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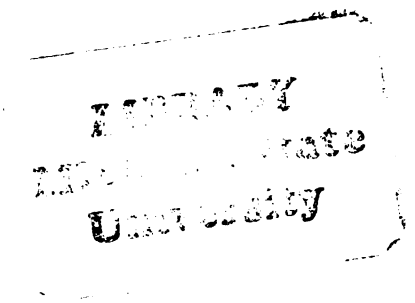

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REPRODUCTIVE CONTROL SYSTEMS IN THE AGING RAT

By

Anna E. Miller

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Submitted to
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Department of Physiology

ABSTRACT

REPRODUCTIVE CONTROL SYSTEMS IN THE AGING RAT

By

Anna E. Miller

Nine separate experiments were performed in order to examine the effects of age on the hypothalamic-pituitary-gonadal unit in male and female Long-Evans rats. Aged male rats demonstrate lower serum testosterone and LH concentrations and a reduced response to acute stimulation by HCG or LHRH as compared to young adult male rats. However, chronic Leydig cell stimulation by HCG results in similar increases in serum testosterone in young and aged male rats and restores testicular responsiveness to acute gonadotropin, indicating that the testis of the aged male rat remains capable of significant response. An in vitro study suggests similar hypothalamic content of biologically active LH-releasing hormone activity in young and aged groups. An experiment to characterize serum testosterone concentrations during different times of the day indicates the present of a diurnal pattern of serum testosterone in young male rats which was not present in aged male groups and also of small

episodical surges of pituitary LH and testicular testosterone secretion which show lower average concentrations and less variability with increased age.

Experiments in the female rat support a hypothesis of multiple factors affecting changes in the estrous cycle and fertility in the aging female rat. The magnitude of the LH and progesterone surge is progressively decreased with increased age and this reduction presumably ultimately results in increased estrous cycle irregularities and contributes to the development of the constant estrous state.

A longitudinal breeding study demonstrates decreased fertility at nine to ten months of age in female rats, which is prior to the loss of normal ovarian cyclicity. An increased gestation length accompanied by longer maintenance of elevated serum progesterone concentrations in aged compared to young rats is also demonstrated.

Although hypothalamic LH-releasing content in vitro is not found to be decreased with age in the female rat, multiple LHRH injections could increase serum LH in aged rats to concentrations which were similar to those found following similar hormone treatment in young groups. A hypothesis of altered neurotransmitter function in the hypothalamus contributing to decreased LH-releasing hormone secretion is discussed.

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INTRODUCTION

Aging is believed to affect the function of all physiological systems. Recognized aging effects on body systems include anatomical degeneration, reduced functional reserve, reduced responsiveness to control inputs and loss of synchrony between and within physiological systems. A substantial amount of information about the biology of aging has been collected in recent years. However, answering the fundamental questions concerning specific molecular causes of aging changes in body function and whether the effects of aging can be reversed remains the challenge.

As major regulators of body function which are recognized to influence all aspects of physiology, the endocrine systems have always fascinated gerontologists. Early investigators often thought that aging of the endocrine systems could be directly linked to the effects of aging on all body systems. Aging changes in the endocrine control systems that regulate reproduction are particularly well documented. In fact, many early investigators postulated that age deterioration of gonadal function was the primary cause of biological aging.

Major advances in our understanding of the biochemistry, physiology and pharmacology of reproductive control systems

have been made in recent years. Significant age-related alterations in resting hormone concentrations, and decreases in the ability of components of reproductive control systems to respond to control input have been identified in several species. Current biochemical-physiological techniques offer promise for clinical identification and treatment of age-related alterations in reproductive function.

The primary goals of most studies of the biology of aging are twofold: to develop a better understanding of the etiology of age effects on human function; and to identify potential clinical procedures to treat or reverse adverse aging effects on function. The effects of aging on human reproductive function are particularly dramatic. No other known biological change with age is as finite or as irreversible as the menopausal changes which occur in women. A significant problem for scientists attempting to relate their work to aging problems of human significance concerns appropriate selection of animal models. To date, no animal species has been identified that shows identical aging effects on reproductive function as found in humans.

The laboratory rat has been extensively utilized as a model of mammalian aging. In addition, the rat has been a primary model for basic studies of endocrine and reproductive control systems. Although the effects of age on reproduction in the rat are somewhat different from that occurring in humans, particularly related to menopause, I believe that my studies and experiments reported from other

laboratories indicate that the rat can be an appropriate model for the study of premenopausal aging in women and as a model for the decline in reproductive function in aging men.

Aging and Reproduction

Although the effects of aging on reproduction received extensive study by early biologists, modern scientists and clinicians have not given sufficient attention to the effect of age on human sexuality and reproductive control systems. When dealing with the elderly, current clinicians are much more likely to concentrate their thinking in terms of age effects on hearing, sight, arthritis and senility than on age effects on human sexuality. Behavioral scientists have likewise been concerned with the effectiveness of health care delivery systems, nursing homes, and pension and social security systems, also at the expense of a better understanding of sexuality in the aged (Butler, 1978). Schneider (1978) pointed out societal attitudes toward the elderly tend to identify them as relatively sexless. Butler (1978) has summarized our societal expectations toward sexual stereotypes in the aged. Men over the age of 50 are typically considered sexually impaired. However, Butler points out that most aging men do not have physiological bases for failure of sexual performance and indicates a high success rate of treatment of sexual impotence in aged men.

The societal attitude toward aging women in our society is even more troublesome than with men. Butler describes the sexual metamorphosis of women from desirable, sexy young

things, to sexually interesting mature women which at about the age of 50 are expected to decline into sexual oblivion. In addition, women are often victimized by the myths of the menopause. These myths include the onset of mental alterations including depression, defeminization and the loss of sexual desire. These attitudes toward the sexuality of older men and women are carried into our institutions. The fact that no provision for sexual privacy normally exists in most homes for the aged, hospitals or other forms of public institutions for the aged may be a reflection of these attitudes.

Schneider (1978) has hypothesized that our failure to give attention to reproductive function in the aged is related to a mistaken belief that pathologies of these organs and tissues do not have life threatening potential in the elderly when compared to cardiovascular, renal, pulmonary or other physiological systems. However, Schneider pointed out that pathological involvement of the reproductive system such as prostatic or breast carcinomas is one of the leading causes of morbidity and mortality in the older patient.

Aging changes in reproduction and reproductive endocrine control systems have been extensively studied in the past few years. A wealth of new information and understanding regarding the anatomical and physiological parameters that are associated with aging effects in reproductive control mechanisms have been obtained.

Although the loss of reproductive function with increased age is of concern for many animal breeders, the effect of age on human reproduction has not been as much of a concern in our society in the past because most men and women had completed their desired procreation before fertility was significantly affected by aging. In recent years, however, fertility problems have become of increasing interest and concern, largely because of the growing trend toward postponing motherhood until the thirties and even forties. Thus, the desired childbearing years are increasing coinciding with the premenopausal years. Recent statistics (Schwartz and Mayaux, 1982) indicate that fertility declines significantly in women after age 30. However, despite growing medical knowledge of reproductive mechanisms, the precise reasons for this decrease in fertility cannot be readily explained. Increasing age can certainly be related to increasing incidence of uterine disease, tubal obstruction and ovulatory disorders, but aging processes affecting the hypothalamic-pituitary-ovarian axis are also implicated. Thus, although the changes in reproductive function occurring with the menopause in women are dramatic, it must be remembered that aging effects on reproduction are continuous and important changes in reproductive control systems occur throughout the biological lifespans of humans and in all mammals.

Major advances have recently been made in our understanding of the relationship of the hypothalamus-pituitary

regulation of the gonadotropin secretion and the mechanisms of gonadal gametogenesis and steroidogenesis in the aging mammal. It has become clear that there are significant differences in the effect of age on parameters of the reproductive control systems among various species. It is my objective in this thesis to summarize current thought concerning the mechanisms of aging effects on the reproductive control system and to specifically consider the results of my experiments concerning the effect of aging on reproduction in the rat in the context of our understanding of aging effects on reproduction in other mammals, particularly humans.

Effect of Age on Reproductive Control Systems in the Male

The most consistent effect of aging on reproduction in males is the progressive decrease in sexual interest, libido and sexual activity from maturity into old age (Bishop, 1970). Although aged males of all mammalian species have decreased sexual function, individual males have been shown to be fertile at advanced age (Bishop, 1970). The most complete studies of the effect of age on male sexuality and reproduction have been done in men and were first reported by Kinsey et al. (1948). Additional studies of the effect of age on sexual activities in men have been made by Newman and Nichols (1960) and Martin (1975). These workers have reported a progressive decrease in orgasmic frequency from age 35 onward. The numbers of sexual encounters decrease with increasing age in men until by age 65 to 79 a majority

of men in their study reported less than 20 sexual encounters per year with considerable numbers of men reporting no sexual activity. Similar observations have been made with domestic mammals. Fraser (1968) and Rowson (1959) reported sexual apathy and impotence in older, domestic, male mammals which cause problems in breeding programs. In summary, Martin (1975) pointed out that a large number of variables, including environmental, psychological and physiological may be involved in determining the level of human male sexual fuction. Certainly all these variables affect reproductive function during aging. It is the intent of this reviewer to focus on the physiological variables.

The anatomy of the testes is significantly affected by aging. Bishop (1970) points out the inconsistencies between measures of sexual activity and anatomical evidence of testicular degeneration and decreased spermatogenesis which occur in males. Some males show little sexual interest with near normal spermatogenesis, some continue sexual activity with little or no spermatogenesis and others lose both sexual interest and spermatogenesis with increasing age.

Degenerative changes in the testes of aging men include seminiferous tubule fibrosis, decreased spermatogensis and thickening of the basement membrane of the seminiferous tubule (Nelson and Heller, 1945; Engle, 1952; and Balze, et al., 1954). Decreased interstitial cell size and number often accompanies seminiferous tubule degeneration in aging males. Similar degenerative testicular changes occur in the

aging bull (McEntee, 1958). MacLeod and Gold (1953) found increased sperm counts and ejaculate volumes and decreased sperm motility with age in men ranging from 25 to 50 years of age. Natoli, et al. (1972), studied seminal characteristics in men ranging from 45 to 91 years of age. Although these workers found a decrease in the numbers of normal sperm per ejaculate with aging, they found no age effect on the total number of sperm in each ejaculate. These studies suggest that decreased ejaculatory frequency tends to compensate for the decrease in spermatogenesis in older men. In addition, these studies imply age-related alterations in the normalcy and fertility of sperm produced in aging seminiferous tubules.

Testosterone is recognized to be essential for the maintenance of the male reproductive system and the stimulation of sexual behavior. Many of the previously mentioned alterations in sexual activity and spermatogenesis in aged males are consistent with a hypothesis of decreased testosterone synthesis in the aged. Several investigators have measured blood testosterone in aging men. Although the early work of Hollander and Hollander (1958) showed decreased testosterone concentrates in aged human testicular venous blood, several subsequent early studies failed to find age-related alterations in serum testosterone concentrations in blood samples collected from adolescence to old age (Coppage and Cooner, 1965; Kent and Acone, 1966; Gandy and Peterson, 1968). In more recent years several

investigators have completed major studies of large numbers of men indicating decreased blood testosterone concentrations with increasing age (Baker, et al., 1976; Greenblatt, et al., 1976; Mazzi, et al., 1974; Rubens, et al., 1974; Stearns, et al., 1974; Vermeulen, 1976). Vermeulen (1976) found a progressive decrease in plasma testosterone concentrations from 50 years of age. This decrease in total blood testosterone was accompanied by an increase in plasma testosterone binding capacity which resulted in a major decrease in free testosterone from 10.6 ng/100 ml in 20 to 50 year old men to only 3.6 ng/100 ml in men over age 65.

The effect of age on hormone receptors is another variable which can affect endocrine control systems. Alterations in tissue responsiveness to hormone regulation, including androgen stimulation of reproductive accessory tissue, is a common alteration that can occur with biological aging. Decreased androgen receptors in the aging male rat have been reported in the liver (Roy, et al., 1974) in the prostate (Shain, et al., 1973) and in the hypothalalmus, cerebral cortex, pituitary and testis (Chauknyiska and Vassileva-Popova, 1977).

Relatively less is known about the effect of age on testicular endocrine function in other species. A study by Eleftheriou and Lucas (1974) found no changes in plasma testosterone in two strains of aging mice. In addition, Nelson, et al. (1975) failed to show decreased plasma testosterone concentration in C57BL/6J male mice at 28 months

of age. On the other hand Leathem an Albrecht (1974) found sharply reduced testicular Δ^5-3β hydroxysteriod dehydrogenase activity, suggesting decreased testosterone secretion in Long-Evans male rats at age 18 and 24 months of age.

This suggestion of decreased serum testosterone in aging male Long-Evans rats was confirmed by my M.S. thesis which concerned blood testosterone concentrations and testicular responsiveness to human choronic gonadotropin (HCG) in the aging male rat (Miller, 1976). My study showed sharply decreased resting serum testosterone in 22 to 30 month old male rats compared to 4-month old controls. Separate groups of young and aged males received intravenous injections of 0, 1, 5, or 20 IU of HCG. Serum testosterone was measured from serial blood samples collected at 45, 90, and 150 minutes after HCG injection. Serum testosterone was increased in both young and aged groups following all HCG treatments. However, the increase was smaller in the aged compared to the young rat. The result of our initial experiment did not identify whether the decrease in basal testosterone and testicular responsiveness to HCG was due to interstitial cell pathologies in aged rats or an age-related failure in testicular stimulation.

Several groups have measured the effects of HCG treatment in aged men. Although Mazzi, et al. (1974) found lower basal blood testosterone in the older man, they reported similar percentage increases in testosterone after HCG treatment. On the other hand, Rubens, et al. (1974) found

both lower blood testosterone and reduced testicular responsiveness to HCG stimulation in aged compared to young men.

Changes in endocrine control systems of aged males could also involve steroids other than testosterone. Pirke and Doerr (1975) and Rubens, et al. (1974) have reported increased free and total plasma estradiol and estrone in aged men. The increase in estrogen is thought to be responsible for the increase in testosterone binding globulin reported in the blood of aged men (Vermeulen, 1976) and the resultant decrease in free testosterone in these men.

If the gonadal control system remains functional with age, the decrease in testicular secretion and serum testosterone concentration should be detected by hypothalamic-hypophyseal negative feedback systems and result in increased pituitary gonadotropin secretion. This relationship between testicular endocrine fuction and gonadotropin secretion in aging males has received considerable attention. Early studies of this control system in aging men showed that the increase in urinary gonadotropins with increasing age was much smaller than the postmenopausal increase in gonadotropins in women (Pedersen-Bjergaard and Jonnesen, 1948). Recent studies by Baker, et al. (1976), Rubens, et al. (1974) and Stearns, et al. (1974) have demonstrated increased circulating concentrations of luteinizing hormone (LH) and follicle stimulating hormone (FSH) in aging men. The increase in FSH reported in these studies was

consistently greater than that reported for LH. This increase in gonadotopin secretion by aged men suggest the pituitary is responsive to the decrease in testosterone in these subjects. However, it is not clear why the increase in gonadatropin secretion is not of sufficient magnitude to effectively restore testicular secretion of testosterone to the concentrations of this hormone that are present in young men.

In contrast to the findings in aged men there is no evidence for increased LH secretion in the aged rat. Previous studies in our laboratory and by others have demonstrated decreased serum LH concentrations in the aged male rat (Riegle, et al., 1976; Riegle and Meites, 1976; Meites, et al., 1978). In contrast to the data from men, these studies indicate that the aged male rat does not increase LH secretion in response to the decrease in testosterone, suggesting a neuroendocrine imbalance in the rat rather than the primary testicular alteration believed to contribute to decreased testosterone concentrations in men.

The responsiveness of the pituitary to hypothalamic stimulation has been tested in aged men and rats. Studies of pituitary responsiveness to LH-releasing hormone (LHRH) in aged men have consistently showed increased LH secretion. Mazzi, et al. (1974) found similar increases in LH after LHRH in young and aged men over 70 years. Rubens, et al. (1974) and Hashimoto (1973) reported a relatively reduced responsiveness of aged men to LHRH. However, this reduced

responsiveness reflects high pretreatment LH concentrations since both groups had similar maximal serum LH concentrations after LHRH were similar in young and aged men. On the other hand, Haug, et al. (1974) and Snyder, et al. (1975) reported decreased LH responsiveness to LHRH in older men in terms of both magnitude of the increase in blood LH after LHRH stimulation and the maximum concentration of LH attained. Snyder, et al. (1975) also found decreases in the increase in plasma FSH following LHRH stimulation in the older men. These data support a hypothesis of altered neuroendocrine function as well as Leydig cell failure in aged men.

My M.S. thesis showed that the pituitary of aged male rats is capable of increasing serum LH concentrations after LHRH stimulation. The magnitude of the increase in LH secretion after a single LHRH injection was smaller in aged compared to young male rats (Riegle and Meites, 1976). However, we found similar serum LH in young and aged male rats following multiple LHRH injections. This finding was supported by our report of decreased pituitary LH content and reduced LH release after LHRH treatment in pituitary incubates from aged compared to young male rats. (Riegle, et al., 1976).

Reported aging changes in the gonadal control system of male mice are not similar to what we found in the rat. Finch (1978) reported similar serum LH concentrations in 10 and 28 month old male C57BL/6J mice. In addition, this

study showed similar increases in testosterone secretion from young and aged mice testis slices, incubated with variable concentrations of LH. This study suggests no functional impairment of the male reproductive control system with aging in this strain of mice.

In recent years there has been increasing recognition in the role of the hypothalamus in the regulation of reproduction and its possible role in the effect of age on reproductive function in the male. It is believed that the hypothalamus synthesizes LHRH primarily in the preoptic region. The gonadotropin releasing hormone is transported to the median eminence where, upon stimulation, the pituitary stimulation factor is released into the hypothalamic-hypophyseal portal vascular system. A large number of experiments suggest that the secretion of hypothalamic hormones is regulated by the neurotransmitters of the central system. Fuxe and Hokfelt (1969) demonstrated the presence of dopamine containing neurons in the external layer of the median eminence by histochemical techniques. These workers also reported that dopamine release from these neurons was correlated with pituitary gonadotropin release. In recent years numerous laboratories have attempted to establish the role of specific hypothalamic neurotransmitters with the control of hypothalamic gonadotropin releasing hormone secretion, often with conflicting evidence and conclusions. Kamberi, et al. (1970) showed that dopamine could stimulate FSH release from pituitaries incubated with

hypothalamic fragments, and Schneider and McCann (1970) found third ventricle injection of catecholamines stimulated pituitary gonadotropin and inhibited prolactin secretion. Results of other experiments have suggested that hypothalamic gonadotropin stimulating hormone secretion may be specifically controlled by the activity of norepinephrine (Sawyer, et al., 1974; Cocchi, et al., 1974). Although Fernstrom an Wurtman (1977) summarized the evidence for neurotransmitter regulation of LHRH secretion as inconclusive, many other investigators believe that neurotransmitter function is directly involved with the regulation of hypothalamic function.

Several experimenters working on the effect of aging on endocrine function have postulated significant age related alterations in hypothalamic sensitivity to control input. Dilman (1976) has suggested decreased hypothalamic sensitivity to multiple hormone inputs. Aschheim (1976) hypothesized increased hypothalamic sensitivity to estrogens in aged rats. A series of experiments from our laboratory (Riegle and Miller, 1978), and from Dr. Meites' group (Meites, et al., 1978) suggest altered hypothalamic responsiveness to testosterone feedback related to castration, stress and the catecholamine precursor L-Dopa.

The decrease in hypothalamic responsiveness to control inputs is consistent with the hypothesis of decreased hypothalamic neurotransmitter function in aged rats. In a preliminary experiment included in my M.S. thesis, we found

decreased whole hypothalamic dopamine and norepinephrine content in 24-26 month old compared to 4 month old male rats (Miller, et al., 1976). Although this reduction in content does not prove there are functional deficiencies in these neurotransmitters at specific hypothalamic neurons involved in gonadotropin control, these findings are consistent with the hypothesis of involvement of neurotransmitters with the reduction of hypothalamic-pituitary function in the aged rat. Our report of reduced catecholamines content in aged male rats has been confirmed and expanded by the report of Simpkins, et al. (1977) showing decreased hypothalamic content and turnover of dopamine and norepinephrine and increased serotonin turnover in old compared to young male rats and our recent report of hypothalamic and median eminence function in aged rats (Riegle, et al. 1979). It is assumed that these changes in hypothalamic neurotransmitter function are related to the age-related decrease in hypothalamic responsiveness to changes in testosterone negative feedback in the aged male rat.

In summary, aging has been reported to affect reproductive function in males of all mammalian species studied. On the other hand, the specific effects of biological aging varies markedly among the species studied. Decreased spermatogenesis and fertility seems characteristic of all species. Aging men have been reported to have alterations in both testosterone secretion and hypothalamic responsiveness to control input. On the other hand, the aged male rat

has decreases in both testosterone and LH which seem reversible with LH and LHRH stimulation which suggests that age alterations in hypothalamic responsiveness may be the primary lesion in the reproductive control system in this species.

Effect of Age on Reproductive Control Systems in the Female

The most fundamental effect of aging on the mammalian female reproductive control system is the irreversible decline in oocytes which begins during fetal and neonatal life and continues through the reproductive lifespan of the individual (Jones, 1970). The best known and most widely documented consequence of aging on female reproductive control systems is the loss of ovarian function which occurs in women. Although the number of normal oocytes is exhausted at, or shortly after, menopause in women (Jones, 1970), other species experience reproductive failure with substantial numbers of oocytes remaining in the ovaries (Talbert, 1978). In many instances, estrous cycles, and at least in some instances, ovulation, can be induced by hormone or drug therapy in aging females that have experienced loss of ovarian cycles or infertility (Finch, 1978). However, decreased fertility precedes ovarian cycle loss in all species which have been studied to date. These findings suggest that the loss of oocytes is not the primary factor involved in the failure to reproduce in these species.

The effect of age on reproductive function has been most extensively studied in the laboratory rodent, particularly

in the rat. Finch (1978) has proposed two phases of reproductive senescence in aging female rodents. The first phase, which is characterized by the loss of fertility in animals which retain apparently normal ovarian cycles, begins before the rat reaches the middle of its normal lifespan. The changes in reproductive control systems in the rat during this interval appear to be similar in many respects to the premenopausal decline of fertility in women. The second phase begins later in the rat's lifespan and is characterized by alterations in the patterns of the rat estrous cycle.

Aschheim (1976) has extensively studied the effect of aging on estrous cycles in the rat. The first change in vaginal cytological patterns in the rat is the development of increased incidence of cornified cells in vaginal lavages which leads to the development of constant estrous states. At more advanced ages, increased numbers of the aging female rats develop vaginal cytological patterns characteristic of repetitive pseudopregnancies or they may eventually enter a noncyclic, diestrous state. A large number of studies have shown that the loss of ovarian function in the aged rat is not accompanied by the large increase in serum gonadotropin (Riegle and Miller, 1978; Meites, et al., 1978; Finch, 1978) which is characteristic of postmenopausal women (Heller and Heller, 1939).

A great deal of attention has recently been directed toward the identification of specific lesions occurring in

the aging hypothalamic-pituitary-gonadal control system of the laboratory rodent. Many studies have shown decreased litter size prior to decreased ovulatory rates in a variety of species (Adams, 1970; Fekete, 1946; Harman and Talbert, 1970). Age-related alterations in reproduction which result in decreased fertilization and inhibition of the development of the preimplanted egg has been proposed. The normalcy of oocytes from aged mammalian females has been studied by transplanting fertilized eggs from old donors into the uteri of young recipients. Although an initial study of ova transplanted from aged to young female hamsters by Blaha (1964) indicated decreased fertility from the eggs from aged hamsters compared to eggs transported from young donors to young recipient controls, subsequent studies by Gosden (1974), Jones (1970) and Talbert and Krohn (1966) utilizing somewhat different postovulatory transplant times have shown similar viability of aged compared to young donor ova transplanted to young recipient mice and rabbits. In addition, these same studies show sharply decreased survival of eggs transplanted from young donors to aged recipients compared to transplants between young donors to young recipients.

These data indicate that the decrease in fertility of aged mammalian females occurs much earlier than any known alteration in ovarian reproductive potential and implicate a deterioration in the ability of the reproductive tract, presumably the uterus, to sustain normal gestation with increased age. Age-related alterations in uterine function

have been implicated in the decrease in fertility of both premenopausal women and in mid-aged rodent females. Failure of the uterus to sustain pregnancy could involve both anatomical changes in reproductive tract tissue which impair their ability to sustain the conceptus and age changes in endocrine stimulation of uterine function. Blaha (1967) and Holinka and Finch (1977) have reported decreased decidual tissue development after uterine endometrial stimulation in aged hamsters and mice. Since this decrease in decidualization in aged females could reflect declines in either the magnitude of hormone stimulation or endometrial responsiveness, several investigators have considered the effect of age on ovarian steroid secretion.

Both direct and indirect estimates have been made on the effect of aging on luteal progesterone secretion. Fekete (1946), Green (1957), and Harman and Talbert (1970) have reported reduced numbers of luteal cells in the ovaries of postovulatory aging mice. In addition, attempts to increase blood progesterone concentration in aged mice by surgical ovarian implants from young donors (Blaha, 1970) or progesterone injections (Gosden, 1975) have resulted in increased numbers of fertilized eggs undergoing implantation. On the other hand, Spilman, et al. (1972) found similar plasma progesterone concentrations during pregnancy in young and aged rabbits and Larson, et al. (1973) showed no effect of postovulatory progesterone injection on the fertility of aged rabbits. The effect of age on progesterone secretion

and the biological activity of progesterone is of fundamental importance and warrants additional consideration in the search for understanding the effect of aging on reproductive control systems.

The influence of progesterone in pregnancy could be influenced by age-related alterations in uterine responsiveness to the hormone. For instance, normal cytosol receptor function is essential for progesterone function. Feil, et al. (1972) found induction of progesterone receptors in estrogen treated ovariectomized rat. Since estrogen and progesterone receptors can be influenced by the presence of these hormones, a preexisting deficiency in either of these hormones could result in impairment of uterine responsiveness to the steroids. Additional experiments need to be conducted to clarify these important areas.

Information is currently available concerning aging effects on estrogen secretion in laboratory rodents (Lu, et al., 1979; Steger, et al., 1979; Page and Butcher, 1982). Our preliminary data indicate reduced serum estradiol concentrations in aged constant estrous rats (45 pg/ml) compared to young proestrous (122 pg/ml) or estrous (93 pg/ml) rats (Miller and Riegle, unpublished). Age related alterations in estrogen secretion could be important in many of the changes occurring in reproduction in rodent species and warrant further experimentation.

Age alterations in estrogen receptor function could also affect the biological activities associated with the

hormone. Peng and Peng, (1973) reported decreased estradiol uptake in aged rat hypothalami and pituitaries compared to young controls. Estimates of estradiol receptor concentration indicate progressively reduced receptor control in the hypothalamus and cerebral cortex (Haji, et al., 1981; Kanungo, et al., 1975) but not in the pituitary (Haji, et al., 1981). Estradiol receptor content is also progressively reduced in the uterus of the aged rat (Hsueh, et al., 1979) with the concentration at 30 months of age only about 15% of that at 4 months of age.

Although there has been considerably more data collected related to estrogen and progesterone function in aging women than in rodent species, most of these studies have concerned changes in the menopausal rather than the premenopausal years. Talbert (1978) points out that as women approach menopause, failure of ovulation and corpus luteum formation becomes an increasingly common occurrence. Preliminary studies on premenopausal women (Sherman and Korenmen, 1975) suggest that blood estradiol levels are reduced by about 50% throughout their ovarian cycle. There is also evidence that corpora lutea formed in these premenopausal women may be anatomically abnormal and may secrete less progesterone. The decrease in progesterone in these women is hypothesed to result in shortened menstrual cycles or early abortion of the fetus if pregnancy has occurred (Collett, 1954; Novak, 1970).

Mean values for pregnandiol of 24 hour urine samples (Klapper and Wilson, 1962), luteal progesterone secretion (Sherman, et al., 1976), and plasma 17-dehydroxy-progesterone (Abraham, et al., 1969) have all been reported to be decreased in postmenopausal women. The work of Greenblatt, et al. (1976) and others has also established that ovarian estrogen secretion, particularly estradiol, decreases in the menopausal woman. Poortman, et al. (1973) have shown that peripheral metabolism of androgens, including those of ovarian origin, is a major source of estrogen in postmenopausal women. These workers showed that ovarian vein concentrations of estrogens in postmenopausal women were only about one third that found in premenopausal women while androgen synthesis is increased to about twice the level of premenopausal ovarian secretion. In summary, there are major endocrinological alterations occurring with the menopause. Most of the studies of this system have concerned the loss of ovarian gamete and endocrine function. There have been only minimal attempts to consider specific age-related lesions which may occur in the hypothalamic-pituitary-gonadal control system in the premenopausal woman.

Our laboratory and others have proposed an alteration in hypothalamic-pituitary unit sensitivity to steroid feedback in the aging mammal. However, the sharp increase in post-menopausal FSH and LH secretion reported by Odell and Swerdloff (1968) and others and their demonstrated ability to induce ovulatory-like gonadotropin surges using steroid

treatments in postmenopausal women challenges this theory when it is applied to women. Another proposed mechanism to explain the decline in fertility in premenopausal again women was the proposed exhaustion of normal oocytes in the aging ovary. However, the previously mentioned fertility of transplanted eggs from aged rodents and the description of anatomically normal primordial follicles in non-ovulatory menopausal women (Costoff and Mahesh, 1975) challenges this theory. These findings offer partial support to Longcope's (1974) hypothesis of an age-related change in ovarian sensitivity to gonadotropin in the menopausal years. Ovarian factors associated with this apparent insensitivity to gonadotropins, including differences in gonadotropin receptors in the human ovary, remain to be identified.

Pituitary gonadotropin secretion is sharply increased in postmenopausal women. FSH and LH concentrations were reported to increase and to lose their typical cyclicity during the menstrual cycles of the early climacteric (Adamopoulos, et al., 1971; Yahia, et al., 1964). Everitt (1976) hypothesized that increased gonadotropin secretion in premenopausal women could contribute to the loss of oocytes in the menopausal ovary. However, a premenopausal increase in gonadotropin secretion has not been uniformly reported. Kohler, et al. (1968) have not shown increased gonadotropin secretion in women until after the climacteric. The increase in gonadotropin secretion in postmenopausal women appears to occur as a direct result of reduced negative

feedback associated with alteration in ovarian estradiol and progesterone secretion since Odell and Swerdloff (1968) showed that restoration of these hormones reduced the post-menopausal increase in gonadotropin secretion.

Although aged female rodents lose their reproductive capacity long before they reach their maximal longevity, there is minimal evidence for increased gonadotropin secretion associated with this infertility. Wilkes, et al. (1979) reported increased serum FSH at 10 a.m. in proestrus in 12 month old rats with regular estrous cycles compared to 6 month old controls. Although Clemens and Meites (1971), Wilkes, et al. (1978), and Lu, et al. (1979) found modest increases in serum FSH in older acyclic rats, the magnitude of this increase does not approach the FSH concentrations characteristic of postmenopausal women. Aschheim (1976) has reported the presence of deficiency cells in ovarian interstitial tissue whose anatomical appearance could be restored by LH injections. This suggestion of inadequate gonadotropin secretion in aged female rats is supported by direct measurements of serum LH concentrations in aged groups. Several laboratories have shown that the aged female rat maintains increased serum prolactin but reduced LH concentrations (Huang, et al., 1976; Shaar, et al., 1975; Watkins, et al., 1976). In addition, Shaar, et al. (1976) showed smaller increases in serum LH following ovariectomy in aged compared to young female rats. These data could reflect decreased pituitary capacity for gonadotropin secretion.

Although Watkins, et al. (1976) reported decreased pituitary responsiveness in terms of increased serum LH after a single LHRH injection in aged compared to young female rats, the increase in LH stimulated by LHRH indicates that the aging rat is capable of sustaining greater pituitary gonadotropin secretion than normally occurs. This observation of similar pituitary responsiveness to LHRH in young and aged rats is similar to the reports of Tsai and Yen (1971) and Wentz, et al. (1975), showing no difference in pituitary response to LHRH stimulation in pre and postmenopausal women.

Many investigators believe the hypothalamus is a primary site of age-related alterations in female reproductive control systems in mammalian species. Changes in hypothalamic responsiveness to control systems input have been reported in aging females by several laboratories (Dilman, 1976; Meites, et al., 1978; Riegle and Miller, 1978). Aging effects on the ability of hypothalamic neurons to synthesize LHRH could contribute to the decrease in gonadotropin secretion. However, in a preliminary study, we found no age differences in biological LHRH activity in hypothalamic extracts from young and aged rats (Riegle, et al. 1976). These data were confirmed by the experiments of Steger, et al. (1979) who found similar immunoassayable LHRH in hypothalamic extracts from young and aged rats. These data suggest that age alterations in the ability of the rat hypothalamus to release LHRH, rather than aging effects on

LHRH synthesis, contribute to the decrease in pituitary LH secretion.

