Aging of the Brain-Testicular Axis: Reproductive Systems of Healthy Old Male Rats with or without Endocrine Stimulation

(43953)

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Abstract. **To test the hypothesis that endocrine declines in males are incidental to disease, 24 gonadally intact old (22–24 months) rats were selected on the basis of good general health and assigned to one of three groups. One group of aged males was left untreated for comparison with an untreated control group of young adult males. Results from multiple measures of sociosexual behavior and reproductive physiology indicated that endocrine declines in males are not simply a by-product of increased disease incidence with aging. The untreated old animals showed clear decrements on all 13 measures of hypothalamic-pituitary-testicular (HPT) activity. The other two groups of old males were used to compare responsiveness of the aging HPT axis in healthy males to supplements with a typical exogenous (ExT) androgen regimen (300 μg testosterone/kg body wt/SC/daily/6 weeks) or to social stimulation (brief daily exposure to an inaccessible estrous female) for additional episodes of endogenous (EnT) hormone. Neither treatment restored our disease-free old male rats to levels approximating those of untreated young adults. Nonetheless, both treatments activated the aging HPT axis. EnT males showed increases in sociosexual behaviors, growth of androgen-sensitive bulbospongiosus muscle, and elevation of epididymal sperm reserves. ExT males, on the other hand, experienced a more foreboding hypertrophy of the ventral prostate gland. Our conclusion is that endocrine aging in males is ubiquitous and inevitable. Still, aged androgen-sensitive systems of healthy old rats retain notable capacity, particularly, for *endogenous* activation. Evidence points to functional responses by healthy aged males to the presence of sexually receptive females that, although not quantitatively the same, are qualitatively similar to the responses of young adult males.** [P.S.E.B.M. 1996, Vol 211]

An important goal of geriatric medicine is distinguishing preventable from inescapable changes accompanying normal aging. There is little doubt that endocrine aging and reproductive senescence are components of normal ontogenetic processes in females (1). Yet findings with elderly males, particularly healthy members of a group, have questioned the inevitability of aging in the hypothalamic-pituitary-testicular (HPT) axis (2–4). The implication is that declines in male reproductive function can be thwarted by promoting disease-free aging (5, 6).

The typical subject is selected randomly from groups of old men and nonhuman animals. As a group, these males show clear evidence of HPT disruption. Circulating androgen titers decrease during old age (7–9), and the characteristic episodic release of steroidal and neuropeptide hormones are altered (10–12). Functional consequences to HPT target systems are pro-

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Received December 2, 1994. [P.S.E.B.M. 1996, Vol 211]
Accepted September 6, 1995.

0037-9727/96/2111-0069$10.50/0

found and ubiquitous. Size and function of primary and secondary sex structures undergo marked change (13, 14). Sexual dimorphism is lost in other androgen-dependent tissues both inside (15) and outside (16) the central nervous system. Frequencies of sociosexual behaviors decline, often to complete cessation (17, 18), while interest wanes for initiating contact with a sexually receptive female (19, 20).

On the other hand, researchers often report wide individual variation in the HPT axis of elderly subjects (21). Some, presumably healthy, old males show a pattern of endocrine integrity comparable to that of young adults (3, 4, 22). The suggestion is that vulnerability of the elderly to disease, rather than HPT aging, is responsible for the dysfunction typically reported among groups of randomly selected aged males (1). There are a few published studies in which subjects have been selected on criteria of healthfulness, but the results have proved equivocal (10, 12, 23, 24).

For the present study, only gonadally intact male rats in good overall health were employed as subjects. Our interest was regarding two questions. The first was whether evidence of biologically meaningful endocrine declines could be detected in males who maintained good overall health into old age. Second was how sensitive the HPT axis of disease-free old males was to exogenous androgen therapy or to social stimulation of endogenous hormone release (20, 25). To address the first question, one group of healthy old males was left untreated and compared with young adult males on multiple indices of HPT integrity. For the second question, groups of healthy old males were either provisioned with a daily androgen supplement or exposed to an inaccessible estrous female repeatedly for 6 weeks. Social behavior and reproductive physiology were assessed at the end of the treatments.

