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Age-Related Decreases in Gonadal Hormones in Long–Evans Rats

Relationship to Rise in Arterial Pressure

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Sex steroids modify sexual behavior and autonomic function. The gradual decline in circulating levels is correlated with several diseases in humans and animals. However, little is known about age-related changes that occur in the availability of these steroids. In the current studies, we characterized age-related changes in (1) circulating levels of estradiol (females) or testosterone (males), (2) reproductive function (estrous cyclicity in females; erectile reflexes in males), and (3) blood pressure in a longitudinal study. In a separate study, we characterized the estrous cyclicity of sex steroids in female, and diurnal periodicity in male, Long–Evans rats. Young females exhibit regular estrous cycles, transition to irregular cycles at about 10 mo of age, then to cycles characterized by extended periods of estrous, and to persistent estrous. Despite the loss of cyclicity, circulating 17β-estradiol in middle-aged females was maintained at levels similar to those in young females during diestrous. Males display an age-related decline in testosterone, circulating levels decrease by about 25% during the period from 8 to 16 mo of age. Also, during any 24 h period testosterone levels in young males vary from a peak of about 3.5 ng/mL (late light period) to a trough of 0.7 ng/mL (early dark period). In middle-aged males the rhythm amplitude is greatly blunted (1.4 to 0.7 ng/mL). Males exhibit age-related decrements in erectile reflexes. In females and males systolic blood pressure is relatively stable until 8 mo of age, but significantly increases during the next 5 mo of age. In males, the increase in arterial pressure is gradual from about 8 mo of age. Young females have lower blood pressures than age-matched males, but by 14 mo of age this sex-related advantage is lost. Thus, by middle age, male and female rats are exposed to less gonadal hormone/altered patterns of availability, exhibit decrements in reproductive function, and display an increase in systolic blood pressure.

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Introduction

Hypertension is one of the most common medical syndromes in developed countries *(1)*. With increasing age humans experience an increased incidence of hypertension *(2,3)* in association with reductions in sexual function *(4)*. Young women typically have lower arterial blood pressure than age-matched men. This advantage is no longer evident in the decades after menopause *(2,3)*. Sex steroids modify sexual behavior and autonomic function in animals as well as in humans, and the age-related decline in the circulating levels is correlated with several age-related diseases, such as hypertension and sexual dysfunction, in both humans and animals *(5–8)*. Despite these observations, little is known about age-related changes that occur in the availability of these steroids. There are limited data available on longitudinal changes in blood pressure in aging male and female rats, and none on longitudinal changes in sex steroids in relationship to blood pressure and reproductive function. Therefore, in the current studies, we characterized the age-related changes in circulating levels of estradiol (females) or testosterone (males), reproductive function (estrous cyclicity in females, erectile reflexes in males), and blood pressure in a longitudinal study. In a separate cross-sectional study, we characterized the estrous cyclicity of sex steroids in young and middle-aged female, and the diurnal periodicity in young and middle-aged male, Long–Evans rats. The data indicate that, by middle age, male and female rats are exposed to less gonadal hormone and altered patterns of availability, and display increased systolic blood pressures in conjunction with decrements in reproductive function.

Results

Longitudinal Study

Survival

Of the 60 rats (30 male and 30 female) that began the study, three males and two females died during the course of the study (3–16 mo of age).

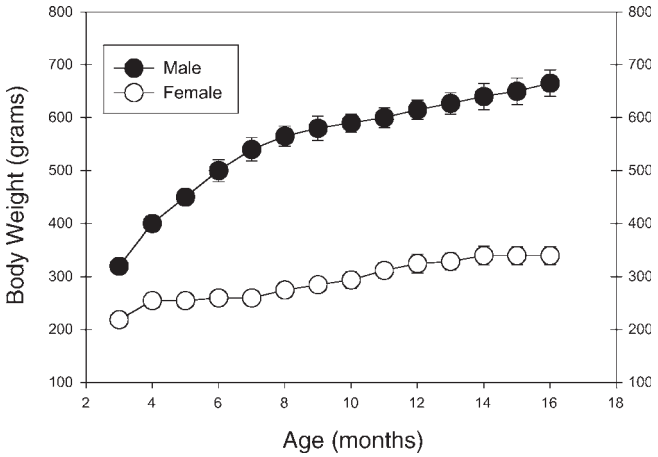

Fig. 1. Effects of aging on body weight in male and female Long–Evans rats. Male ($n = 27$) and female ($n = 28$) rats gained weight over the course of the study (repeated measures ANOVA $p < 0.001$ for males and females). At every time point, males weighed more than females ($p < 0.001$). Data are presented as mean ± SEM.