Many researchers have shown that the release of hypothalamic hormones, including LHRH, into the hypothalamic-hypophyseal portal vessels in the median eminence can be influenced by alterations in hypothalamic neurotransmitters. In particular, Fuxe and Hokfelt (1969) and Sawyer, et al. (1974) and others have shown an association with hypothalamic dopamine and norepinephrine function with LHRH secretion. The decrease in hypothalamic dopamine and norepinephrine content and turnover previously described in the male rat suggests that loss of catecholamine function could contribute to the decrease in hypothalamic LHRH release and the loss of fertility in the female rat. This hypothesis was at least in part challenged by the report of Wilkes, et al. (1979) indicating increased median eminence norepinephrine content on the morning of proestrus in regularly cycling 12 month compared to 6 month old controls. In recent experiments, our laboratory has extended study to the aged female rat and have found decreased dopamine content and turnover in the hypothalamus and median eminence of aged pseudopregnant-like or constant estrous rats compared to young controls with normal estrous cycles (Riegle, et al., 1979; Demarest, et al., 1982).

In addition, there is considerable indirect evidence which supports the hypothesis of decreased catecholamine function as a primary cause of reproductive failure in aging

rats. Riegle and Meites (1976) and Watkins, et al. (1976) showed decreased pituitary responsiveness of L-Dopa inhibition of prolactin secretion in aged male and female rats compared to young controls. As mentioned previously, one of the first alterations in the ovarian cycle of the aging rat is the development of the constant estrous state. Clemens and Meites (1971) and Wilson (1974) showed resumption of estrous cycles and ovulation in aged constant estrous rats receiving controlled electrical stimulation of the preoptic region of the hypothalamus. Finch (1978) has summarized a large number of studies which have shown that a variety of experimental treatments, including progesterone, ACTH, L-Dopa, lergotrile, iproniazid or epinephrine injections or stress treatments, were capable of apparent reinitiation of estrous cycles in aging constant estrous rats. Although some of these treatments may reinitiate estrous cycles by affecting hypothalamic neurotransmitter and LHRH secretion, direct evidence of effects of these agents on hypothalamic monoamines is lacking. Several of these treatments, including progesterone, ACTH and epinephrine injections or stress, could act by increasing progesterone secretion which, in turn could be involved in the regulation of gonadotropin secretion by the hypothalamic-pituitary unit.

In summary, current experimental evidence suggests more than a single mechanism involved with the effects of aging on female reproductive control systems. Aging rats and the menopausal woman are reported to have decreased ovarian

steroid secretions. The primary decrease in ovarian steroid secretion results in sharply increased gonadotropin secretion in postmenopausal women. Although the relationship between ovarian steroid and gonadotropin secretion in premenopausal women is not clear, current experimental data in the aging rat suggest that decreased steroid secretion may be associated with reduced pituitary gonadotropin secretion. Similarly, there is minimal evidence for hypothalamic involvement with decreased fertility in aging women whereas decreased sensitivity of hypothalamic gonadotropin control mechanisms to control input seems to be unquestionably a significant alteration of the reproductive control system of the aging female rat.

EXPERIMENTAL

Introduction

The focus of my graduate research has been to identify age-related alterations in reproductive control systems of the laboratory rat. The data collected in the Ph.D. research have been organized and presented in eight separate manuscripts. Seven of these papers are already published. The other one is currently undergoing editorial review. The remaining portion of this thesis is organized to reflect these research publications. My approach to this presentation is to first describe materials and procedures which are common to all of the experiments, then to summarize my findings related specifically to the aging male rat, and finally to present my findings concerning age effects of the female rat reproductive control system.

Materials and Methods

Experimental Animals

Rats used in these studies were of the Long-Evans strain. All of the rats utilized were bred and raised in our colony from genetic stock obtained from Blue Spruce Farms, Altamont, New York and Charles River Breeders, Wilmington, Massachusetts. All rats were housed in the

Endocrine Research Unit's rat colony under controlled light (12 hour light cycle) and temperature (22 $\pm$ C^{o}) and were allowed free access to Wayne Lab-Blox (Allied Mills, Chicago, Illinois) and tap water. Only rats which were maintaining stable body weights and were free of obvious tumors or disease were used in these studies. Animals known to be sick or diseased were removed from the colony and destroyed. None of the experimental animals received antibiotic or other drug therapies to combat respiratory disease or other maladies.

Blood Collection

Most of the experiments included in this thesis involved serial blood collections from individual rats. Our standard procedure for blood collection involved orbital sinus puncture using a capillary tube under light ether anesthesia. After sinus puncture, venous pressure causes blood to flow through the capillary tube and into 12 x 75 mm disposable culture tubes for collection. In recent years we and others (Euker, et al., 1975; and Ruisseau, et al., 1978) have shown that acute stress can affect blood hormone concentrations. In our experiments we attempted to minimize nonspecific stress effects on blood hormone concentrations by standardization of rat handling techniques associated with blood collection. Rats were removed from their cages and placed in ether-saturated desiccator jars. The rats were then quickly transported to a surgery room where blood samples were collected. The volume of each blood sample collected

ranged from 0.5 to 1.5 ml depending on the number of serial blood collections included in the experimental design and the amount of serum needed for the hormone analyses.

Blood samples were allowed to clot at room temperature from 30 to 60 minutes and then refrigerated overnight. Serum was separated by centrifugation, decanted into clean 10 x 75 mm disposable culture tubes and was stored at -20°C until used for steroidal or protein hormone radioimmunoassays.

Radioimmunoassay for Testosterone and Progesterone

The radioimmunoassay procedure for testosterone determination was a modification of the technique validated by Mongkonpunya, et al. (1975) and that for progesterone a modification of the progesterone radioimmunoassay outlined and validated for the Niswender antibody by Gibori, et al. (1977).

A single serum sample large enough to furnish duplicates of two serum volumes for final assay (i.e., if we wished to assay duplicates of 25 and 100 ul of serum, we would initially extract at least 250 ul of serum) was decanted into a glass stoppered 15 ml extraction tube. Three ml of N-hexane was added to each tube, the tubes were securely stoppered and vigorously agitated for two minutes. After this extraction, the tubes were stored at -20°C for about thirty minutes in order to freeze the aqueous phase. The hexane was then decanted into clean 12 x 75 mm culture tubes. The extract was then taken to dryness in a water bath evaporator

by passing a slow stream of air over the solvent with the tubes maintained at 42 $\pm$ 2°C. The extraction procedure was then repeated for each serum sample with the solvent from the second extraction added to the dried culture tube that contained the first extract residue and then dried down again. Extraction efficiency was estimated by the addition of tritiated testosterone or progesterone (about 3000 dpm) to serum samples subjected to the same extraction procedure. The solvent from these recovery extractions was decanted into scintillation vials and the radioactivity compared to similar amounts of tritiated hormone pipetted directly into counting vials. Aliquots of pooled rat serum were included in each assay to determine interassay variability. In addition, solvent blanks were included in each steroid radioimmunoassay.

At the end of the extraction procedure a known volume of phosphate buffered saline was added to each culture tube to solubilize the extraction residue. After addition of the saline, the contents of the tubes were mixed by vortexing for ten seconds and the tubes were covered and stored overnight at 30°C. The next day duplicates of two serum equivalents were pipetted from the extract-containing culture tubes and transferred to clean 12 x 75 mm culture tubes. The content of these tubes was then adjusted to 200 ul total volume by the addition of appropriate amounts of phosphate buffered saline.

All tubes then received 200 ul of phosphate buffered saline which contained progesterone or testosterone antibody. The antibodies used were Dr. Niswender's # 666 anti-testosterone and # 337 anti-progesterone (Dr. G.D. Niswender, Colorado State University, Fort Collins, Colorado). The concentration of antibody was adjusted to optimize assay conditions for binding and sensitivity (anti-progesterone use used at 1:2000 and anti-testosterone at 1:15000 dilutions). After addition of the antibody, the content of the tubes was stirred by vortexing for ten seconds and the tubes were stored at room temperature for thirty minutes to allow equilibration. At this time 100 ul of phosphate buffered saline containing about 20,000 dpm of chromatographically purified H^3 -1,2,6,7 Testosterone or H^3 -1,2,6,7 Progesterone was added to each tube. The content of each tube was again mixed by vortexing for ten seconds and the tubes were stored overnight at 3^oC to allow equilibration of the antibody-antigen complex.

To separate free from antibody bound steroid, 1 ml of cold 25% polyethylene glycol 4000 (Fisher Scientific, Fair Lawn, New Jersey) was added to each tube. The content of each tube was mixed by vortexing and the tubes were placed in an ice bath for a thirty minute equilibration. At the end of the equilibration, the antibody bound and free hormone were separated by centrifugal separation of the polyethylene glycol, which binds the free hormone, in a refrigerated centrifuge.

After centrifugation, a 1.0 ml aliquot of the supernatant fluid of each tube was decanted into mini liquid scintillation vials into which 5 ml of aqueous counting scintillant (Amersham Corporation, Arlington Heights, Illinois) was added. The content of the scintillation vials was mixed, allowed to equilibrate in light shielded boxes and counted on a liquid scintillation counter (Searle Analytical Delta 300 Liquid Scintillation System).

The counts from the unknown serum extracts were arithmetically corrected for hormone loss during the extraction procedures and for the solvent blanks. Hormone content (ng/ml) of the unknown serum samples was determined by comparison of the counts of the unknown with standard curves which were constructed as the log of hormone concentration from three sets of assay tubes which contained known amounts of progesterone or testosterone. Separate sets of standard curves were prepared for each centrifuge run.

Radioimmunoassay of LH

The basic radioimmunoassay procedure for LH determination was that described in the NIAMDD kits (Dr. A. F. Parlow, University of California, Los Angeles). At least two serum volumes in duplicate (from 10 to 200 ul, depending on the expected serum LH content) were pipetted into 12 x 75 mm disposable culture tubes. Sufficient phosphate buffered saline, containing 0.1% gelatin, was added to bring the total volume to 400 ul. One hundred ul of NIAMDD anti-rat LH, diluted to 1:10,000 in 3% normal rabbit serum in

phosphate buffered saline was added to each tube. The content of each tube was mixed by vortexing for ten seconds and the tubes were stored overnight at 3°C. The next day, 100 ul of iodinated rat LH diluted to a concentration of about 30,000 dpm was added to each tube. The iodinated LH was generously supplied by Dr. Meites. The content of each tube was mixed by vortexing for 10 seconds and the tubes were stored overnight at 3°C. On the following day, the antibody-antigen complex was precipitated by the addition of 100 ul of a second antibody produced by specific immunization of sheep against rabbit gamma globulin. The second antibody was produced in our laboratory and titrated for maximal assay efficiency (usually from 1:60 to 1:80 dilutions). The content of each tube was again mixed by vortexing and the tubes were returned to the cold (3°C) for three more days of equilibration. On the third day, 3 ml of cold phosphate buffered saline was added to each assay tube. The antibody-antigen precipitate was concentrated by centrifugation at 2800 rpm (5,500 g) in a refrigerated centrifuge (Sorvall, RC-5). At the end of the centrifugation, the supernatant fluid containing the free hormone fraction was poured off and discarded. Each assay tube was counted in our automatic gamma counting system (Searle Model 1197, Automatic Gamma System).

Unknown serum LH concentrations were determied by comparison of the dpm of the precipitates of each assay tube with standard curves constructed as dpm as a function of the

log dose of standard LH (NIAMDD rat LH RP-1). Samples of pooled rat serum were run in each assay to allow estimation of interassay variation in LH determination. Individual assay variables in terms of nonspecific binding and total antibody binding were determined for each LH assay. The nonspecific binding tubes contained only the diluent for the LH antibody in place of the antibody containing diluted rabbit serum. The total antibody binding tubes were equivalent to zero hormone standards. The counting time for each assay was calculated as that interval required to record 10,000 disintegrations from the total count tubes.

Experiment 1: The Effect of Age on Reproductive Control Mechanisms in the Male Rat

A. Responsiveness of the Aged Male Rat Testis to Chronic HCG Stimulation

The studies included in my Master's thesis (Miller, 1976) showed sharply reduced basal serum testosterone concentrations in aged compared to young male rats. In addition, these studies showed that the aged rat had smaller increases in testosterone following intravenous injection of 1, 5 or 20 IU of HCG than was found in young male rats.

These data were of interest in that they seemed similar to the reported effects of age on testicular function in men. Although Coppage and Cooner (1965) and Gandy and Peterson (1968) found similar blood testosterone concentrations in the human male from adolescence to old age, most recent studies have shown sharply reduced blood testosterone

in aged human males (Kirscher and Coffman, 1968; Persky, et al., 1971; Longcope, 1973; Vermeulen, 1976; Baker, et al., 1976). In addition, Longcope (1973) reported a smaller increase in blood testosterone after HCG stimulation in aged compared to young men and Vermeulen (1976) reported an increase in plasma testosterone binding protein capacity in aged men which, coupled with the decreased total testosterone concentrations, resulted in a dramatic reduction in free testosterone in the older men.

The similarity in our initial data concerning the effect of age on Leydig cell function in the rat and data collected from older men suggested that the rat may be a particularly useful model to study age effects on reproductive function in men. However, it is recognized that an acute injection of tropic hormone may not demonstrate the secretory potential of an endocrine tissue, particularly one that has not recently received normal stimulation. Consistent with our findings in rats, Rubens, et al. (1974) found that a group of 65 to 90 year old men treated with 1500 IU of HCG per day for three days had much smaller increases in plasma testosterone than a similarly treated group of men 20 to 50 years of age. These data were interpreted as further evidence of an inherent age decline in Leydig cell function in these men. However, these types of acute experiments fall short of elucidating whether the aged testis can be stimulated to greater secretion under more chronic conditions. The following experiment was designed to consider the effect of

chronic HCG stimulation on Leydig cell function in aged male rats compared to similarly treated young male groups.

Materials and Methods

Separate groups of 16 young (5 months) and aged (22 to 26 months) male rats received subcutaneous injections of physioloigical saline containing 0 or 5 IU of HCG/100 gm bw (Ayerst Laboratories, New York) for seven consecutive days. Testosterone was measured in blood samples collected before the treatment was started and 24 hours after the final HCG injection.

The effect of the chronic HCG treatment regime on an acute Leydig cell response was measured by determining serum testosterone concentration in serial blood samples taken before and at one, two and three hours after intravenous injection of 0.2, or 2.0 IU of HCG/100 gm bw. This test of acute responsiveness after chronic HCG stimulation was started 24 hours after the final daily subcutaneous treatment.

Differences within and between age groups were tested by multivariant analysis of variance and analysis of variance for repeated measurements. Only differences with a probability of error of less than 0.05 were considered as significant.

Results

The effect of seven consecutive days of treatment with 5 IU of HCG/100 gm bw on basal serum testosterone are shown in Figure 1. Serum testosterone concentrations in the

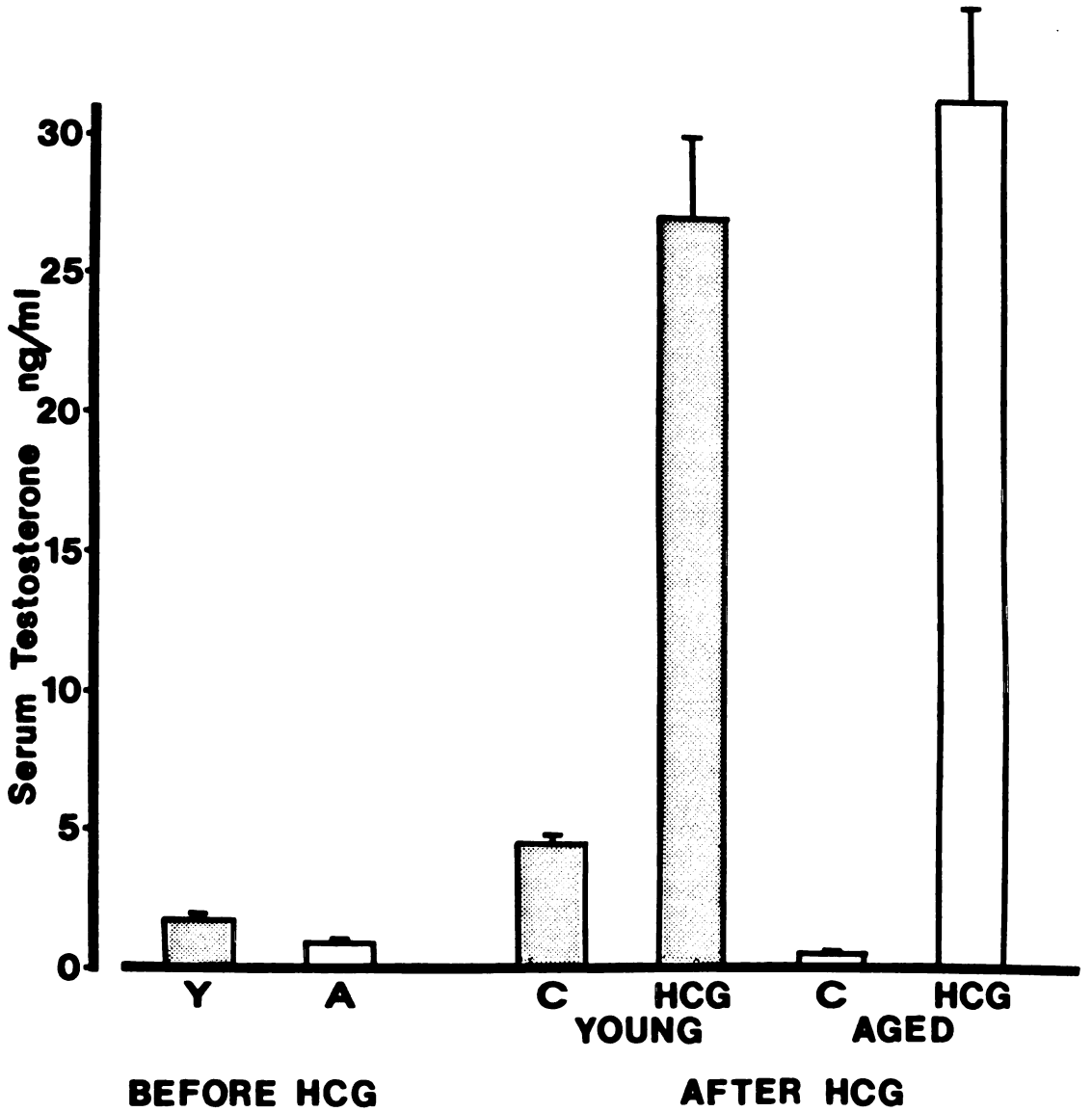


Figure 1. The Effect of 7 Days of HCG Treatment on Serum Testosterone in Young and Aged Male Rats. Seven days of daily subcutaneous injection of 0.5. ml. of saline containing 5 IU of HCG/100 gm bw or 0.5 ml of saline alone were given to young (n = 32) male rats. Testosterone is expressed as group means (ng/ml serum) with the indicated standard error of the means from blood samples collected under light ether anesthesia before HCG injections and 24 hours after the last HCG treatments (n = 16 in control and HCG treated groups).

pretreatment blood sample were greater in young than in aged rats ($P<0.01$). The difference between young and aged groups receiving the saline vehicle control injections was sustained throughout the treatment regime. On the other hand, both groups of rats receiving the chronic HCG treatment had similar large increases in testosterone concentration 24 hours after the final HCG injection ($P<0.01$). Serum testosterone concentrations were not different in young and aged rats following the HCG injections.

Figure 2 illustrates the effect of intravenous HCG injection on serum testosterone concentrations in young and aged groups which had received 0 to 5 IU of HCG/100 gm bw for seven days previous to this experiment. Although the 0.2 IU/100 gm bw injection of HCG stimulated increased testosterone concentrations in both the young and aged control groups, this level of acute HCG stimulation did not cause a further increase in serum testosterone above the elevated initial testosterone levels in either age group which had received the chronic HCG pretreatment. Intravenous injection of 2.0 IU of HCG/100 gm bw stimulated increased serum testosterone in young and aged HCG treated and control groups ($P<0.05$). The increase in testosterone following 2.0 IU of HCG injections was greater in the young control than in the aged group which was not pretreated with chronic HCG. However, this level of HCG stimulation resulted in similar stimulation of serum testosterone concentration in both young and aged groups which had received HCG pretreatment.

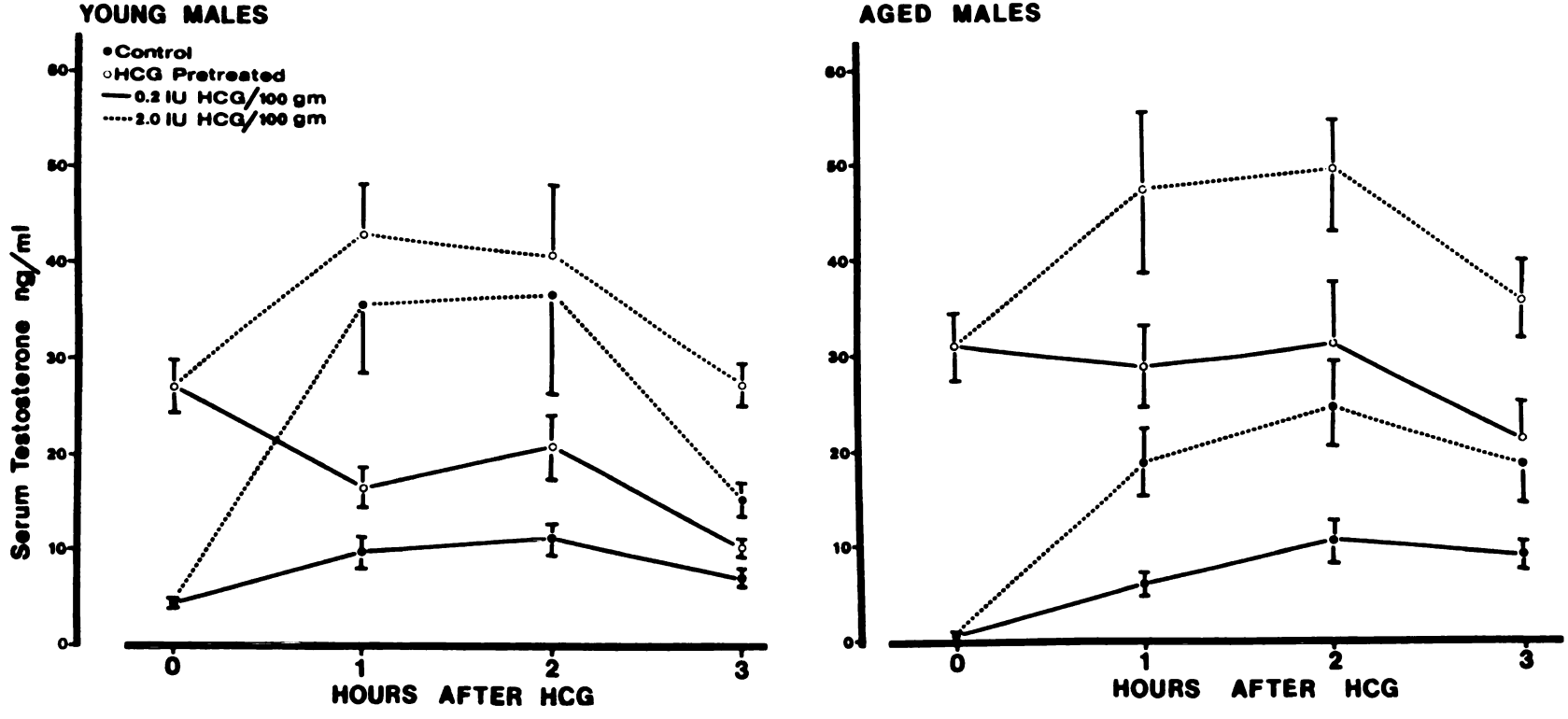


Figure 2. The Effect of Injection of 0.2 and 2.0 IU of HCG on Serum Testosterone in Young and Aged Male Rats. Intravenous injection of 0.2 and 2.0 IV of HCG on serum testosterone were given to young and aged male rats (n = 8/group) following 7 days of daily subcutaneous injection of saline containing 5 IU of HCG/100 gm bw. Testosterone is expressed as group means (ng/ml serum) with indicated standard error of the means from blood samples collected under light ether anesthesia before injection and at 1, 2, and 3 hours after the HCG injection.

Discussion

The results of this experiment suggest that the decrease in serum testosterone found in the aged male Long-Evans rat is not due to an impairment of Leydig cell steroidogenesis or to decreased sensitivity to gonadotropin stimulation. Although the aged male rat has sharply decreased basal serum testosterone and reduced responsiveness to acute testicular stimulation (Miller, 1976), the increase in blood testosterone concentrations following either acute or chronic gonadotropin stimulation suggests the Leydig cells of the aged male rat are capable of sustaining much greater testosterone secretion than normally occurs. The similarity of testicular response to seven days of HCG treatment indicates that there may be no significant decline in steroidogenic capacity of the Leydig cells. These results are consistent with the report of Leathem and Albright (1974) that Δ^5-3β- hydroxysteroid dehydrogenase activity of the aged male rat testis could be restored by similar HCG treatments as were employed in this study and other recent reports (Steger, et al., 1979; Geisthovel, et al., 1981; Kaler and Neaves, 1981) showing no decrease in LH binding by aged male rat Leydig cells. In addition, Kaler and Neaves (1981) found that diminished testosterone in aged male rats could not be attributed to functional or numerical deficits in the Leydig cell population. These data suggest that the primary factor involved with decreased testosterone secretion of the aged male rat is reduced gonadotropin stimulation of the testis.

Several recent studies have shown decreased serum LH in the aged male rat (Riegle and Meites, 1976; Bruni, et al., 1977; Gray, 1978; Pirke, et al., 1979). If the gonadal control system of the aged male rat was functioning normally, we would expect that the decrease in testosterone negative feedback would stimulate the hypothalamic-pituitary unit to increase LH secretion. Although the aged male rat has somewhat smaller increases in serum LH following a single LHRH injection than young male rats (Riegle and Meites, 1976; Bruni, et al., 1977), the increase in LH secretion is sufficient to stimulate increased Leydig cell steroidogenesis (Miller, 1976) and to maintain normal serum testosterone concentrations. These data suggest that age changes in neuroendocrine control of hypothalamic LHRH may be a major factor in the reduction in pituitary and testicular function in the male rat.

Earlier studies have found decreased neuroendocrine responsiveness to acute stress stimulation of LH secretion (Riegle and Meites, 1976) in aged compared to young male rats. These experiments suggest that a primary effect of age on hypothalamic regulation of gonadotropin secretion involves a change in its responsiveness to regulatory input. Considerable recent evidence suggests that changes in hypothalamic neurotransmitters, particularly involving catecholamine function, are associated with the regulation of LHRH secretion. My Master's thesis indicated decreased hypothalamic content of both dopamine and norepinephrine in aged

male rats. This observation has been confirmed by Simpkins, et al. (1977), Riegle, et al. (1979) and Demerest, et al. (1980) who also found decreased catecholamine turnover in the hypothalamic and median eminences of aged male rats.

In summary, these data indicate that the decrease in blood testosterone of the aged male rat is not due to an alteration in steroidogenic capacity of the testicular Leydig cell. Our results suggest that reduced testosterone secretion occurs secondarily to an age-related reduction in LH secretion which, in turn, may be associated with an alteration in the control of hypothalamic secretion of LHRH.

B. Hypothalamic LH-Releasing Activity in the Aged Male Rat

Several previous studies from our laboratory have shown decreased hypothalamic-pituitary responsiveness to gonadectomy (Shaar, et al., 1975), L-dopa (Riegle and Meites, 1976), and stress (Riegle and Meites, 1976). The increase in serum LH following LHRH injection (Riegle and Meites, 1976; Bruni, et al., 1977) suggests that the pituitary can sustain sufficient LH secretion to support testicular steroidogenesis and implicates failure of hypothalamic LHRH secretion as a major lesion in the gonadal control system of the aged male rat. An age-related alteration in the capacity of hypothalamic peptidergic neurons to synthesize and store LHRH could contribute to this alteration in neuroendocrine function. The following experiment was designed as a preliminary attempt to determine the effect of age on

hypothalamic content of LH-stimulating activity in intact and orchidectomized male rats.

Materials and Methods

Young adult (3 and 5 months) aged (22 to 26 months) intact and gonadectomized male rats were used in these studies. The gonadectomized rats had been surgically prepared eight weeks prior to the estimation of hypothalamic LH-releasing activity.

The procedures for handling hypothalamic tissue and the pituitary incubations were modifications of the techniques of Shaar and Clemens (1974). Groups of fifteen or sixteen young and aged rats were decapitated as rapidly as possible after removing them from their cages. Trunk blood was collected at decapitation for subsequent measurement of serum LH and testosterone. The skull was quickly opened, the hypothalamus exposed, and the hypothalamic island was dissected out and pooled by groups in tissue homogenizers containing 0.1 ml of cold 0.4 N perchloric acid for each hypothalamus included in the group. The weight of the hypothalamic tissue removed ranged from 17 or 22 mg and was not different between intact and gonadectomized, young or aged groups. After homogenization, the homogenate was transferred to a centrifuge tube and the homogenizer was rinsed with an additional 0.1 ml of cold 0.4 N perchloric acid per hypothalamus extracted. The homogenate from each group was centrifuged at 20,000 x g for 30 minutes at $3^{o}C$. The supernatant was decanted and brought to a pH of 7.25 by

the addition of cold 1 N NaOH. After neutralization, the volume of the hypothalamic extract was adjusted to 0.25 ml for each hypothalamus in each group.

Hypothalamic LH-releasing activity was measured by adding 0.25, 0.5 or 1.0 hypothalamic equivalents to young male rat paired hemisectioned pituitary incubates. The pituitaries were incubated at 37.5°C in medium 199 (Difco, Detroit, Michigan) which was maintained at pH 7.25 to 7.35 by the addition of 0.165 gm of $NaHCO_2$ /100 ml of culture medium and constant gasing with 95% O_2 -5%/CO_2 in a Dubnoff incubator at 60 cycles/min. Anterior pituitary halves were incubated in 2 ml of medium 199 in glass 12 x 75 mm culture tubes. After a one hour preincubation, the culture medium was decanted and discarded and 2 ml of fresh culture medium was added. The control pituitary half received only medium 199. The paired treated pituitary half received either 0.25, 0.5, or 1.0 hypothalamic equivalent in the 2 ml of medium 199. Average culture medium osmolarity ranges were from 305 to 330, 325 to 350, 355 to 380 and from 385 to 430, mOsm/L for the control tubes, and the tubes containing 0.25, 0.5 and 1.0 hypothalamic equivalents, respectively (5120 Vapor Pressure Osmometer, Wescor, Inc., Logan, Utah). At the end of a four hour incubation, the culture medium was decanted, diluted with phosphate buffered physiological saline, and frozen until the LH assays could be completed.

These data were analyzed using the "t" test for paired observations to compare the response of the treated

pituitary halves with their corresponding controls. Differences between groups treated with hypothalamic extracts were tested by multivariant analysis of variance.

Results

The effect of age on serum testosterone and LH from the trunk blood samples is shown in Table 1. Both LH and testosterone were higher in intact young male rats than in aged groups ($P<0.01$) in both age groups. However, the young gonadectomized rats maintained higher serum LH than did the aged gonadectomized group. ($P<0.05$).

TABLE 1

Serum LH and Testosterone Concentration in Intact and Gonadectomized Young and Aged Rats

Group	Age (mo)	No.	Serum Testosterone (ng/ml)[a] Intact	Serum LH (ng/ml)[a] Intact	Serum LH (ng/ml)[a] Gonadectomized[b]
Young male	3, 5	31	3.62 ± 0.35	14.9 ± 2.9	484.1 ± 31.8
Aged male	22-26	32	1.26 ± 0.12	8.1 ± 1.5	290.0 ± 31.9

[a]Serum LH and Testosterone expressed as group means ± SEM

[b]Eight weeks after gonadectomy

The responsiveness of incubated pituitaries to hypothalamic extracts is shown in Table 2 and is plotted as percent increases in LH release from hypothalamic extract treated compared to pituitary halves in Figure 3. Hypothalamic extract addition to the incubation medium increased LH release in all groups ($P<0.05$). Age differences in the effect of hypothalamic extracts on LH release were not significant in either intact or gonadectomized groups. Although the intact male group appeared to have greater hypothalamic LH-releasing activity than was found in the castrate group, the experimental design required separate incubation on separate days for each group which makes direct comparison of response between intact and orchidectomized male groups unreliable.

Discussion

The decrease in serum testosterone in aged male rats and reduced serum LH in intact and gonadectomized aged male rats are in agreement with previous reports from our laboratory (Shaar, et al., 1975,; Watkins, et al., 1975; Miller and Riegle, 1978a) and with other more recent reports (Geisthovel, et al., 1981; Gray, 1978; Pirke et al., 1979).