Materials and Methods

Animals. Males (n = 32) were chosen from a colony of Wistar rats bred and raised in a well-maintained facility at Heidelberg Universität. The 22 to 24-month-old males (n = 24) were selected "blind" by two veterinarians based on clinical signs of good overall health. The same criteria were applied to select a group of 5-month-old males (n = 8). None of the animals had been used previously in an experimental protocol, and all had been housed in same-sex, same-age pairs until 2 weeks before the beginning of the experiment. Animals were housed individually for the experiment in Makrolon III cages.

Sets of 4 to 6-month-old Wistar males (n = 15) and females (n = 30) served as "opponent" and "stimulus" animals, respectively, for behavior testing. Food and water were available *ad libitum* to all animals. Colony and experimental rooms both were maintained at controlled temperatures (20°–22°C) and relative humidity (50% ± 5%). Lighting was on a 12:12-hr light: dark cycle (lights on at 7:00 hr).

Experimental Design and Procedures. There were two control and two experimental groups (n = 8/group). One control group was composed of old males, and the other controls were young adult males. The remaining old rats were assigned at random to receive daily treatment for 6 weeks with either exogenous (ExT Group) or endogenous (EnT Group) testosterone. Each animal was assayed for systemic testosterone and given a battery of behavioral tests prior to sacrifice and evaluation of androgen-sensitive physiological systems.

Exposure routines. Procedural details have been published for hormone injections (14) and for social stimulation (26). Here, ExT males received daily sc injections for 6 weeks of 300 μg testosterone/kg body wt in 0.2 ml olive oil. Males in the other three groups were handled briefly and injected with vehicle only.

To induce endogenous HPT activity, males in the EnT group were exposed to inaccessible receptive females at the same time each day males in the ExT group were administered testosterone. Female rats 4–6 months of age were ovariectomized and induced to estrus with sc injections of 100 μg estradiol benzoate and, 48 hr later, with 400 μg progesterone. Each EnT male was exposed in his home cages to the sight, sounds, and odor of the receptive female. The animals were prevented from direct contact by fitting a mesh-wire screen across the breadth of the male's home cage. After a 10-min exposure period, the female was returned to her home cage, and the screen was removed from the male's cage.

Response Measures. Blood serum was obtained from each male on three occasions during the experiment. Samples were used to determine between-group differences in androgen levels and to confirm that an endocrine episode had been successfully induced from our experimental treatments. Targets of the HPT axis (27) were examined during the week after treatments to the experimental groups had ended. Tests of sociosexual behaviors were conducted over 3 days following the final hormone exposure to prevent confounding influences of current systemic hormone. Androgen-sensitive physiology was examined when the animals were sacrificed the next day.

Behavior. Young adult male and female rats were used as "stimulus animals" for behavior testing. Our methods are published for quantifying both sexual motivation (14, 20) and intermale aggression (28). Testing was scheduled to ensure that stimulus animals were unfamiliar to the subject males, and order of the tests was counterbalanced within and between groups.

Intermale aggressive behaviors were examined twice. On separate days, hostile interactions were ob-

served between subject and opponent males for 10 min in a neutral Makrolon III cage that contained clean bedding. Specifically, a subject male from one of the four groups was paired with one of the males randomly selected from a pool of 15 young adult stimulus males. Frequencies of the various forms of aggression (pushing, aggressive grooming, aggressive posturing, attacks with biting) initiated by a subject male and his opponent male were recorded.

Sexual motivation was evaluated on the day aggression was not tested. A single motivation test was used to minimize the confounding influence to the ExT group with female exposure. Our previous work with young adult rats has pointed to development of maximal response in males with relatively few presentations of an estrous female. The same concerns dictated our choice to assess sexual responsiveness by a test that prevented physical contact with the female.

We believe motivation represents a sensitive measure of sexuality in old males. Interest in a receptive female was measured in a paradigm (14) that, unlike tests of copulation, is not rate-dependent. This is a decided advantage to distinguish changes in the HPT axis from general motor deficits commonly observed in old animals.

The motivation test allowed the male a choice between approaching an area containing an estrous female versus areas that were empty or contained a nonestrous female (18). Interest in the receptive female was defined by time in proximity to each of four compartment located at the ends of a specially constructed "plus" maze. The end compartments were constructed with mesh wire to keep the male separated from the estrous female housed in one compartment. Nonestrous females were in two compartments, and the fourth compartment was empty. Location of the male was monitored automatically. A cumulative recorder timed the male as he moved freely about the apparatus. After 3 hr, animals were removed, and the apparatus was cleaned thoroughly before new females were placed randomly into end compartments for the next male.