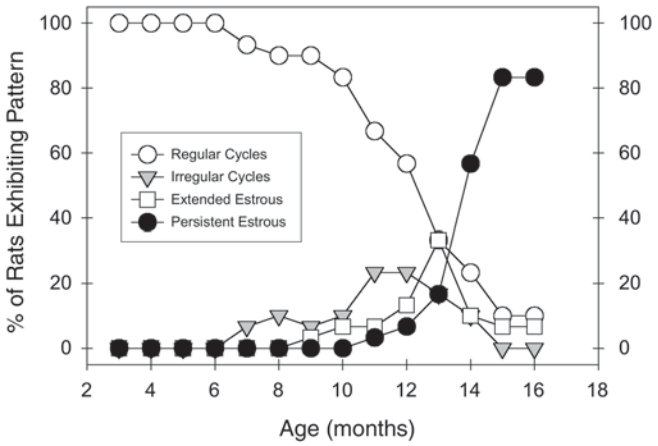

Fig. 2. Age-related changes in estrous cyclicity in female Long–Evans rats. Females were selected for regular 4-d estrous cycles ($n = 28$) and subjected to daily vaginal smears on 14 consecutive days each month. With aging the incidence of regular cycles decreased and the incidence of a state of persistent estrous. Data are presented as the percentage of females exhibiting regular cycles, irregular cycles, cycles with periods of extended estrous, and persistent estrous increased (see text for further details).

Age-Related Changes in Body Weight (Fig. 1)

Female Long–Evans rats gained weight slowly over the period from 3 to approx 13 mo of age, remaining almost constant during the last 3 mo of the study period. Female body weight increased from 219 ± 5 g at 3 mo of age to 340 ± 17 g at 16 mo of age. Male Long–Evans rats consistently weighed more than females. From an initial weight (3 mo of age) of 320 ± 12 g, males rapidly gained weight over the next 5 mo (565 ± 17 g at 8 mo of age). Subsequently, males gained weight more slowly over the remaining 8 mo of study (665 ± 25 g).

Age-Related Changes in Estrous Cyclicity (Fig. 2)

As these female Long–Evans rats aged, reproductive function changed. These virgin Long–Evans females were selected for regular 4-d cycles, and this pattern continued until 7 mo of age. After 7 mo of age, the number of females exhibiting regular estrous cycles (as evidenced by vaginal cytology) steadily decreased. As the incidence of regular cycles decreased, the incidence of irregular cycles and cycles with periods of extended estrous increased. After 10 mo of age, the incidence of persistent vaginal cornification increased, with this being the predominant pattern at 15–16 mo of age. By 15 mo of age approx 80% of the females exhibited vaginal smears indicating a state of persistent vaginal estrous, with no regular estrous cycles seen.

Age-Related Changes in Erectile Reflexes (Fig. 3)

As male Long–Evans rats aged, erectile reflexes remained relatively consistent from 3 to 9 mo of age and then declined. By 16 mo of age they were exhibiting approx 50% of the number of erections per test. Although there was a small decline in the latency to the first erection at 6 mo (8.7 ± 0.9 min) of age relative to 3 mo of age (11.2 ± 1.2 min), the latency to the first erection gradually increased thereafter and is equivalent to 3 mo values at 16 mo of age (11.6 ± 1.7 min). There was no decrement in the number of rats exhibiting erections at any time point tested (data not shown). There were no age-related differences in the number of cups or flips (data not shown).

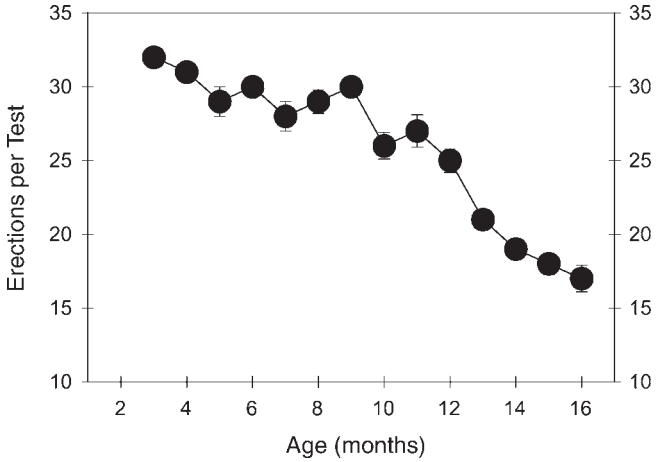

Fig. 3. Age-related decreases in erectile function. In *ex copula* tests of erectile reflexes male Long–Evans rats ($n = 27$) exhibit an age-related decline in the number of erections (repeated measures ANOVA $p < 0.001$). Erectile reflex tests were administered twice during one week of each month, and data are presented as mean ± SEM of the second test each month.