These data indicate that the aged male rat hypothalamic-pituitary unit is less responsive to alterations in steroidal feedback than the young male rat. We have previously reported that the aged rat pituitary can increase LH secretion after LHRH injection (Riegle and Meites, 1976). If the aged male rat hypothalamus was functionally intact, its

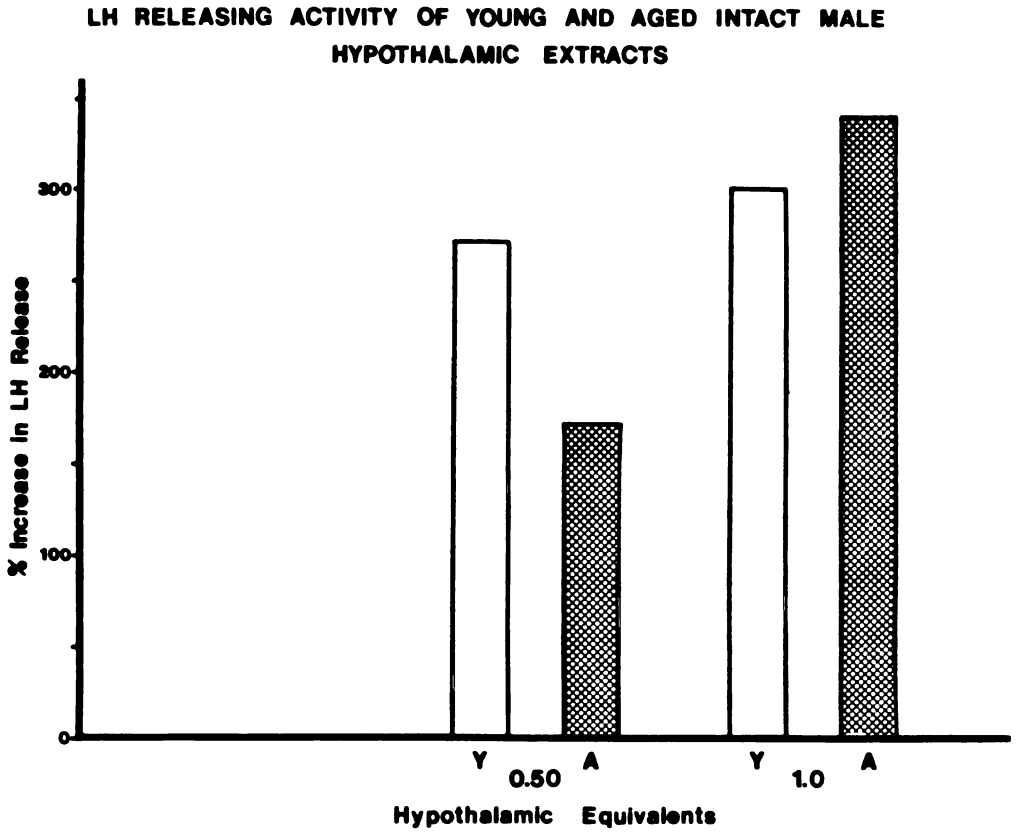
TABLE 2

Effect of Hypothalamic Extracts on LH Release from Incubated Rat Pituitaries[a]

Group	n	H.E.	LH Release (ng/mg pituitary)[b]			
			Young[c]		Aged[d]	
			Control	Treated	Control	Treated
Intact Male	8	0.50	1770 ± 376	7275 ± 1244	1436 ± 255	4671 ± 462
	8	1.00	1842 ± 171	7877 ± 983	1788 ± 231	7547 ± 770
Gonadectomized Male	8	0.25	649 ± 73	804 ± 141	947 ± 124	1629 ± 212
	8	0.50	1001 ± 182	1280 ± 152	1041 ± 148	1778 ± 133
	8	1.00	714 ± 78	1489 ± 186	730 ± 130	1490 ± 257

[a]Hypothalamic Extracts were incubated with paired hemisectioned 5 mo male rat pituitaries in medium 199.
[b]LH release expressed as group means ± SEM
[c]Young rats were 3 to 5 mo of age.
[d]Aged rats were 22 to 26 mo of age.

Figure 3. The Effects of 0.25, 0.5 and 1.0 Young and Aged Hypothalamic Equivalents from Intact and Gonadectomized Male Rats on LH Release from Incubated Pituitary Halves. Young male pituitary halves were incubated 4 hours in medium 199. LH release is plotted as the average % increase of 8 hypothalamic extract-treated pituitary halves compared to their paired control pituitary halves.


LH RELEASING ACTIVITY OF YOUNG AND AGED INTACT MALE HYPOTHALAMIC EXTRACTS
% Increase in LH Release
300
200
100
0
Y
A
0.50
Y
A
1.0
Hypothalamic Equivalents

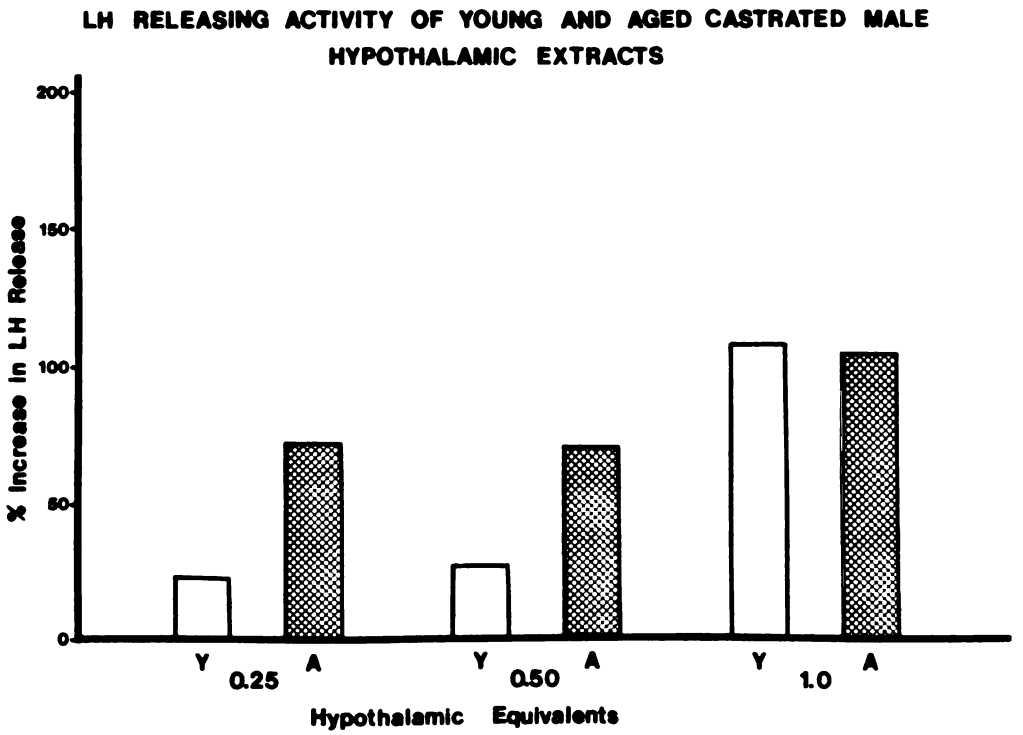

LH RELEASING ACTIVITY OF YOUNG AND AGED CASTRATED MALE HYPOTHALAMIC EXTRACTS
% Increase in LH Release
200
150
100
50
0
Y
A
0.25
Y
A
0.50
Y
A
1.0
Hypothalamic Equivalents

Figure 3.

release of LH-stimulating substances should be increased when serum testosterone concentrations are reduced. Although hypothalamic secretion of LH-releasing substances in aged intact male rats was not sufficient to maintain concentrations of blood LH and testosterone which are similar to that found in young adult male rats, further reduction of serum steroid concentration by gonadectomy results in sharply increased LH secretion. The magnitude of the increase in serum LH concentration was smaller in aged than in the young rats. However, the proportion of LH increase between the young and aged groups is roughly similar when pre- and post-gonadectomy hormone concentrations are compared. Although these data suggest some reduced ability of aged male rat hypothalamic-pituitary units to sustain LH after gonadectomy, the control system is responsive to sustaining higher serum LH concentrations than normally found in the aged rat. The similarity of LH-releasing activity of hypothalamic extracts between young and aged groups supports this hypothesis that the aged rats are capable of stimulating greater pituitary LH secretion than was measured both before castration when serum testosterone and LH concentrations were both low and after gonadectomy when pituitary LH secretion was sharply stimulated.

This experiment implies a significant age reduction in the responsiveness of the hypothalamus leading to decreased secretion of factors which regulate pituitary gonadotropin secretion. Several alterations in neuroendocrine function

could be associated with aging changes in hypothalamic responsiveness. The possibility of age-related changes in neurotransmitter regulation of LHRH secretion is receiving a great deal of current attention. The ability of major alterations of hypothalamic biogenic amines function to affect hypothalamic release of pituitary regulating substances has been established. The hypothalamus contains large amounts of dopamine and norepinephrine (Palkovits, et al., 1974). Alterations in the activity of these catecholamines has been associated with gonadotropin control mechanisms (Fernstrom and Wurtman, 1977). We and others have reported decreased hypothalamic content and turnover of catecholamines in aged male rats (Miller, et al., 1976; Simpkins, et al., 1977). Although absolute proof of the relationship between hypothalamic catecholamine function and the effect of aging on gonadal control mechanisms is not firmly established, the decrease in hypothalamic catecholamine function is consistent with the reduced gonadotropin secretion found in this experiment.

In summary, these data indicate no significant change in hypothalamic LH-releasing activity in the intact or orchidectomized aged male rat. This experiment confirms the hypothesis of significant alteration in the responsiveness of the hypothalamus to control input in the aged rat. Although the hypothalamus contains sufficient LH-releasing activity to stimulate higher levels of pituitary and gonadal endocrine function, aging effects on the neuroendocrine

control system are hypothesized to result in reduced hypothalamic hormone secretion.

C. Temporal Patterns of Serum LH and Testosterone and Pituitary and Testicular Responsiveness to LHRH in the Aged Male Rat

Recent studies have shown decreased serum testosterone concentrations in aged men (Kirschner and Coffman, 1968; Baker, et al., 1976). One study showed that the decreased plasma testosterone after age fifty in men was accompanied by an increase in plasma testosterone binding capacity which resulted in a sharply reduced free testosterone concentration (Vermeulen, 1976). The decreased serum testosterone in aged men is accompanied by increased serum concentrations of FSH and LH (Baker, et al., 1976; Vermeulen, 1976). Although the increase in gonadotropin secretions are believed to partially compensate for decreased Leydig cell function, it has been shown that the Leydig cells of aged men have reserve capacity for still further testosterone secretion (Rubens, et al., 1974) and that pituitaries of aged men can maintain similar serum LH concentrations following LHRH injection as pituitaries from young men (Hashimoto, et al., 1973; Rubens, et al., 1974). These data indicate probable aging effects in Leydig cell function and hypothalamic-pituitary unit responsiveness to negative feedback in aging men.

Although the aging male rat is also characterized by decreased serum testosterone concentrations (Miller and Riegle, 1978a; Pirke, et al., 1978; Steger, et al., 1979),

the aged male rat has decreased serum LH concentrations (Shaar, et al., 1975; Riegle and Meites, 1976; Gray, 1978; Geisthovel, et al., 1981) rather than the increased blood gonadotropins found in aged men. Both Leydig cell secretion of testosterone and pituitary secretion of LH in aged male rats can be stimulated to serum concentrations similar to those after similar treatment in young males if aged male rats receive chronic HCG or LHRH treatments (Miller and Riegle, 1978a; Miller and Riegle, 1978b). These findings suggest significant age effects of hypothalamic-pituitary LH secretory control mechanisms in the aging rat.

It is accepted that testosterone secretion occurs in episodical bursts reflecting patterns of LH secretion, rather than in continuous, sustained secretion. Previous studies of aged male rat testicular function have usually measured serum testosterone concentrations at only one sampling interval. The objective of the present study was to determine the effect of aging on patterns of testosterone secretion in the male rat by sequentially measuring serum testosterone and LH in aging rats through a 24 hour period. In addition, we determined the patterns of pituitary and testicular responsiveness of LHRH injection in young, medium and aged male rats.

Materials and Methods

Temporal patterns of serum testosterone and LH. Two experimental procedures were used to determine daily patterns of LH and testosterone secretion. In the first

experiment, groups of ten young (4 months), medium (13 months) and aged (20 months) male rats were subjected to serial blood collections every two hours for 24 hours beginning at 5 a.m. In the second experiment the interval between blood collections was extended to minimize effects of hypovolemia and anesthesia-blood collection stress on hormone control mechanisms. In this study, groups of 14 young (4 months), medium (13 months) and aged (22 months) male rats were subjected to serial blood collections at 38 hour intervals beginning at 1 p.m. This bleeding schedule also produced a more random sequence of blood sampling times (1 p.m., 3 a.m., 5 p.m., 7 a.m., et cetera.)

LHRH stimulation of LH and testosterone secretion

Groups of 20 young (3 months), medium (12 and 13 months) and aged (22-26 months) male rats received jugular vein injections of 1 or 5 ng of synthetic LHRH (Lot 628059, Calbiochem, San Diego, California)/gm bw. Blood samples were collected from all rats just before the LHRH injections. After the hormone treatment (0 time) the rats in each age group receiving each level of LHRH stimulation were randomly divided into two subgroups (10 rats/subgroup). Serial blood samples were collected from one subgroup 15 and 90 minutes after LHRH stimulation. Blood samples from the second subgroup were taken at 45 and 150 minutes after the intravenous injection. Serum LH and testosterone concentrations were measured from each blood sample.

Differences between and within age groups were tested by multivariant analysis of variance and analysis of variance for repeated measurements by use of the program BALANOVA supplied by the Michigan State University Computer Laboratory. Only differences with a probability of error of less than 0.05 were considered as significant.

Results

Temporal changes in serum testosterone are illustrated as group means in Figures 4 and 5. Individual and groups means and ranges of testosterone for the twelve sampling intervals are included in Tables 3 and 4. In the first experiment, in which rats were serially sampled 12 times in a 24 hour period (Figure 4), average testosterone concentrations in the first blood samples were higher in young than in aged male rats, with the medium aged males having intermediate hormone concentrations. Serial blood sampling reduced serum testosterone concentration ($P<0.05$) in all three age groups. Average testosterone values were not different between the age groups by the third sampling interval (9 a.m.). Serum testosterone concentrations were decreased proportionally greater by this experimental procedure in the young compared to the aged rats.

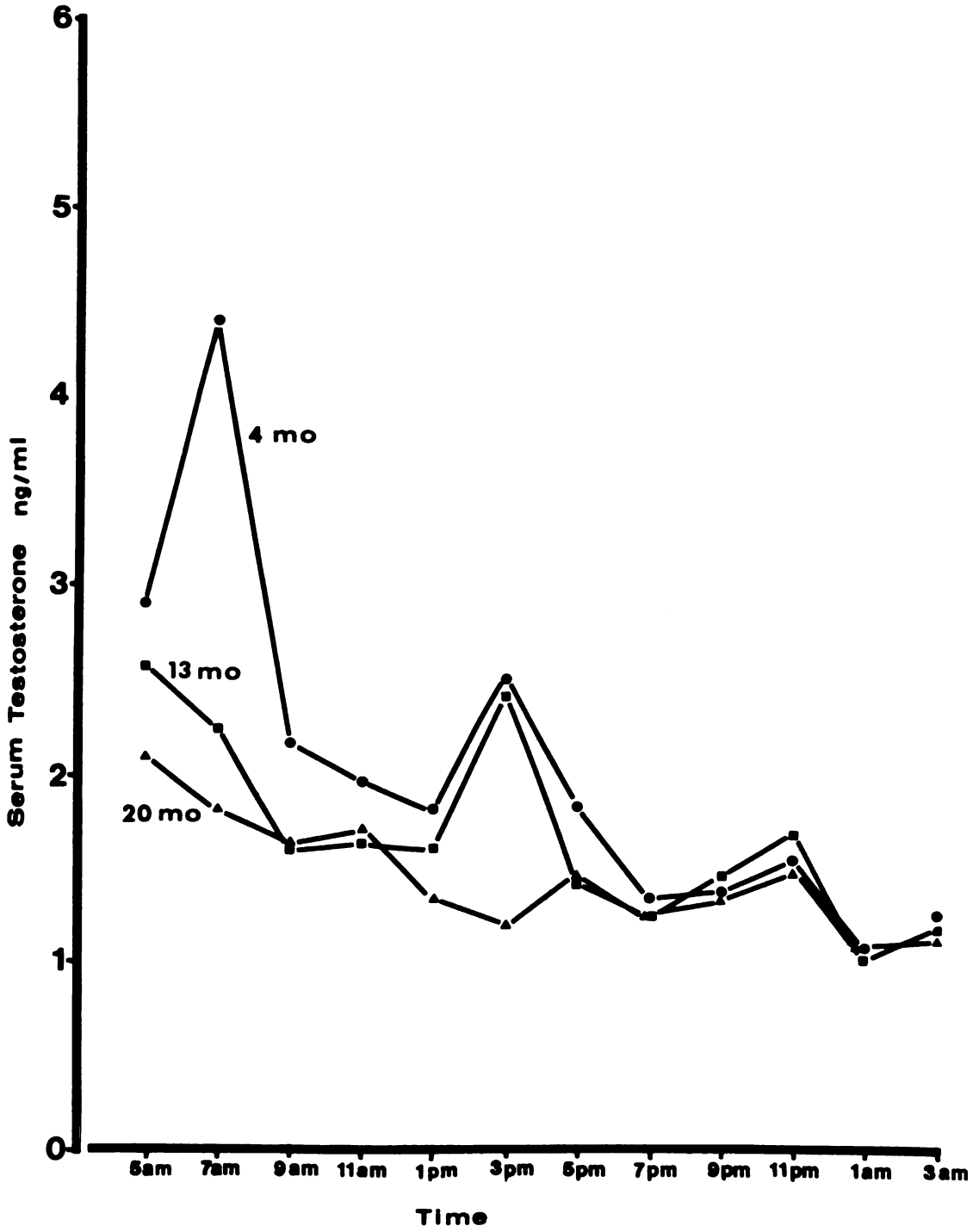


Figure 4. Serum Testosterone from Serial Blood Samples Collected at 2 Hour Intervals in Young, Middle-Aged and Aged Male Rats. Blood samples were collected under light ether anesthesia beginning at 5 a.m. Testosterone is expressed as the group means (ng/ml serum, N = 10)

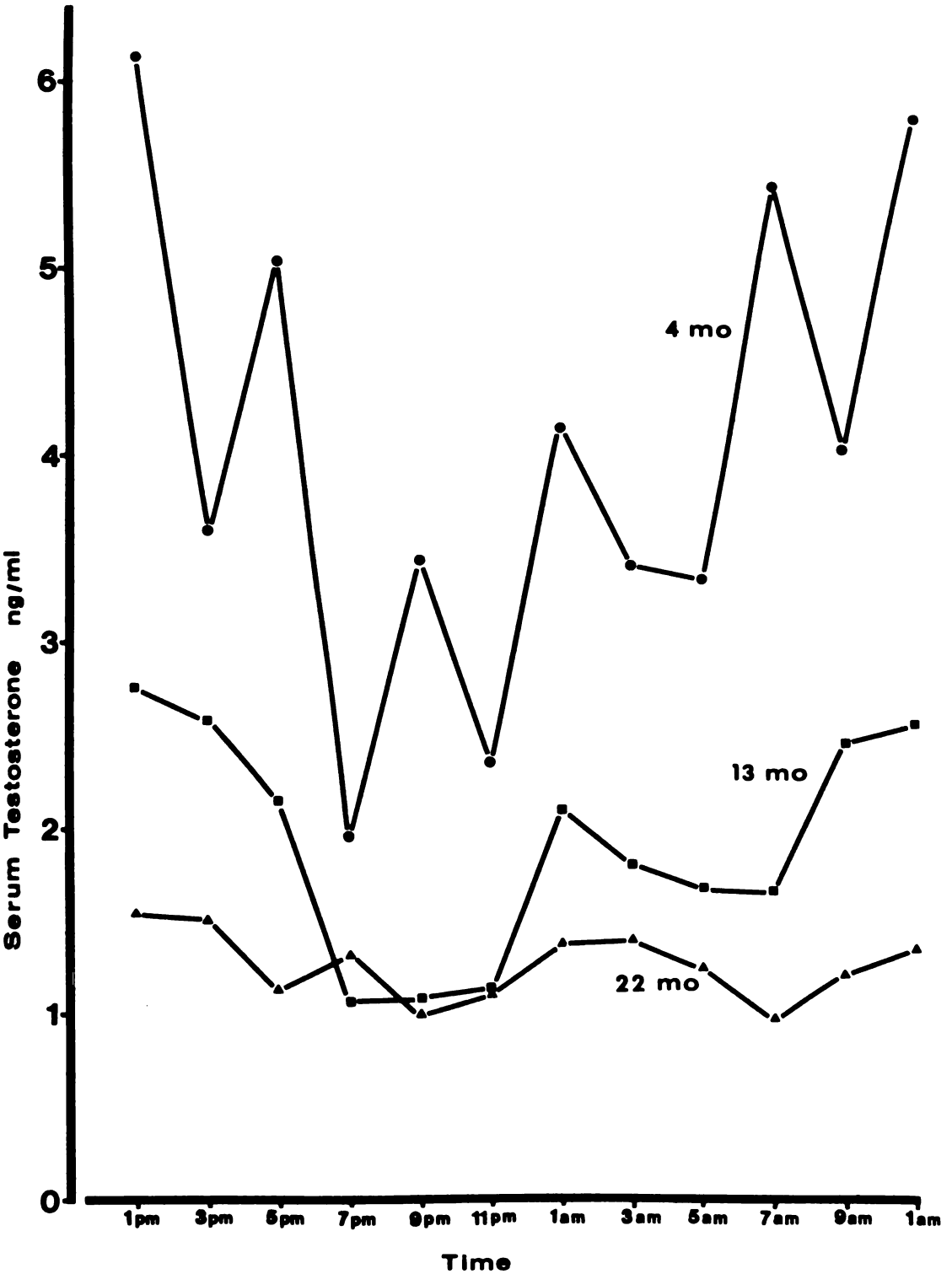


Figure 5. Serum Testosterone from Serial Blood Samples Collected at 38 Hour Intervals in Young, Middle-Aged and Aged Male Rats. Blood samples were collected under light ether anesthesia. Testosterone is expressed as the group means (ng/ml serum, N = 10/group).

Table 3

Group Means and Range of Individual Serum Luteinizing Hormone and Testosterone from Serial Blood Samples in Male Rats[a]

		Testosterone		Luteinizing hormone	
Age	N	M	Range	M	Range
Young	14	4.09 ± .26	.67-17.70	36.6 ± 2.8	4.0-300.0
Medium	14	1.91 ± .07	.58- 7.21	18.6 ± 1.6	2.0-208.0
Aged	14	1.26 ± .08	.41- 4.10	11.3 ± .9	2.0- 58.0

[a]Twelve blood samples were collected from each rat at 38-hour intervals. Young rats were 2 months, medium-aged 13 months, and aged 22 months of age. Testosterone and luteinizing hormone reported as mean ± SEM ng/ml of serum. Range reported as ng/ml of serum within the 168 blood samples for each age group.

Table 4.

Individual Rat Mean and Range of Serum Testosterone (ng/ml) from Serial Blood Samples in Male Rats[a]

Young			Middle-Aged			Aged		
Rat	M	Range	Rat	M	Range	Rat	M	Range
1	5.67	1.06-10.20	2	2.00	.75-4.22	1	1.16	.55-2.18
2	2.76	1.11- 4.63	3	2.03	.82-4.51	2	.94	.74-1.51
3	4.16	1.44- 9.38	4	2.27	.79-5.04	3	1.82	.86-2.96
4	2.95	1.15- 6.22	5	1.53	.63-2.84	4	1.20	.83-2.10
5	4.46	1.50-13.25	6	2.02	.80-3.19	5	1.26	.63-4.45
6	3.29	.79- 9.44	7	1.53	.69-4.46	6	.72	.46- .99
7	2.77	.81- 9.62	9	1.54	.80-3.45	7	1.41	.67-3.60
8	4.08	.67-11.84	16	1.96	.58-4.52	8	1.61	.64-4.10
9	4.61	1.01- 9.32	17	1.58	.66-5.34	9	1.40	.66-3.13
10	4.75	1.30- 9.97	18	1.86	1.02-3.14	10	1.35	.73-2.44
11	4.28	1.11-11.26	19	2.04	.65-4.05	11	1.24	.68-2.03
12	3.59	1.56- 8.56	20	1.84	.87-3.40	12	.73	.41-1.23
13	3.61	1.41- 8.56	21	2.38	.90-7.21	13	1.55	.71-4.10
14	5.84	.92-17.70	24	2.11	1.10-6.75	14	1.26	.72-2.10

[a]Blood samples were collected from each rat at 38-hour intervals. Young rats were 2 months, medium-aged 13 months, and aged 22 months of age.

In the second experiment with the extended blood collection schedule (Figure 5, Tables 3, 4, and 5), average testosterone and LH concentrations of the young group were higher than the medium or aged groups of rats ($P<0.05$). The younger rats also showed more fluctuation between average hormone concentrations (Figure 5) than did either the medium or the aged groups. A diurnal pattern of testosterone concentration with reduced serum testosterone in the early evening hours was present in the young and medium aged rats but absent in the aged group. The aged group did not have significant differences in testosterone concentration at any of the blood sampling intervals.

Serum concentrations of LH and testosterone following intravenous LHRH injection are plotted in Figures 6 and 7. The young groups had higher pretreatment LH concentration than the medium or aged groups ($P<0.01$). Pretreatment LH was also higher in medium than in aged groups. The 1 ng LHRH/gm bw treatment (Figure 6) stimulated increased serum LH concentration in all age groups at both the 15 and 45 minutes post-injection blood sampling intervals. Serum testosterone concentrations were higher in the young group than in either the medium or aged group of rats receiving the 1 ng LHRH treatment at all blood collection times ($P<0.05$). Although this level of LHRH treatment stimulated significant increases in LH secretion in all age groups, only the young male rats had increased testosterone which was present in the 45 minutes post-injection blood sample.

TABLE 5

Serum LH from Serial Blood Samples[a] in Aging[b] Male Rats Experiment 2

Sampling Time	LH (ng/ml)[c] Young	Medium	Aged
1 p.m.	115.0 ± 17.5	32.7 ± 6.7	24.0 ± 3.3
3 p.m.	15.1 ± 2.6	11.8 ± 1.6	7.4 ± 1.5
5 p.m.	32.6 ± 7.3	16.2 ± 2.9	11.6 ± 2.6
7 p.m.	69.6 ± 13.6	62.1 ± 20.6	24.2 ± 6.4
9 p.m.	22.5 ± 4.7	9.5 ± 1.8	9.7 ± 1.5
11 p.m.	28.5 ± 3.5	11.3 ± 2.2	7.2 ± 1.0
1 a.m.	20.5 ± 3.6	12.9 ± 2.3	7.5 ± 1.2
3 a.m.	32.1 ± 4.2	15.4 ± 2.4	13.2 ± 4.3
5 a.m.	20.9 ± 5.6	13.0 ± 2.5	8.9 ± 2.6
7 a.m.	34.2 ± 4.7	9.1 ± 1.3	5.4 ± 0.6
9 a.m.	24.0 ± 3.7	20.1 ± 3.3	9.5 ± 1.6
11 a.m.	24.4 ± 4.9	9.6 ± 1.3	7.1 ± 1.1

[a]Blood samples were collected from each rat at 38 hour intervals.
[b]Young rats were 2 mo, medium 13 mo, and aged 22 mo of age.
[c]LH expressed as group means (n = 14) ± SEM

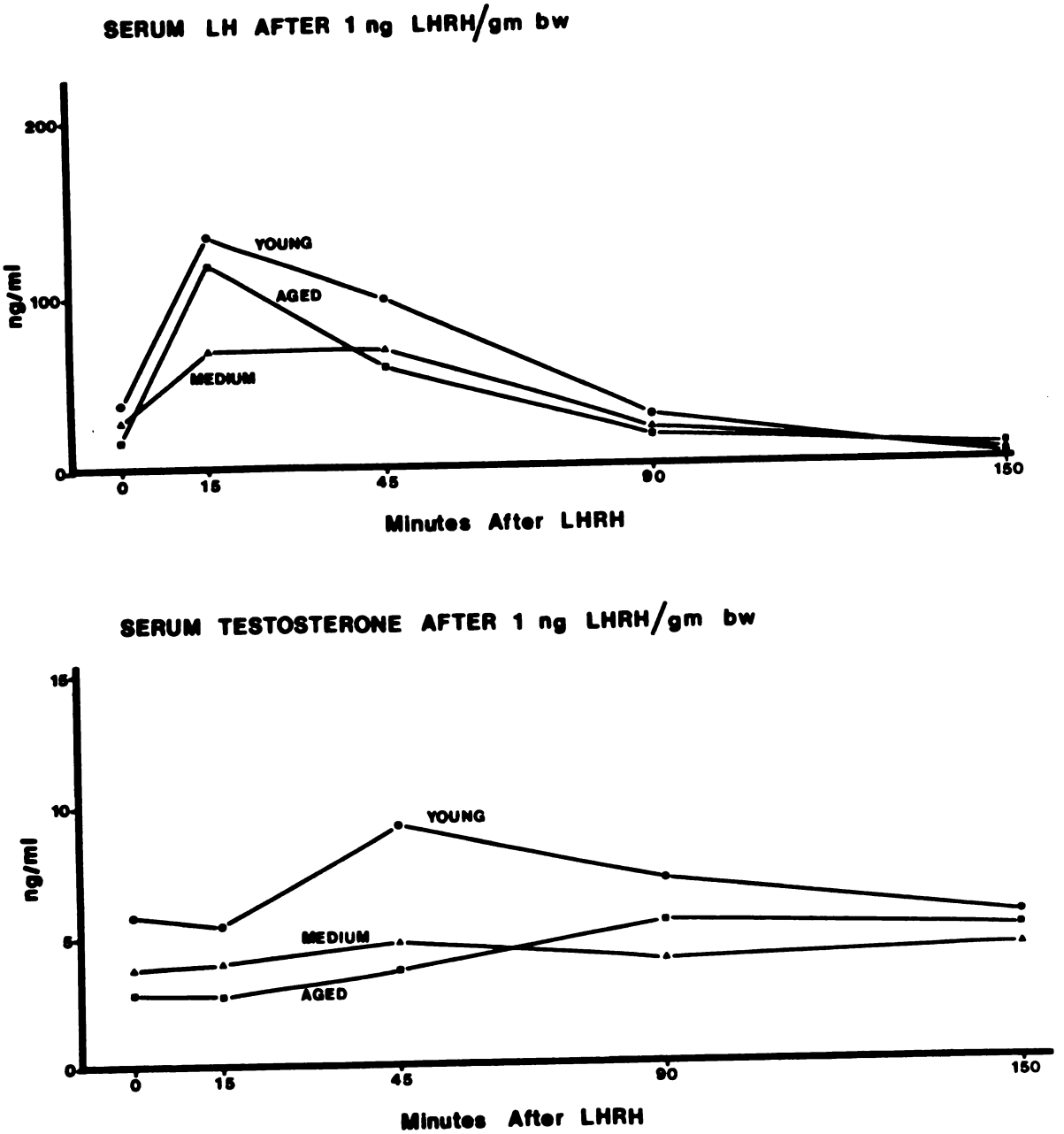


Figure 6. The Effect of Injection of 1 ng/g bw LHRH on Serum LH and Testosterone in Young, Middle-Aged and Aged Male Rats. Blood samples were collected under light ether anesthesia before and at 15, 45, 90 and 150 min. after intravenous injection of LHRH in all three groups (N = 10/group).

Figure 7. The Effect of Injection of 5 mg/g bw LHRH on Serum LH and Testosterone in Young, Middle-Aged and Aged Male Rats. Blood samples were collected under light ether anesthesia before and at 15, 45, 90, and 150 min. after intravenous injection of LHRH in all three groups (N = 10/group).

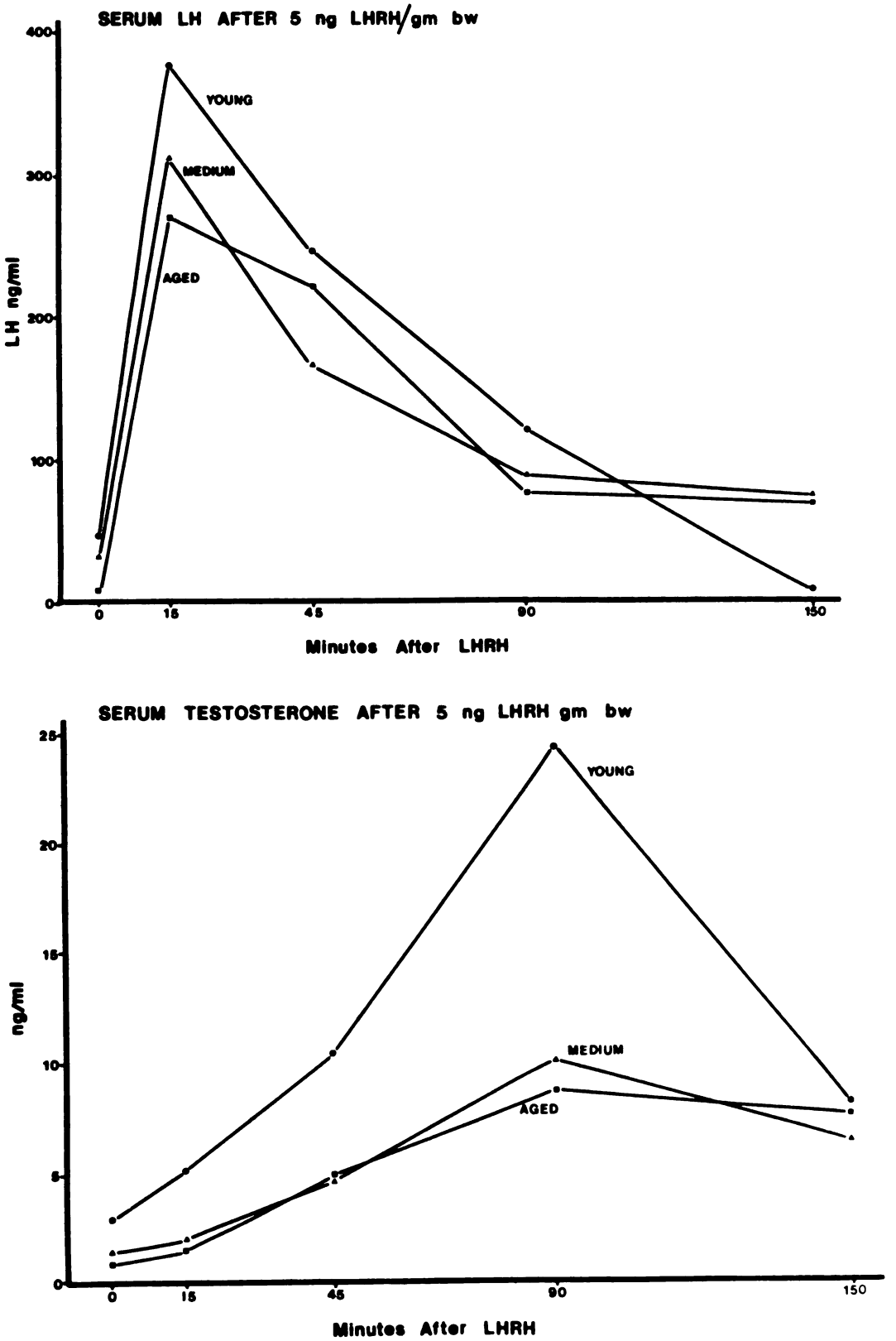

SERUM LH AFTER 5 ng LHRH/gm bw
LH ng/ml
400
300
200
100
0
YOUNG
MEDIUM
AGED
0
15
45
90
150
Minutes After LHRH
SERUM TESTOSTERONE AFTER 5 ng LHRH gm bw
ng/ml
25
20
15
10
5
0
YOUNG
MEDIUM
AGED
0
15
45
90
150
Minutes After LHRH

Figure 7.

