH dose. In C.R. ♂ -II and C.R. ♂ -III there was no difference in LH response to LHRH between stress and control male rats. C.R. ♂ -I rats achieved a peak value at 30 minutes for control rats, while the stress group had reached peak serum LH secretion at 15 minutes and by 30 minutes was returning towards the resting concentrations. Although preinjection serum LH concentrations were less in the stressed rats, peak values measured at 15 minutes and duration of LH release were similar, except for the one control group mentioned. These three experiments suggest that the pituitary of the stressed male rat retains its responsiveness to LHRH, implying that stress may be affecting LHRH availability to the pituitary.

A bolus injection of 500 ng LHRH was also used to determine chronic stress effects on the control of LH release in female rats. Resting serum LH was suppressed by the chronic stress in female rats and exogenous LHRH was used to help determine if stress exerted its effects at the hypothalamus or the pituitary. The first restraint group, C.R. ♀ -I, released 31.90% less LH in response to LHRH following 2 weeks of restraint stress than they did before the stress. Peak release was measured at the 15

minute sample, and by 45 minutes postinjection both groups were returning towards the preinjection LH values while peak response following injection with 500 ng LHRH for the stress group C.R.♀ -II was not significantly different from the control groups value, overall secretion may have been less. By 45 minutes postinjection stress rats serum LH concentration had decreased to half of the control serum LH levels, which suggests a possible decrease in pituitary responsiveness in female rats following two weeks of chronic restraint.

The in vitro LHRH response of male rats supported the in vivo LH data from C.R.♂ -II and C.R.♂ -III. Chronic stress had no effect on LH content or release of LH from the pituitaries of control and LHRH stimulated pituitaries. The amount of LH released from the pituitary was related to the dose of LHRH used and not to the stress. From the in vivo and in vitro LHRH data from C.R.♂ -II and C.R.♂ -III, it can be hypothesized that chronic restraint stress does not effect the ability of the pituitary to synthesize or secrete LH in male rats. The effects of stress to lower resting serum LH concentrations in male rats does not appear to be controlled by a change at the pituitary, but at higher centers. Stress can alter serum glucocorticoids and adrenal catecholamines (Scheiber, 1963; Mountcastle, 1974) which can feed back at the pituitary and hypothalamus directly or indirectly and can alter LH

and prolactin secretions (Swerdloff, Jacobs and Odell, 1972). Injection of hydrocortisone, prednisolone or ACTH during follicular development blocked the estrogen rise and delayed ovulation in sows (Liptrap, 1970) which indicates interference in LH secretion. In 1972 Ganong suggested that catecholamines may regulate hypothalamic function but conflicting data as to catecholamines stimulating or inhibiting ability has not been elucidated (Hiroshige and Abe, 1973, 1974; Kaplanski, VanDelft, Nyakas, Stoof and Smelik, 1974; Eisenberg, 1975; Palkovits, 1975). It has also been shown that stress can change serum LH directly in adrenalectomized rats, who have lowered levels of adrenal glucocorticoids or adrenal catecholamines (McKay, Wood and Riegle, 1976). Thus stress may also be mediated in some manner at higher centers, by changing neural input at the brain or by changing the sensitivity of the hypothalamus to LH or testosterone negative feedback.

LHRH treatment stimulated similar LH release from pituitary halves of the control and stressed female rats. However pituitary LH content was higher in stressed compared to control groups. Although these studies show that the primary effect of stress on gonadotropin control mechanisms is at the level of the hypothalamus, the increase in pituitary LH content suggests that stress may also be affecting LH synthesis and release at the level of the pituitary. Since LH release following LHRH stimulation was

similar in control and treated groups, the increase LH content may be caused by storage granules not available for secretion. These experiments do not indicate whether stress effects on pituitary LH content are mediated by unidentified hypothalamic factors or other neural factors or alterations in adrenal function affect the pituitary directly. While the data suggests that the hypothalamus or some higher center and not the pituitary is changed by chronic stress in male rats, it also suggests that both the pituitary and higher centers are affected by stress in female rats.

Prolactin is a hormone that is also affected by stress. Acute stress such as new environment, ether stress or handling results in an increase in serum prolactin in male rats (Euker, Meites and Riegle, 1975; Seggie and Brown, 1975; Brown, Uhler, Seggie, Schally and Kastin, 1974). Two hours of restraint stress increased serum prolactin in male rats (Euker, Meites and Riegle, 1973; Riegle and Meites, 1976). In the chronic restraint experiments two hours of restraint gave mixed results. C.R. ♂-I exhibited the previously established increase in serum prolactin, while two hours of restraint for C.R.♂-III serum prolactin either decreased or remained unchanged from control values. It is possible that the C.R. ♂-III stress group serum prolactin did initially rise in response to the stress, but had begun to return towards

nonstressed control values. This suggests that there is a limit to the stress induced release of prolactin. Following the first and second week of stress serum prolactin was not significantly affected by two hours of restraint. Treatment with dexamethasone is known to suppress stress-induced CRF and ACTH release (Harms, Langlier and McCan, 1975; Euker, Meites and Riegle, 1975), and can block stress-induced prolactin release caused by distrubance (Harms, Langher and McCann, 1975) or serial bleeding (Euker, Meites and Riegle, 1975). It is possible that stress-induced alterations of adrenal glucocorticoid secretion can influence pituitary prolactin release. Apparently the animals experiencing chronic stress adapt to the procedure, or high stress-induced levels of glucorticoids may have inhibited hypothalamic-hypophyseal mechanisms allowing increased prolactin release.