Age-Related Changes in Systolic Blood Pressure (Fig. 4)

Systolic blood pressure was relatively stable until 8 mo of age, with males exhibiting higher pressures than females.

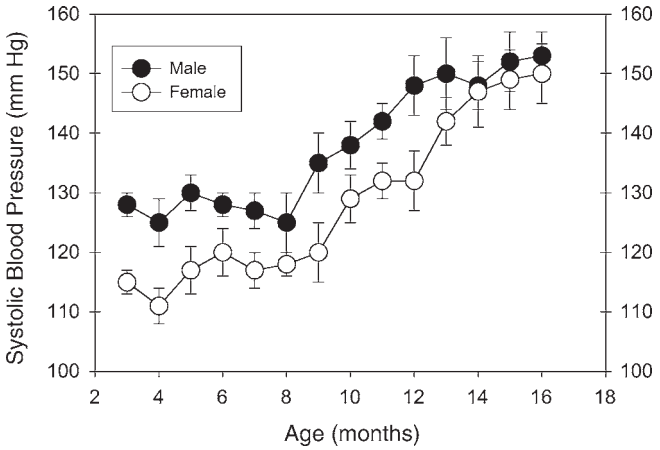

Fig. 4. Age-related increases in blood pressure in male and female Long–Evans rats. Systolic blood pressure was measured indirectly one week per month. Systolic blood pressure was relatively stable until 8 mo of age, with males exhibiting higher pressures than females. In males and females, arterial pressure increased from 9 to 13 mo of age. From 14 to 16 mo of age (end of study) arterial pressure in females increased to be equivalent to that of age-matched males.

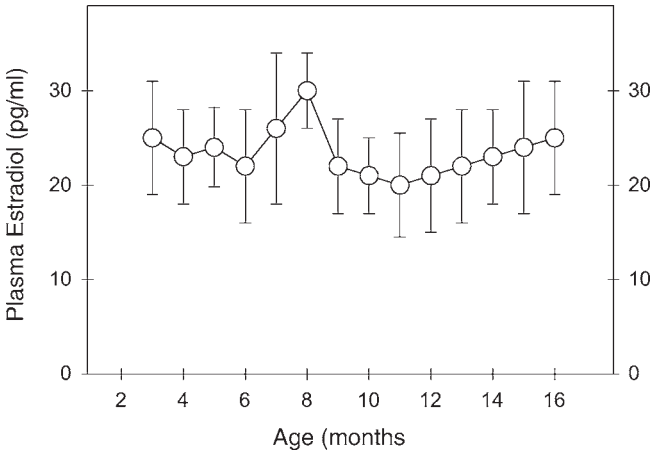

Fig. 5. Plasma levels of estradiol in middle-aged females are similar to those in young animals on diestrous. Monthly blood samples were obtained on diestrous in rats exhibiting estrous cycles, or during persistent estrous in older rats. Plasma estradiol levels were determined by radioimmunoassay.

In males and females, arterial pressure increased from 9 to 13 mo of age. From 14 to 16 mo of age (end of study) arterial pressure in females increased to be equivalent to that of age-matched males. In females the incidence of hypertension (systolic blood pressure ≥ 140 mm Hg) was markedly lower when estrous cycles were normal when compared to periods of persistent vaginal estrous ($p < 0.0001$).

Age-Related Changes in Circulating Sex Hormones (Figs. 5 and 6)

Plasma levels of estradiol in rats on diestrous d 1 are relatively constant at approx 25 pg/mL. Despite the loss of estrous cyclicity (see above), circulating levels of estradiol in rats exhibiting persistent vaginal estrus are similar to those in young rats on diestrous d 1, at least until 16 mo of age (Fig. 5). Plasma testosterone declines slowly with age in male rats. In male rats, testosterone levels were relatively constant until 8 mo of age. Afterward, testosterone levels decreased by about half from 8 to 16 mo of age (Fig. 6).

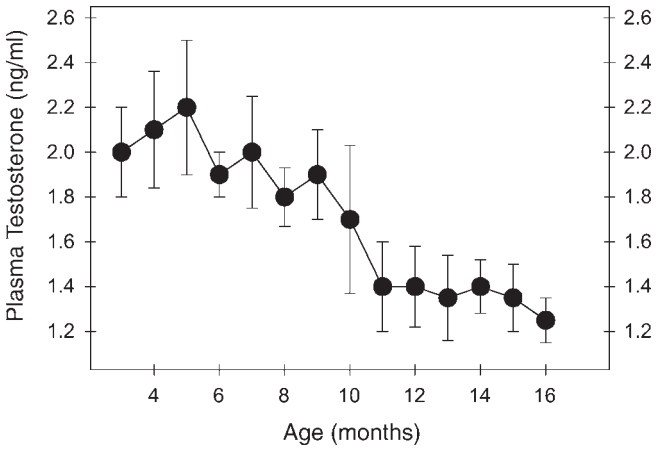

Fig. 6. Age-related decline in plasma testosterone in male Long–Evans rats. Monthly blood samples were obtained in the late part of the light cycle. Plasma testosterone declines slowly with age (repeated measures ANOVA $p < 0.005$; $n = 27$).