Physiology. Circulating testosterone levels were assayed three different times using a serial phlebotomy procedure described earlier (29). The rat was lightly ether anesthetized, and blood was collected by cardiac puncture. Serum was obtained by centrifugation (15 mins at 4000*g*, Haereus-Christ type 4123) and frozen (−20°C) until assayed for testosterone.

Blood was collected twice from each male during Week 3–4 and once at necropsy. The former two collections were used to probe for hormone episodes. Serum was obtained from blood collected 1 hr before and, on another day, 1 hr after administration of the animal's daily experimental treatment.

Reproductive anatomy and physiology were examined at necropsy performed 72 hr after the final experimental treatments. Using methods already published (14, 26), animals were euthanized with an overdose of pentobarbital sodium. Indices of end-organ responsiveness to androgens were obtained. Reproductive structures excised and weighed included glans penes, bulbospongiosus muscles, seminal vesicles, ventral prostate, testes, and epididymides. The left epididymis from every animal was further prepared for counting of sperm (30). Epididymal sperm reserves provide a measure of functional changes from HPT activation, as well as a direct indicator of reproductive capacity.

Histology. Qualitative evaluations of tissues were used to corroborate clinical indices of health. Histological evaluations were made of all structures obtained at necropsy. Gross evidence of disease was recorded as a binary, yes or no, evaluation.

Prostates and testes also were sectioned and stained for histological analyses (31). Tissue sections were provided without group identifications to colleagues in the Histodiagnostic Division, German Cancer Research Center (Heidelberg). The pathologists were instructed to report any indications of pathology.

Statistical Analyses. One-way analyses of variance (ANOVAs) were used to assess differences among groups. Means ± SEM were calculated for each group for presentation in tables and figures. An aggression score was determined by totaling the frequencies of aggressive behaviors initiated by the old male and by the young male serving as his opponent for the behavioral test. Sexual motivation was quantified by timing the male in each compartment. Data analyzed were the percentage of the time spent in the compartment of the estrous female relative to the other compartments of the plus maze. Wet weights of structures were calculated as mg/100 g body wt. Testosterone titers are presented as ng/100 ml serum and total sperm numbers as $\times 10^{-6}$/g epididymis. Finally, post-hoc Tukey's Honestly Significant Difference tests (HSD, $P < 0.05$) were used for pair-wise comparisons of mean group differences.

Results

Behaviors. Findings for sociosexual responsiveness and reproductive physiology are summarized in Table I–III. ANOVA results were statistically reliable for both aggression and sexual motivation (range of F values = 4.57–36.58, $P < 0.01$).

Aggressiveness was estimated from data obtained during paired encounters between subject males and young adult opponent males. Sexual motivation was evaluated by interest shown an inaccessible estrous versus nonestrous female. Post-hoc group comparisons confirmed clear differences between 5- and 24-

Table I. Androgen-Sensitive Behaviors (n = 8/Group) of Healthy Old (22–24 months) or Young (5 months) Male Rats Following No Treatment or Repeated Exposure Over 6 Weeks Either to Exogenous Testosterone (ExT) or to a Receptive Female (EnT)

Group	Intermale aggression: subject	Intermale aggression: opponent	Sex motivation (% time near female)
Young controls	44 ± 4[a]	24 ± 2[a]	64 ± 9[a]
Old controls	5 ± 1[c]	7 ± 1[d]	27 ± 5[c]
Old ExT males	8 ± 1[c]	13 ± 1[c]	33 ± 4[c]
Old EnT males	16 ± 2[b]	18 ± 1[b]	45 ± 7[b]

Note. Findings reported are group Means ± SEM, first, of the frequencies of aggressive behaviors initiated by old male and by the young male serving as his opponent for the behavioral test, and, second, of the percentage of a consecutive 3-hr period in a plus maze spent near the compartment housing an inaccessible estrous female.
Different superscript letters denote statistically reliable differences (analyses of variance and post-hoc Tukey's tests, both $P < 0.05$); read down columns for group comparisons on each measure.

month-old controls on all measures. EnT males had higher frequencies of aggression and spent more time in proximity to the estrous female than either ExT or old controls. Still, the EnT group was far less responsive than young adult males. ExT and old control males showed similar levels of intermale aggression and interest in the estrous female.