The pretreatment serum LH and testosterone concentrations were again greater in the young than in the aged groups receiving the 5 ng/gm bw LHRH treatment (Figure 7). This LHRH treatment markedly stimulated serum LH concentrations in all age groups ($P<0.01$). This increase in LH was also uniformly greater than that stimulated by 1 ng/gm bw ($P<0.01$) and was sustained through the 90 minutes post-injection blood sampling interval for all groups and through 150 minutes for the two older groups. Differences in LH concentration between groups were significant before LHRH injection, between the young and aged groups at the 15 minute interval and between the young and aged groups 150 minutes after LHRH treatment.

Discussion

The results of these experiments confirm and extend our previous findings and hypotheses concerning age effects on gonadal control systems in the male rat (Miller and Riegle, 1978a). These data show that young male rats maintain consistently higher serum testosterone and LH concentrations than do mid-aged or aged male rats. The young male group also had much more variability among sampling time means and a greater range of hormone concentration among serial blood samples collected from individual rats. This variability was progressively decreased in the mid-aged and in the aged group. If the gonadal control system remained functionally intact, the decrease in testosterone in the aged male rat should stimulate increased gonadotropin secretion due to the

loss of negative feedback (Davidson, 1969). However, serum LH concentrations were consistently decreased in the aged rat. These findings are in agreement with our previous reports of reduced serum LH concentrations (Riegle and Meites, 1976) and smaller increases in serum LH following gonadectomy in aged compared to young male rats (Shaar, et al., 1975). On the other hand, several gonadal control system components of the aged male rat remain responsive to stimulation. We have shown that the reduced serum testosterone of the aged male rat could be stimulated to concentrations similar to young rats by several days of HCG treatment (Miller and Riegle 1980a), the low serum LH of the aged male rat can be stimulated to concentrations similar to that found in young groups following serial LHRH treatment (Miller and Riegle, 1978b) and hypothalamic content of LH stimulating activity was similar in young and aged rats (Miller and Riegle, 1978c).

These findings offer strong support to the hypothesis of significant age-related change in the responsiveness of the neuroendocrine mechanisms which regulate pituitary gonadotropin secretion. Although hypothalamic neurons contain biologically active LHRH, their responsiveness to a variety of factors which stimulate LH release, including acute stress stimulation of LH release (Riegle and Meites, 1976), reduced testosterone negative feedback after castration (Shaar, et al., 1975), and LH release after L-dopa treatments (Riegle and Meites, 1976) are reduced in the aged male

rat. An early study on the responsiveness of the hypothalamic-pituitary unit to negative feedback inhibition by testosterone after gonadectomy suggested increased sensitivity to testosterone inhibition in aged compared to young orchidectomized rats (Shaar, et al., 1975). This finding was confirmed by a similar study in young and old orchidectomized rats which received testosterone from silastic capsule implants (Pirke, et al., 1978). These observations of apparent increased neuroendocrine sensitivity to a negative feedback inhibitor are contrary to the developing evidence of significant reductions in specific neurotransmitter function (Carlsson, 1978; Finch, 1978; McGear and McGear, 1978) and reduced hypothalamic testosterone receptor concentrations (Chouknviska and Vassileva-Popova, 1977) and warrant further consideration.

Although acute stress has been shown to increase serum LH in young male rats (Euker, et al., 1975), the results of our first attempt to study temporal hormone changes clearly indicate that the combined stress of serial anesthesia, handling, and blood collection result in sharply reduced serum testosterone concentrations. The decrease in testosterone occurs in association with suppression of LH secretion. Although the initial serum LH concentrations from the rats subjected to serial blood sampling at two hour intervals were similar to those measured in the second temporal experiment with the 38 hour sampling intervals, LH concentrations in the first experiment were sharply reduced by the

third blood sample (5 p.m.) and remained at near baseline assay sensitivity for the remaining sampling times in all groups.

Although precise mechanisms regulating secretory activity of LHRH synthesizing neurons in the brain are unknown, direct and indirect evidence implicates neurotransmitter involvement within the hypothalamus and in other brain regions (Fernstrom and Wurtman, 1977). Decreased hypothalamic and median eminence content and turnover of dopamine and norepinephrine and increased serotonin turnover occurring with increased age have been reported by several laboratories (Miller, et al., 1976; Simpkins, et al., 1977; Finch, 1978). It is widely presumed that alterations in these or other yet unidentified neurotransmitters affect LHRH neuron synthesis and secretion.

Factors which regulate the episodical bursts of LH secretion in the young male rat are also unknown. Our blood sampling intervals were much too infrequent to show close associations with LH release followed by stimulation of testosterone secretion. However, our data do show consistent progressive decreases in the range of LH concentrations measured in individual blood samples and in the overall average LH values measured from the young compared to the middle, compared to the aged groups (LH concentrations from all blood samples averaged 36.6, 18.6 and 11.3 ng/ml serum from the young, middle, and aged groups respectively; individual serum samples ranged from 4 to 300 ng/ml in the

young, from 2 to 208 ng/ml in the middle, and from 2 to 58 ng/ml in the aged group).

The increase in serum LH following LHRH treatments indicate that the aged male rat maintains a high degree of pituitary responsiveness to hypothalamic gonadotropin stimulatory activity. These data are in agreement with our previously reported findings (Miller and Riegle, 1978b) and suggest that although the serum LH concentrations in the 15 minutes post-injection blood sample of the aged group is statistically less than that from young males, this difference probably reflects chronically low levels of pituitary stimulation and is not considered to be of major biological significance. The higher serum LH concentrations in the medium and aged groups compared to the young rats at 150 minutes is in agreement with our earlier work in aged females (Miller and Riegle, 1978b) and suggest age-related differences in the duration of pituitary LH secretion or in the metabolism and removal of LH from the circulation.

These data indicate decreased Leydig cell responsiveness to LH stimulation in the medium and aged groups. However, our earlier experiments (Miller and Riegle, 1978a) indicated that the decrease in testicular responsiveness to acute stimulation was due to consistently low levels of LH stimulation in the aged rat, rather than actual biological differences in responsiveness to the product of pituitary secretion as measured by radioimmunoassay. These findings together now suggest that a functional impairment of normal

neuroendocrine responsiveness to gonadotropin control input in the aged male rat results in decreased pituitary LH secretion and markedly reduced testicular responsiveness to LH. The decreased testicular responsiveness results in a consistently low level of testosterone secretion without the episodical bursts of secretion which are characteristic of younger males.

Experiment 2: The Effect of Age on Reproductive Control Mechanisms in the Female Rat

A. Hypothalamic LH-Releasing Activity in Intact and Ovariectomized Rats

The effect of age on gonadotropin secretion in the female rat is not as marked as for the aged male which was described in the first section of this thesis. The effect of age on blood LH concentration appears to be related to the reproductive state of the old rat. Previous work in our laboratory indicated reduced LH concentration in aged pseudopregnant and noncyclic constant diestrous rats compared to young rats in the diestrous phase of their estrous cycle (Watkins, et al., 1975). On the other hand, aging constant estrous rats maintain higher serum LH concentrations than that measured in young rats at estrous or diestrous stages of their ovarian cycle. However, the aged constant estrous rats apparently secrete insufficient LH for full follicular maturation and ovulation. Additionally, aged rats had smaller increases in serum LH following ovariectomy than young rats (Shaar, et al., 1975). These

experiments indicate that at least in some situations of gonadotropin stimulation, aged female rats secrete less LH than do young rats. In addition, Clemens and Meites (1971) and Lu, et al., (1979) found elevated serum FSH concentrations in aged constant estrous rats. These reports suggest that the effect of age of FSH control mechanisms in the rat may be different than age effects on LH secretion.

It is hypothesized that failure of adequate LH secretion during the proestrus surge may be a factor contributing to the decrease in fertility of the aging female rat. The ability of hypothalamic neurons to synthesize and store LH-releasing hormone is one factor which could affect hypothalamic stimulation of pituitary LH secretion. The following study was undertaken to measure LH-releasing activity of hypothalamic extracts from young and aged intact and ovariectomized female rats.

Materials and Methods

Young adult (3 and 5 months) and aged (22 to 26 months) intact and gonadectomized female rats (n = 15 and 16/group) were used in this experiment. The gonadectomized groups were surgically prepared eight weeks prior to the estimation of hypothalamic LH-releasing activity. The methods and procedures used for collection of trunk blood, for preparation of the hypothalamic extracts and the incubation of the extracts with paired male pituitary halves was identical to that described in section 1-B for the experiment with male rat hypothalamic extracts.

Results

Average serum LH concentrations for the experimental groups are shown in Table 6. Serum LH was higher in the young intact than in the aged group ($P<0.05$). LH concentration was increased after ovariectomy in both age groups ($P<0.01$). Although the increase in LH following gonadectomy was nearly proportional in both groups, the magnitude of the increase was greater in the young group.

TABLE 6

Serum LH Concentrations in Intact and Gonadectomized Young and Aged Female Rats

Group	Age (Mo)	No.	Serum LH (ng/ml)[a] Intact	Gonadectomized[b]
Young Female	3-5 mo	31	47.4 + 6.7	407.1 + 48.1
Aged Female	22-26 mo	32	21.8 + 6.4	139.3 + 11.6

[a]Serum LH expressed as group means ± SEM

[b]Eight weeks after gonadectomy

The responsiveness of incubated rat pituitaries to hypothalamic extracts is shown in Table 7 and is plotted as percent increases in LH secretion over control pituitary half LH secretion in Figure 8. Addition of increased hypothalamic extract stimulated increased LH release from incubated pituitaries for all groups. The effect of age on

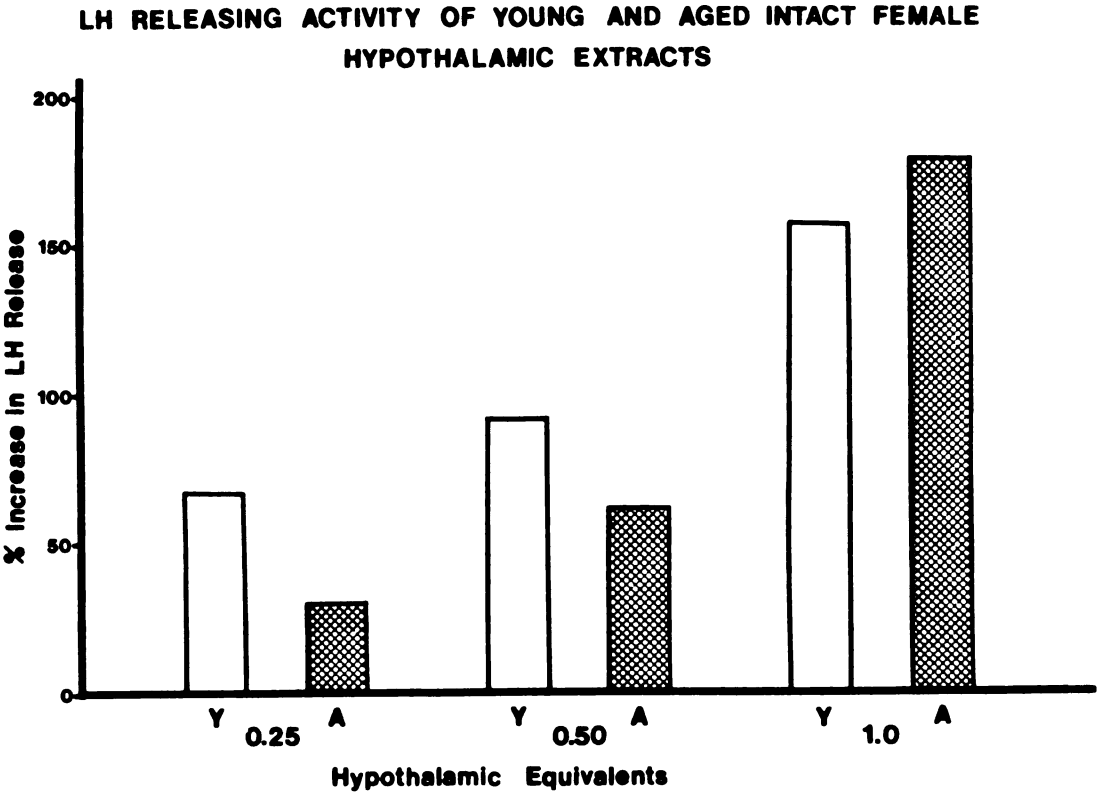
TABLE 7

Effect of Hypothalamic Extracts on LH Release from Incubated Rat Pituitaries[a]

Group	n	H.E.	LH Release (ng/mg pituitary)[b]			
			Young[c]		Aged[d]	
			Control	Treated	Control	Treated
Intact Female	8	0.25	909 ± 175	1510 ± 318	884 ± 98	1156 ± 158
	8	0.50	1161 ± 221	2235 ± 434	1130 ± 197	1831 ± 277
	8	1.00	940 ± 229	2420 ± 354	1050 ± 285	2929 ± 473
Gonadectomized Female	8	0.25	1226 ± 82	2355 ± 332	1249 ± 100	2123 ± 247
	8	0.50	1169 ± 96	2236 ± 255	945 ± 93	2384 ± 409
	8	1.00	1589 ± 310	4111 ± 494	1052 ± 130	2813 ± 362

[a]Hypothalamic Extracts were incubated with paired hemisectioned 5 mo male rat pituitaries in medium 199.
[b]LH release expressed as group means ± SEM
[c]Young rats were 3 and 5 mo of age.
[d]Aged rats were 22 to 26 mo of age.

Figure 8. The Effects of 0.25, 0.5 and 1.0 Young and Aged Hypothalamic Equivalents from Intact and Gonadectomized Female Rats on LH Release from Incubated Pituitary Halves. Young male pituitary halves were incubated 4 hrs. in medium 199. LH release is plotted as the average percent increase of 8 hypothalamic extract-treated pituitary halves compared to their paired control pituitary halves.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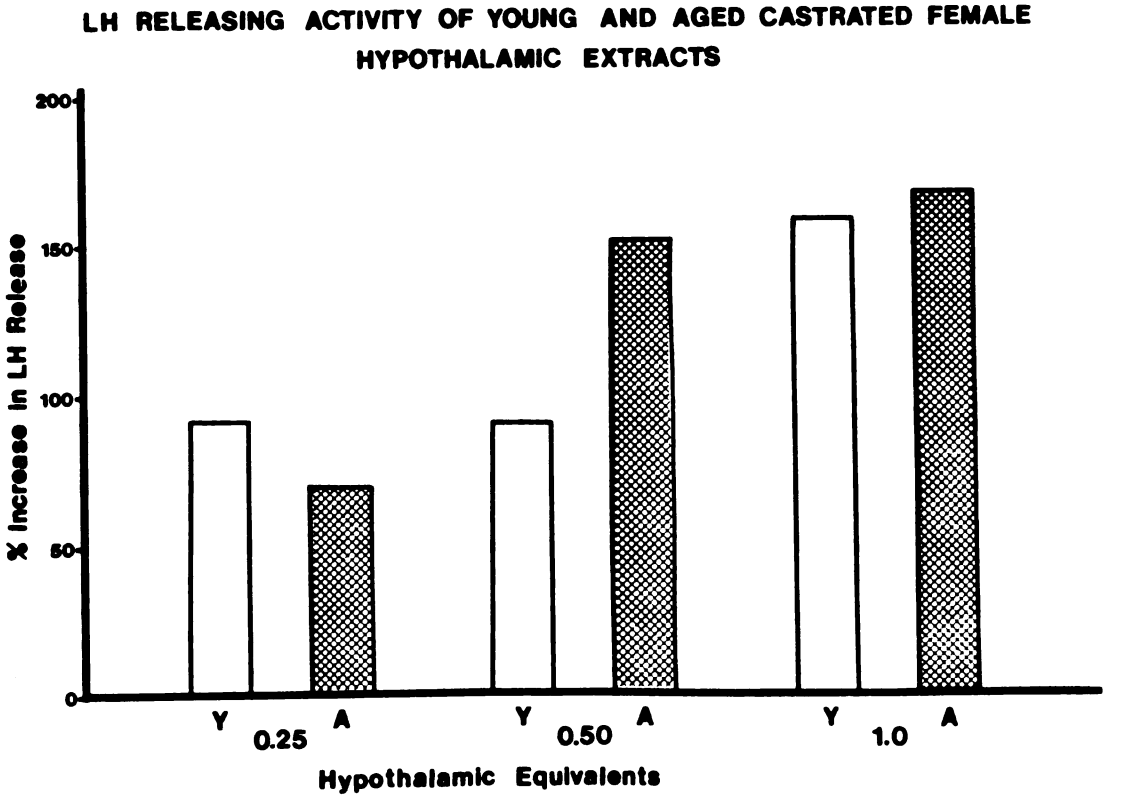

LH RELEASING ACTIVITY OF YOUNG AND AGED CASTRATED FEMALE HYPOTHALAMIC EXTRACTS
% Increase in LH Release
200
150
100
50
0
Y
A
0.25
0.50
1.0
Hypothalamic Equivalents

Figure 8.

hypothalamic LH-releasing activity content was not significant for either the intact or gonadectomized groups.

Discussion

These data confirm the previous experiment on the aged male rat and indicate that the aged rat hypothalamus contains substances (presumably LHRH) capable of stimulating LH secretion. These results reaffirm the hypothesis that aged rat hypothalami have sufficient LHRH synthesizing capacity to stimulate greater pituitary gonadotropin in both intact and ovariectomized female rats and emphasize the importance of age changes in neuroendocrine responsiveness to gonadotropin control input as a major alteration of the reproductive control mechanisms of the aging rat.

A growing body of experimental data implicate age-related changes in catecholaminergic neurotransmitters with the regulation of LHRH secretion from hypothalamic peptidergic neurons. There remains considerable controversy concerning the relative importance of stimulatory and/or inhibitory activity of specific neurotransmitter substances related to LHRH control (Fernstrom and Wurtman, 1977). Negro-Vilar, et al. (1979) have shown that <u>in vitro</u> hypothalamic LHRH secretion is influenced by both dopamine and norepinephrine and normal hypothalamic function of both neurotransmitters is essential for optimal secretory processes. The reported decreases in hypothalamic catecholamine content and turnover in aged rats (Demerest, et al., 1982) are consistent with the hypothesis that impairment of

catecholamine function results in reduced LHRH secretion. However, it must be emphasized that the relationship between the changes in hypothalamic catecholamine function and aging alterations in reproductive control mechanisms will require a great deal more experimentation before the hypothesis can be regarded as dogma.

B. Serum LH Following Multiple LHRH Injections in Aging Female Rats

Although the precise neuroendocrine mechanisms involved with age-related alterations in reproduction remain unresolved, current information from various species suggests that age may affect several components of the hypothalamic-hypophyseal-gonadal control system.

Although the effect of age on ovarian endocrine function is not as dramatic as that measured for testosterone in the old male rat, there is ample evidence of alterations occurring in the female with increased age. Huang and Meites (1975), Aschheim (1976), and others have shown that normal ovarian cyclicity is replaced by periods of constant estrous and repetitive pseudopregnancies. The effects of age on gonadal function and the reduction in gonadal steroid production with age in men and women is accompanied by increased blood gonadotropin concentrations (Adamopoulos, et al., 1971; Baker, et al., 1976; Lazarus and Eastman, 1976). On the other hand, there is no evidence for similar increases in gonadotropin release in aged female rats. Riegle and Meites (1976) reported decreased serum LH levels in aged male rats. In female rats the effect of age on serum LH

levels is variable and related to the variable ovarian states of the aged rat. Although aged constant estrous rats have higher serum LH concentrations than do young cycling female rats in estrous or diestrous stages of their ovarian cycles, the LH level in aged constant estrous rats is non-cyclic and is much less than that found in young rats at proestrus (Watkins, et al., 1975). This study also showed that aged rats with repetitive pseudopregnancies or aged anestrous rats have serum LH levels which are lower than LH levels in young rats at diestrus. In addition, several studies have shown that ovaries from young rats grafted into aged female rats resume the endocrine state of the recipient before the transplant (Aschheim, 1976).

An age associated decrease in pituitary responsiveness to LHRH could contribute to the decrease in LH secretion, affecting gonadal control and fertility in aged rats. Previous experiments from our laboratory have shown smaller increases in serum LH following single intravenous LH releasing hormone (LHRH) injections in aged compared to young rats of both sexes (Watkins, et al., 1975; Riegle and Meites, 1976). The responsiveness to acute LHRH injection in aged rats indicates that their pituitaries are capable of secreting greater amounts of LH than they normally maintain. However, suppressed endocrine function leads to a functional disuse atrophy in most endocrine systems. The long-term reduced LH secretory activity which is characteristic of the aged rat could at least partially account for the reported

decrease in pituitary responsiveness to acute LHRH in aged compared to young rats. The current study was designed to consider the effects of more sustained LHRH stimulation on serum LH concentrations in aging female laboratory rats in order to ascertain whether or not this reduction in secretion is indeed due to a primary dysfunction of the pituitary.

Materials and Methods

Young adult animals used in these studies were four and five months of age and aged groups ranged from 24 to 28 months of age. The aged females were multiparous rats obtained as retired breeders at nine months of age (Blue Spruce Farms, Altamont, New York). Experimental groups included aged females with either constant estrous or persistent diestrous (repetitive pseudopregnant) vaginal cytology and young females at estrous or diestrus day two of their ovarian cycles. Old rats were considered to be constant estrus or persistent diestrus if they had at least eight consecutive days of cornified or leucocytic vaginal smears, respectively.

Experiments were begun at 10:00 a.m. to minimize any unrecognized diurnal variation in pituitary responsiveness. A pretreatment blood sample was taken by orbital sinus puncture under light ether anesthesia. All experimental groups (12 rats/group) received three serial intravenous injections of LHRH. Initially, 500 ng of LHRH (Eli Lilly, Inc., Indianapolis, Indiana) was injected into the exposed

jugular vein. Serial blood samples averaging about 1 ml were taken from each rat 15, 75, 90, 150 and 165 minutes after the first LHRH injection. Similar intravenous LHRH treatments (500 ng) were also administered at the 75 and 150 minutes blood sampling intervals.

The statistical analysis of the data included analysis of variance of the pretreatment LH concentrations and multivariant analysis of variance to determine interaction between age groups, treatments, and treatment responses.

Results

Figure 9 compares serum LH levels in young, day two diestrous and aged pseudopregnant (persistent diestrous) female rats. Serum LH concentration in the samples taken prior to LHRH administration (0, 75, and 150 minutes) were different in the aged compared to the young groups ($P<0.05$). LH was higher in the young group than the aged group at 0 time (16.3 vs. 8.6 ng/ml). However, in the 150 minute sample, LH levels in the aged group far exceeded that of the young rats (141.0 vs. 35.7 ng/ml). Serum LH concentration was increased after each LHRH injection in both age groups ($P<.0001$). The increase in serum LH 15 minutes following each injection was greater in both the young and aged groups with each successive LHRH injection ($P<.01$). Although the increase in serum LH following the first LHRH injection was greater ($P<.05$) in the young than the aged group (112 vs. 64 ng/ml), the apparent differences between pretreatment and the 15 minute post injection LH values in the young and aged

Figure 9. The Effect of Serial Intravenous LRH Injections on Serum LH Concentration in Young Diestrous and Aged Pesistent Diestrous Female Rats. Serial samples were collected under light ether anesthesia before LRH injection (0, 75, and 150 min.) and 15 min. following LRH stimulation (15, 90, and 165 min.). LH is expressed as ng/ml of serum with indicated standard error of the means (N = 12/group).

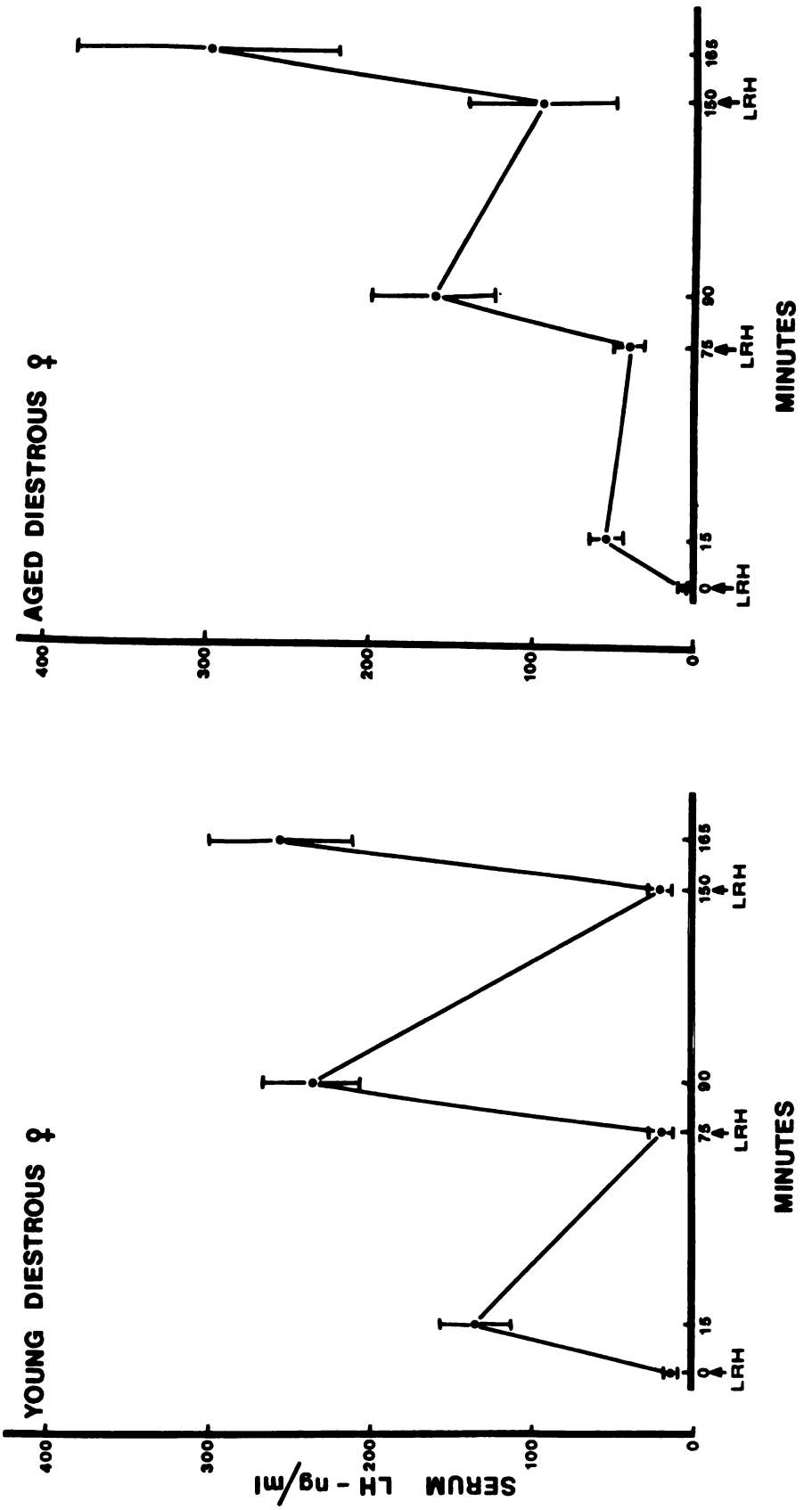

YOUNG DIESTROUS ♀
AGED DIESTROUS ♀
SERUM LH - ng/ml
400
300
200
100
0
0
15
75
90
150
165
LRH
MINUTES

Figure 9.

groups following the second and third LHRH injections were not statistically different.

The effects of multiple LHRH injections on serum LH concentrations in young estrous and aged constant estrous female rats are illustrated in Figure 10. LH levels in the blood samples taken from the aged group before each LHRH injection (0, 75 and 150 minutes), increased with each sampling interval ($P<.01$). Mean LH concentrations increased from 13.5 to 79.1 to 207.9 ng/ml from the 0 minute to the 75 minutes to the 150 minutes sampling interval. Serum LH was increased after each LHRH injection in both age groups ($P<.0001$). The increase in LH in the 15 minutes sample was greater for the young than the aged groups ($P<.01$). Although the increases in serum LH concentrations in the young group were similar after the second and third LHRH injections to the response after the first injection, the aged group had greater increases in LH after the second and third LHRH injections (the increase in serum LH after the first injection was 163 ng/ml; after the second, 474 ng/ml; and after the third, 479 ng/ml). In addition, the increases in LH were similar after the second and third LHRH injections in the young and aged groups.

Figure 10. The Effect of Serial Intravenous LRH Injections on Serum LH Concentration in Young Estrous and Aged Constant Estrous Female Rats. Serial samples were collected under light ether anesthesia before LRH injection (0, 75, and 150 min.) and 15 min. following LRH stimulation (15, 90, and 165 min.). LH is expressed as ng/ml of serum with indicated standard error of the means (N = 12/group).

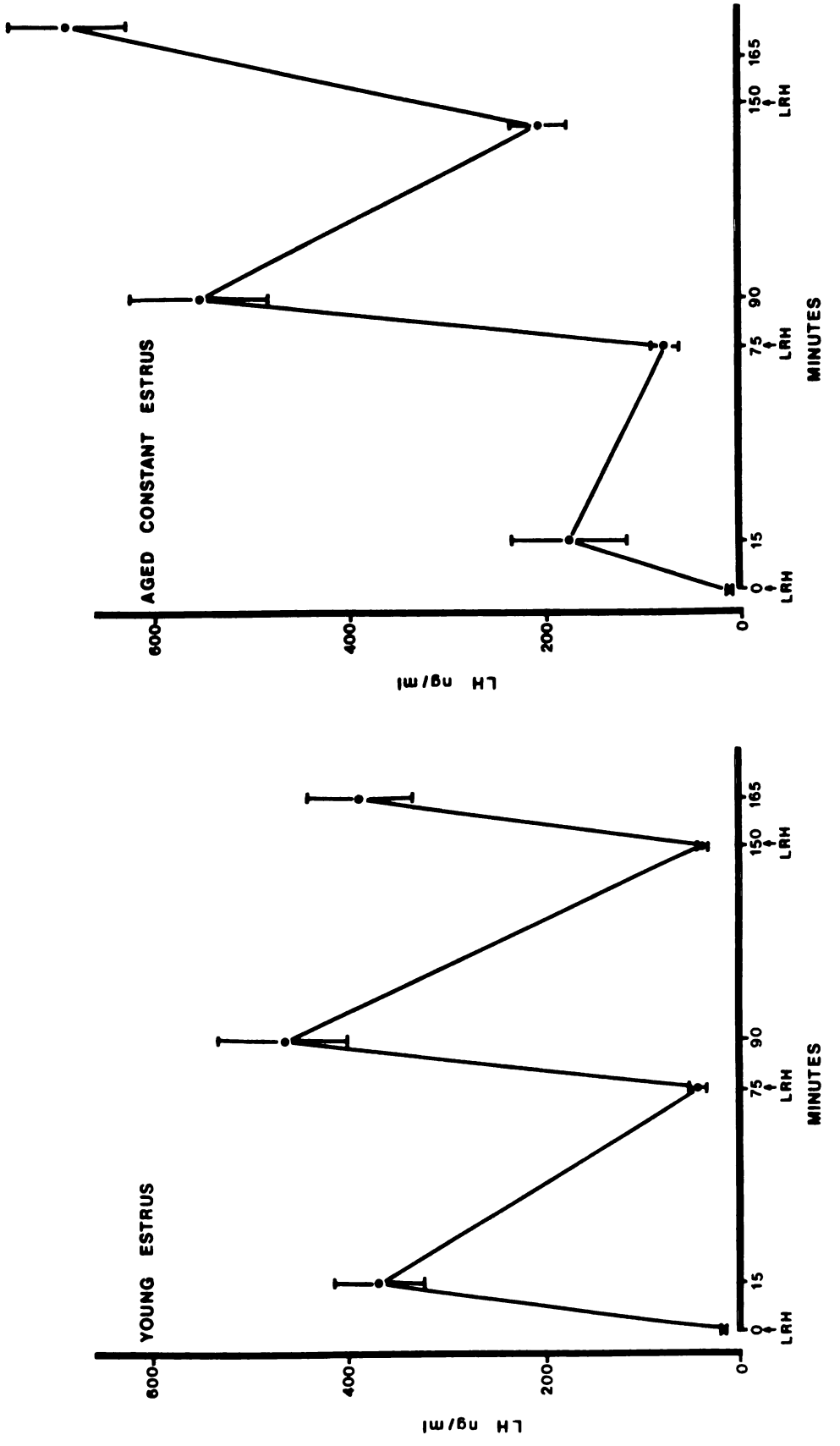

AGED CONSTANT ESTRUS
YOUNG ESTRUS
LH ng/ml
600
400
200
0
0 LRH
15 LRH
75 LRH
90 LRH
150 LRH
165 LRH
MINUTES

Figure 10.