Cross-Sectional Study

Cyclic Changes in Estradiol in Young, but not in Middle-Aged, Females

Young females exhibited cyclic increases in estradiol, whereas persistent estrus females do not (data not shown). Estradiol levels on diestrous d 1 and 2 are low (20–30 pg/mL), rise to a peak on proestrous (105–120 pg/mL), and drop again on estrous (20–25 pg/mL; all vaginal smears were performed and blood samples were obtained 3–4 h prior to lights off). In rats that exhibited a persistent estrous pattern of vaginal smears there was no discernable daily fluctuation in estradiol levels.

Circadian Periodicity of Testosterone Is Attenuated in Middle-Aged Males (Fig. 7)

Young male rats showed a marked circadian periodicity in testosterone levels, with a peak level (3.37 ± 0.3 ng/mL) achieved about 2 h prior to the onset of darkness, and a trough level (0.59 ± 0.24 ng/mL) achieved about 2 h after the onset of darkness. In contrast, middle-aged males showed only very slight diurnal variations (1.47 ± 0.19 to 0.65 ± 0.19 ng/mL) in testosterone.

Discussion

To our knowledge, these data are unique in that we utilized an extensive longitudinal approach to determine how changing sex hormone levels are related to increments in

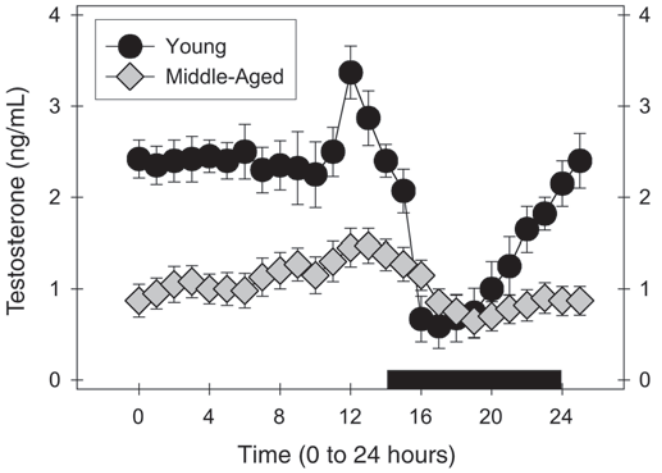

Fig. 7. Circadian periodicity of testosterone is attenuated in middle-aged male rats. Lights were on from "0" to "14" h and off from "14" to "24" h (indicated by dark bar on the horizontal axis). Young male rats exhibited a marked circadian periodicity in circulating testosterone levels, with a peak achieved about 2 h prior to the onset of darkness, and a trough level achieved about 2 h after the onset of darkness. In contrast, middle-aged males showed only very slight diurnal variations. Rats were decapitated and trunk blood collected hourly (n = 5–7 rats per time point).

blood pressure. In agreement with others (e.g., refs. *9–14*) we report that, with aging, the incidence of regular estrous cycles decreases in Long–Evans female rats (Fig. 2). Thus, although female rats do not undergo menopause *per se*, they do transition from regular estrous cycles to irregular cycles, to periods of extended and persistent estrus. Earlier reports indicated that they later transition from persistent estrus to irregular cycles (approx 20 mo of age) and, finally, to anestrus (approx 22 mo of age). Females in persistent estrus have plasma levels of estradiol that are similar to those seen in young cycling rats on diestrous (Fig. 5). Many physiological parameters exhibit circadian periodicities in rats as in man. In contrast to humans, in rats activity and ingestive behaviors are largely restricted to the dark period. In rats, blood pressure is typically higher during the dark period. Several other groups have investigated the effects of aging on blood pressure in normotensive rat strains with ambivalent results *(15,16)*. Sei et al. *(15)* compared 3- and 24-mo-old male Wistar rats on a 12:12 light:dark cycle and reported no major differences in circadian patterns of blood pressure, heart rate, or body temperature although circadian patterns of activity were attenuated in the old rats. In contrast, Zhang and Sannajust *(16)* compared 6- and 24-mo-old male Wistar rats on a 12:12 light:dark cycle and reported that the old rats showed reduced differences between diurnal and nocturnal blood pressures. These studies used relatively small numbers of animals (seven or eight per age group) and were cross-sectional in design. We report that systolic blood pressure measured during the late part of the light phase (3–4 h prior to lights off) was relatively stable until 8 mo of age, with males exhibiting higher pressures than females. Subsequently, in males and females, arterial pressure increased from 9 to 13 mo of age. Finally, from 14 to 16 mo of age (end of study) arterial pressure in females increased to be equivalent to that of age-matched males (Fig. 4). Our animals were maintained on a long-day schedule (14L:10D). The absence of changing light cycles may have contributed to our results. It is interesting to note that our females appear to have shifted to irregular cycles and persistent vaginal estrus at an earlier age than reported by others whose rats were maintained in 12L:12D conditions *(9–12)*.