Physiology. Analyses of end-organ response obtained at necropsy yielded statistically significant differences (F ranges = 6.07–262.77, $P < 0.01$). Post-hoc group comparisons of results indicated that none of the aged animals was the functional equal of the young males. Of the two control groups, young adult had significantly larger primary and secondary sex structures than the old controls. The latter animals had epididymal stores of spermatozoa that were less than half the numbers sported by young males.

The two experimental treatments influenced both size and function of reproductive structures of old males. Compared with old controls, EnT males experienced growth of testes, epididymides, and bulbospongiosus muscles, as well as greater epididymal sperm reserves. The ExT animals had the largest seminal vesicles and prostate glands.

Testosterone was determined from serum obtained 1 hr before (denoted as T_1 in Table III) and, on a different day, 1 hr after (Table III, T_2) a daily treatment, as well as at necropsy after treatments had ended. A repeated, factorial ANOVA of those data yielded a statistically reliable value for the interaction of group × time (F = 8.75, $P < 0.01$). Subsequent statistical comparisons indicated that young males typically had double the circulating testosterone of untreated old males.

Within and between groups comparisons of old males revealed three notable results. First, comparisons between pre *versus* post-administration confirmed an elevation of systemic hormone in old males with both injection and exposure to a female. Second, endocrine data at 1 hr postadministration indicated ExT treatments maintained elevated titers of testosterone in circulation longer than EnT treatment. Third, there were no statistically reliable differences in circulating testosterone among the aged groups at necropsy 72 hr after the last experimental exposure.

Histology. Reexamination of the animals at necropsy by the veterinarians revealed no evidence of preexisting or current pathology. Old males were essentially disease-free at the end of the study.

Organ samples obtained at necropsy were forwarded to pathologists without group identity. Histodiagnostic reports noted that tissues from old rats were easily distinguished from young adult males. There was indication of hyperactivity in the hypertrophic ventral prostate glands of the ExT group. Endothelial thickening and increased numbers of nucleoli in prostate samples were reported in five of eight ExT males. Nevertheless, the reports indicated no indications of disease in the prostates or other structures from any of the old males.

Table II. Androgen-Sensitive Physiology of Healthy Old Male Rats Exposed Chronically to Exogenous Testosterone (ExT), to a Receptive Female (EnT), or to Neither (Old or Young Controls)

Group	Ventral prostate	Seminal vesicles	Testes	Bulbospongiosus muscle	Epididymides	Epidid. sperm nrs.
Young controls	173 ± 7[a]	494 ± 23[a]	630 ± 25[a]	334 ± 11[a]	332 ± 9[a]	386 ± 15[a]
Old controls	115 ± 8[b]	330 ± 22[c]	373 ± 15[c]	209 ± 5[c]	128 ± 4[c]	191 ± 14[c]
ExT	193 ± 18[a]	428 ± 26[b]	432 ± 20[b,c]	228 ± 5[c]	125 ± 6[c]	175 ± 13[c]
EnT	127 ± 10[b]	359 ± 18[c]	463 ± 14[b]	269 ± 17[b]	158 ± 4[b]	266 ± 8[b]

Note. Data are means ± SEM of wet weights (mg tissue/100 g body wt) and of sperm reserves ($\times 10^{-6}$) per gram epididymis.

Table III. Circulating Testosterone (ng/100 ml Serum) of Healthy Old Male Rats Prior to Experimental Manipulation (T_1) and within 1 hr (T_2) of Experimental Manipulation in the Form of an Injection of Testosterone (ExT) or Exposure to a Receptive Female (EnT)

Group	T_1	T_2	T_3
Young controls	353 ± 22^{a}	$327 \pm 29^{a,b}$	$307 \pm 25^{a,b}$
Old controls	149 ± 20^{c}	164 ± 12^{c}	140 ± 12^{c}
ExT	146 ± 12^{c}	431 ± 60^{a}	177 ± 17^{c}
EnT	153 ± 14^{c}	292 ± 23^{b}	146 ± 12^{c}

Note. The final value (T_3) was obtained at necropsy, 72 hr after the final exposure.

Discussion

Little support can be found in our data on sociosexual behaviors and reproductive physiology for speculating that disease-free stature exempts old males from age-typical declines in the hypothalamic-pituitary-testicular axis (2–6). There is no question that a lifestyle promoting good health into old age is sure to accrue benefits (16, 22, 32). For example, our rats showed clear signs of maintaining the capacity for considerable responsiveness to experimentally induced HPT activation. Nonetheless, even these healthy old males showed equally clear signs of endocrine aging and biologically meaningful declines in HPT function.