It was not surprising to find chronic stress had no effect on pituitary content or release of prolactin, as serum prolactin was unchanged with chronic stress. Prolactin release from pituitary halves from stress and control animals were not significantly different nor was pituitary content of prolactin, which suggests that chronic stress had no effect on prolactin release at the pituitary. The data from both the <u>in vivo</u> and <u>in vitro</u> experiments indicate that chronic stress does not alter prolactin release from the pituitary and agrees with the hypothesis

of Harms et al. (1975). The data from these experiments do not indicate whether hypothalamic factors that inhibit prolactin secretion are changed by chronic stress.

The unique distribution of brain biogenic amines in the hypothalamus suggests an important role of catecholamines in the neuroendocrine regulation of anterior pituitary ACTH secretion (Fuxe and Hokfelt, 1969). The adrenegic system was hypothesized to inhibit pituitary secretion and cholenergic as stimulatory (Ganong, 1972). While Abe and Hiroshige (1973, 1974) found catecholamines increased pituitary release. Catecholamines, especially dopamine can inhibit release of pituitary prolactin by affecting hypothalamic release of factors inhibiting prolactin secretion or by direct hypophyseal inhibitors (Shaar and Clemens, 1974). Hypothalamic content of dopamine and norepinephrine were found unchanged in chronically stressed rats when compared to control rats. This data suggests that hypothalamic catecholamines are unaffected by chronic stress which is reflected in unchanged pituitary content and serum concentrations of prolactin with chronic restraint stress. Although the whole hypothalamic content of catecholamines appears to be unaltered by stress, it may not be the best way to measure the effect of chronic stress on brain catecholamines. Whole hypothalamic catecholamine content may not be as important as specific loci within the hypothalamus, as both acute and repeated stress was found

to decrease norepinephrine and dopamine content in the arcuate nucleus without changing catecholamine content in the rest of the hypothalamus (Palkovits et al., 1975). Since the arcuate nucleus is only 5% of the hypothalamic weight, measurement of whole hypothalamic catecholamines may fail to detect alteration in such a small area. A change in turnover of catecholamines may also be important as a stress effect, as the enzyme tyrosine hydroxylase used in aminergic synthesis increases in the arcuate nucleus with stress (Palkovits et al., 1975). This suggests that norepinephrine and/or dopamine are being synthesized and secreted at an increased rate with stress. Studies measuring catecholamine turnover within areas of the hypothalamus may be beneficial in understanding hypothalamic catecholamines role in stress on gonadotropin and prolactin regulation.

Serum testosterone was measured in male rats and daily vaginal cytology monitored in the female rats as an indication of reproductive capabilities. While restraint stress had no effect on serum testosterone on day 1 of stress by day 7 resting levels of testosterone before and after stress treatment were less than one-third of control animal values. Although resting serum LH levels of stressed groups were not significantly different from serum LH concentrations in control rats, the decrease in serum testosterone levels indicate decreased gonadotropin

stimulation of the testes. Serum LH was lowered during the twice daily stress periods. Perhaps this interfered with the release of LH necessary for testosterone synthesis and release, as the pulsatile release of LH was blocked by immobilization stress (Blake, 1975). While stress appears to have a direct effect at the testes as serum LH increased following LHRH injection and testosterone did not, it is more likely that the testes did not respond because it had been inactive for two weeks and needed time to respond to reestablish synthesis of testosterone. This hypothesis is supported by the data of Riegle and Miller (unpublished) that in aged male rats serum testosterone responds less dynamically to an injection of HCG than serum testosterone of young male rats. Aged male rats primed for several days with HCG could release similar amounts of testosterone as unprimed young male rats, suggesting constant or tonic gonadotropin secretion is necessary for testicular response to bolus injection of LHRH or HCG.

Chronic restraint stress similarly shut down reproductive function in female rats as they ceased to cycle and showed a persistent diestrus vaginal cytology. Vaginal cytology is considered an indication of the stage of the estrous cycle and reflects relative levels of estrogen and progesterone as well as help detect ovulation in the rat (Swartz, 1968). Since LH release was suppressed by chronic stress, apparently little or no follicular

growth took place as no estrogen-induced cornified cells were seen. The only exceptions noted were following the second LHRH challenge when LH was present in the blood in high concentrations. At this time vaginal cytology of 30% of the rats was either nucleated or cornified, proestrus or estrus respectively, which indicates LH stimulation.

It was expected that the rats in the stress groups would lose weight. Whether stress itself was directly a factor or through stress effects of glucocorticoid and epinephrine serum concentrations effect on appetite and metabolism was not explored. Stress animals were also removed from food and water for a total of four hours a day, while control rats had continued access to food and water. Although rats normally do not eat during the day, they do have sleep and activity periods that are interrupted. It also took 45 minutes to an hour following a stress period for the stressed rats to groom and settle down, which could further upset their rest-activity schedule.

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