Discussion

These data suggest substantial differences in young and aged rat pituitary response to multiple LHRH stimulations. Levels of serum LH measured after the initial LHRH injection were uniformly lower in aged compared to young experimental groups. This finding is in agreement with our previous reports in aged rats of both sexes (Riegle and Meites, 1976; Watkins, et al., 1975). However, the results of this study show that age-related differences in serum LH are much less prominent following multiple LHRH injections than after an acute stimulation, in agreement with the report of Wise and Ratner (1980) indicating similar LHRH responsiveness in 8-12 month compared to 3-4 month old female rats. The marked increase in serum LH following the second and third LHRH treatments in the aged female rats and the young diestrus female group is similar to the accepted self-priming effects of LHRH previously shown to be involved in the proestrus LH surge. It is presumed that the increased responsiveness to LHRH involves changes in gonadal steroids which can affect pituitary responsiveness to LHRH, the self-priming effects of LHRH on pituitary LH secretion which results in increased LH synthesis, or changes in the secretory processes within pituitary gonadotrophs resulting in more rapid LH secretion response. The marked increase in serum LH levels in the second and third preinjection blood samples of the aged female groups suggests either a more sustained LH release

following LHRH or more likely, a slower rate of removal of the hormone from the circulation in these animals.

Although this study indicates that the aged rat can sustain substantial serum LH levels under experimental conditions of high LHRH stimulation for limited time periods, there is ample evidence that in fact, the secretory activity of the pituitary of the aged rat is significantly reduced. We and others have consistently found low basal serum LH levels in aged male rats and in aged female rats in various reproductive states. Van der Schoot (1976) and Cooper, et al. (1980) reported reduced serum LH levels during the proestrous surge in 10-12 month old cycling rats compared to 3-5 month old cycling rats which we confirmed in section 2D of this thesis. These low blood gonadotropin concentrations have been implicated in the decrease in reproductive performance of aged rats of both sexes by several laboratories.

We and others have hypothesized that the most fundamental age-related alteration in the reproductive control system occurs in the neural regulation of hypothalamic hormone release. In the previous section of this thesis, we showed that hypothalami of aged rats contain sufficient gonadotropin releasing activity to stimulate LH release from incubated rat pituitaries. Studies by Shaar, et al. (1975) and Huang, et al. (1976) demonstrate smaller increases in serum LH and FSH following orchidectomy and ovariectomy in aged male and female rats compared to young control groups.

These findings indicate that although the reproductive control system in aged rats does respond to decreased negative feedback following gonadectomy, the response is less than that which occurs in the young adult rat. These data suggest that decreased responsiveness of the LHRH-release mechanism, rather than loss of LHRH content or pituitary responsiveness to LHRH, is the major contributor to age effects in this control system.

The constant estrous state in aged female rats seems to be involved with failure of hypothalamic mechanisms responsible for the proestrous hormone surge. Crighton and Schneider (1969) showed that the preoptic area of the rat hypothalamus contains gonadotropin releasing hormone. Electrical stimulation of this region can cause ovulation in rats (Everett and Quinne, 1966) and lesions of the preoptic area have been shown to reduce gonadotropin releasing hormone content in this region (Mess, 1969) and cause persistent vaginal cornification in young female rats (Crighton and Schneider, 1969). In addition, the preoptic area has been reported to be the site of the stimulatory effect of estrogen on gonadotropin release. Clemens et al. (1969) showed that electrical stimulation of the preoptic area will stimulate ovulation in constant estrous rats. Aschheim (1976) concluded that aged constant estrous rats have sufficient serum LH levels to stimulate minimal follicular development and estrogen secretion. Although the hypothalamic mechanisms have apparently become less responsive

to endogenous estrogen or other hormone stimulation of gonadotropin release. The results of the current study show that aged rats can sustain high levels of serum LH if the hypothalamic regulation of LH release is circumvented by the injection of LHRH, which reinforces the concept that the ability of the hypothalamus to secrete gonadotropin releasing factors is impaired in the aged rat.

Although the precise mechanisms involved in the deterioration of hypothalamic function in the aged rat are not understood, a substantial amount of experimental data suggests that hypothalamic catecholamines may be involved. Hypothalamic catecholamines have been shown to influence anterior pituitary secretions presumably either by affecting the hypothalamic pituitary-regulating hormones' secretory mechanisms or by the catecholamines acting directly on the pituitary, as has been demonstrated for control of prolactin. Increased hypothalamic catecholamine function has been implicated in the release of pituitary LH and the inhibition of pituitary prolactin release (Sawyer, 1975). The decrease in serum LH and increase in serum prolactin that we have found in the aged rat is consistent with a hypothesis of decreased hypothalamic catecholamine function. This hypothesis is supported by reports of decreased hypothalamic catecholamine content and neuronal catecholamine turnover rates in aged rats and mice (Riegle and Miller, 1978; Meites, et al., 1978; Finch, 1978). On the other hand, Wilkes, et al. (1979) found increased median eminence

norepinephrine content in 12 month old rats with regular estrous cycles compared to six month old controls. A great deal more experimentation is required to understand the molecular basis for changes in hypothalamic catecholamine function in aging rats and to understand how changes in hypothalamic catecholamines may be related to alterations in hypothalamic responsiveness to the multiple stimulatory and inhibitory inputs it receives.

C. The Effect of Age on Reproduction in Repeatedly Mated Female Rats

In the past few years we and others have studied the effects of increasing age on several parameters of the reproductive control system of the laboratory rat. The aging female rat is characterized by loss of regular ovarian cycles (Huang and Meites, 1975; Aschheim, 1976; Lu et al., 1979). Although some rats remain cyclic throughout their lifespan, these authors report increased incidence of constant estrus and repetitive pseudopregnant states in the vaginal cytologies of aged rats. It has also been demonstrated that aged constant estrous rats will return to regular estrous cycles following stimulation of the hypothalamus directly or with presumably centrally acting drugs or hormones (Finch, 1978; Lehman, et al., 1978; Meites, et al. 1978). Previous work from our laboratory and others has shown decreased serum LH and increased serum prolactin in both male an female rats of increasing age (Watkins, et al., 1975; Riegle and Meites, 1976; Takahashi, et al., 1980). The previous section of this thesis showed that the

decreased serum LH in aged female groups could be restored with chronic administration of LHRH (Miller and Riegle, 1978). These data have been interpreted as evidence for age-related alterations in hypothalamic sensitivity to control inputs (Aschheim, 1976; Meites, et al., 1978; Riegle and Miller, 1978). Although our bioassay study showed that the hypothalamus of the aged rat contains similar LH-releasing activity as that of young rats (Miller and Riegle, 1978), the aged hypothalamus apparently releases less of this factor than young rats since the hypothalamic-pituitary unit of the aged rat appears to be less responsive to physiological changes in sex steriod concentrations in various reproductive states. In addition, the hypothalamic-pituitary unit of the aged rat is less responsive to castration-induced decreased gonadal steroids (Shaar, et al., 1975; Gray and Wexler, 1980) or systemic L-dopa injections (Riegle and Meites, 1976).

These experiments have been interpreted to indicate that neuroendocrine alterations in the aging rat may be associated with alterations of the estrous cycle normalcy in the female rat. Although neuroendocrine mechanisms undoubtedly contribute to the loss of reproductive function in the aged rat, there are other factors which also contribute. The most characteristic effect of aging on mammalian ovarian function is the decline in the number of oocytes remaining in the ovary with increasing age (Talbert, 1978). Aging laboratory rodents show decreased litter size (Asdell, 1941;

Blaha, 1964). Although the decrease in oocyte numbers may ultimately contribute to the decreased reproduction in aged mammals, Adams (1970) showed that litter size decreased prior to a decrease in ovulation rate. These observations suggest increased post-ovulatory interruption of reproduction in the aged mammal.

Arvay (1976) reported that multiple pregnancies accelerated certain indices of biological aging in the female rat during the reproductive period, but contributed to overall longevity. Similarly, Gonzalez-Lima (1981) found that pregnancy and lactation retarded the subsequent onset of sterility. The study described here represented our initial attempt to study age effects on reproductive control systems during the interval when the rat loses its ability to reproduce. The experiment was undertaken to determine the interaction between age and repeated pregnancies on longevity, the maintenance of normal ovarian cyclicity and the ability of the laboratory rat to successfully reproduce.

Materials and Methods

Twenty-four two month old female rats were randomly assigned to two groups of twelve each. Both groups of rats sustained serial pregnancies, beginning at two months of age for the first group and at nine months of age for the second group. To accomplish this, rats with proestrus vaginal smears were individually placed in cages containing two reproductively experienced male rats. Male rats used in this study ranged from four to ten months of age. We have

not found decreased fertility in male rats over this age frame in our colony. Successful mating was ascertained by the presence of sperm in the vaginal smear on the morning of estrus. Mated rats were placed in individual cages for littering. The number of pups born, number of live pups and litter weights at birth and on days 7, 14, 21 and 24 were recorded. At 24 days the litters were weaned and the mother's vaginal smears were taken until proestrus was detected and the rat was again mated for the next pregnancy. This procedure was continued from two months of age through nine gestational cycles when, at seventeen months of age, only one rat littered.

At 4 p.m. on the day of proestrus a single blood sample was taken by orbital sinus puncture under light ether anesthesia. Serum was collected from this blood sample for subsequent LH radioimmunoassay.

Results

The percentage of rats successfully mated and the percentages of rats littering are plotted as a function of age and number of pregnancies in Figure 11. All of the rats showed normal ovarian cyclicity and were successfully mated through six months of age. The percentage of rats cycling and mated ranged from 73% to 83% between 7 1/2 and 14 1/2 months of age. Sixty percent of the rats were still cycling and were mated at seventeen months of age when the experiment was discontinued.

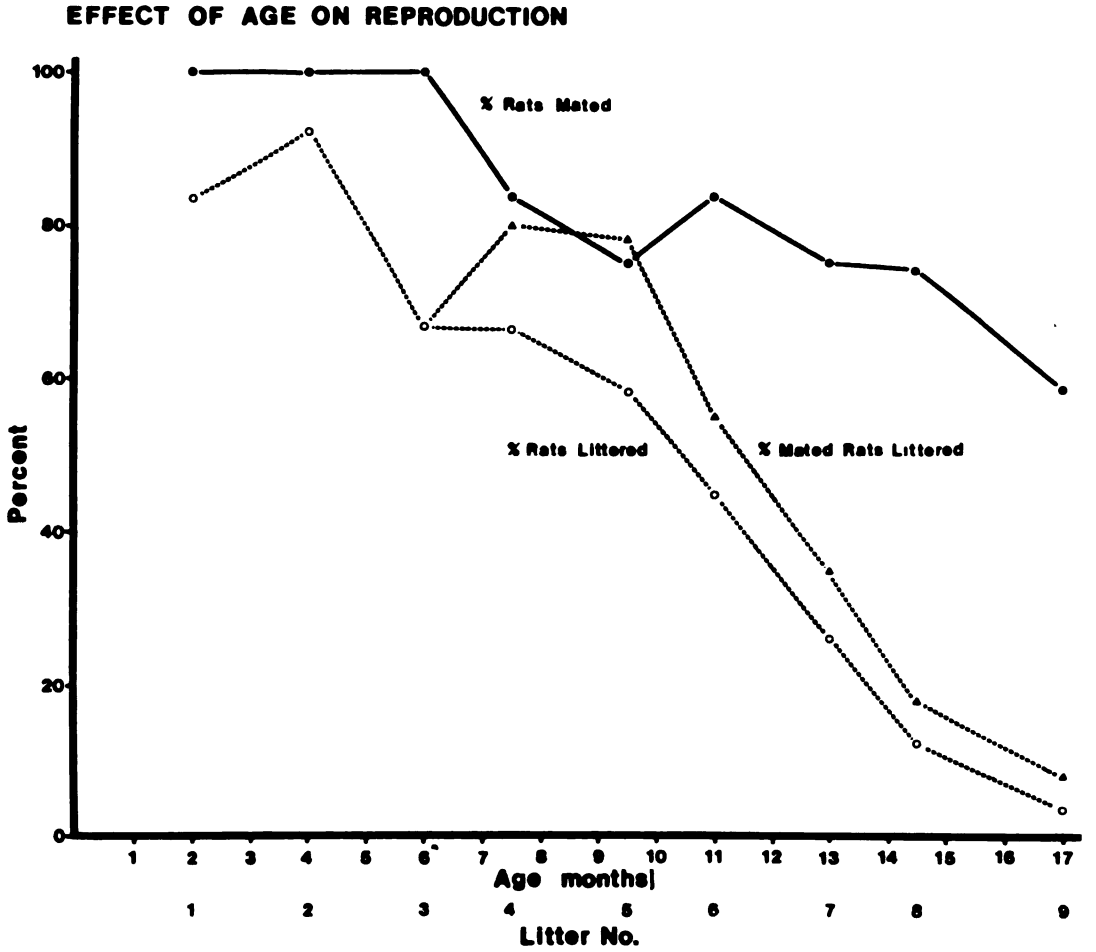


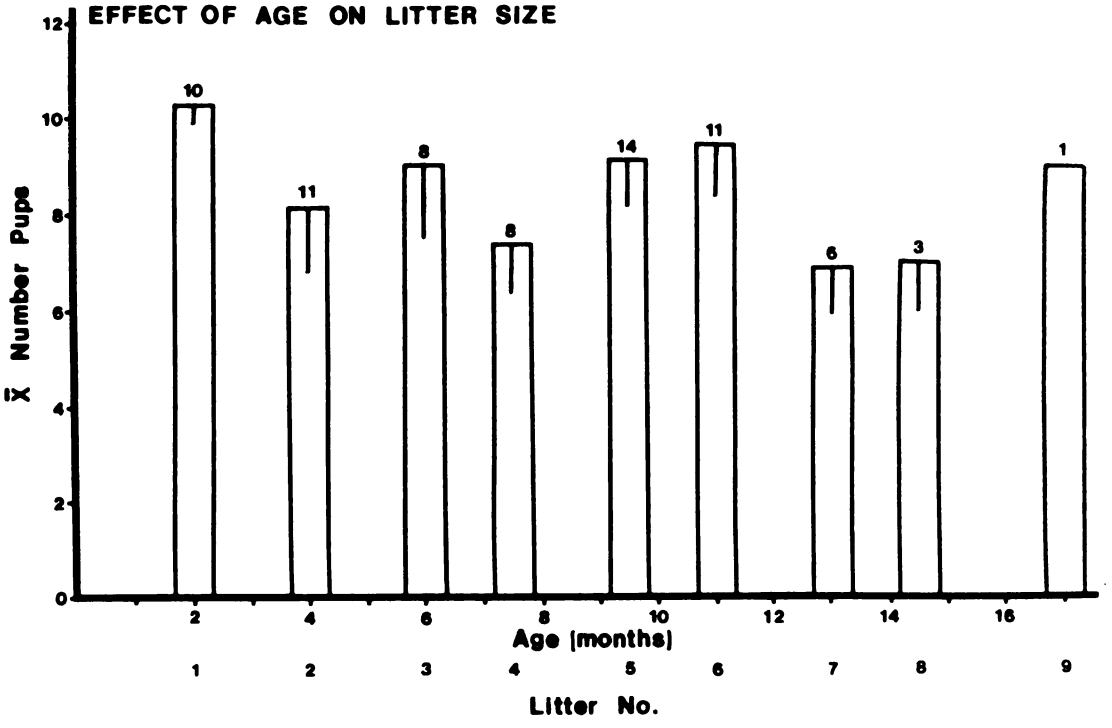
Figure 11. The Effect of Age and Repeated Pregnancies on Fecundity. The percentage of rats with normal ovarian cycles which were successfully mated and the percentages of both total and mated rats producing litters are plotted as a function of rat age and litter number.

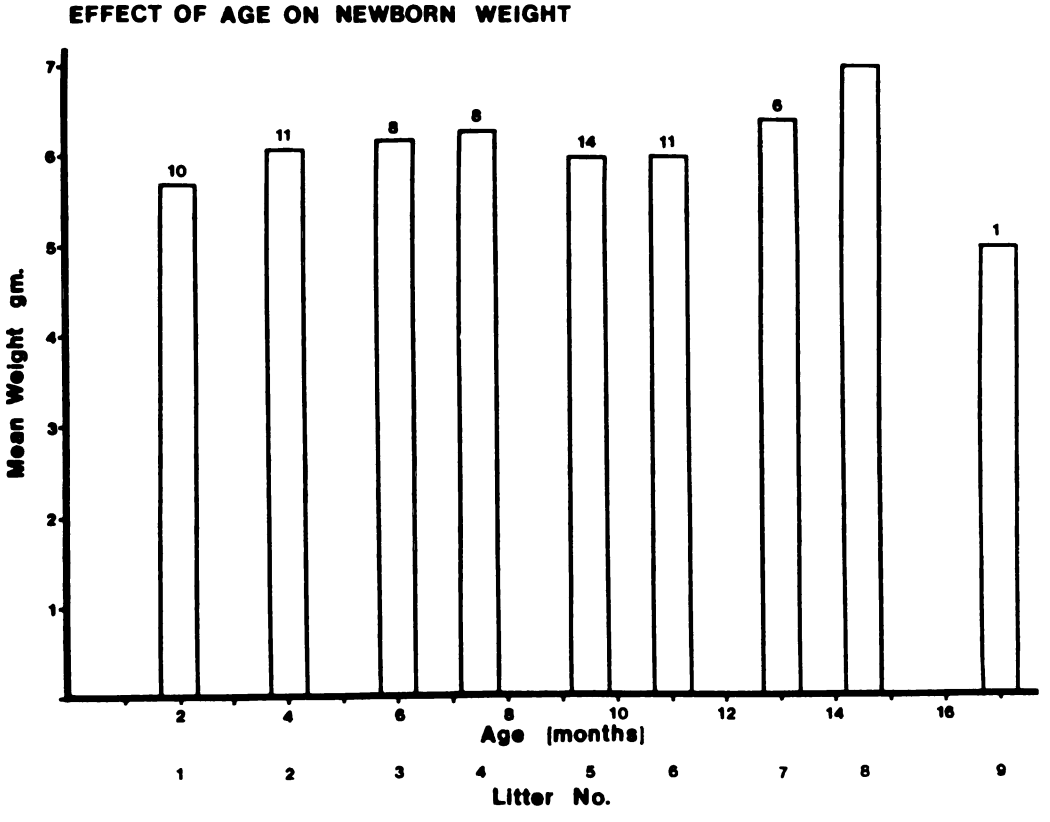
The percentage of rats delivering litters (Figure 11) are plotted both as the proportion of successfully mated rats and as the percentage of all rats included at the various stages of the study (twelve rats for litters sequence numbers 1-4, twenty-four rats beginning at litter five). The reduction in successful pregnancies with increased age was much more dramatic than the previously described loss of ovarian cyclicity in this study. The numbers of rats which delivered normal litters fell progressively from eleven of twelve rats at four months of age to only one of twenty-two in seventeen month old rats.

The effect of age and repeated pregnancy on litter size and average pup weight are shown in Figure 12. Neither age nor number of previous pregnancies affected the numbers or weight of the pups born. The range of average litter size was from 7 to 10.3 pups/litter and mean pup weight at birth ranged from 5 to 7 gms.

Mean serum LH concentrations of rats exhibiting an LH surge at 4 p.m. on proestrus are plotted as a function of age and litter number in Figure 13. Serum LH concentration was not affected by age or serial pregnancy number. All rats did not show a proestrus LH surge in the 4 p.m. blood sample. Only serum LH concentrations greater than 50 ng/ml were considered indicative of an LH surge and were included in the calculation of mean LH surge levels. The numbers above each column indicate the fraction of the normally cycling-mated rats with 4 p.m. LH surges. It should be

Figure 12. The Effect of Age and Repeated Pregnancies on Litter Size and Average Newborn Pup Weight. Group means are plotted with indicated standard errors of the means. The numbers above each column indicate the number of litters born at each interval.


EFFECT OF AGE ON LITTER SIZE
X̄ Number Pups
0
2
4
6
8
10
12
10
11
8
8
14
11
6
3
1
2
4
6
8
10
12
14
16
Age (months)
1
2
3
4
5
6
7
8
9
Litter No.


EFFECT OF AGE ON NEWBORN WEIGHT
Mean Weight gm.
1
2
3
4
5
6
7
10
11
8
8
14
11
6
1
2
4
6
8
10
12
14
16
Age (months)
1
2
3
4
5
6
7
8
9
Litter No.

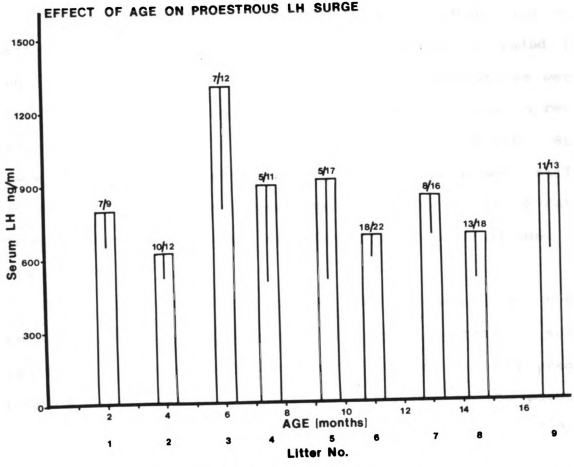


Figure 13. The Effect of Age and Repeated Pregnancies on the Proestrous LH Surge. Serum LH concentrations from 4 PM proestrous blood samples are plotted as group means (ng/ml serum) with indicated standard errors of the means. The numbers above each column indicate the proportion of rats with detectable LH surges (serum LH greater than 50 ng/ml at each breeding interval).

noted that these fragmentary data are not totally representative of the characteristics of the proestrus LH surge. There was no correlation between rats with demonstrable 4 p.m. LH surges and successfully mated rats which delivered normal litters.

The age when serial pregnancies were started did not affect any of the parameters of reproduction included in this study (Table 8). The age when serial pregnancies were initiated also had no effect on rat longevity. Eleven rats from each group were alive at eighteen months of age. Six rats from group one and seven rats from group two were still in the colony at twenty-four months of age and three rats from each group were still alive at thirty months of age.

Discussion

These data indicate that there is a sharp, continuous reduction in the ability of aging Long-Evans rats to successfully reproduce and that the age when repeated pregnancies was initiated did not influence any of the parameters of reproduction in the aging rat which were included in this study. The aging female rat exhibits a gradual increase in estrous cycle irregularities including the development of persistent vaginal cornification, pseudopregnancies of varying lengths, or diestrous states (Huang and Meites, 1975; Aschheim, 1976; Lu, et al., 1979). In previous experiments we and others have shown increased serum prolactin and a smaller increase in LH following ovariectomy in aged compared to young adult female rats

TABLE 8

The Effect of Age on Initiation of Repeated Pregnancies on Reproduction in the Rat

Litter Number	Group Number[1]	Age (mo)	n	Number Mated	Number Littered	Average No. Pups Born	Average Pup Weight (gm)
5	1	9 1/2	12	10	6	8.5	6.0
1	2	9 1/2	12	8	8	9.8	6.0
6	1	11	12	12	6	10.2	6.5
2	2	11	12	8	5	8.8	5.5
7	1	13	11	8	2	6.0	6.4
3	2	13	12	9	4	7.8	6.4
8	1	14 1/2	11	9	1	8.0	5.3
4	2	14 1/2	12	8	2	6.0	7.8
9	1	17	11	8	1	9.0	5.0
5	2	17	11	5	0	---	---

[1]Repeated pregnancies initiated at 2 mo of age in group 1 and at 9 mo of age in group 2.

(Riegle and Miller, 1978). Our studies (Miller and Riegle, 1978a; 1978b; 1978c) and the work of other investigators (Meites, et al., 1978; Finch, 1978; Gray and Wexler, 1980) suggest that aging changes in hypothalamic function contribute to the loss of normal estrous cycles in the rat. However, most of the changes in ovarian cyclicity and the previously documented neuroendocrine changes in reproductive control systems of the aging rat occur after the end of the rat's normal fertile lifespan (Jones, 1970). The results of this study confirm that aging effects on neuroendocrine mechanisms of reproductive control systems reduce the proportion of aging rats that are cycling and mated and contribute to the loss of reproduction. However, the sharper reduction in percentage of rats littering compared to percentages of rats with normal estrous cycles indicate that other post-ovulatory factors are major contributors to the loss of reproduction in the aged rat.

Van der Schoot (1976) reported decreased proestrous LH secretion in twelve month old rats. Cooper, et al., (1980) demonstrated that the LH surge may be more variable, lower and appear later in ten month old cycling rats. In a following section of this thesis we have also shown reduction in the magnitude of the proestrous LH surge in 12-16 month old compared to 4 month old control rats. Young rats in our colony show peak proestrous LH concentrations from 3 to 7 p.m. The similarity of LH concentrations with increasing age in this experiment was unexpected. However, our data

also indicate significant numbers of rats did not have a proestrous LH surge at the 4 p.m. sampling interval. Although the measurement of serum LH from a single blood sample does not preclude the possibility of aging effects on the timing of the LH surge and ovulation, the results of this limited experiment do not support the hypothesis that changes in the neuroendocrine control of the proestrous LH surge contribute to the loss of reproductive function.

The studies of Talbert (1978) and Mandl and Shelton (1959) suggest that normal ovulation was occurring in the cycling, mated rats used in this study. This hypothesis is supported by the report of Harman and Talbert (1970) which showed no difference in the number of eggs ovulated per ovarian cycle in mice from four to thirteen months of age. The hypothesis of normal ovulation rate occurring at the age when fertility is lost is also supported by our data presented in section 2 F of this thesis. In addition, studies by Talbert and Krohn (1966), and Gosden (1974) showed no difference in the survival of ova from aged mice compared to that of ova from young mice when both were transplanted to young multiparous uterine hosts. On the other hand, ova collected from young donors which were transplanted into aged recipients showed much smaller rates of survival, indicating that the aged uterus was less capable of sustaining normal pregnancies. Although these experiments clearly implicate uterine relationships to infertility in aging rats, additional experimentation will be required

before conclusions can be made concerning the cytogenetic normalcy of ova from aging rats and possible roles of aging ova in the decline of reproductive function.

Several investigators have suggested that the decrease in post-ovulatory reproductive capacity of the aging mammal could be due to alterations in endocrine support of uterine function. In a review of corpora luteal function in aging rodents, Talbert (1978) concluded that although the evidence was contradictory, most studies do not suggest that the decrease in reproduction in aged mammals could be contributed to by decreased progesterone secretion. This conclusion is supported by our recent experiments reported in section 2 E of this thesis which show no difference in serum progesterone in young compared to aged rats made pregnant or pseudopregnant by mating. The ability of the uterus to sustain pregnancy could also be affected by estrogen function.

Less is known about the effect of aging on estrogen secretion and its role in the decline in reproduction in mammalian species. Estrogen is markedly decreased in postmenopausal women and Sherman and Korenmen (1975) showed nearly a 50% reduction in estradiol during the premenopausal years in women. In the middle aged female rat, Lu, et al., (1979) report serum levels of estradiol (17 ± 1 to 18 ± 2 pg/ml) which were significantly lower than young rats during diestrus day two or proestrus. Serum levels of estradiol in aged rats that were repetitive pseudopregnant or constant

diestrus were also relatively low. However, Page and Butcher (1982) found no significant difference in the serum estradiol concentrations found in young (3 to 6 month) and middle aged (12 month) normally cycling rats.

The aforementioned observations have led most investigators to conclude that age effects on uterine structure and fuction are major contributors to age-related decreases in fertility. Biggers, Finn and McLaren (1962) concluded that increased deposition of collagen in the aging mouse uterus contributed to the loss of reproductive capacity. In the aged rat, the uterine connective tissue stroma enlarges to comprise a large part of the myometrium (Soriero, 1978). Finn, et al. (1963) suggested that the high collagen content of the aging mouse uterus may impair fertility by decreasing uterine vascularity, which in turn could result in decreased nutritional support of the conceptus. Although Burack, et al. (1941) reported that the rate of collagen deposition in the aging rat uterus is slower in fertile breeders than in nulliparous females of comparable age, the results of the present study suggest that the loss of reproductive capacity is not affected by the numbers of pregnancies experienced in the reproductive lifespan of the rat.

Another factor which could contribute to the loss of reproductive function in the aged rat is failure of normal implantation. Maibenco and Krehbiel (1973) found reduced uterine response to hormones in aged ovariectomized rats and increased leucocytic infiltration and infections in

naturally mated eighteen month old rats. Shapiro and Talbert (1974) reported decreased decidualization in the aged mouse uterus. Soriero (1978) concluded that the aged female has decreased uterine tissue response and delayed implantation. Although the design of this study prevented detection of the stage of reproductive failure, it is assumed that the loss of function occurred in the early stages of pre- and post- implantation development since no aborted fetuses were detected in the study.

The similarity of average litter size and pup weight in litters born of aged compared to young females in this study suggests an "all or none" type of interference with reproduction. Rather than producing litters of smaller size or impairing the growth of fetuses, age effects on reproduction in the Long-Evans rat seem to be manifested in total loss of reproductive function. This observation is consistent with our observation of similar "all or none" effects of stress on post-ovulatory reproduction in this strain of rats (Euker and Riegle, 1973). Our findings conflict with the reports of Asdell, et al. (1974) and Ingram, Mandl, and Zuckerman (1958). These studies showed similar age-related decreases in numbers of rats littering as found in our work. However, they report decreased average litter size in the aged rat. These findings suggest significant strain difference in this variable in the aging rat or that the relatively small numbers of older rats producing litters in this

study may not be representative of a larger population of aging rats.

D. Temporal Changes in Serum Progesterone in Aging Female Rats

Aging changes in the reproductive control system are well documented in mammalian females. During the past several years, we and others have considered the effect of aging on the hypothalamic-pituitary-ovarian axis in the laboratory rat. Although the postmenopausal human ovary does not contain sufficient number of normal oocytes to maintain reproductive function (Novak, 1970), the aged rat ovary has been shown to retain considerable numbers of oocytes throughout the lifespan of the rat (Mandl and Shelton, 1959). Since the rat loses normal reproductive capacity at 9-12 months of age (Miller, et al., 1979) the decline in oocyte numbers does not appear to be the primary factor involved with the failure to reproduce.

The aging female rat is characterized by loss of regular ovarian cycles (Huang and Meites, 1975; Aschheim, 1976; Lu, et al., 1979) with increased incidence of constant estrous and repetitive pseudopregnant patterns of vaginal cytology. The aged constant estrous rat will return to regular ovarian cycles following several types of hypothalamic stimulation (Meites, et al., 1978). Although we previously showed that hypothalamic extracts from the aged rat have similar LHRH activity as those from young rats (Miller and Riegle, 1978), the hypothalamic-pituitary unit of the aged rat was less

responsive to castration-induced decreases in gonadal steroids (Shaar, et al., 1975). These data have been interpreted to indicate decreased hypothalamic sensitivity to control inputs with age (Meites, et al., 1978; Riegle and Miller, 1978).

Secretion of gonadotropin by the hypothalamic-hypophyseal unit is influenced by steroid hormones. The negative feedback relationship between gonadal steroids and gonadotropin secretion is well established. However, increased gonadotropin secretions in response to LHRH stimulation have been induced by controlled increases in both estrogen and progesterone in intact proestrous and gonadectomized rats (Vilchez-Martinez, et al., 1974; Aiyer, et al., 1976). We and others have consistently shown decreased serum gonadotropin concentrations in intact and gonadectomized aged female rats which could be related to reductions in sex steroid availability. The purpose of the present study was to measure temporal changes in serum progesterone in young and aged cycling rats and in aged constant estrous, pseudopregnant and diestrus female rats in order to begin to identify changes in the pattern of sex steroid secretion occurring with age which may be related to alterations in ovarian cycles and decreased fertility.

Materials and Methods

The first experiment was designed to measure serum progesterone concentration at 1600 hours in aging rats

during proestrous, estrous and diestrous day two stages of their ovarian cycle. Rats included in this study were 3, 12, 18 or 24 months of age (n = 12/group). Only rats with at least two previous regular ovarian cycles were used in the study. Repetitive blood samplings were scheduled at least five days apart.

The second experiment considered temporal serum progesterone and LH changes during the proestrus hormone surge. Serial blood samples were taken from groups (n = 12/group) of young (3 mo.) and aged (12 - 24 mo.) regularly cycling proestrous rats at 1400, 1800, 2200 and 0200 hours.

Temporal serum progesterone concentrations were also measured in the third experiment. Groups of twenty young (3 mo.) and twenty aged (12 - 24 mo.) regularly cycling rats at proestrous, estrous and diestrous day two stages of their ovarian cycles were randomly divided into two groups. In an effort to minimize blood sampling effects on neuroendocrine control mechanisms, serial blood samples were collected from one subgroup at 0400, 1200, and 2000 hours and from the other subgroup at 0800, 1600 and 2400 hours.