There are distinct male–female differences in hemodynamics that putatively depend on circulating levels of gonadal steroids. In general, females react to nonsexual stimuli with less distinct increments in blood pressure than do males *(14)*. Relative to females, males are thought to react not only more distinctly, but also more frequently with increases in blood pressure in response to "stress" than females *(17)*. In a study involving 34 normotensive women with normal menstrual cycles and studied over the course of four cycles, von Eiff and Piekarski *(18)* reported that there was no distinct difference in average values from pre- vs post-ovulatory phases, that systolic and diastolic blood pressure at rest decreased in conjunction with increasing blood levels of estrogen, and that the rise in systolic pressure under stress was decreased with increasing estrogen levels. A more recent study in young female rats using continuous telemetric monitoring of blood pressure *(19)* reported daily and estrous cycle variations in activity, blood pressure, and heart rate. Arterial blood pressure was lowest during diestrous d 2, when light–dark differences were least evident *(18)*.

A recent report *(20)* used hypertensive female rats (SHR) that were ovariectomized or left intact at 8 mo of age and were subsequently monitored for 10 mo. These animals were compared with young females (4 or 8 mo of age) and middle-aged males (18 mo). Estradiol levels were decreased in the 18-mo-old females to levels not different from young females in proestrous or from old males *(19)*. These data are similar to ours, except that we report that estradiol levels in middle-aged Long–Evans are not different from young rats on diestrous, and that peak levels of estradiol are seen on proestrous. Furthermore, Fortepiani et al. *(20)* reported that arterial pressure increased progressively with age (8 to 18 mo of age) in intact female SHR, but not in ovariectomized or in male rats, and that the gender difference in hypertension disappeared by 18 mo of age. In male SHR, the age-related increase in blood pressure occurs peripubertally with little further age-associated changes (Clark, unpublished data). Our current data indicate that in "normotensive" females there is a similar age-related increase in blood pressure. Thus, in Long–Evans rats, as in humans, blood pressure increases with age. In young rats, females have lower

blood pressure than age-matched males, but at 14 mo of age there are no differences between the sexes (Fig. 4).

Norepinephrine (NE) is an important neurotransmitter in the regulation of hypothalamic–pituitary–gonadal function. One brain area that we have studied extensively is the anterior hypothalamic nucleus (AHN), which exerts a tonic sympathoinhibition *(21)*. Several stimuli that augment central catecholamine neuronal activity have been reported to reinitiate estrous cycles in old constant estrous rats which suggests that catecholamine neuronal function is impaired with advanced age. In the preoptic–anterior hypothalamic region (which includes the AHN) NE content was reduced in old (25–26 mo constant estrous) relative to young (3-mo-old normally cycling) female Long–Evans rats *(22)*. Additional studies examined the effects of age on NE levels and turnover rates in previously normally cycling young (3–4 mo old) and middle-aged (10 mo old) and constant estrus old (20–22 mo old) Long–Evans rats 2 wk after ovariectomy *(23)*. Steady-state NE concentrations were decreased in old vs young rats in the the preoptic–anterior hypothalamic region, the medial forebrain bundle, and other areas *(23)*. The rate constant of NE loss progressively decreased with increasing age only in the preoptic–anterior hypothalamic region and was unchanged or augmented in other regions. Turnover rate of NE was decreased in old animals *(24)*. The observed age-related alterations in CA turnover may contribute to impaired LH response and the persistent hyperprolactinemia in old constant estrus rats. In males, the steady-state concentration of NE in the hypothalamus, and the hypothalamic NE depletion rate, were significantly lower in old (21 mo) vs young (3 mo) animals, suggesting that a decrease in catecholamine turnover occurred in the hypothalamus of the old male rats *(24)*. They suggested that these changes may be related to the decrease in release of gonadotropins and the increase in release of prolactin observed in these old male rats *(24)*.