Gonadally intact male rats were selected for good general health, a conclusion confirmed by histodiagnostic evaluations at necropsy. The healthy old males were assigned to one of three conditions. One group of old animals was left untreated for comparison with a group of untreated 5-month-old males. Statistical analyses confirmed differences between the aged and young males on each of 13 quantitative measures of HPT function. Compared with young males, circulating titers of testosterone were lower in the untreated older group (8). Interest in a sexually receptive female and intermale aggressive exchanges were substantially lower (18, 21, 33), and primary and secondary sex structures were smaller (11, 20). Reduced reserves of epididymal sperm from the untreated males provided direct evidence for functional declines accompanying the structural changes with aging (13, 16).

The other old rats were administered one of two experimental treatments to assess response capabilities of the healthy aged HPT axis. One treatment was a standard pharmacologic method, exogenous hormone supplements, employed in both clinic and laboratory as attempts to reverse an endocrine deficiency (25, 34). The second treatment was to induce an additional endocrine event laid over the characteristic episodic nature of endogenous hormone releases during a day (12, 35). Males in the ExT group received a bolus injection of testosterone at the same time males in the EnT group were being exposed to inaccessible receptive females. The latter is a technique we have used often in studies of young males to induce a reliable burst of endogenous endocrine activity (26, 28). It is important to note that the old males received repeated exposures to testosterone injections or to the female prior to behavior testing. Daily exposures for 6 weeks were designed to ensure maximal response to both experimental manipulations.

Findings indicated neither regimen rejuvenated the HPT axis of our old males to anything approximating functional equivalence with young adult rats (27). Nevertheless, the treatments were not unnoticed by target systems of the healthy old males (34, 36, 37).

Maintaining good general health appears to have left the aged HPT axes of our old males with notable response capacities (20, 38, 39). Similar to reports with young males (26, 40, 41), all EnT males released a testosterone pulse to presentation of an estrous female. Previous findings were that only a portion of randomly selected old male rats showed full endocrine response to females (32, 42). The young males used as opponents in tests of aggressiveness also took notice of the changes in our experimental males. Compared to their interactions with control old males, opponent males behaved more aggressively toward experimental males. The sexual behavior and physiological findings provided additional evidence of HPT activation to social exposure and to hormone administration.

Patterns of results for EnT and ExT groups, however, were markedly different. Response to hormone administration was dissimilar to the response marshalled to environmental stimulation. The lesson suggested is that exogenous therapy with a single steroid is unlikely to successfully mimic the complexity of endogenous endocrine activation (43, 44). Species-typical social stimulation induces an array of endogenous events in young adult males (26, 45, 46).

Here, exposure to females left a broad stamp on androgen-sensitive systems of healthy old males. Indeed, the EnT group showed sociosexual vigor not observed in untreated old nor in the ExT group. EnT males spent more time in proximity to the estrous female and had higher intermale aggression scores (20, 21, 37). Their elevated epididymal sperm reserves point to adoptive functional changes accompanying social activation of the HPT axis (30).

At the same time that EnT males showed signs of enhanced reproductive potential, ExT males had larger seminal vesicles and prostate glands. However, treatments that promote prostatic hypertrophy and hyperactivity are of special concern for elderly men (47). The conclusion points to endogenous endocrine activation as being less perilous for the prostate gland of aged males than testosterone injections.

These conclusions, admittedly, are based on a sin-

gle ExT dosage and a specific presentation of females. For example, endocrine findings comparing ExT and EnT groups suggest pharmacokinetics of our oil-suspended testosterone dosage extended half-lives (48) beyond those characteristic of a pulse of endogenous steroid (35). Still, the distinctively different functional patterns to the two endocrine manipulations remain a notable result.

In summary, the implication is that disease-free old males retain capacity for HPT activation that, if not quantitatively equal, is qualitatively similar to young adult males. Healthy old male rats show significant endocrine arousal to changes in the social environment. Nonetheless, we are left with the inescapable conclusion that males and females alike are subject to age-related endocrine declines. HPT axis changes are fundamental to the aging process rather than being incidental to diseases associated with old age. It is those changes underlying the age-related declines in sociosexual responsiveness and reproduction in males.

This research was supported, in part, by grants from the Alexander von Humboldt Foundation (Taylor/Weiss) and NIMH (Bardgett).

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