In addition, temporal serum progesterone was measured in groups of aged constant estrous, pseudopregnant and diestrous rats. Rats were considered to be constant estrous if they had at least eight consecutive days of cornified vaginal smears. The pseudopregnant group consisted of rats having between eight and twelve consecutive days of leucocytic vaginal smears which followed one or two days of

cornified vaginal cytology. The diestrous rats had at least twenty-four days of consecutive leucocytic vaginal smears without cornification. Thus, groups of twenty twelve month old and twenty twenty-four month old constant estrous, sixteen twenty-four month old pseudopregnant, and sixteen twenty-four month old diestrous rats were also divided into subgroups and serially bled at either 0400, 1200, and 2000 hours or at 0800, 1600, and 2400 hours.

Differences within and between age groups were tested by multivariant analysis of variance and analysis of variance for repeated measurements. Only differences with a probability of error of less than 0.05 were considered significant.

Results

Figure 14 illustrates serum progesterone concentration in 3, 12, 18, and 24 month old rats at 1600 hours on proestrous, estrous, and diestrous day two stages of their ovarian cycles. Mean progesterone concentrations were higher in the three month old group than in any of the aged groups at all three stages of the ovarian cycles. Serum progesterone concentrations were not different among any of the three higher aged groups at this sampling time.

We next considered the effect of age on the increase in serum progesterone which accompanies the proestrous hormone surge (Figure 15). Serum progesterone was increased on the afternoon of proestrus in both age groups. The magnitude of

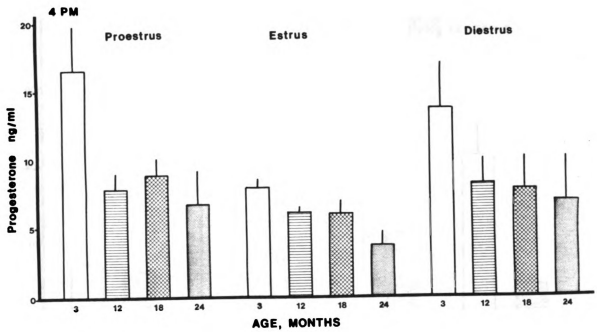


Figure 14. The Effect of Age on Serum Progesterone at Proestrous, Estrous, and Diestrus Day 2 Stages of the Ovarian Cycle. Progesterone is expressed as the group mean (ng/ml serum) with the indicated standard errors of the means from blood samples collected under light ether anesthesia at 1600 h in 3, 12 and 18, and 24 mo. old rats (N = 12/group).

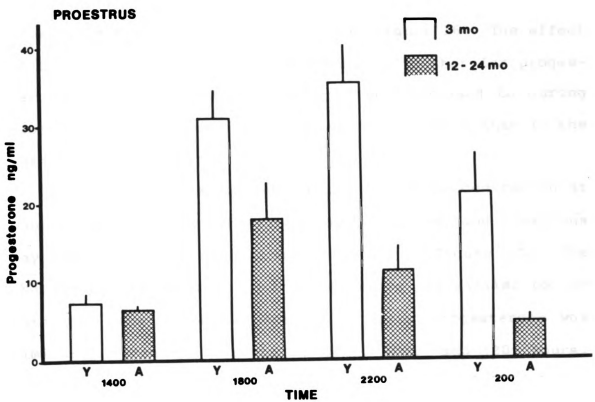


Figure 15. Serum Progesterone Concentrations During the Proestrus Hormone Surge in Young and Aged Female Rats. Progesterone is expressed as the group mean (ng/ml serum) with the indicated standard errors of the means from blood samples collected under light ether anesthesia at 1400, 1800, 2200, and 0200 h in 3 and 12 to 24 mo. old rats (n = 12/group).

the increase and the proportional increase over basal progesterone levels were both greater in the young than in the aged group. The sustained increase in progesterone at 0200 hours also indicates a longer duration of elevated progesterone in young compared to aged proestrous rats. Serum LH concentration from these young and aged groups during the proestrous hormone surge are shown in Figure 16. The effect of age on LH concentrations was similar to that for progesterone. Although both age groups had increased LH during proestrus, the increase was greater in the young than in the aged group.

Temporal changes in serum progesterone concentration at four hour intervals from proestrous, estrous, and diestrous day two young and aged rats are shown in Figure 17. The changes in progesterone during proestrous are similar to the data from the second experiment. Serum progesterone was similar between aged groups at 0400, 0800, and 1200 hours. The increase in progesterone at 1600, 2000, and 2400 hours was much greater in the young compared to the aged group. The higher progesterone concentrations in the young compared to aged groups was sustained through the estrous stage of the ovarian cycle. Mean serum progesterone levels were of greater magnitude in the young than in the aged group during estrous at all sampling intervals except at 2000 hours. Additionally the young group showed a mid-day increase in progesterone which was not detected in the aged group. Both age groups showed a surge in serum progesterone on diestrous

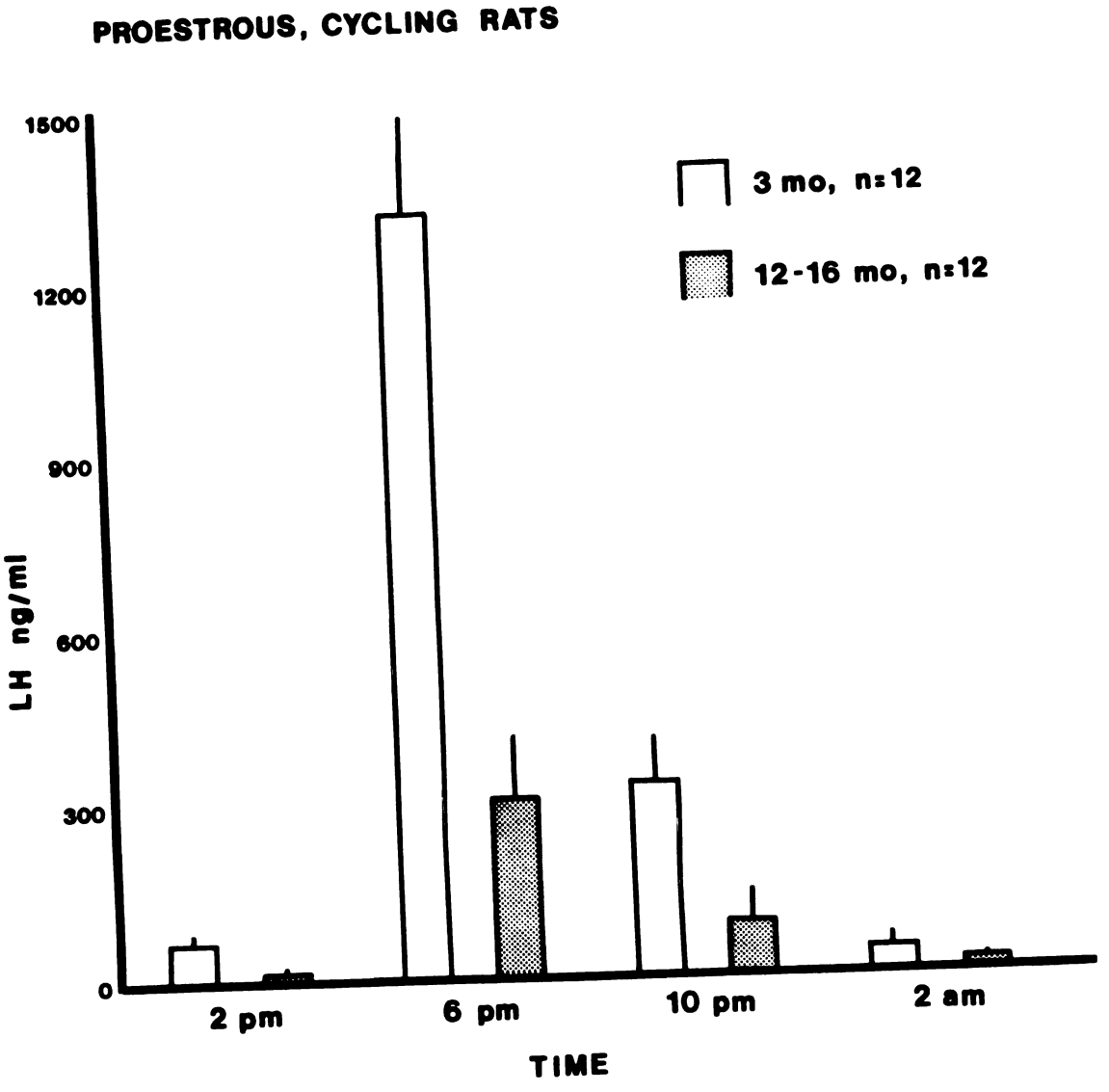


Figure 16. Serum LH Concentrations During the Proestrous Hormone Surge. LH is expressed as the group mean (ng/ml serum) with the indicated standard errors of the means from serial blood samples collected under light ether anesthesia.

Figure 17. Temporal Changes in Serum Progesterone in Young and Aged Female Rats at Proestrous, Estrous, and Diestrus Day 2 Stages of Their Ovarian Cycles. Progesterone is expressed as the group mean (ng/ml serum, N = 10) with the indicated standard errors of the means from serial blood samples collected under light ether anesthesia at 0400, 0800, 1200, 1600, 2000 and 2400 h (N = 10/group).

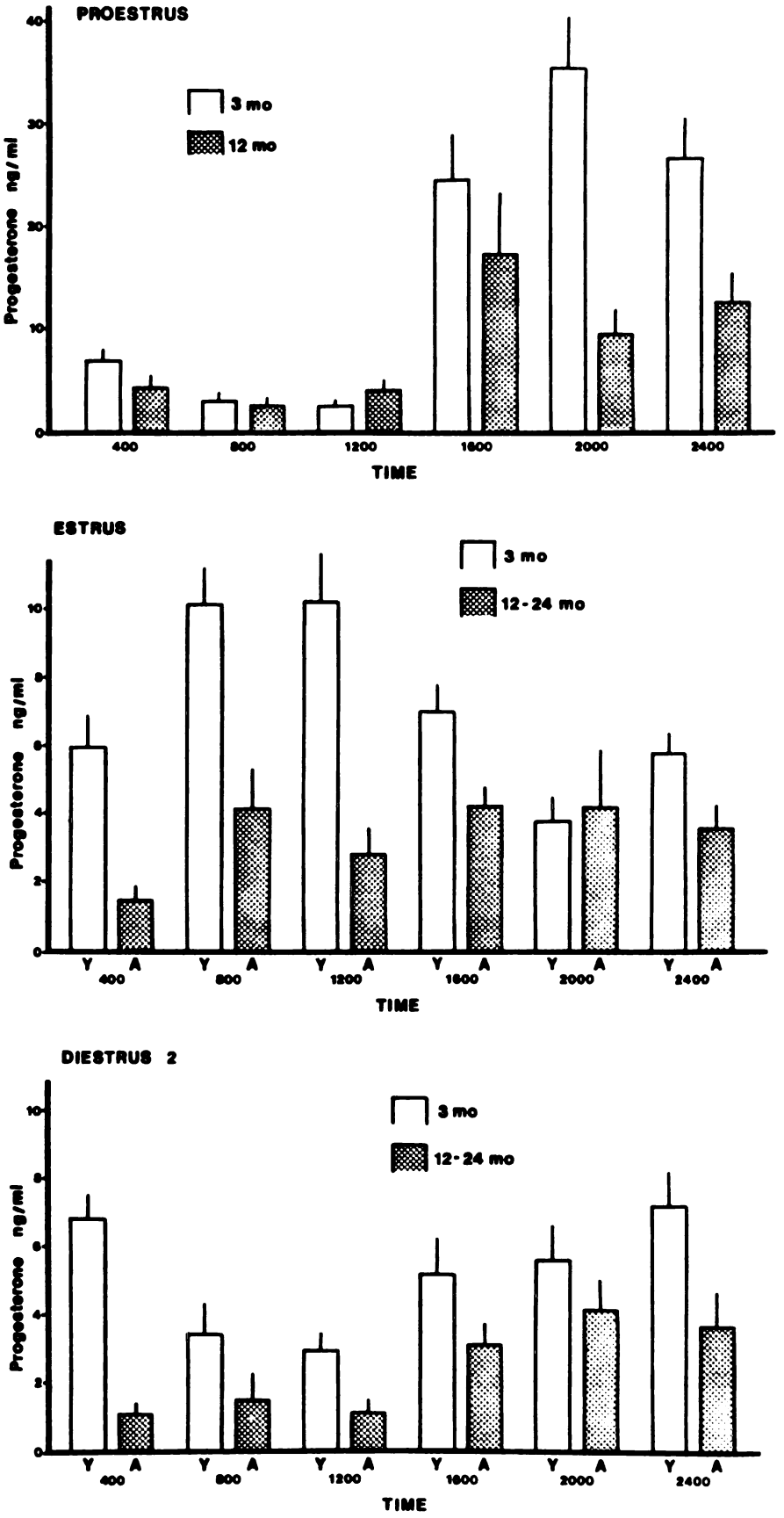

PROESTRUS
3 mo
12 mo
Progesterone ng/ml
0
10
20
30
40
400
800
1200
1600
2000
2400
TIME
ESTRUS
3 mo
12-24 mo
Progesterone ng/ml
0
2
4
6
8
10
Y
A
400
800
1200
1600
2000
2400
TIME
DIESTRUS 2
3 mo
12-24 mo
Progesterone ng/ml
0
2
4
6
8
10
Y
A
400
800
1200
1600
2000
2400
TIME

Figure 17.

day two which was of greater magnitude and of longer duration in the young group.

The patterns of serum progesterone concentration found in both young and aged cycling rats were not found in any of the noncycling aged groups (Figure 18). Serum progesterone concentrations were uniformly low in both twelve and twenty-four month old constant estrous groups. Mean progesterone concentrations were higher in aged repetitive pseudopregnant and constant diestrous rats than progesterone levels during estrous or diestrous day two in young or aged cycling rats. However, progesterone levels were not significantly different between aged (twenty-four month) rats classified as repetitive pseudopregnant or during the leucocytic period of those designated constant diestrus by vaginal cytological characteristics.

Discussion

The results of these experiments indicate substantial alterations in serum progesterone with age in cycling rats. The aged cycling rat had lower progesterone concentrations at most stages of the estrous cycle which were sampled in this study. The aged groups had smaller increases in serum progesterone and shorter duration of increased progesterone concentrations during both the proestrous and diestrous day two hormone surges. This decrease in serum progesterone could contribute to age-related decreases in reproduction in the rat and could be associated with the decrease in LH

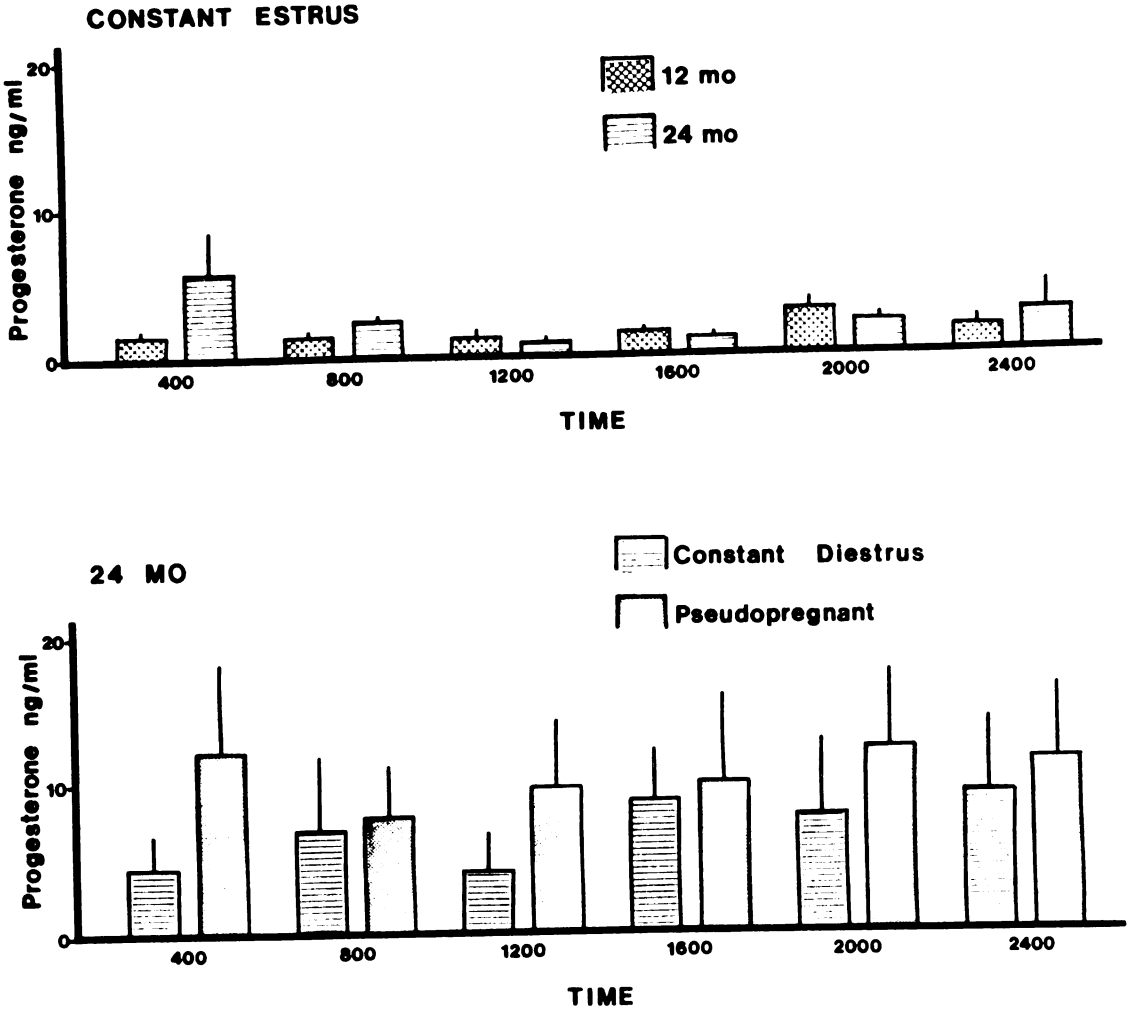


Figure 18. Serum Progesterone Concentrations in Noncycling Constant Estrous, Pseudopregnant, and Constant Diestrous Aged Female Rats. Progesterone is expressed as the group means (ng/ml serum) with the indicated standard errors of the means from serial blood samples collected under light ether anesthesia at 0400, 0800, 1200, 1600, 2000, and 2400 h from 12 and 24 mo. old constant estrous (N = 10) and 24 month old pseudopregnant and constant diestrous (N = 8) rats.

secretion found in these studies and commonly associated with aging in the rat.

It is agreed that the rat and most other rodent species retain considerable numbers of oocytes long after they fail to successfully reproduce (Mandl and Shelton, 1959; Talbert, 1978). However, the number of follicles that develop to the vesicular stage is somewhat related to the numbers of primordial follicles remaining in the ovary (Erickson, 1966) which can limit the numbers of follicles maturing and forming potential ovulatory oocytes (Talbert, 1976). Although reproductive failure does not occur in the rat because of a lack of oocytes, defective oocytes or changes in ovulatory conditions could contribute to the inability of the aged female to reproduce. The development of more large non-ovulatory follicles has been hypothesized to be responsible for the secretion of additional estrogen which could be related to alterations occurring with age in the development of the reproductive tract and the control of gonadotropin secretion (Foote, 1967).

The origin of the proestrous increase in progesterone and its relationship to the proestrous gonadotropin surge has received considerable attention. Several studies have shown an adrenocortical role in the increased progesterone secretion during proestrus (Mann and Barraclough, 1973a; Shaikh and Shaikh, 1975). Increased adrenocortical progesterone secretion has been shown to occur before the proestrous gonadotropin surge (Shaikh and Shaikh, 1975).

The increase in serum progesterone, on the morning of proestrous, has been shown to facilitate gonadotropin secretion and induce earlier mating behavior and ovulation in the rat (Nequin, et al. 1975) and to cause LH release in Nembutol blocked rats (Kobayashi, et al. 1973). It is also clear that increased estradiol concentrations can augment pituitary responsiveness to LHRH (Vilchez-Martinez, et al. 1974). It has recently been reported that increased blood estradiol, presumably of ovarian origin can increase the duration of the adrenal progesterone secretion during proestrus in the rat (Campbell, et al. 1977). On the other hand, this study also showed a stress-induced increase in adrenal estradiol secretion in proestrous but not in metestrus cycling rats suggesting that the proestrous hormone milieu can have specific effects on adrenocortical steroidogenesis.

On the other hand, adrenalectomized rats have normal estrous cycles and ovulate (Feder, et al. 1971). Decreased progesterone in intrauterine device-bearing hamsters caused a delay in LH secretion (Saksena and Shaikh, 1974) and the critical period for LH release was extended in adrenalectomized rats (Feder, et al. 1971). Interruption of the proestrous LH surge with Nembutol also affects the increase in progesterone during proestrus, indicating that the ovulatory LH surge increases ovarian progesterone secretion during and after the peak of gonadotropin release (Everett, 1967). It has been proposed that the primary role of adrenocortical progesterone secretion is to facilitate LH secretion and

estrous behavior at specific time intervals (Mann and Barraclough, 1973b).

These findings suggest several possible variables in steroid-gonadotropin interaction that could affect reproduction in the aging rat. The smaller increase in serum progesterone concentration during diestrous day two and proestrus could reflect an age-related decrease in adrenal or ovarian progesterone secretion with age. Additionally, the decrease in both magnitude and duration of the progesterone increase could be due to interactions with undetected changes in adrenocortical or ovarian estrogen secretion in the aged rats. The reported effects of decreased progesterone secretion on gonadotropin control systems are consistent with the decrease in basal serum LH levels and the reduced proestrous LH surge in the aged rats from this study. This reduction in serum LH is in agreement with previous reports of smaller increases in LH secretion following acute LHRH injections or gonadectomy (Meites, et al. 1978; Riegle and Miller, 1978; Gray and Weler, 1980) in the aged compared to the young female rat.

It is not clear whether the reduced progesterone concentrations found on diestrous day two are characteristic of post-coital progesterone secretion. There is considerable evidence of decreased uterine capacity to sustain pregnancy in aging rodents. An increase in degenerate-appearing corpora lutea has been associated with decreased numbers of implantation sites in mice (Harmon and Talbert, 1970) and

the pregnancy rate of mice has been improved by progesterone injections on days 2-9 of pregnancy (Gosden, 1975). However, no decrease in blood progesterone was found in aged pregnant rabbits (Spilman, et al. 1972) and we recently reported no differences in serum progesterone between young and aged rats on days one through six and on days eleven and sixteen of pregnancy (Riegle and Miller, 1978; Section 2 E of this thesis).

These findings suggest that if differences in progesterone availability contribute to loss of fertility in the aging rat, the effects are related to the control of gonadotropin secretion which stimulates ovulation or involve the ability of the uterus to respond to progesterone. This latter hypothesis could reflect differences in progesterone receptors. In addition, estradiol has been shown to induce uterine progesterone receptors (Feil, et al. 1972). Therefore, a pre-existing estrogen deficiency could markedly alter uterine responsiveness to circulatory progesterone.

With increasing age, rat estrous cycles become irregular and increasing proportions of aging female rats develop a constant cornified vaginal cytology, commonly referred to as the constant estrous state (Aschheim, 1976). A large variety of treatments, including the use of certain drugs, hormones and stress, have been shown capable of restoring vaginal cytologic changes characteristic of ovarian cycles in these rats (Finch, 1978; Lehman, et al. 1978). Although it is agreed that aged constant estrous rats are

anovulatory, the alterations in the neural, pituitary, gonadal mechanisms which account for this endocrine state remain unclear. Our results confirm the previous hypothesis that progesterone secretion was reduced in the senile constant estrous rat (Weisz and Lloyd, 1965). Several investigators have shown an increase in the incidence of ovulatory ovarian cycles in constant estrous rats following progesterone injections (Everett, 1940; Meites, et al. 1978). However, at this time it is not clear whether the effectiveness of progesterone is due to increased hypothalamic LHRH secretion, increased pituitary responsiveness to LHRH, progesterone effects on hypothalamic monoamine availability or other neuroendocrine mechanisms.

The similarity of serum progesterone concentrations which we found in repetitive pseudopregnant and constant diestrous rats was not expected. The ovarian cycles of aged pseudopregnant rats have previously been shown to be ovulatory followed by development of functional corpora lutea (Huang and Meites, 1975; Lu, et al., 1979). Although the presence of corpora lutea was not ascertained in this study, it is presumed that the pseudopregnant state in these rats was due to luteal maintenance of high serum progesterone concentrations. Our data do not support the hypothesis that the aged rats classified as pseudopregnant by vaginal cytologic criteria consistently ovulate and form functional corpora lutea. We found serum progesterone concentrations in these rats to be highly variable. Average

progesterone concentrations for the group classified as pseudopregnant was considerably less than the 40-100 ng/ml concentrations we and others (Feder, et al. 1971) have reported in induced pseudopregnancy in younger aged groups. Although some of these rats had high progesterone concentrations typical of pseudopregnancy in younger rats, others had low progesterone which could suggest failure of ovulation and luteinization. Resolution of this discrepancy will require further measures of progesterone secretion coupled with anatomical observation of ovarian tissues or establishment of the magnitude or the nocturnal-diurnal pattern of prolactin secretion in these rats classified as pseudopregnant on the basis of vaginal cytological changes.

On the other hand, constant diestrous rats do not show evidence of estrous cycles or ovulation by changes in vaginal cytology. It has been assumed that these rats would have minimal serum progesterone concentrations. However, our data suggests significant progesterone secretion, presumably from adrenocortical or nonluteal ovarian sources. Our findings also suggest that vaginal cytological data may not always clearly indicate the functional state of the hypothalamic hypophyseal-ovarian-axis.

In summary, these experiments demonstrate that significant decreases in serum progesterone concentration occur during the estrous cycle of aging female rats. In addition, progesterone concentrations were reduced in aged constant estrous rats and generally elevated in experimental groups

identified as pseudopregnant or constant diestrous. These data suggest that alterations in progesterone secretion could contribute to the decline in gonadotropin secretion, ovulation and fertility in aging rats which have sustained their ovarian cyclicity and decreased progesterone secretion could be associated with the induction of aberrant ovarian cycles and the absence of ovarian cycles in aging female rats. These experiments do not indicate whether the primary alteration between sex steroid secretion and gonadotropin control mechanisms involve failure of gonadal and adrenocortical steroidogenic tissues to secrete adequate hormone which facilitates gonadotropin release or a failure of gonadotropin stimulation of progesterone secretion.

E. Serum Progesterone During Pregnancy and Pseudopregnancy and Gestation Length in the Aging Rat

Decreased reproductive function is well documented in aging laboratory rats and other mammalian species (Arvay, 1976 and Talbert, 1978). During the past few years we and others have considered the effect of aging on the hypothalamic-pituitary-gonadal control system in the laboratory rat. Although postmenopausal women are characterized by substantial increases in serum gonadotropins (Odell and Swerdloff, 1968) which occurs as a consequence of reduced ovarian steroid secretion, the aging rat has reduced serum LH and increased prolactin concentrations when compared to young rats with normal reproductive function (Riegle and

Miller, 1978). The aged rat has reduced serum LH concentrations following a single LHRH stimulation (Riegle and Meites, 1976; Watkins, et al., 1975) and smaller LH increases after gonadectomy (Shaar, et al., 1975; Gray and Wexler, 1980). However, we found similar LH concentrations in young and aged rats following multiple LHRH injections (Miller and Riegle, 1978, Section 2 B of this thesis) which is in agreement with an experiment studying pituitary responsiveness to LHRH in aging C57BL/56 mice (Finch, et al., 1977). These data suggest that the aged female rat retains sufficient pituitary function to stimulate ovarian function and that alterations in hypothalamic gonadotropin control systems could contribute to changes in ovarian cycles and the decreased fertility characteristic of this species.

To date, most studies of aging effects on laboratory rodent female reproductive control systems have considered changes in very aged (after 24 months of age or older) animals with obvious alterations in ovarian cycles. However, rodents characteristically begin to show a reduced ability to reproduce at a much earlier age, when most are still exhibiting regular estrous cycles (Thung, et al., 1956). Several studies have shown that reproductive failure first occurs in rodents while their ovaries contain considerable oocytes (Mandl and Shelton, 1959), and they are capable of producing normal numbers of oocytes at ovulation (Harman and Talbert, 1970). Oocyte transfer experiments have shown that eggs from old donors are capable of normal

development when transplanted into young recipients (Finch, 1978) suggesting that uterine failure contribues to the decline in fertility. Morphological studies (Harman and Talbert, 1970) have suggested that an increase in degenerate-appearing corpora lutea may be associated with implantation failure in aged mice. In addition, some investigators have found improved reproduction in aging mice receiving progesterone treatments (Gosden, 1975). These studies suggest that decreased progesterone secretion could contribute to the failure of the uterus to sustain pregnancy in aged mammals.

The purpose of the present study was to consider the effect of increasing age on serum progesterone concentrations in mated normally cycling female rats and in aged constant estrous rats made pseudopregnant by mating.

Materials and Methods

The reproductive status of the rats were established by daily vaginal lavage. Only rats with at least two consecutive four or five day ovarian cycles were considered to be regularly cycling. Rats maintaining at least eight days of consecutive cornified vaginal smears were considered to be in the constant estrous state. Rats to be mated were individually placed in a cage of two males (four to eight months of age) in the late afternoon on the day of proestrus or designated day of constant estrus. Successful mating was

determined by the presence of sperm in the vaginal lavage performed the following morning.

The first experiment considered the effect of pregnancy and pseudopregnancy on serum progesterone in separate groups of cycling rats at 4, 7, 9, 11, 13, 15, 20 and 22 months of age and groups of constant estrous rats at 11, 15, 20 and 22 months of age. Serum progesterone was measured in serial blood samples taken between 2 and 4 p.m. on days 1, 6, 11, and 16 after mating. All rats were maintained in the colony for the entire gestation period. The number of pups born and parturition time was recorded for each rat successfully completing gestation. Most young rats in our colony delivered their litters in the late afternoon of the twenty-second day of gestation. In this study 6 p.m. was designated as the end of the twenty-second day of gestation. Pregnant rats were checked for the presence of pups at twelve hour intervals to estimate parturition time.

The second experiment considered the effect of age on serum progesterone concentration during early and late gestation. In this study progesterone was measured in serial blood samples taken from young (4 mo., n = 14) and aged (13 - 16 mo., n = 26) cycling rats at 4 p.m. on days 1, 2, 3, 4, and 5 of early pregnancy and at 8 a.m. on days 19, 20, 21, and 22 of pregnancy. Daily vaginal smears were taken from all rats throughout their expected gestation period. Five of the aged rats resumed estrous cycles between days fourteen and nineteen of their gestation period

and were not included in the rats serially bled in late gestation. The number of pups born and parturition time was recorded for each rat successfully completing gestation.

Results

The effects of increasing age on successful pregnancy, mean litter size and average gestation length in groups of regularly cycling rats ranging from four to twenty-two months of age are illustrated in Table 9. The effect of age on the proportion of rats producing litters was apparent by eleven months of age. In this study, no successful reproduction was obtained in rats older than thirteen months. Increased maternal age was also associated with increased gestation length. Average gestation intervals progressively increased from 22.2 days in four month old rats to 23.7 days in the thirteen month old females.

Changes in serum progesterone on days 1, 6, 11, and 16 of pregnancy and pseudopregnancy are plotted in Figures 19 and 20. The data are shown on histograms which include progesterone concentrations in mated cycling rats which delivered litters, mated cycling rats which did not deliver litters (considered pseudopregnant), and aged mated constant estrous rats made pseudopregnant by mating. Average serum progesterone concentrations on day one of pregnancy ranged from 1.8 to 9.7 ng/ml and were not different between cycling rats which littered, cycling rats that did not produce litters and mated constant estrous rats. Average progesterone concentrations were markedly increased in all groups

TABLE 9

Effect of Age on Pregnancy in Cycling Female Rats

Age (mo)	No. Mated	No. Littered	Litter Size	Gestation (days)
4	12	11	11.6	22.2
7	14	13	10.5	22.6
9	13	10	7.2	23.1
11	14	6	9.4	23.3
13	12	3	4.7	23.7
15	12	0	----	----
20	8	0	----	----
22	5	0	----	----

Figure 19. Serum Progesterone Concentrations from Groups of Aging Female Rats on Days 1 and 6 After Mating. Each age group is subdivided into rats with regular ovarian cycles that produced litters (Cycling, Littered), those with regular ovarian cycles that did not produce litters (Cycling, Pseudopregnant), and mated constant estrous rats (Constant estrous, Pseudopregnant). Serial blood samples were collected between 1400 and 1600 h under light ether anesthesia. Progesterone is expressed as group means (ng/ml serum) with indicated standard errors of the means.