Our previous studies demonstrate that in intact, 3-mo-old, female SHR, chronic dietary NaCl excess causes a modest rise in arterial pressure (approx 10 mm Hg). Conversely, in chronically estrogen-depleted, young SHR the high NaCl diet elicits a very large arterial pressure increase (> 40 mm Hg) *(25)*. More recently, SHR were ovariectomized at 10 mo of age and placed on a phytoestrogen-free diet, which contained either basal or high NaCI. Each rat was then implanted with a Silastic tube containing 17β-estradiol or vehicle. Three months later arterial pressure and AHN norepinephrine metabolite (MOPEG) release was measured. On the basal NaCI diet, estrogen depleted rats exhibited increased arterial pressure (12 mm Hg) and decreased AHN MOPEG (by 20%) *(26)*. Both effects were reversed by estrogen treatment. In all groups, the high NaCI diet increased arterial pressure by over 35 mm Hg and reduced AHN MOPEG by > 60%. In the rats on the high NaCI diet, chronic estrogen depletion (compared to intact controls) modestly increased the NaCI-sensitive hypertension and AHN norepinephrine decrease, and estradiol replacement prevented these changes. Across all groups, there was a significant inverse correlation between arterial pressure and AHN MOPEG *(26)*. These data suggest that both dietary NaCI excess and estrogen depletion raise arterial pressure in middle-aged female SHR by decreasing hypothalamic norepinephrine. We suggest that aged-associated decrements in AHN NE contribute, at least in part, to the age-related increases in arterial pressure, as well as to the age-related decreases in reproductive function.

In contrast to the accepted effects of estrogen, androgens are generally believed to be hypertensinogenic. Thus, treatment of female rats (uninephrectomized and given 1% sodium chloride as the sole fluid source) with androgens—methylandrostenediol *(27)*, methyltestosterone *(28)*, or testosterone *(29)*—induces hypertension. Furthermore, prepubertal castration of male spontaneously hypertensive rats is associated with an attenuated development of hypertension (e.g., ref. *30*). But, aging is associated with increased blood pressure in males as well as females. We suggest that the increased blood pressure seen in aging males is secondary to the loss of circadian periodicity. However, it is also possible that with the reduced available androgen, the conversion of testosterone to estradiol in the aging male hypothalamus is reduced. Thus, the decline in the protective effects of estrogens may contribute to age-related increases in blood pressure in males.

We previously conducted a longitudinal study in male Long–Evans rats from 3 through 27 mo of age, with blood samples obtained for hormonal determinations *(31)*. At 23 mo of age, cross-sectional studies were conducted comparing results to those obtained in 5-mo-old males. Changes in parameters of mating behavior became evident in early middle-age (by 11 mo). Small decrements in circulating testosterone levels (1–3 h prior to the light:dark transition) were also evident at this age. A decline in LH levels was not evident until 19 mo of age. Correlational analyses revealed small ($r \leq 0.29$) but significant negative correlations between testosterone levels and parameters of mating behavior with age, but plasma testosterone levels were not predictive of behavioral performance. At 28 mo of age, only testosterone levels showed an effect of age; prolactin, estradiol, and LH levels were not different from those seen in 5-mo-old males *(31)*. Chambers and Phoenix *(32)* reported on the effects of castration plus testosterone replacement in middle-aged (10 mo) and old (25 mo) male rats. In middle-aged males testosterone was administered as either continuous or periodic testosterone replacement; continuous testosterone was associated with an increased interval between the initial mount and intromission (increased latency to intromission). Old males were unaffected by testosterone. They concluded that testosterone does not prevent or attenuate the rate of decline in sexual behavior, and that the degree of decline in sexual

performance does not depend on whether testosterone is continuously present *(32)*. Phoenix and Chambers *(33)* later reported that, in rhesus macaques, an age-related decrease in sexual activity occurs without a decline in testosterone and that levels of sexual activity were not increased by administration of testosterone to older males. Hsu et al. *(34)* reported that when serum testosterone levels were maintained at 0.78–0.87 ng/mL for 6 mo (from 4 mo of age) sexual activity was equivalent to intact males, except for a decrease in the number of males ejaculating, and a decreased total mount frequency at 10 mo of age. Also, long-term elevation of testosterone levels attenuated further decrements in intromission frequency in spite of an age-related decline in copulatory activity. Roselli et al. *(35)*, using F344 rats, documented age-related deficits in estrogen receptors in the medial nucleus of the amygdala in conjunction with lower levels of sexual behavior, testosterone, and LH. They suggested that the capacity of neural tissue to bind estrogen (presumably derived from plasma testosterone) may be a limiting factor in determining androgen responsiveness in aging males *(35)*.

In agreement with our earlier longitudinal study (*31*; see above), as male rats aged testosterone levels slowly declined (Fig. 6), as did erectile function (Fig. 3). Although decreased relative to young males, circulating testosterone levels are above those needed to restore copulatory and erectile function in castrated male rats (e.g., ref. *36*). Young female rats exhibit estrous cycle–related increases in plasma estradiol, whereas middle-aged rats in persistent estrus do not (data not shown). Others (e.g., ref. *37*) have reported similar estrous cycle–related changes in estradiol. Sexual behavior in female rats is dependent on sex steroids, with the display of receptivity coordinated with ovulation and occurring approx 24 h after the proestrous peak in estradiol *(38)*. Furthermore, circadian periodicities in circulating levels of testosterone in young male rats *(39,40)*, and a modification of pulsatile hormone secretion with aging *(41–43)*, have been reported. Based on the suggested loss of diurnal changes with aging, it is not surprising that some reports indicate that testosterone changes little, if at all, with aging, whereas others report large differences between young and aged male rats (as a function of time of day of sampling). We measured testosterone by RIA in trunk blood samples obtained after decapitation from groups of rats (n = 5–7 per group) at hourly intervals. Samples were obtained from 4- and 17-mo-old rats that were sexually naive. Our data (Fig. 7) demonstrate that young males exhibit distinct diurnal patterns of testosterone release and that diurnal patterns of plasma testosterone are greatly attenuated in middle-aged males.

In summary, with aging, the incidence of regular cycles in "normotensive" female rats decreases. Thus, although female rats do not undergo menopause *per se*, they do transition from regular cycles to irregular cycles, to periods of extended and persistent estrus. Young female rats exhibit estrous cycle–related increases in plasma estradiol, whereas middle-aged rats in persistent estrus do not. Females in persistent estrus have plasma levels of estradiol that are similar to those seen in young cycling rats on diestrous. As male rats age, testosterone levels slowly decline. In rats, as in humans, blood pressure increases with age. In young rats, females have lower blood pressure than males, but at 14 mo of age there are no differences between the sexes. Young male rats exhibit a marked circadian periodicity for plasma testosterone, which is lost with aging. Thus, our data clearly indicate that "normotensive" rats exhibit age-related increases in blood pressure in association with decreases in reproductive function and circulating sex steroid levels. Thus, the situation appears analogous to that observed in humans.

In conclusion, in "normotensive" rats, blood pressure increases with age as the pattern of gonadal hormone exposure changes. We suggest that the changing pattern of hormone secretion contributes to the age-related increase in systolic blood pressure. Thus, the dramatic loss of cyclic (at least fivefold) increases in estradiol may underlie the greater increase in blood pressure seen in middle-aged female rats, and by inference post-menopausal women. The middle-age increases in blood pressure in males may also be due, at least in part, to the dampening of diurnal periodicities in testosterone, or the reduced conversion of testosterone to estradiol.

Materials and Methods

Animals

Male and female Long–Evans rats (Simonsen Laboratories, Gilroy, CA) arrived in the laboratory at 6–8 wk of age and were housed two per cage. They were provided food and water *ad libitum* and maintained in a temperature and light (lights off, 1200 h; lights on, 2200 h) controlled environment within an AALAC accredited facility. The 14 h of light and 10 h of darkness was used for longitudinal and cross-sectional studies.

Longitudinal Study

In our laboratory we have repeatedly observed that manipulations performed in the late light phase (3–4 h prior to lights off) does not affect changes in activity and ingestive behavior normally seen with the light:dark transition. These manipulations have included vaginal smears, tail-cuff blood pressure measurements, erectile reflex tests, and blood sampling. Furthermore, this period is a period of stable blood pressure *(16,44)* and previous reports indicate that diurnal–nocturnal differences in blood pressure are reduced with aging *(16)*. Thus, in the present longitudinal study all manipulations were done during the late part of the light phase, being completed at least 2 h prior to the light:dark transition.

Females: After a 2 wk acclimation period daily vaginal smears were obtained from the females for 21 consecutive days during the late part of the light phase (3–4 h prior to

onset of darkness). Only females (30 of 36) that exhibited at least four regular 4 d cycles were included in the study (of the six not exhibiting 4 d cycles two had consecutive 3 d cycles, two had consecutive 5 d cycles, and two had cycles varying from 3 to 6 d). Monthly thereafter, vaginal smears were obtained for at least 14 consecutive days. Rats were considered to be (a) regular—if regular cycles of 3–5 d were observed; (b) irregular—displaying estrous cycles of 6–12 d in duration; (c) extended estrous—if multiple consecutive proestrous/estrous smears were observed separated by 2–5 d of diestrous smears; or (d) persistent estrous—exhibiting persistent vaginal cornification for at least 12 consecutive days. The criteria for classification of estrous cyclicity were modified from those of Lu et al. *(10,12,13,45)*. Females were maintained in same-sex pairs (two rats per cage) and were never mated.

One week each month, blood pressure and body weight were measured 3–4 h prior to lights off. Systolic blood pressure was measured in young rats exhibiting diestrous vaginal smears (see below).