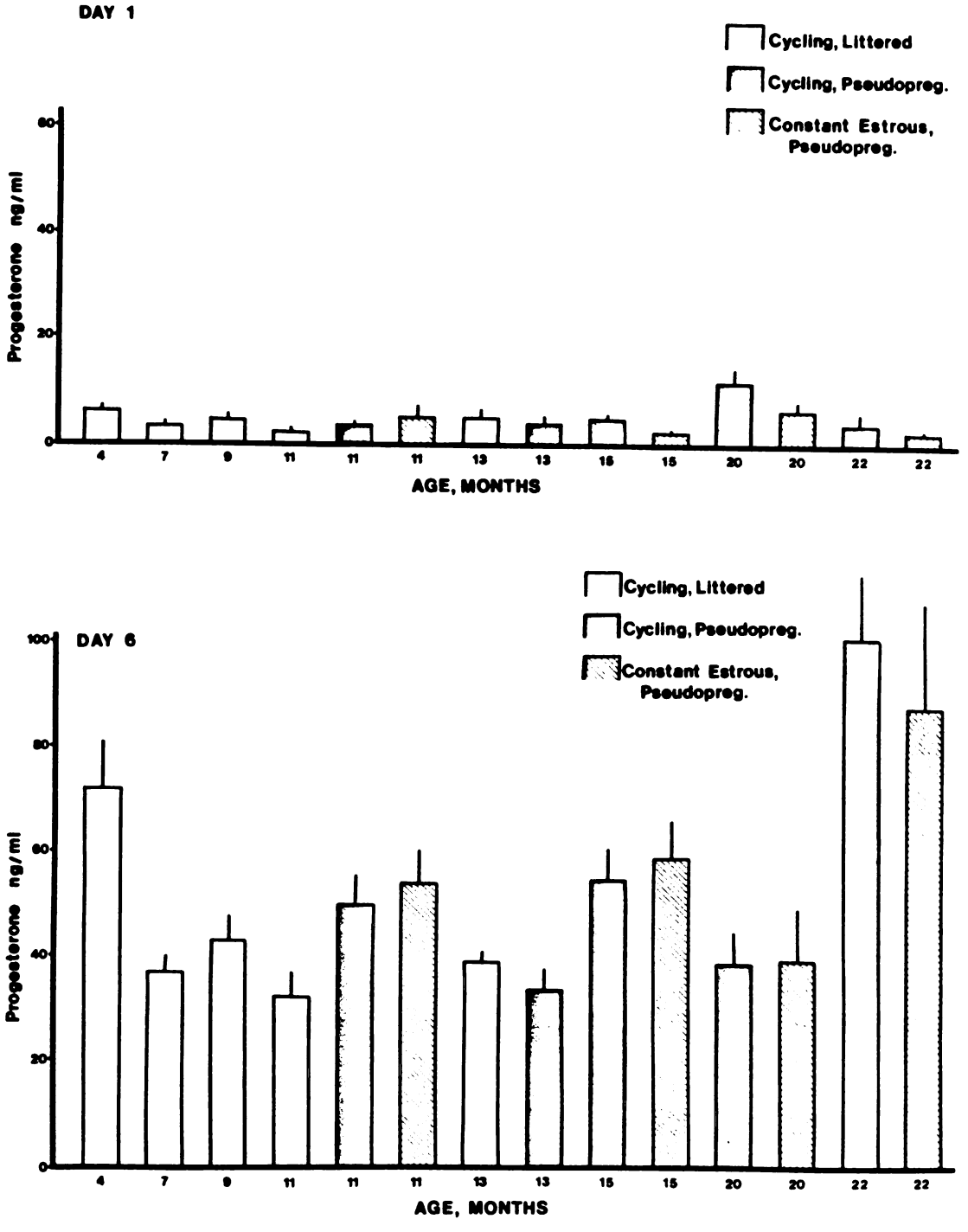

DAY 1
Cycling, Littered
Cycling, Pseudopreg.
Constant Estrous, Pseudopreg.
Progesterone ng/ml
0
20
40
60
4
7
9
11
11
11
13
13
15
15
20
20
22
22
AGE, MONTHS
DAY 6
Cycling, Littered
Cycling, Pseudopreg.
Constant Estrous, Pseudopreg.
Progesterone ng/ml
0
20
40
60
80
100
4
7
9
11
11
11
13
13
15
15
20
20
22
22
AGE, MONTHS

Figure 19.

Figure 20. Serum Progesterone Concentrations from Groups of Aging Female Rats on Days 11 and 16 After Mating. Each age group is subdivided into rats with regular ovarian cycles that produced litters (Cycling, Littered), those with regular ovarian cycles that did not produce litters (Cycling, Pseudopregnant), and mated constant estrous rats (Constant estrous, Pseudopregnant). Serial blood samples were collected between 1400 and 1600 h under light ether anesthesia. Progesterone is expressed as group means (ng/ml serum) with indicated standard errors of the means.

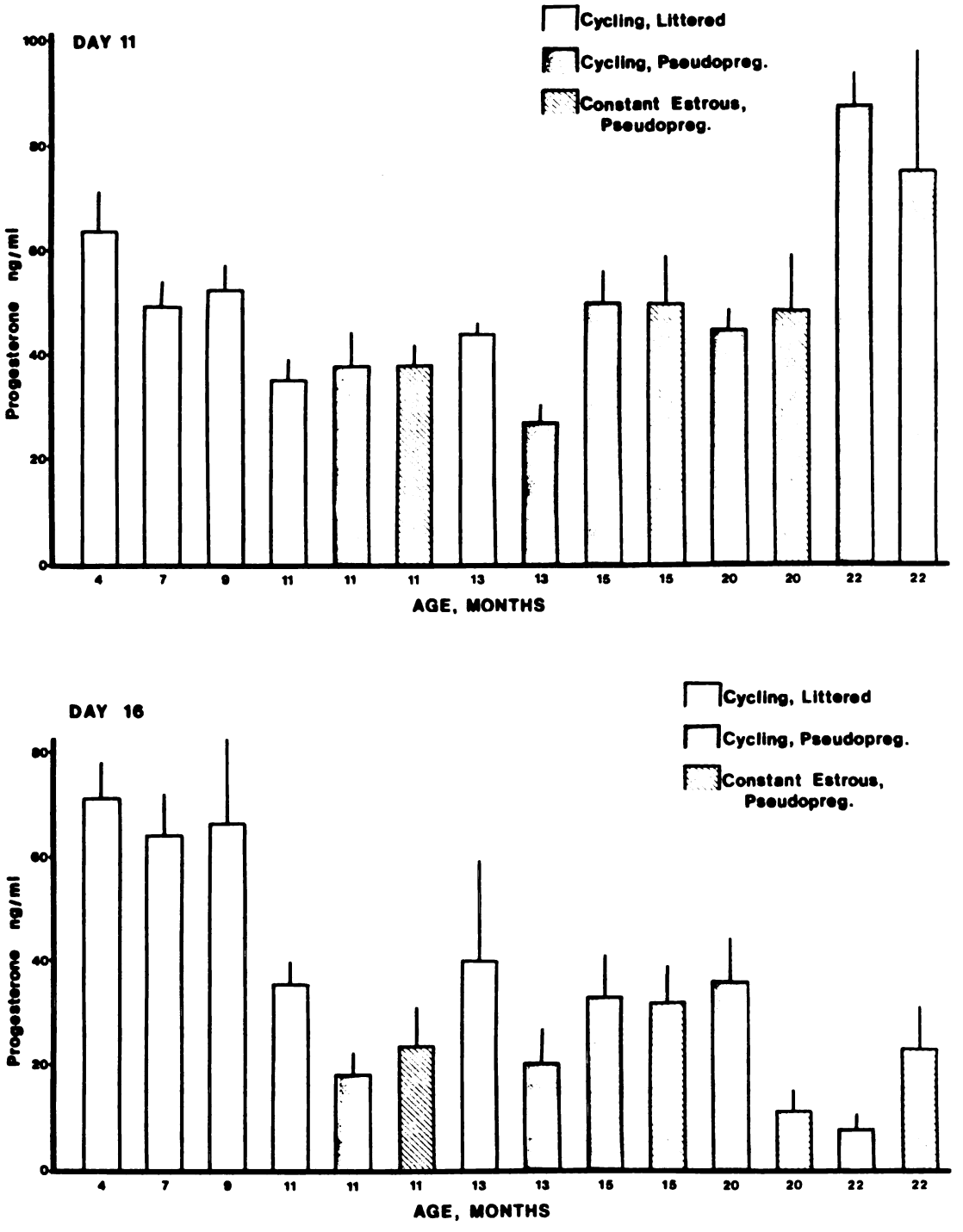

DAY 11
Cycling, Littered
Cycling, Pseudopreg.
Constant Estrous, Pseudopreg.
Progesterone ng/ml
0
20
40
60
80
100
4
7
9
11
11
11
13
13
15
15
20
20
22
22
AGE, MONTHS
DAY 16
Cycling, Littered
Cycling, Pseudopreg.
Constant Estrous, Pseudopreg.
Progesterone ng/ml
0
20
40
60
80
4
7
9
11
11
11
13
13
15
15
20
20
22
22
AGE, MONTHS

Figure 20.

by day six of pregnancy or pseudopregnancy. Although there were substantial variations between individual age group means, progesterone concentrations were not consistently different between littering-cycling females, nonlittering-cycling rats and constant-estrous-pseudopregnant groups.

The similarity between groups and the absence of age associated variations in serum progesterone was still apparent in the data from day eleven after mating. No differences were found in progesterone between littering groups, cycling-pseudopregnant or constant-estrous-pseudopregnant groups. The average progesterone concentration on day eleven of pregnancy from the 43 rats that subsequently littered was 55.2 ng/ml. The 64 rats of the cycling and constant estrous pseudopregnant groups at the same interval after mating averaged 48.0 ng progesterone/ml.

By day sixteen after mating, average progesterone concentrations were higher in the groups producing litters than in the pseudopregnant groups ($P<.05$). Average progesterone concentrations were 60.8 ng/ml on day sixteen in the pregnant groups, but were decreased to only 25.7 ng/ml in the cycling pseudopregnant group, and 24.0 ng/ml in the constant estrous pseudopregnant group.

The second experiment considered the effect of age on serum progesterone in early and late pregnancy. Average serum progesterone concentration from regularly cycling mated young and aged groups are shown as a function of the days of early pregnancy in Figure 21. Serum progesterone

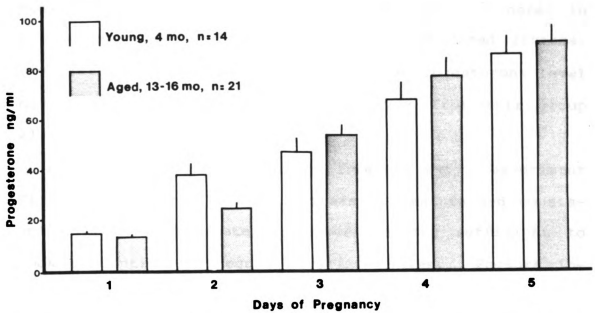


Figure 21. Serum Progesterone Concentrations from Young and Aged Female Rats on Days 1, 2, 3, 4 and 5 After Mating. Serial blood samples were collected at 1600 h under light ether anesthesia. Progesterone is expressed as group means (ng/ml serum) with the indicated standard errors of the means.

concentrations were progressively increased with developing pregnancy in both aged groups. Average serum progesterone concentrations were not different between the two age groups during this interval. Although the mean progesterone concentration of the young group was somewhat higher than that of the older group on day two, elevated progesterone on day two was not related to successful pregnancy in the aged rats. Progesterone concentrations averaged 27.2 ng/ml in the twelve rats in the aged group that produced litters, which was not different from the average progesterone level in the non-littering pseudopregnant rats from this group (21.4 ng/ml).

Eleven of the mated aged rats from the second experiment (Figure 22) did not produce litters but maintained consistently elevated progesterone concentrations sufficient to block resumption of regular ovarian cycles. Factors involved with continued progesterone secretion (presumably of luteal origin) in these rats were not identified in this study. However, the duration of pseudopregnancy in this group did exceed that normally induced by cervical stimulation alone, inferring possible corpora lutea stimulation by uterine factors, which suggests postnidation reproductive failure in this group.

Changes in serum progesterone on days 19, 20, 21, and 22 of pregnancy are plotted in Figure 22. Average progesterone concentrations were decreased in late pregnancy in both the young group and the aged group which produced litters. The

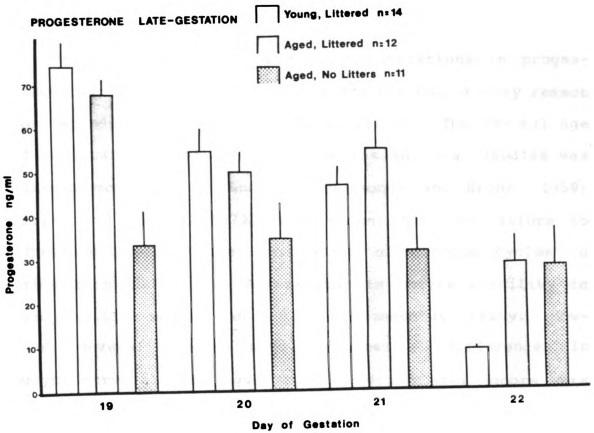


Figure 22. Serum Progesterone Concentrations from Young and Aged Female Rats on Days 19, 20, 21 and 22 After Mating. The aged group was subdivided into a group that produced litters and another group that did not. Serial blood samples were collected at 0800 h under light ether anesthesia. Progesterone is expressed as group means (ng/ml serum) with indicated standard errors of the means.

reduction in progesterone on day twenty-two of pregnancy was greater in the young than in the aged group which produced litters. The aged group which did not produce litters had similar progesterone concentrations at each blood sampling interval. Average length of gestation was 22.1 days in the young group and 22.9 in the aged group.

Discussion

These experiments suggest that alterations in progesterone availability to the uterus are not the primary reason for reproductive failure in the aging rat. The maximal age of successful reproduction in the rat in these studies was sixteen months. We and others (Jones and Krohn, 1959; Miller and Riegle, 1979) have shown that the failure to reproduce precedes the termination of estrous cycles in aging rats and mice. Alterations in oocyte viability in aging mammals may be related to decreased fertility. However, several studies have suggested no differences in oocyte survival when eggs from young and aged donors were transplanted into young host uteri (Talbert and Krohn, 1961; Mauer and Foote, 1971) and drastically reduced egg survival when oocytes were transplanted from young donors to aged uterine environments.

These findings suggest that failure of the aged uterus to sustain gestation contributes to the decline in fertility of aging female mammals. Experiments considering whether this uterine failure is due to changes in hormonal support

or aging effects in uterine function have produced conflicting data. Reduced decidualization responses have been reported from ovariectomized estrogen and progesterone primed aging mice and hamsters compared to decidualization in young adult control animals (Finn, 1966; Blaha, 1967). The decline in fertility of aging mice has been associated with morphological evidence of corpora lutea degeneration (Harman and Talbert, 1970). Other experiments have shown smaller corpora lutea of pregnancy in aged mice and hamsters compared to young controls (Thorneycroft and Soderwall, 1969; Gosden, 1974).

The possibility of reduced luteal progesterone secretions contributing to loss of fertility in aging is supported by experiments showing increased implantation rates in aging hamsters which have received young ovarian implants beneath the kidney capsule (Blaha, 1970) and increased survival of fetuses in mice receiving progesterone injections on days two through nine of pregnancy (Gosden, 1975).

The results of the present study do not support the hypothesis of reduced corpora lutea progesterone secretion during pregnancy in aged rats and suggest that the cycling or constant estrous female rats of any age can form functional corpora lutea. Similar serum progesterone concentrations were found from days one to eleven after mating in aging rats with regular estrous cycles that did not produce normal litters, aged constant estrous groups which did not produce litters, and the younger age groups which produced

normal litters. In addition, these data indicate substantial differences in serum progesterone from pseudopregnancies induced by cervical stimulation in this study compared to progesterone concentrations in similarly aged females showing spontaneous vaginal cytology which has been classified as persistent pseudopregnancy, as described in the previous section of this thesis. In the rat, cervical stimulation by mating on the evening of proestrous establishes two daily surges of prolactin secretion, the nocturnal surge from 3-5 a.m. and a diurnal surge from 4-7 p.m., which will support luteal secretions characteristic of pregnancy or pseudopregnancy (Freeman and Neill, 1972; Smith and Neill, 1976). The results of this study indicate that coital stimulation of constant estrous female rats induces similar effects on endocrine control systems. The luteotropic function of prolactin in the mated rat is augmented by LH stimulation of luteal cells which is obligatory for normal progesterone secretion between days seven and twelve after mating and is replaced by placental luteotropic secretions in the latter half of pregnancy (Morishige and Rothchild, 1974).

The similarity of serum progesterone during the early postcoital period in groups producing litters and those not producing litters suggests that alterations in neuroendocrine factors related to pituitary hormone stimulation of luteal tissue formation and function were not primary causes for infertility in the aged groups. Reports from our

laboratory have previously indicated decreased serum LH concentration after ovariectomy in aged compared to young female rats (Shaar, et al., 1975) and decreased sensitivity of the hypothalamic-pituitary unit to L-dopa inhibition of prolactin secretion in the aged female rat (Watkins, et al., 1976). We and others have interpreted these and similar data to indicate age-related alterations in hypothalamic-pituitary sensitivity and responsiveness to control input in the rat. Although we did not measure serum LH or prolactin concentrations in these experiments, the results of this study suggest that changes in hypothalamic-pituitary responsiveness to control input in the aging rat with regular ovarian cycles or in aging constant estrous rats are not sufficient to interfere with pituitary stimulation of luteal function. The decreased serum progesterone at postcoital day sixteen in the groups which did not deliver litters indicates that these rats did not have normal placental luteotropic stimulation of progesterone secretion.

The design of this experiment does not allow determination of the stage of reproductive failure in the aging rat. Failure of ovulation is not generally considered to be a primary alteration of reproductive systems in aging rodents. No change in post-ovulatory corpora lutea numbers have been reported in aging mice (Harman and Talbert, 1970) or rabbits (Adams, 1970). In a separate experiment we have assessed the ovulation rate of twelve month old Long-Evans rats with regular ovarian cycles by counting the numbers of eggs

recovered from the ballooned section of the fallopian tube on the day of estrus. In our study, fifteen of eighteen animals ovulated. The average number of eggs recovered from ovulatory rats was 11.1 and was not different from three month old rats. These data indicate that most rats are continuing to ovulate at the age interval when fertility rates are declining rapidly and support the hypothesis of post-ovulatory fertility loss in aging rats. Coital cervical stimulation is believed to be sufficient to stimulate prolactin secretion and maintain the nocturnal and diurnal surges of prolactin secretion required for early luteal function. The failure of normal uterine development results in a loss of corpora lutea support in the later stages of pregnancy.

Average gestation length was increased about 1.5 days in the aged rats in this study. This increase in gestation length is in agreement with age effects on gestation lengths in hamsters (Soderwall, 1960) and mice (Holinka, et al., 1978). Increased gestation length could be associated with reduced fertility in aging rats, since experimentally induced prolonged gestation has been shown to cause fetal mortality in mice (Kroc, et al., 1959) and rats (Moore, 1963). Serum progesterone concentrations in the present study were similar on days 19, 20, and 21 of gestation in young and aged rats which produced litters. Although average progesterone was decreased on day twenty-two of gestation in both age groups, the decline in progesterone was

less in the aged compared to the young group. This difference in the rate of decline of progesterone in late pregnancy in aging rats is in agreement with a report in aged mice (Holinka, et al., 1978) and suggests that maintenance of luteal progesterone secretion was related to the increased length of gestation in the aged rat since a major decrease in plasma progesterone is thought to be essential for the onset of parturition in the rat.

Factors controlling reduction of luteal progesterone secretion and the onset of parturition are poorly understood. Changes in blood concentration or biological activity of a large number of hormones and uterine factors, including progesterone, estrogen, prostaglandins and fetal glucocorticoids, have been implicated in controlling the onset of parturition (Ryan, 1977). All these factors act at least in part by decreasing serum progesterone concentrations or decreasing the biological activity of progesterone.

Biological actions of hormones depend on target cell receptor function as well as extracellular hormone concentrations. Estrogens have been shown to influence uterine progesterone receptors (Leavitt, et al., 1977). It is possible that undetected alterations of estrogen secretion are affecting progesterone function in late gestation in the aging rat. The specific role of factors believed to influence the onset of parturition in the aging rat as well as consideration of other prepartum gestational parameters such as the time of implantation remains to be resolved.

In summary, these data indicate that the loss of fertility in the aged rat is not due to failure of luteal tissue formation or progesterone secretion during early gestation. Our experiments suggest anatomical failure of fertilization, egg development or implantation or functional alterations in the ability of the uterus to sustain pregnancy. In addition, we have shown increased gestation length in the aged rat indicating age-related changes in the timing of endocrine changes regulating pregnancy in the rat. Identification of the specific alterations in pregnancy control systems which affect reproduction in the aged rat warrant further consideration.

F. Endocrine Factors Associated with the Development of the Constant Estrous State in Aging Female Rats

Decreased reproductive function with increased age is especially well documented in the female laboratory rat and inbred mouse (Arvay, 1976; Talbert, 1978; Finch, 1978). The first effect of age on fertility in these species occurs at a time when most females have normal, regular estrous cycles (Thung, Boot and Muhlback, 1956), and substantial numbers of oocytes in their ovaries (Mandl and Shelton, 1959) which produce normal numbers of eggs at ovulation (Harman and Talbert, 1970). Oocyte transfer experiments have shown that eggs from aged hamster (Blaha, 1964), mouse (Gosden, 1974) and rabbit (Adams, 1970) donors have higher survival rates when transferred to young compared to aged recipients.

These data have been interpreted to indicate significant post-ovulatory decreases in the ability of aged females to reproduce.

The aging female rodent also has significant changes in the regulation of estrous cycles which is also thought to contribute to the loss of fertility. Although some aging rats retain essentially normal estrous cycles throughout their lifespan, others show marked changes in ovarian cycles beginning at about nine to ten months of age. At this age increased numbers of rats show increased occurrence of cornified vaginal epithelial cells typical of continuous estrogen stimulation of this tissue (Huang and Meites, 1975; Aschheim, 1976). This condition often persists for several days or weeks and is commonly classified as constant estrous. With increasing age, some rats show occasional irregular 1-3 day intervals of cornified vaginal epithelium and on the basis of vaginal lavage characteristics have been classified as repetitive pseudopregnant. At more advanced ages, increased proportions of rats show no evidence of estrous cycles or periodic vaginal cornification and have been classified as persistent diestrus.

Changes in reproductive control mechanisms associated with the decline in fertility have received considerable recent attention. Increased serum FSH concentrations have been reported in ten to fourteen month old and twenty-four month old constant estrous and pseudopregnant female rats (Clemens and Meites, 1971; Wilkes, et al., 1978; Lu, et al.,

1979). On the other hand, no studies have shown increased serum LH in the aged female rat. Previous experiments from our laboratory (Shaar, et al., 1975; Watkins, et al., 1975) and studies by other groups (Aschheim, 1976; Meites, et al., 1978) indicate reduced serum LH and smaller increases in LH secretion after gonadectomy or single LHRH injections in aged compared to young female rats. On the other hand, aged female rats receiving multiple LHRH treatments had increases in serum LH which were similar to young rats receiving the same treatment (Miller and Riegle, 1978) and aged rats can be stimulated to ovulate (Meites, et al., 1978; Lehman, et al., 1978).

To date, many of the experiments concerning age effects on reproduction in the rat have considered very old (twenty-four months) compared to young (two to four months) groups, without giving major attention to intermediate ages when fertility is first affected. One of the changes which occurs at this early interval is the development of constant estrous. Endocrine factors associated with the pattern of vaginal cytology (8 or more consecutive days of cornified epithelial cells) constant estrous state are not well understood. The following experiments were designed to consider changes in the reproductive control system associated with the development and maintenance of this alteration in reproductive function.

Materials and Methods

Rats used in these experiments ranged from eleven to sixteen months of age. The reproductive status of the rats was established by daily vaginal lavage. Only rats with at least two previous consecutive four or five day ovarian cycles were considered to be regularly cycling. Rats were considered to be in the constant estrous state if they showed at least eight consecutive days of cornified epithelial cells in their daily vaginal lavage (the duration of two normal estrous cycles).

The first experiment considered the effect of coital stimulation of rats in the constant estrous state on LH secretion and ovulation. In one study, a group of constant estrous rats were individually placed in cages containing two reproductively experienced six month old males at 7:30 a.m. Mating was verified by the presence of sperm in vaginal lavages collected at noon. The ability of coital stimulation to activate ovulatory mechanisms was determined by the presence of oocytes in oviducts removed at 11 a.m. on the following day. A second study was designed to consider the possibility that the time sequence for ovulation could be different in the constant estrous rat. In this study a group of eighteen rats received coital stimulation as previously outlined. Evidence of ovulation in this group was based on measurement of LH in blood samples collected at 3 and 5 p.m. on the day of mating and by the presence of fetuses in the uterus on day eight following coital

stimulation. In addition to these experiments on rats in the constant estrous state, ovulatory rate was determined by oviductal egg collection at 11 a.m. on the day of estrus in a group of eighteen, twelve month old rats with regular estrous cycles.

Stress is one of the several factors which has been employed to induce estrous cycles in constant estrous rats (Finch, 1978). The basic experimental design utilized in the remainder of this study was to interrupt constant estrous by subjecting rats to two hours of restraint stress and then to measure serum progesterone and LH concentrations during the following ovarian cycle which could lead to ovulation and the formation of corpora lutea or the reinitiation of the constant estrous state.

The effect of the stress treatment on progesterone secretion was determined by measuring progesterone in blood samples taken before the stress was initiated and at the end of the two hour restraint. In addition, progesterone concentrations were measured in blood samples collected at 4 p.m. on the first day of leucocytic cell-dominated vaginal lavage following the stress and at 4 and 8 p.m. on the day the stressed rats exhibited their first characteristic proestrous vaginal lavage following the stress treatment (the vaginal smear is dominated by nucleated epithelial cells). Similar blood samples were collected at the same times on diestrous day one and on proestrous from similarly aged rats which were maintaining regular estrous cycles.

The final experiment included in this study considered the effect of progesterone treatment on the proestrous LH surge. In this study separate groups of ten, fifteen month old rats with normal estrous cycles received a subcutaneous injection of 0, 0.5 or 2 mg of progesterone (Sigma Chemical Company, St. Louis, Missouri) at 8 a.m. on proestrus. LH was measured in blood samples collected at 5 and 8 p.m. on proestrus.

Differences between groups was determined by analysis of variance. Only differences with a probability of error of less than 0.05 were considered significant.

Results

The effects of increasing age and reproductive state on ovulation rates in these rats are illustated in Tables 10 and 11. Ovulation rates in 12 month old rats with normal estrous cycles were similar to three month old controls. Although the constant estrous rats all were successfully mated, as determined by the presence of sperm in their vaginal lavage, none of the rats exhibited normally ballooned oviducts or had oviductal oocytes on the day after mating. In addition, only two of ten mated constant estrous rats had implanted fetuses eight days after mating. The mated rats showed a modest increase in serum LH concentration at 3 and 5 p.m. after coital stimulation.

Figure 23 illustrates the effect of stress on serum progesterone concentrations. The two hour stress treatment

TABLE 10

Evidence for Ovulation in Aged Long-Evans Rats

Group	Age (mo)	n	Number Ovulating	Oviductal Eggs[a]	Implanted Fetuses
Young, cycling	3	12	12	11.2	----
Aged, cycling	12	18	15	11.1	----
Aged, constant estrous[c]	14	8	0	----	----
Aged, constant estrous[d]	13	10	2	----	7.0

[a]Average oocyte number from ovulating rats teased from the ballooned portion of the oviduct at 11 a.m. one day after mating.
[b]Average implantation sites from pregnant rats, counted after laparotomy on day eight after mating.
[c]Constant estrous rats were caged with males at 7:30 a.m., egg collection was attempted at 11 a.m. the following day.
[d]Constant estrous rats were caged with males at 3 p.m., implantation sites were counted eight days after mating.

TABLE 11

The Effect of Mating on Serum LH
in Aging Constant Estrous Rats

Group	Age (mo)	n	Time	LH (ng/ml)[a]
Constant Estrous, Control	12-15	24	4 p.m.	41.3 ± 2.6
Constant Estrous, Mated[b]	13	10	3 p.m.	180.6 ± 28.2
			5 p.m.	162.6 ± 27.1

[a]Average LH concentration ± SEM
[b]Serial blood samples were collected at 3 and 5 p.m. on the day of coital stimulation.

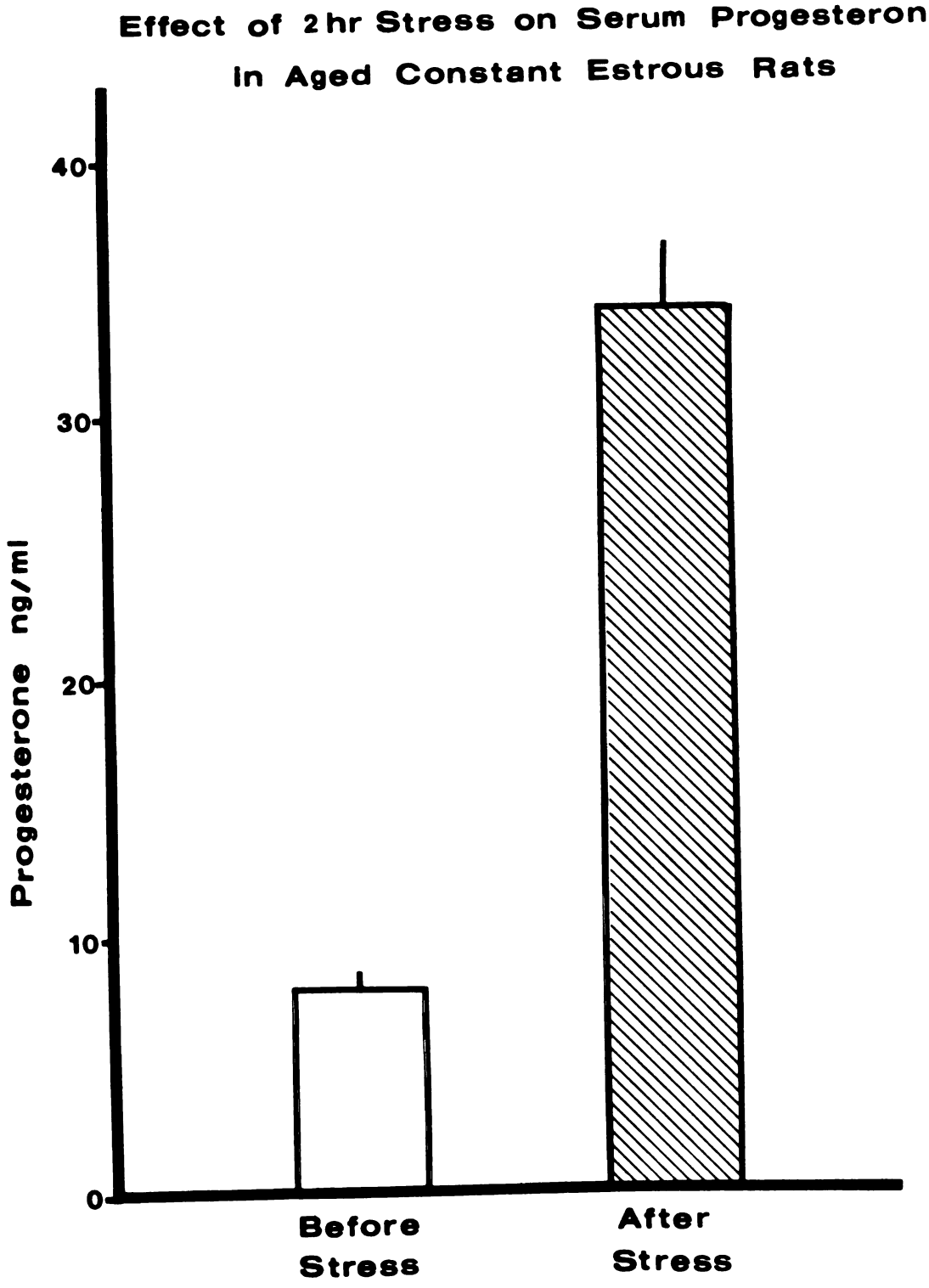


Figure 23. The Effect of 2 Hour Stress on Serum Progesterone in Aged Constant Estrous Rats. Serial blood samples were collected under light ether anesthesia prior to and immediately following the stress treatment. Progesterone is expressed as group means (ng/ml serum) with indicated standard errors of the means.

increased serum progesterone from 8.0 ng/ml before the stress to 34.2 ng/ml at the end of the treatment regime. All 26 rats included in this group exhibited an interruption in their estrous state. After one additional post-stress day of cornified cells in their vaginal lavage, the rats had two to four days of leucocytic cells in their vaginal smear which was followed by one day of nucleated cells typical of proestrous and a resumption of the constant estrous state by 23 of the 26 rats. In summary, these rats had vaginal cytological changes characteristic of a normal ovarian cycle followed by presumed failure of ovulation and resumption of the constant estrous state.

The character of the vaginal lavage following the stress treatment suggests normal luteal function in these rats. Average progesterone concentrations at 4 p.m. on apparent diestrous day one from stress-interrupted constant estrous rats and similarly aged normally cycling rats are plotted in Figure 24. In this experiment, 44 of 47 rats receiving stress treatments had their constant estrous state interrupted. Progesterone averaged 44.0 ng/ml following the stress treatment in these rats. Average progesterone concentration on apparent diestrous day one was only 4.8 ng/ml which was less than diestrous day one progesterone in similarly aged cycling rats (15.6 ng/ml) ($P<0.01$).