During the remaining week, females exhibiting diestrous smears were anesthetized with ether and a blood sample (1.5 mL) obtained by jugular venisection, 3–4 h prior to lights off. Blood was collected in heparinized syringes, and right and left sides were alternated for jugular sampling. Approximately 3 min elapsed between the initial disturbance of the animal to completion of sampling. Animals received 500 µg gentamycin, im, after each venesection. Plasma was separated and stored at –20°C for subsequent determinations of estradiol.

Males: Beginning at 3 mo of age, erectile reflexes were assessed in four tests separated by 3–5 d, 1–5 h prior to lights off. Erectile reflexes were assessed as described previously *(46,47)*. Briefly, the rat was placed in a clear plastic cylinder with the penile sheath retracted using a wooden applicator. Males were allowed 15 min to show the first erection, and observed for an additional 20 min from the first erection, if an erection occurred. The following reflex events were recorded: erection—increased tumescence of the penis followed by detumescence; cup—maximal flaring of the engorged glans penis followed by detumescence; quick flip—a rapid movement of the penis toward the abdomen; and long flip—a more gradual and sustained dorsiflexion of the penis. Thus, parameters quantified included latency to first erection, and the numbers of erections, cups, quick flips, and long flips. Thirty males exhibiting erectile reflexes in the last two tests were included in this study. Subsequently, erectile reflex tests were administered twice during one week each month. As for females, males were maintained in same-sex pairs (two rats per cage) and were never mated.

As with females, during one week each month, blood pressure and body weight were recorded. Systolic blood pressure was measured 3–4 h prior to lights off indirectly using tail-cuff plethysmography (see below),

During the remaining week, males were anesthetized with ether and a blood sample obtained by jugular venisection, 3–4 h prior to lights off. Blood was collected in heparinized syringes, and right and left sides were alternated for jugular sampling. Approximately 3 min elapsed between the initial disturbance of the animal to completion of sampling. Animals received 500 µg gentamycin, im, after each venesection. Plasma was separated and stored at –20°C for subsequent determinations of testosterone.

Cross-Sectional Study

Females: Venous samples were obtained from young (3 mo of age; $n = 32$) female Long–Evans rats that had exhibited at least three consecutive 4-d estrous cycles. Seven to nine animals were sampled 3–4 h prior to lights off on each day for two complete cycles (diestrous 1, diestrous 2, proestrous, estrous—each rat was sampled twice with 3 d between samples). Blood samples were also obtained from middle-aged females (15 mo of age; $n = 28$) exhibiting vaginal smears indicating a state of persistent estrous for eight consecutive days (seven rats per day, two samples per rat, samples separated by 3 d).

Males: Previous studies indicated that young male rats exhibit circadian periodicities in circulating levels of testosterone. Young (3 mo of age) and middle-aged (15 mo of age) male Long–Evans rats (seven to nine per time point) were decapitated and trunk blood collected into heparinized tubes at hourly intervals. In an attempt to simplify interpretation, we have organized the data such that "0" time was when the lights came on and "14" was the time the lights went off. Thus, lights were on from 0 to 14 and off from 14 to 24.

Circulating Hormone Levels

Estradiol and testosterone were quantified using tritiated standards and specific antibodies (ICN), as previously described (e.g., refs. *26, 36, 47–51*). Assays were performed at the conclusion of the studies, and all samples from any individual animal in the longitudinal study were measured in the same assay. Intra- and interassay coefficients of variation were: estradiol, intraassay = 6.5%, interassay = 9.3%; testosterone, intraassay = 5.6%, interassay = 7.1%.

Measuring Systolic Blood Pressure

Systolic blood pressures were obtained using tail-cuff plethysmography, as previously described by us *(49–52)*. Briefly, indirect arterial pressures were monitored using a tail-cuff plethysmography system. Systolic arterial blood pressure was measured in conscious animals with an automated system (Model 179; IITC Life Science Instruments, Woodland Hills, CA) that utilizes tail cuffs and photoelectric sensors to detect tail pulses *(49–53)*. Rats are restrained in holders appropriate for their body weight in a test chamber maintained at 29°C. Blood pressure was evaluated in

three sessions during a 5 d period, and values will represent the mean of those obtained in the last two sessions. Each session involved at least five blood pressure determinations.

Statistics

Data are presented as mean ± SEM and were evaluated using Friedman's two-way analysis of variance (repeated measures), and *post hoc* Newman–Keuls and/or Duncan's tests. At each time point, male and female data were compared using two-sample *t*-tests. For the longitudinal study, the incidence of hypertension (systolic blood pressure ≥ 140 mm Hg in females as a function of normal cycles or persistent vaginal estrus was assessed using chi-square analysis.

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