Serum progesterone and LH concentrations at 4 and 8 p.m. on proestrous from stress interrupted constant estrous and similarly aged, normally cycling rats are plotted in Figures

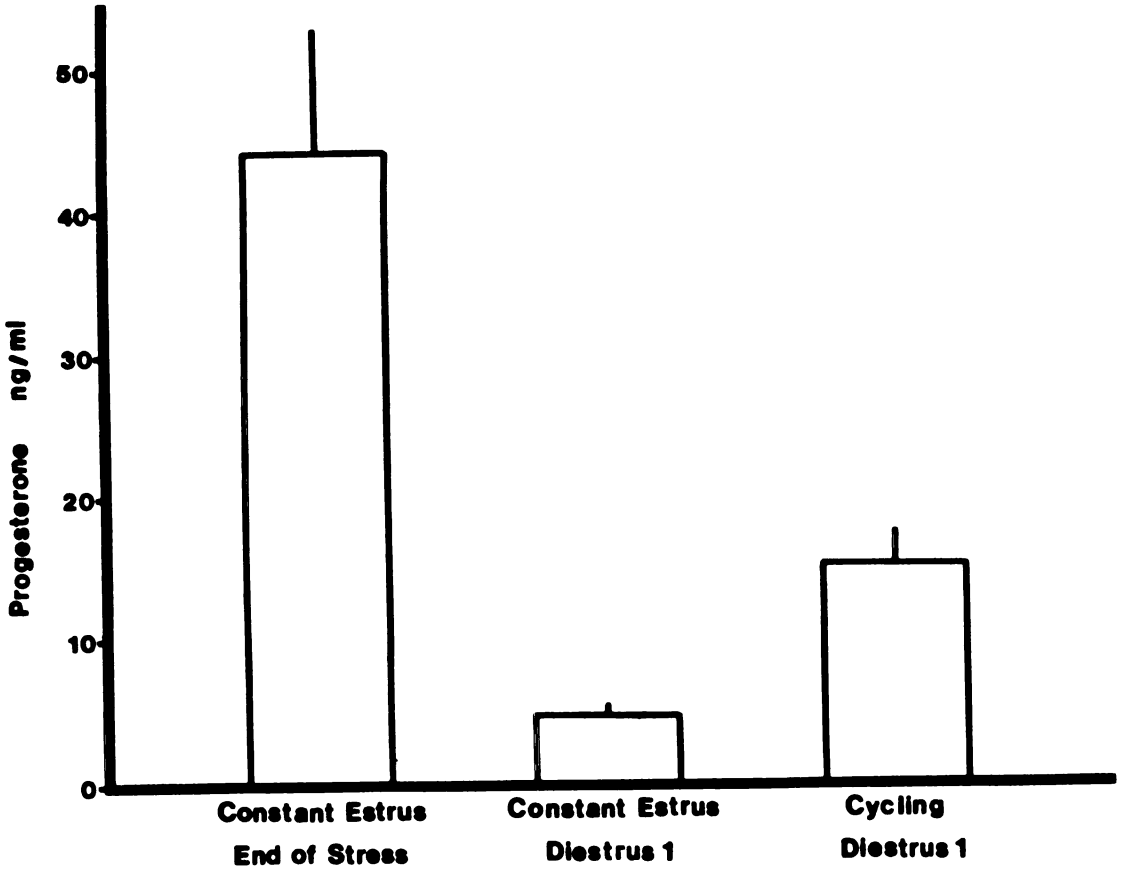


Figure 24. Serum Progesterone Concentrations from Stress Interrupted Constant Estrous Female Rats and Similarly Aged Normally Cycling Female Rats on Diestrus Day 1. Blood samples were collected under light ether anesthesia immediately following the stress treatment and at 1600 h on diestrus Day 1 in both the stress interrupted constant estrous rats and the regularly cycling female rats of similar age. Progesterone is expressed as group means (ng/ml serum) with indicated standard errors of the means.

25 and 26. Average progesterone concentrations were higher at both 4 and 8 p.m. ($P<0.01$) in the cycling compared to the constant estrous stress-interrupted group. Average progesterone from the cycling group was 24.2 ng/ml at 4 p.m. and 58.3 ng/ml at 8 p.m. compared to 7.2 ng/ml at 4 p.m. and 15.7 ng/ml at 8 p.m. from the constant estrous group. LH concentrations were similarly higher in the same blood samples collected from cycling rats compared to the constant estrous group ($P<0.01$). Serum LH averaged 377 ng/ml at 4 p.m. and 579 ng/ml at 8 p.m. in the cycling group compared to 107 ng/ml at 4 p.m. and 166 ng/ml at 8 p.m. in the constant estrous group (Figure 26).

The effect of progesterone injection on the proestrous LH surge is plotted in Figure 27. Both 0.5 and 2.0 mg of progesterone injected at 8 a.m. increased serum LH concentrations at 5 p.m. ($P<0.01$) and the 2.0 ng treatment increased LH at the 8 p.m. blood sampling interval ($P<0.05$).

Discussion

The results from these experiments indicate that inadequate proestrous LH secretion may be associated with the failure to ovulate and the initiation of the constant estrous state in aging female rats. Since ovulation can be stimulated in aging rats by gonadotropin injection, and estrous cycles, with presumed ovulation, can be induced by treatment of constant estrous rats with a wide variety of hormones, drugs or treatments known or presumed to stimulate hypothalamic-pituitary release of gonadotropins in the aging

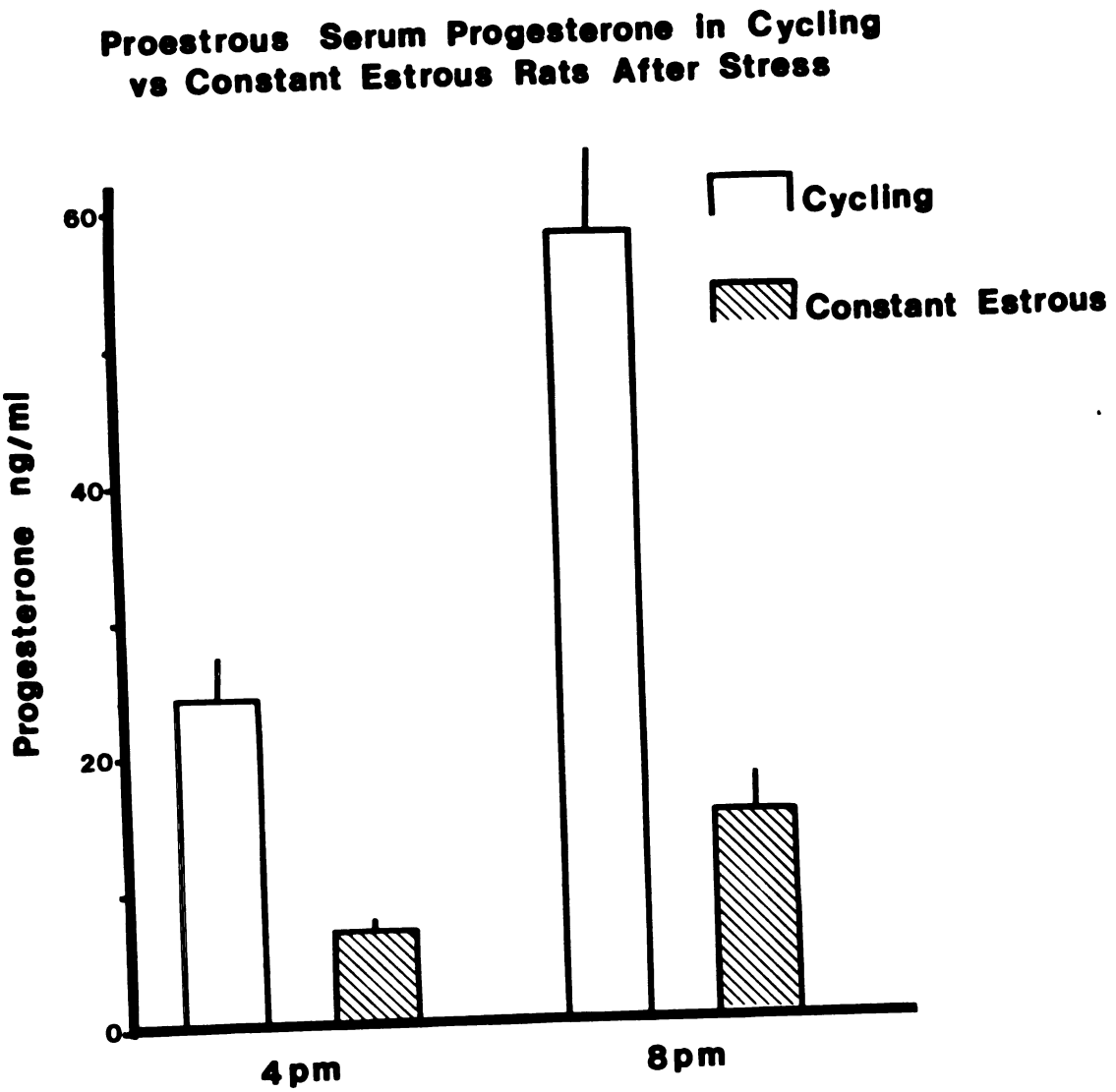


Figure 25. Proestrous Serum Progesterone in Cycling vs. Constant Estrous Rats After Stress. Blood samples were collected under light ether anesthesia at 1600 h and at 2000 h on the day of proestrus which occurred following the stress treatment in the constant estrous rats and on the day of proestrus in normally cycling female rats of similar age. Progesterone is expressed as group means (ng/ml serum) with indicated standard errors of the means.

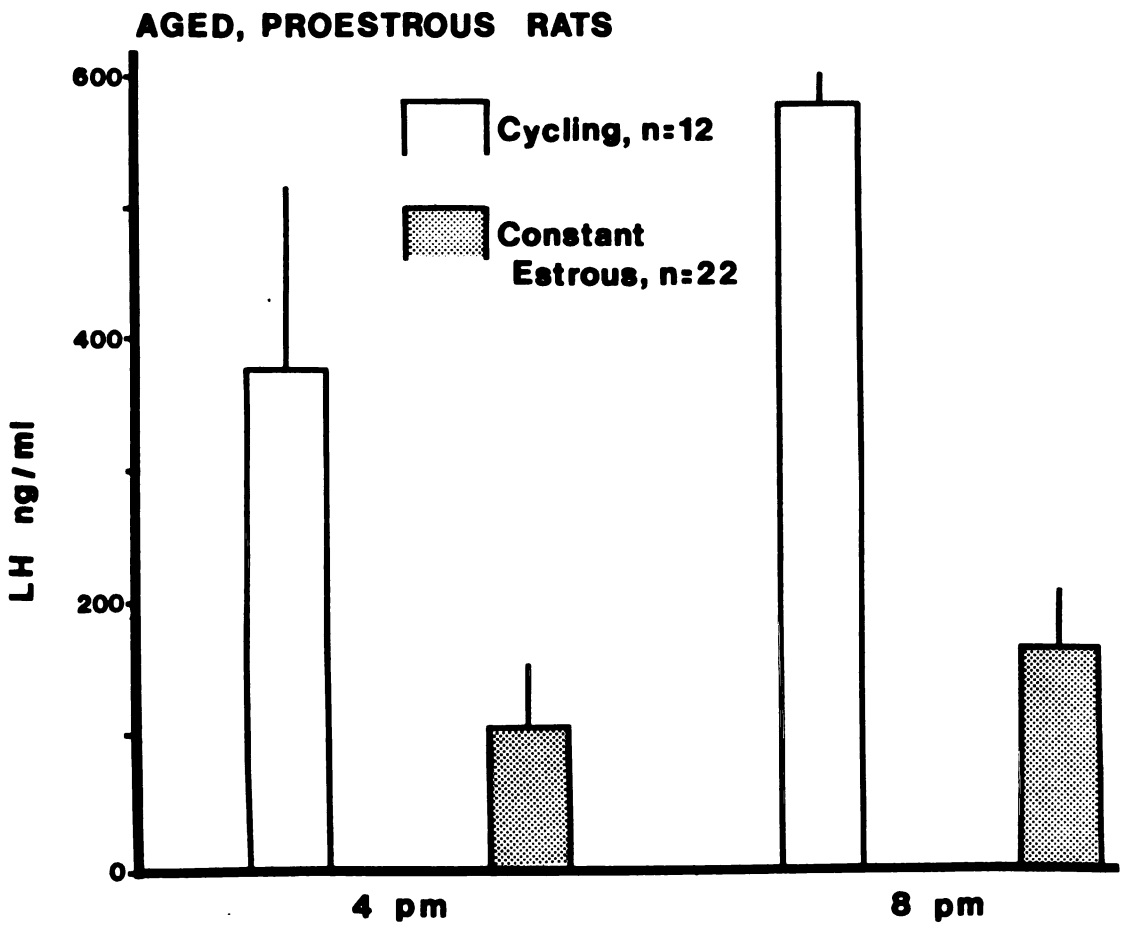


Figure 26. Proestrous Serum LH in Cycling vs. Constant Estrous Rats After Stress. Blood samples were collected under light ether anesthesia at 1600 h and at 2000 h on the day of proestrus which occurred following the stress treatment in the constant estrous rats and on the day of proestrus in normally cycling female rats of similar age. LH is expressed as group means (ng/ml serum) with indicated standard errors of the means.

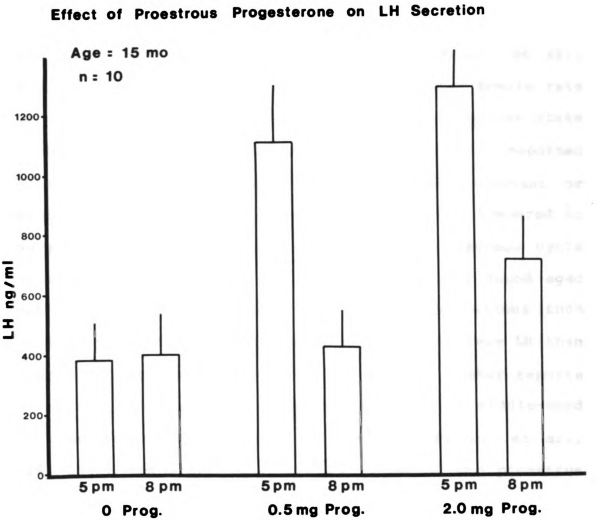


Figure 27. The Effect of Injection of 0, 0.5 or 2 mg. of Progesterone on Proestrous Serum LH in Aged Cycling Female Rats. Injection of progesterone was performed at 0800 h and blood samples were collected at 1700 h and 2000 h on the day of proestrus. LH is expressed as group means (ng/ml serum) with indicated standard errors of the means.

rat (Finch, 1978; Lehman, et al., 1978; Meites, et al., 1978), failure of adequate gonadotropin stimulation of follicular maturation and ovulation is believed to be involved with the age-related loss of normal ovarian cycles and reproduction function.

Although decreased LH has been consistently reported in aged male rats (Riegle and Meites, 1976; Bruni, et al., 1977; Riegle and Miller, 1978), data from aged female rats are more variable and are related to the reproductive state of the aged female. A report from our laboratory reported decreased serum LH in aged rats with pseudopregnant or persistent diestrous vaginal cytological states compared to young rats in the diestrous stage of their estrous cycle (Watkins, et al., 1975). On the other hand, we found aged constant estrous rats have higher LH concentrations than found in young diestrous or estrous groups but less LH than present during the proestrous hormone surge. Other reports have found unchanged LH concentrations in both middle-aged and aged female rats (Wilkes, et al., 1978; Lu, et al., 1979) and a temporary delay in the onset of the proestrus gonadotropin surge (Cooper, et al., 1980; Wise, 1982). Serum FSH concentrations have been reported to be increased in aging constant estrous and pseudopregnant rats (Clemens and Meites, 1971; Wilkes, et al., 1978; Lu, et al., 1979) and during the proestrous surge in 12 month old rats with regular estrous cycles (Wilkes, et al., 1979). These data

offer strong evidence that gonadotropin secretion is not uniformly depressed in aged female rats.

We have reported a smaller proestrous LH surge in cyclic 12 to 16 month old compared to three month old young rats (Section 2 D of this thesis). In that study, peak LH concentration measured in the aged group at 6 p.m. (310 ng/ml) was less than one-quarter that of the young rats. However, the ovulation data from the current experiment implies that the reduced proestrous LH surge in aging cycling rats is sufficient for follicular maturation and normal ovulation. Other important variables that could affect gonadotropin stimulation of the ovary include possible age differences in the biological activity of the pituitary hormones measured in peripheral blood samples and age-related decreases in gonadal responsiveness to gonadotropins. Age-related alterations in the biological activity of blood TSH and insulin have been reported (Klug, et al., 1978), however this has not been shown to be true of LH in rats (Parkening, et al., 1982). There are several reports of no change in LH binding to the Leydig cells of aged male rats (Steger, et al., 1979; Geisthovel, et al., 1981; Kaler and Neavens, 1981) and similar data on female rats indicates that there is also no significant difference in LH binding or aromatase activity in granulosa cells (Erickson, et al. 1979).

Our data support the concept of a gradual reduction in the magnitude of the proestrous LH surge in aging rats which is hypothesized to lead to the development of constant

estrous. Since the aged rat pituitary retains a high capacity for LH secretion following LHRH stimulation (Miller and Riegle, 1978; Meites, et al., 1978; Tang and Tang, 1981), it is less likely that an increasing inability to secrete LH, inherent in aging pituitary gonadotrophs, contribute significantly to this reduced LH secretion. These findings are supportive of the hypothesis that age effects on the neuroendocrine regulation of hypothalamic secretion of LHRH is the primary factor contributing to the loss of estrous cycles and fertility.

Available evidence concerning the regulation of the proestrous gonadotropin surge suggests that controlled increases in the secretion of both estradiol and progesterone during proestrus play prominent roles in this process. Increased estradiol secretion has been shown to augment pituitary responsiveness to LHRH (Vilchez-Martinez, et al., 1974) and progesterone has been one of multiple factors associated with reinitiation of estrous cycles in aged animals. In the previous section of this thesis, we reported smaller increases in the proestrous increase in serum progesterone in aging compared to young female rats. The origin of the proestrous increase in progesterone and its relationship to the proestrous gonadotropin surge has received considerable attention. Several studies have shown an adrenocortical role in the increased progesterone secretion at proestrous (Mann and Barraclough, 1973; Shaikh and Shaikh, 1975). The increase in serum progesterone occurs

before the gonadotropin surge (Shaikh and Shaikh, 1975). Progesterone has been shown to facilitate gonadotropin secretion and induce earlier mating behavior and ovulation in the rat (Neguin, et al., 1975) and to stimulate LH release in pentobarbital-blocked rats (Kobayaski, et al., 1973). It has also been shown that increased blood estradiol, presumably of ovarian origin, can increase the duration of proestrous adrenal progesterone secretion (Campbell, et al., 1977). These findings suggest that decreased estrogen secretion during proestrous or the recognized decreases in estrogen uptake and receptor numbers could be associated to the age-related reduction in the proestrous LH surge (Peng and Peng, 1973; Kanungo, et al., 1975). In addition, Campbell, et al., showed a stress-reduced increase in adrenal estradiol secretion in proestrous but not in metestrous cycling rats, suggesting that the proestrous hormone milieu can have specific effects on adrenocortical steroidogenesis. These experiments indicate that the decrease in serum LH during the proestrous hormone surge in stress-interrupted constant estrous rats could be related to the decrease in progesterone. This concept is supported by the increase in proestrous LH secretion we found in aged cycling rats injected with progesterone on the morning of proestrous.

On the other hand, adrenalectomized rats have normal estrous cycles and ovulate (Feder, et al., 1971) and interruption of the proestrous LH surge with pentobarbital

also decreases the increase in serum progesterone during proestrous, indicating that the ovulatory LH surge affects ovarian progesterone secretion during and after the release of gonadotropins (Everett, 1967). These reports suggest that reduced serum progesterone during proestrous in aging rats can contribute to the smaller LH release but indicate that the reduction in progesterone may not be the only or the primary factor involved in the age-related alteration in proestrous gonadotropin release.

The increase in progesterone in the post-stress blood sample presumably occurs due to stress-induced ACTH stimulation of the adrenal gland. The magnitude of this increase in progesterone suggests normal adrenocortical secretory response and implicates age-related alteration in gonadotropin stimulation of ovarian or adrenal progesterone secretion or altered ovarian responsiveness to gonadotropin as primary factors involved with the reduced proestrous progesterone secretion in the aged rat.

A wide variety of treatments have been reported to be capable of reinitiating estrous cycles in aging rats (Finch, 1978). In many of these studies evidence for the reinitiation of ovarian cycles is based on cytological characteristics of vaginal lavages rather than measured stimulation of gonadotropin release or by verified ovulation. The results from this study clearly indicate that these indirect criteria are inadequate. We found that although rats receiving stress treatments have apparently normal post-stress estrous

cycles, there is no evidence of normal luteal progesterone secretion, proestrous surges of LH and progesterone are sharply reduced, and a majority of animals resume their constant estrous state without ovulating or developing functional luteal tissue. It is presumed that several of the factors associated with the restoration of estrous cycles in aged rats, including progesterone, ACTH, epinephrine or various other stressors, could act similarly.

We found similar responses in mated constant estrous rats. Although average serum LH concentrations at 3 and 5 p.m. in the mated rats were increased (7 and 10 hours after initiation of copulatory behavior) over that in non-mated constant estrous rats, these LH concentrations are lower than average LH measured in similarly aged proestrous cycling rats and, together with the failure to recover eggs from the fallopian tube, suggest that the increased LH secretion induced by coital stimulation was insufficient to stimulate ovulation.

In the past few years, several laboratories have shown significant age-related alterations in hypothalamic neurotransmitter content and turnover in the aging rodent. These studies have shown decreased norepinephrine content and turnover in the hypothalamus (Miller, et al., 1976; Simpkins, et al., 1977), reduced hypothalamic and median eminence dopamine content and turnover (Finch, 1973; Miller, et al., 1976; Simpkins, et al., 1977; Riegle, et al., 1979) and increased hypothalamic serotonin turnover in aged

compared to young groups. It has been proposed that these neurotransmitters regulate LHRH secretion from the peptidergic hypothalamic neurons. Our current understanding of neurotransmitter content of LHRH release indicates that these agents may be involved in the age-related alterations in the reproductive control system.

In summary, these experiments indicate that the loss of estrous cycles in the aging rat involves multiple components. Although the stress interrupted constant estrous aging rat shows vaginal cytological changes which are typical of the normal rat estrous cycle, these rats show decreased proestrous progesterone and LH secretion and failure of normal ovulation and luteinization. Our preliminary studies suggest that the proestrous release of LH can be increased in the aging rat by progesterone administration. These findings suggest a neuroendocrine alteration with increasing age which reduces hypothalamic LHRH stimulation of pituitary gonadotropin secretion. The decrease in proestrous progesterone secretion, presumably of ovarian origin, is hypothesized to be one of the factors involved in the reduction of hypothalamic stimulation of pituitary gonadotropin secretion. Other neuroendocrine factors including age-related decreases in hypothalamic and median eminence catecholamine function are believed to contribute to the loss of estrous cycles and fertility.

SUMMARY AND CONCLUSIONS

The Reproductive Control System of the Aging Male Rat

The data reported here indicates major alterations in the endocrine reproductive control system of the aging male rat. Specifically, these studies:

1. affirm our previous experiments showing a major reduction in serum testosterone in aging male Long-Evans rats.
2. indicate that the Leydig cells of the aged male rat remain responsive to gonadotropin stimulation.
3. show that chronic Leydig cell stimulation by HCG administration stimulates similar increases in serum testosterone in young and aged male rats and restores testicular responsiveness to acute gonadotropin stimulation.
4. suggest similar hypothalamic content of biologically active LH-releasing hormone activity in young and aged, intact and gonadectomized male rats.
5. indicate the presence of a diurnal pattern of serum testosterone concentrations in young male rats which was not present in aged male groups.
6. imply that the aged male rat has small episodical surges of pituitary LH and testicular testosterone

secretion which result in reduced average serum LH and testosterone concentrations and less variability in individual blood sample content of LH and testosterone in older male rats.

7. show that although the aged rat pituitary and testes respond to LHRH stimulation, the magnitude of the increase in LH and testosterone is reduced in the older animals.

The aged male rat is recognized to have smaller testes, reduced rates of spermatogenesis, reduced male accessory gland size, decreased sexual libido and decreased fertility. In addition, the aged male rat shows reduced skeletal muscle mass, less physical vigor, reduced aggression and progressive thinning of his hair coat and skin. Although some of the aforementioned changes in structure and function of the aged male rat are undoubtedly associated with specific age effects on these tissues, all of these alterations are consistent with the biological effects expected to accompany the decrease in testosterone concentrations reported in this thesis. This progressive reduction in the biological availability and activity of testosterone with increasing age undoubtedly contributes to the deterioration of function in a variety of physiological systems with age.

Although our results clearly indicate reduced testicular testosterone secretion with increasing age in the Long-Evans rat, it is also apparent that the reduction in serum testosterone concentration occurs secondarily to a chronic

reduction in pituitary LH secretion rather than an inability of testicular Leydig cell to respond to gonadotropin stimulation. Our experiments confirm the hypothesis that the pituitary and testis are capable of sustaining much higher levels of endocrine function than that which normally occurs in the aging male rat.

In addition, these experiments suggest that hypothalamic peptidergic neuronal content of LH-releasing activity is not reduced with increased age or increased rate of peptide hormone synthesis and secretion which occurs following gonadectomy. These data support our previous hypothesis of significant age-related alterations in the responsiveness of hypothalamic neurons to control input in the male rat. A large number of recent experiments have shown that significant alteration in hypothalamic and median eminence content and turnover of neurotransmitters, particularly of norepinephrine and dopamine, can profoundly affect gonadotropin secretion. These findings have been interpreted to indicate that age alterations in neurotransmitter function in specific regions of the hypothalamus and median eminence could contribute to the alteration in hypothalamic stimulation of pituitary secretion which occurs with increased age. This hypothesis has been strengthened by the results of our experiments and the work of several other laboratories which show reduced hypothalamic and median eminence content and turnover of dopamine and norepinephrine with increasing age in the male rat. Although the association between changes

in neurotransmitters and hypothalamic endocrine function remains an attractive hypothesis, it needs to be emphasized that this relationship should not yet be accepted as dogma. A large amount of additional experimentation must be completed to establish a quantitative relationship between these two variables.

There are similarities in the effect of age on reproductive control systems in the male rat and men which suggest that the rat can be a useful model for studing many aspects of the effects of age on the reproductive control mechanism in men. Both species commonly encounter decreased peripheral testosterone activities with increased age. Although, there are differences in endocrine reproductive control system responsiveness to the decreased testosterone between rats and men, current data indicates that both species are capable of sustaining sufficient pituitary gonadotropin and testicular secretions to restore testosterone concentrations in the aged to that found in younger males. These data indicate significant age-related alterations in neuroendocrine control mechanisms related to gonadal control. As indicated above, current hypotheses relate these age-related changes in reproductive control systems to alterations in neuronal fuction, particularly to changes involving catecholaminergic neuronal systems. The similarities between control system components between species suggest that a better understanding of age effects on basic neuroendocrine control of hypothalamic secretions

which regulate pituitary function in the rat should contribute to our understanding of fundamental age effects on other neuronal systems in humans.

The Reproductive Control System of the Aging Female Rat

These experiments also indicate significant alterations in the endocrine regulatory systems pertaining to reproduction in the aging female rat. Specifically, the results of these studies:

1. affirm decreased fertility of female rats at nine to ten months of age.
2. confirm evidence that the loss of fertility occurs at an earlier age than the loss of normal ovarian cyclicity in the aging female rat.
3. indicate that total litter loss with increasing age is more characteristic of the decline in fertility than decreased litter size.
4. suggest that hypothalamic content of LH-releasing activity is not different in young and aged intact and gonadectomized female rats.
5. show that serial LHRH injections can increase serum LH in aged female rats to concentrations similar to that measured in young rats with normal estrous cycles.
6. suggest age-related differences in the metabolism and excretion of LH from the blood of aged rats.
7. establish the concept of reduced serum concentrations of progesterone and LH during the proestrous

hormone surge in aging female rats.

8. show that reduced serum progesterone concentrations are also characteristic of aged compared to young rats during estrous and diestrous stages of their estrous cycles.
9. affirm low serum progesterone concentrations in aging constant estrous rats.
10. indicate that vaginal cytology alone is not an adequate index of progesterone secretion in aged rats cytologically classified as constant diestrous or repetitive pseudopregnant.
11. establish that average early pregnancy serum progesterone concentrations are similar in aging female rats with normal estrous cycles compared to aged rats with constant estrous vaginal cytology which were made pseudopregnant by coital stimulation.
12. show similar serum progesterone concentrations during early pregnancy among aging rats which produced normal litters compared to rats which did not produce litters.
13. show that aged pregnant and pseudopregnant rats which did not produce normal litters have reduced serum progesterone concentrations by post-coital day 16, indicating failure of early pregnancy or suggesting age-related alterations in uterine-placenta factors affecting luteal tissue secretion.

14. indicate increased gestation length in aging female rats associated with longer maintenance of increased serum progesterone concentrations in aged compared to young groups.
15. affirm normal ovulation rates in 10 to 16 month old female rats with regular estrous cycles.
16. suggest that stress can interrupt the constant estrous state of aging rats by stimulating progesterone secretion, presumably of adrenal origin.
17. indicate that constant estrous rats whose persistent vaginal cornification is interrupted by stress do not have normal diestrous luteal progesterone concentrations as similarly aged, regularly cycling rats during diestrus.
18. show that constant estrous rats whose persistent vaginal cornification is interrupted by stress have smaller increases in serum LH and progesterone during the post-stress proestrous hormone surge compared to similar aged regularly cycling rats at proestrus.
19. establish the return to the constant estrous state in the majority of untreated stress-interrupted constant estrous aging rats.
20. suggest that failure of progesterone secretion during proestrus in aging rats contributes to the development of the constant estrous reproductive state.

The results of these experiments indicate multifaceted components contribute to the loss of fertility in the aging female rat. The early loss of fertility beginning at about nine to ten months of age in rats with regular estrous cycles seems independent of known changes in pituitary or neuroendocrine control. Although we showed that 10 to 16 month old rats with regular ovarian cycles have a somehwat smaller proestrous LH surge than young rats with regular estrous cycles, they secrete sufficient gonadotropin to stimulate normal ovulation, luteinization, and luteal progesterone secretion. These data imply increased postovulatory loss of reproduction in these rats.

Several experiments from a variety of laboratories have shown that eggs collected from oviducts of aged doner rats can develop normally in young recipient reproductive tracts. These findings indicate that age-related abnormalities in ova or age changes in their ability to be fertilized do not contribute to the loss of fertility. In addition, aged female rat corpora lutea maintain serum progesterone concentrations during early pregnancy which were similar to that measured in normal young rats. These data imply normal control of corpora lutea secretion during early pregnancy in the aging rat and suggest age-related alterations in uterine function contribute to the failure to reproduce.

There are also multiple possibilities for age-related alterations in uterine function. Most experiments suggest some alteration in embryo-placenta development or function

during the first half of gestation in aged animals since normal corpora lutea regulation is lost during the last half of pregnancy, when corpora lutea stimulation is dependent on uterine stimulatory factors. Implantation or placentation mechanisms could be affected in the aging rat. There could also be age-related changes in tissue responsiveness to hormones. In addition, there may be differences in decidua formation or uterine blood flow which are necessary for maintaining normal pregnancy.

My experiments also support a hypothesis of multiple factors affecting the changes in the estrous cycle in the aging female rat. We found that the magnitude of the LH surge is progressively decreased with increased age. It is hypothesized that this reduction in proestrous LH secretion ultimately results in failure of follicular maturation, ovulation and follicle luteinization, and to the development of increased estrous cycle irregularities which are believed to progressively lead to the development of the constant estrous reproductive state. The results of my studies indicate that the decrease in the magnitude of the proestrous LH surge may involve two factors. First there may be reduced LH-releasing hormone secretion from the hypothalamus and secondly, reduced adrenal or ovarian progesterone secretion may result in either decreased hypothalamic-pituitary unit responsiveness to proestrous controlling input or decreased pituitary responsiveness to LHRH.

Our experiments and studies by others indicate that stress-induced increases in progesterone secretion or direct progesterone injections can restore regular ovarian cycles based on cytological changes in vaginal lavages, and in some instances this increase in progesterone is sufficient to induce ovulation and luteinization in aging rats with constant estrous alterations in their estrous cycles. This relationship is strengthened by our finding of increased LH secretion during the proestrous hormone surge in aged rats following progesterone injection.

Although hypothalamic LH-releasing hormone content is not decreased with increasing age in the female rat, the changes in reproductive function which accompany aging are consistent with an alternate hypothesis of decreased LH-releasing hormone secretion. In support of this hypothesis, we showed that multiple LHRH injections could increase serum LH in aged rats to concentrations which were similar to those found following similar hormone treatment in young groups. The hypothesis of decreased LH-releasing hormone secretion is commonly associated with evidence for the alteration of hypothalamic neurotransmitter function in aged rats. The fundamental premise of this hypothesis is that age-associated deterioration of hypothalamic catecholaminergic function results in impairment of peptidergic neuronal secretion of LH-releasing hormone which in turn leads to reduced pituitary gonadotropin secretion and failure of ovarian control mechanisms. However, there are obvious

problems associated with this interpretation. Additional experiments involving study of the effect of restoration of catecholamine function in aging animals, the effects of premature reduction in specific neurotransmitter function, and the interaction of catecholamine regulation of LHRH secretion with peripheral steroid hormone effects on LHRH secretion and pituitary responsiveness are necessary to understand these relationships.

In summary, there are several factors which appear to influence the usefulness of the rat as a model of aging effects on the reproductive control systems of women. The most obvious difference between the species is the lack of a clear menopausal state in the aging rat. Although the aging female rat has a modest increase in blood FSH concentrations, there is no evidence for an increase in LH secretion with age-related decreases in ovarian gametogenic and steroidogenic function. On the other hand, there is ample evidence indicating increased post-ovulatory loss of fertility in the aging rat and in premenopausal women. These findings suggest the mid-aged rat could be a very useful model to study premenopausal changes in reproductive function in women. In addition, both the rat and human show alterations in central nervous system function with increased age. Better understanding of fundamental age effects on neuronal function in the rat should be of major significance for our understanding of and the development of effective prevention or treatment schemes related to the

deterioration of central nervous system function which occurs in aging humans.

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